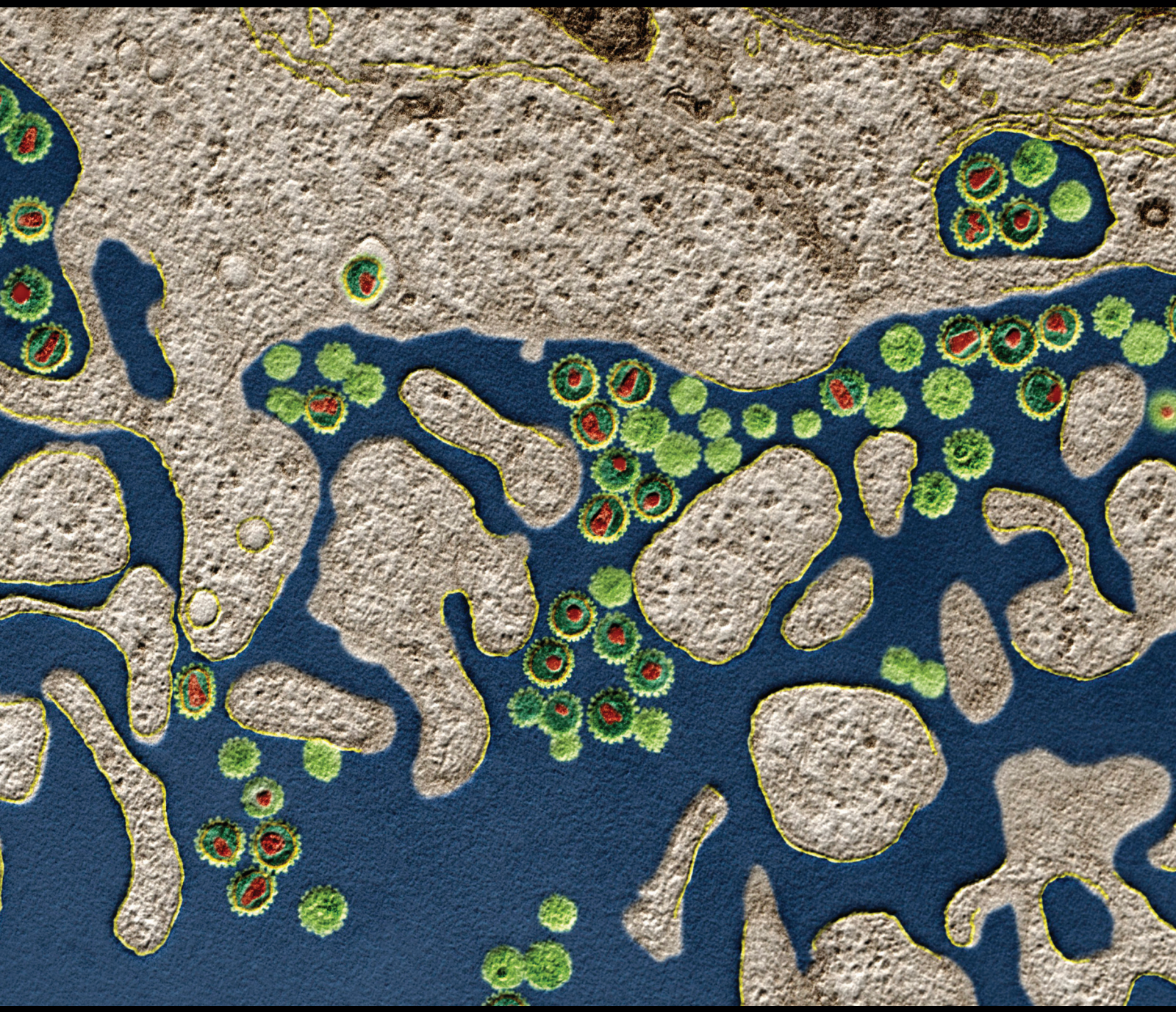
Journal of Immunology Research

Systemic Autoimmune Diseases 2014

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 2014

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Systemic autoimmune diseases are a group of common diseases, including rheumatoid arthritis, systemic lupus erythematosus, spondyloarthropathy, Sjogren's syndrome, polymyositis, and dermatomyositis, etc. They are one of the leading causes of death and disability. With the use of glucocorticoid, immune suppression drugs and new developed biologics, the outcome of this group of diseases has greatly improved, but there is still no cure for them. Knowledge of the pathogenesis, diagnosis, and treatment of those diseases will lead to better understanding of the diseases and better care of patients.

Based on this background, we assembled this special issue for a better understanding of the molecular pathology underlying systemic autoimmune diseases, the development of strategies to treat these conditions, and the evaluation of outcomes.

In this special issue, several review articles discussed many important aspects about autoimmune disease. A. Mastrangelo et al. discussed the role of posttranslational protein modifications in rheumatoid arthritis. C. Gluhovschi et al. made a review about pregnancy associated with systemic lupus erythematosus. A. Kronbichler et al. reviewed the influence and role of microbial factors in autoimmune kidney diseases. T. Shizuma summarized clinical characteristics of concomitant systemic lupus erythematosus and primary biliary cirrhosis. Z. Wu and H. Nakanishi discussed the link between inflammatory bone disorders and Alzheimer's disease. K. R. Sigdel et al. made a review about the functions of long noncoding RNA in immune cells. L. Duan et al. made a review about treatment of bullous systemic lupus erythematosus. R. Hage-Sleiman et al. reviewed recent studies about the novel PKCtheta. L. Zhang et al. made a meta-analysis about interleukin-23R rs7517847 T/G polymorphism and the risk of Crohn's disease in Caucasians.

Beside reviews, many original research studies about the pathogenesis or clinical characteristics of autoimmune disease were also included in this special issue. T. Elisa et al. studied the role of endothelin receptors in the pathogenesis of systemic sclerosis. A. Barbieri et al. analyzed the characterization of CD30/CD30L+ cells in peripheral blood and synovial fluid of patients with rheumatoid arthritis. G. F. Dong et al. researched the effect of leflunomide on the lipid rafts expression in SLE patients. P. Žigon et al. found that antiphosphatidylserine/prothrombin antibodies were associated with adverse pregnancy outcomes. G. Sudzius et al. studied the distribution of peripheral lymphocyte populations in primary Sjögren's syndrome patients. J. Xu et al. showed that autoantibodies affect brain density reduction in nonneuropsychiatric systemic lupus erythematosus patients. B. Shen et al. found that body image disturbances have impact on the sexual problems in systemic lupus erythematous patients. G. Guo et al. and C. Zhao et al. studied mesenchymal stem cells in SLE and RA patients. Y. Liu et al. found a new serological marker in SLE patients. A. E. Ngono et al. found that frequency of circulating myelin oligodendrocyte glycoprotein B lymphocytes was decreased in relapsing-remitting multiple sclerosis patients. J. Amaya-Amaya et al. showed that GDF15 (MIC1) H6D polymorphism does not influence cardiovascular disease in a Latin American population with rheumatoid

arthritis. A. D. Rocha-Muñoz et al. demonstrated that anti-CCP2 antibodies are markers associated with the severity of RA- ILD. M. Lu et al. found that HMGB1 promoted systemic lupus erythematosus by enhancing macrophage inflammatory response. A. D. Rocha-Muñoz et al. studied the influence of anti-TNF and disease modifying antirheumatic drugs (DMARDs) therapy on pulmonary forced vital capacity associated with ankylosing spondylitis. B. Kisiel et al. showed that methotrexate, cyclosporine A, and biologics protect against atherosclerosis in rheumatoid arthritis. L. Wang et al. analyzed clinical characteristics of cerebral venous sinus thrombosis in SLE patients.

This special issue covers many important aspects in autoimmune diseases, which will surely provide us with a better understanding about the pathogenesis, diagnosis, and treatment of autoimmune diseases.

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Research Article

Anti-Phosphatidylserine/Prothrombin Antibodies Are Associated with Adverse Pregnancy Outcomes

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Objective. To determine the prevalence and clinical association of anti-phosphatidylserine/prothrombin antibodies (aPS/PT) in patients with a history of pregnancy complications relevant to antiphospholipid syndrome (APS). *Materials and Methods*. Two hundred and eleven patients with a history of (a) three or more consecutive miscarriages before 10th week of gestation (WG) ($n = 64$), (b) death of a morphologically normal fetus beyond 10th WG ($n = 72$), (c) premature birth of a morphologically normal neonate before 34th WG due to eclampsia, preeclamsia and placental insufficiency ($n = 33$), and (d) less than three unexplained consecutive miscarriages before 10th WG ($n = 42$). Subjects sera were analyzed for lupus anticoagulant (LA), anti-cardiolipin (aCL), anti-β_2-glycoprotein I (anti-β_2GPI), and aPS/PT antibodies. *Results*. 41/169 (24.3%) of patients were positive for at least one measured aPL. The highest prevalence was found for aPS/PT and aCL (13.0% and 12.4%, resp.) followed by LA (7.7%) and anti-β_2GPI (7.1%). 11/169 with APS-related obstetric manifestations had only aPS/PT. 17.8% of patients were positive for LA or aCL and/or anti-β_2GPI; however when adding the aPS/PT results, an additional 7% of patients could be evaluated for APS. *Conclusion*. aPS/PT are associated with recurrent early or late abortions and with premature delivery irrespective of other aPL.

1. Introduction

Patients with elevated levels of antiphospholipid antibodies (aPL) often experience pregnancy complications comprising recurrent spontaneous abortions, intrauterine growth retardation, and preeclampsia, suggesting that these antibodies may influence embryonic implantation and induce thrombosis of the uteroplacental vasculature. The international classification criteria for antiphospholipid syndrome (APS) connect the occurrence of obstetric complications and/or thrombosis together with persistence of aPL in APS [1]. Laboratory criteria for APS include lupus anticoagulants (LA), anticardiolipin antibodies (aCL), and antibodies against β_2-glycoprotein I (anti-β_2GPI). Several other "noncriteria" aPL are believed to be associated with APS; however the lack of evidence confirming their diagnostic applicability so far prevents their inclusion into classification criteria. In recent years, many studies demonstrated the association of antiprothrombin antibodies with the pathogenesis of APS [2–4] and some of them proposed their beneficial role for APS diagnosis [5–7]. These antibodies can be detected by an ELISA targeting prothrombin alone (aPT-A) or targeting phosphatidylserine/prothrombin complex (aPS/PT) [8]; however the latter are more frequently found in patients with APS [4, 9, 10]. Our group reported on an *in-house* aPS/PT ELISA as the optimal method for the determination of clinically relevant antiprothrombin antibodies exhibiting the highest proportion of LA in our population of patients [11]. Clinical relevance of antiprothrombin antibodies was mainly described for patients with APS and thrombosis but very few studies reported their association with adverse pregnancy outcomes. A comprehensive review of "antiprothrombin antibodies" and "pregnancy/obstetric/miscarriages/fetal loss" revealed 12 studies, comprising 1031 patients and 988 controls

Table 1: Review of antiprothrombin antibodies and pregnancy complications.

Author, year (ref.)	Study design	Number of controls (feature)	Number of patients (event)	ELISA	Isotype	Sensit. (%)	OR	*P*
Forastiero et al., 1997 [12]	R	89 (no obstetric complications)	44 (obstetric complications)	aPT-A	IgG	20	1.4	ns
					IgM	12	1.8	ns
Akimoto et al., 2001 [17]	P	12 (healthy nonpregnant)	19 (abortion <13th WG)	aPT-A	**IgG**	**58**		**<0.01**
		36 (normal pregnancy)	28 (severe preeclampsia)	aPT-A	**IgG**	**36**		**<0.01**
Tsutsumi et al., 2001 [16]	R		81 (≥2 recurrent miscarriages)	aPS/PT	IgG	0		
					IgM	0		
Sugiura-Ogasawara et al., 2004 [15]	R		100 (≥2 recurrent miscarriages)	aPS/PT	IgG	1		
					IgM	0		
Bertolaccini et al., 2005 [18]	R(M)	71 (healthy)	40 (recurrent abortions <10th WG)	aPS/PT	IgG	12	1.2	ns
					IgM	9	1.4	ns
				aPT-A	IgG	11	0.9	ns
					IgM	0	0.2	ns
			37 (fetal death >10th WG)	aPS/PT	**IgG**	**17**	**3.3**	**0.005**
					IgM	**13**	**3.7**	**0.006**
				aPT-A	**IgG**	**19**	**4.1**	**0.0007**
					IgM	1	0.5	ns
			29 (prematurity <34th WG preeclampsia/eclampsia/ placental insufficiency)	aPS/PT	IgG	9	1.2	ns
					IgM	5	0.9	ns
				aPT-A	IgG	10	1.4	ns
					IgM	0	0.3	ns
Nojima et al., 2006 [13]	R(M)	74 (healthy)	14 (fetal loss)	aPS/PT		21	1.49	ns
				aPT-A		14	0.25	ns
Sabatini et al., 2007 [21]	RCC	200 (pregnant, gestational/age matched)	100 (pregnant, past ≥3 recurrent abortions <20th WG)	aPT-A	**IgG**	**37**		**<0.001**
					IgM	**18**		**<0.001**
					IgA	4		ns
Marozio et al., 2011 [3]	RCC	163 (uneventful pregnancy)	163 (adverse late pregnancy outcome)	aPT-A	**IgG**	**26**	**9.1**	**<0.001**
					IgM	2	na	ns
Sater et al., 2012 [14]	RCC	288	277 (≥3 miscarriages <12th WG)	aPT-A	IgM	4	14.27	ns
Vlagea et al., 2013 [19]	R	/	71 (obstetric abnormalities)	aPS/PT	**IgG**	**25**	**2.37**	**0.04**
					IgM	27	1.32	ns
Žigon et al., 2013 [20]	R(M)	55 (no obstetric complications)	28 (APS obstetric abnormalities)	aPS/PT	**IgG**	**64**	**9.3**	**<0.001**
					IgM	**54**	**4.0**	**<0.005**

Medline query with keywords "antiprothrombin antibodies" and "pregnancy/obstetric/miscarriages/fetal loss" in various combinations yielded the documented reports. R: retrospective study, R(M): retrospective study with multivariate analyses, P: prospective study, RCC: retrospective case control study, ns: not significant, WG: week of gestation, OR: odds ratio, and Sensit.: diagnostic sensitivity.

(Table 1). Half of these studies failed to find any significant association between antiprothrombin antibodies and pregnancy morbidities [12–16]. On the contrary, Akimoto et al. [17] presented strong and specific association between various types of antiprothrombin antibodies with severe preeclampsia and spontaneous abortion. Only the study from Bertolaccini et al. [18] differentiated among different obstetric complications and showed a significant association of both aPS/PT and aPT-A with unexplained death of a morphologically normal fetus beyond 10th week of gestation. The clinical significance of antiprothrombin antibodies in patients with adverse pregnancy outcome was later confirmed also by Marozio et al. [3], who investigated aPT-A, and Vlagea et al. [19] who investigated aPS/PT. Furthermore, our

Table 2: Patients' clinical features.

Women with obstetric complications $n = 211$ (%)	
Thrombosis	12 (6)
Arterial thrombosis	8 (4)
Venous thrombosis	4 (2)
Pregnancy loss defined by APS criteria [1]	169 (80)
≥3 consecutive miscarriages <10th WG	64 (30)
Fetal death >10th WG	72 (34)
Premature birth <34th WG	33 (16)
Pregnancy loss not defined by APS criteria	42 (20)
<3 miscarriages <10th WG	42 (20)

group has recently reported that aPS/PT is the strongest independent risk factor for obstetric complications compared to LA, aCL, and anti-β_2GPI [20]. However, no reports to date were found describing association of antiprothrombin antibodies with unexplained consecutive miscarriages in the first trimester of pregnancy.

Therefore, the present study aims to investigate the association of aPS/PT with a history of specific idiopathic pregnancy complications in a larger group of patients and to determine whether the presence of aPS/PT is associated with an increased risk of obstetric manifestations relevant for APS.

2. Materials and Methods

2.1. Subjects. This study included 402 sera samples which were prospectively collected from 211 consecutive female patients (median age 33 years, IQR: 7 years) referred between 2005 and 2013 to our clinic due to possible obstetric APS (Table 2). Among them 169 had obstetric manifestations included in the APS classification criteria. Specifically, 64 patients had three or more unexplained consecutive miscarriages before 10th week of gestation (WG), 72 patients experienced unexplained death of a morphologically normal fetus past 10th WG, and 33 patients gave premature birth to a morphologically normal neonate before 34th WG due to eclampsia, preeclamsia, or placental insufficiency. Among 33 preterm deliveries, 10 cases experienced fetal death as a result of either placenta abruptions or growth retardations with any morphological abnormalities being excluded. There were 16 cases with induced deliveries because of preeclampsia or eclampsia and 7 spontaneous preterm deliveries. The remaining 42 patients experienced less than three miscarriages before 10th WG and did not meet the APS classification criteria.

All patients and their partners were investigated for genetic abnormalities. The majority of patients had received the results of genetic analysis at the time of their visit to our clinic and the patients with confirmed abnormalities were excluded from the study.

As a control group we included 87 healthy female blood donors (median age 42 years, IQR: 18 years) without a history of underlying autoimmune disease, bleeding disorders, thrombosis, and/or pregnancy morbidity.

All patients had their sera collected during their clinical examination in the Department of Rheumatology (University Medical Centre, Ljubljana). The samples were aliquoted, stored at −20°C, and subsequently analyzed. This study was conducted as part of the National Research Program "Systemic Autoimmune Diseases" (#P3-0314). Participants signed an informed consent and the study was approved by the National Medical Ethics Committee, Ljubljana, Slovenia.

2.2. LA Determination. Plasma samples were analyzed using coagulation analyzer BCS Siemens according to the guidelines valid at the time the study started [22]. Simplified Dilute Russell's Viper Venom Test (dRVVT) was performed using LA 1 screening reagent and LA 2 confirmatory reagent (Siemens) following manufacturer's instructions [23]. 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).

2.3. An In-House aPS/PT ELISA. This was performed following previously described protocol and validated method [11]. Specifically, the assays average inter- and intra-assay coefficients of variations were <3.3% and <8.2%, respectively. The diagnostic specificity for APS was 92.5% and the diagnostic sensitivity was 59.0%. The diagnostically relevant cut-off of aPS/PT was set on the 99th percentile of 222 blood donors. Briefly, phosphatidylserine was coated on polystyrene microtitre plates (medium binding, Costar, Cambridge, MA, USA) and incubated overnight at 4°C. After blocking with Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA) and 5 mM $CaCl_2$ (1% BSA-TBS-Ca^{++}) plates were washed in TBS containing 0.05% Tween-20. Human prothrombin (10 mg/L) (Enzyme Research Laboratories, UK) and patients' sera diluted 1 : 100 in 1% BSA-TBS-Ca^{++} were applied to wells immediately one after the other and incubated for 1 hour at room temperature (RT). Afterwards, plates were washed and incubated with alkaline phosphatase-conjugated goat anti-human IgG or IgM (ACSC, Westbury, USA) for 30 minutes at RT. After the last wash para-nitrophenylphosphate (Sigma Chemical Company, USA) in diethanolamine buffer (pH 9.8) was applied as substrate and OD_{405} was kinetically measured by microtitre plate reader (Tecan, Grödig, Austria).

2.4. IgG and IgM aCL. These were determined in sera by an *in-house* solid phase aCL ELISA [24]. Briefly, polystyrene microtitre plates (medium binding, Costar, Cambridge, MA, USA) were coated with cardiolipin (Sigma, St. Louis, USA) and blocked with 10% fetal bovine serum (FBS) (Sigma, St. Louis, USA) in phosphate-buffered saline (PBS). After washing with PBS, diluted samples in 10% FBS-PBS were applied and incubated for 2.5 hours at RT. The detection system was the same as in aPS/PT ELISA.

2.5. IgG and IgM Anti-β_2GPI. These were measured by an *in-house* ELISA [25]. Briefly, high binding polystyrene microtitre plates coated with 50 uL/well of β_2GPI (10 mg/L)

Table 3: Prevalence of aPL in patients with obstetric complications and healthy controls.

	Healthy controls $n = 87$ (%)	Non-APS obstetric manifestation $n = 42$ (%)	Pregnancy loss defined by APS criteria [1] $n = 169$ (%)
LA	0	2 (5.3)*	13 (8.7)**
aCL	2 (2.3)	2 (4.8)	21 (12.4)
IgG	1 (1.2)	2 (4.8)	17 (10.1)
IgM	1 (1.2)	0	6 (3.6)
anti-β_2GPI	6 (6.9)	3 (7.1)	12 (7.1)
IgG	6 (6.9)	3 (7.1)	10 (5.9)
IgM	0	0	4 (2.4)
aPS/PT	2 (2.3)	0	22 (13.0)
IgG	1 (1.2)	0	16 (9.5)
IgM	1 (1.2)	0	12 (7.1)

*($n = 38$), **($n = 149$).
aCL: anticardiolipin antibody, anti-β_2GPI: antibodies against β_2-glycoprotein I, aPS/PT: anti-phosphatidylserine/prothrombin antibodies, and LA: lupus anticoagulant.

in PBS were incubated for two hours at RT. The plates were then washed with PBS containing 0.05% Tween-20 (PBS-Tween) and incubated with samples diluted in PBS-Tween for 30 minutes at RT. The detection system was the same as in aPS/PT ELISA.

All aPL tests were performed in the same sera samples. Patients and controls were tested at the same time. None of the patients or controls were pregnant at the time of aPL determination.

2.6. Statistical Analysis. Statistical analysis was performed using the SPSS 15.0 program. The receiver operating characteristic (ROC) analysis and the area under the curve (AUC) 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

Every patient positive for any of the tested aPL was tested again at least 12 weeks after their first visit and only permanently elevated levels of aPL were considered as a positive result. Prevalence of all aPL tested is shown in Table 3. Overall, 169 patients experienced pregnancy morbidity defined by APS criteria and 41 (24%) of them showed permanent positivity for at least one of the measured aPL. The highest prevalence was found for aCL and aPS/PT (13%) while the prevalence for LA and anti-β_2GPI was lower (7%) (Table 3). Eleven patients (6.5%) were aPS/PT positive while being negative for all other tested aPL. Six of them had recurrent abortions before 10th WG, two experienced an unexplained death of a morphologically normal fetus past 10th WG, one delivered prematurely, and two experienced both recurrent abortions and premature birth. Considering Sydney revised laboratory criteria of APS, 17.8% (30/169) of patients were positive for LA or aCL and/or anti-β_2GPI. Among them, 22 were treated with low-molecular-weight heparin (LMWH) and low dose aspirin (LDA) which resulted in a successful pregnancy. Six women had no subsequent pregnancies; three of them experienced successful pregnancy prior to APS pregnancy complications. Two women had unsuccessful subsequent single pregnancies despite the anticoagulant treatment. When adjoined, the aPS/PT results revealed an additional 6.5% (11/169) of patients with adverse pregnancy outcome that was positive for aPL.

The frequency of aPL among 42 patients who experienced less than three miscarriages before 10th WG (and did not meet APS classification criteria) was very low and did not show differences from healthy women.

Statistical analyses could not find any association of higher levels of aPL being more strongly associated to adverse pregnancy outcome.

When analyzing each of the three categories of pregnancy morbidity included in the APS classification criteria only aPS/PT antibodies were statistically significantly associated with either of three types of adverse pregnancy outcome ($P < 0.03$, age adjusted) (Table 4). In fact, these are the only aPL with the significantly higher frequency in patients experiencing recurrent abortion before 10th WG compared to healthy women. On the contrary, anti-β_2GPI antibodies showed no association with any kind of pregnancy morbidity. aCL and LA were associated with obstetric complications appearing late in pregnancy; however they did not show any association with early pregnancy morbidity.

Age-adjusted analyses were performed in order to estimate the relative risk of positive outcome in different aPL tests (LA, aCL, anti-β_2GPI, and aPS/PT) to obstetric complications characteristic for APS presented as OR with 95% confidence interval. As shown in Table 5, only aCL and aPS/PT antibodies presented an elevated risk for obstetric complications (OR 7.4 [95% CI 1.6–34.5] and OR 7.4 [95% CI 1.5–35.2], resp.).

In our group of 169 patients, 12 (6%) had a history of thrombosis and the prevalence of all tested aPL was higher among them as compared to healthy controls ($P < 0.01$). Excluding these 12 patients from logistic regression analyses showed that frequencies of aCL and aPS/PT antibodies were still significantly higher as compared to healthy controls ($P = 0.04$ and $P = 0.015$, resp.).

4. Discussion

While several studies evaluated aPL positivity in APS patients with a history of thrombosis only a few studies established an association of different aPL with individual obstetric abnormalities distinctive for APS. The question arises whether the same profile of aPL occurs in APS patients with a history of thrombosis compared to obstetric APS. There is general consensus to screen for LA, aCL, and anti-β_2GPI, but the role of other autoantibodies remains controversial. Therefore, the

Table 4: Diagnostic accuracy of aPL for different adverse pregnancy outcomes.

	≥3 consecutive miscarriages <10th WG			Fetal death >10th WG			Premature birth <34th WG		
	AUC	OR [95% CI]	*P*	AUC	OR [95% CI]	*P*	AUC	OR [95% CI]	*P*
LA	0.508	1.0 [1.0-1.1]	ns	0.563	1.1 [1.1-1.2]	0.001	/	/	/
aCL	0.516	2.8 [0.5–16.0]	ns	0.572	8.3 [1.8–37.6]	0.002	0.562	9.4 [1.8–49.6]	0.002
IgG	0.512	4.2 [0.4–41.6]	ns	0.558	12.6 [1.6–100]	0.003	0.568	19.1 [2.2–165]	<0.001
IgM	0.503	1.4 [0.1–22.2]	ns	0.52	4.2 [0.5–38.3]	ns	0.494	0.7 [0.7-0.8]	ns
anti-β_2GPI	0.501	0.9 [0.2–3.3]	ns	0.497	0.8 [0.3–2.8]	ns	0.465	0.8 [0.2–4.5]	ns
IgG	0.492	0.7 [0.2–2.8]	ns	0.491	1.1 [0.4–3.6]	ns	0.465	0.9 [0.2–4.6]	ns
IgM	0.509	0.4 [0.4-0.5]	ns	0.513	0.5 [0.4–0.6]	ns	/	/	/
aPS/PT	0.534	5.3 [1.1–26.2]	0.026	0.559	6.8 [1.5–31.4]	0.005	0.544	7.5 [1.4–40.8]	0.008
IgG	0.531	9.0 [1.1–76.3]	0.017	0.533	7.5 [0.9–62.6]	0.030	0.550	15.2 [1.7–135]	0.002
IgM	0.512	4.3 [0.4–42.3]	ns	0.533	7.6 [0.9–63.3]	0.029	0.513	8.6 [0.9–85.9]	0.030

AUC: area under the curve, aCL: anticardiolipin antibody, anti-β_2GPI: antibodies against β_2-glycoprotein I, aPS/PT: anti-phosphatidylserine/prothrombin antibodies, CI: confidence interval, LA: lupus anticoagulant, ns: not significant, OR: odds ratio, and WG: week of gestation.

Table 5: Diagnostic accuracy of aPL for pregnancy loss as defined by APS classification criteria.

	Pregnancy loss defined by APS criteria [1]		
	AUC	OR [95 %CI]	*P*
LA	0.541	0.6 [0.6-0.7]	ns
aCL	0.549	7.4 [1.6–34.5]	0.010
IgG	0.541	12.1 [1.5–97.5]	0.019
IgM	0.514	2.8 [0.3–26.9]	ns
anti-β_2GPI	0.499	1.5 [0.5–4.6]	ns
IgG	0.492	1.2 [0.4–3.9]	ns
IgM	0.514	0.7 [0.6-0.7]	ns
aPS/PT	0.549	7.4 [1.5–35.2]	0.012
IgG	0.535	11.0 [1.3–91.5]	0.012
IgM	0.525	9.0 [1.0–78.9]	0.047

AUC: area under the curve, aCL: anticardiolipin antibody, anti-β_2 GPI: antibodies against β_2-glycoprotein I, aPS/PT: anti-phosphatidylse- rine/prothrombin antibodies, CI: confidence interval, LA: lupus anticoagulant, ns: not significant, and OR: odds ratio.

necessity to perform more cohort studies in order to determine the incidence of noncriteria aPL in pregnancy loss was suggested [26]. Since then, our group focused on evaluating the prevalence of aPS/PT antibodies among female patients experiencing different obstetric complications during their pregnancies.

We found an overall prevalence of aPS/PT of 13.0%, aCL of 12.4%, LA, and anti-β_2GPI less than 8.0% in our group of patients with obstetric complications characteristic for APS. Both aPS/PT and aCL were significantly more prevalent in our cohort of patients compared to healthy blood donors. However, aCL correlated only with late pregnancy morbidity and prematurity while aPS/PT were the only antibodies associated with early recurrent pregnancy loss, as well as with late pregnancy morbidity and prematurity. Our findings are in line with Clark et al. [27] who suggested that aCL-associated early recurrent pregnancy loss be withdrawn from the classification criteria due to inconsistent prevalence of aCL in this population and an increasing body of evidence points to the fact that this clinical manifestation of APS is distinct from late loss or early delivery with placental infarction. However, according to our results, determining aPS/PT in association with early recurrent pregnancy loss may be beneficial. We found 7% (11/169) of patients to be aPS/PT positive and negative for all other tested aPL and 63% (7/11) of them experienced early recurrent pregnancy loss. Only one published study also confirmed association of antiprothrombin antibodies to early pregnancy loss; however it differed from the current study in that they measured aPT-A and included patients whose pregnancies ended spontaneously within 20th WG [21]. Considering the Sydney revised laboratory criteria of APS, only 17.8% (30/169) of patients in our study were positive for LA or aCL and/or anti-β_2GPI, but when aPS/PT was evaluated as an additional parameter, 24.8% (41/169) of patients were aPL positive.

We have also determined the frequency of aPL among 42 patients who experienced less than 3 miscarriages before 10th WG and did not meet APS classification criteria. In line with our expectations the prevalence of all aPL in this group was very low (<7%) and was not different from frequencies found in healthy women.

The review of the literature revealed 12 studies dated from 1997 to 2013. More than half of the articles were published before 2006, when new revised classification criteria were accepted. Five studies tested only aPT-A antibodies, which are (according to newer data) less significant for APS [3, 12, 14, 17, 21]. Among five larger studies, recruiting more than 100 patients, three showed significant association of aPS/PT or aPT-A with obstetric complications [3, 18, 21]; one did not find any significant association; however they only measured aPT-A antibodies [14], and one was performed before 2006 and recruited patients with more than two recurrent miscarriages, instead of three [15]. Conflicting data in the literature and the lack of studies investigating the role of antiprothrombin antibodies in obstetric APS led us to study the occurrence of these antibodies in different early

and late adverse pregnancy outcomes. The overall prevalence of aPS/PT antibodies in our study was 13.0%. This number is higher than the ones reported by Andreoli et al. [28] who reviewed 120 studies investigating LA, aCL, and anti-β_2GPI and provided an overall aPL frequency estimated as 6% for pregnancy morbidity. Rare studies investigated clinical significance of antiprothrombin antibodies in relation to adverse obstetric outcome. Akimoto et al. [17] found the mean titer of antiprothrombin-1 antibodies in patients with spontaneous abortions to be greater than in normal pregnant women. However, subsequent studies failed to show any association of antiprothrombin antibodies (aPT or aPS/PT) with early recurrent pregnancy loss [14–16]. Bertolaccini et al. [18] found correlation between aPS/PT and fetal death beyond 10th WG but not to early pregnancy loss or to prematurity. Marozio et al. [3] reported a positive correlation between aPT-A and adverse late pregnancy outcome. Two recent studies [19, 20] found correlation between aPS/PT and obstetric abnormalities; however neither specified the types of pregnancy complications.

Our study did not confirm the association of either anti-β_2GPI or LA with any of the adverse pregnancy outcomes. This is in line with conclusions of the systematic review [29], implying that there is currently insufficient data to establish any significant link between anti-β_2GPI and pregnancy morbidity. Also, in the study by Clark et al. [30], which included 2257 women attending a high-risk pregnancy clinic, less than 1% of patients with early recurrent miscarriage tested positive for LA, while on the other hand patients positive for LA had a significantly more frequent history of thrombosis. Possibly, LA and anti-β_2GPI are far more associated with thrombotic APS than obstetric APS, while aCL and aPS/PT appear to be associated with both types of APS.

The diagnostic accuracy of individual aPL for pregnancy losses defined by APS classification criteria, determined by AUC, was low, ranging from 0.499 for anti-β_2GPI to 0.549 for aCL or aPS/PT (Table 5). In our previous study [20], AUC for different aPL and APS (either thrombotic or obstetric) varied from 0.88 for IgG aCL to 0.55 for IgM anti-β_2GPI, while Otomo et al. determined AUC (for the revised Sydney criteria) to be 0.688 [31]. Certain patients with clinical symptoms significant for APS, fulfilling clinical criteria, are negative for all tested aPL and within this group aPS/PT despite lower AUC could improve the diagnoses of APS increasing sensitivity of the total aPL.

So far, the mechanism by which antiprothrombin antibodies might be involved in morbidity during pregnancy has not been clarified. However, it is very likely that there are more possible pathways involved. It has been suggested that they may lead to pregnancy loss by the promotion of microvascular placental thrombosis, which was supported by histological findings [32]. Placental trophoblast is the main organ in which phosphatidylserine is highly exposed on the outer leaflet of the membrane. During embryonic and placental differentiation a disruption of the lipid asymmetry occurs, leading to exposure of phosphatidylserine on the outer surface [33]. Antiprothrombin antibodies might crosslink prothrombin on the cell surface where they could complicate pregnancy by complement activation or by interfering with signaling which seems to be an essential factor for disease manifestation from the results of the *in vivo* experiments [34]. An increased number of apoptotic events of giant cells in the phosphatidylserine-exposed ectoplacenta were observed, which may lead to insufficient development of the placenta resulting in embryo small for date or fetal loss [35]. On the other hand, it has been observed that, in mice, prothrombin plays an important role in the development of the embryo and that prothrombin deficiency results in embryonic and neonatal lethality [36]. There is no doubt that clarification of the pathways in which antiprothrombin antibodies are involved in the pathogenesis of APS needs to be further investigated, but nevertheless it has been shown that patients treated for APS have good pregnancy outcomes [37]. A review by Marchetti et al. [38] concluded that screening for high-risk APS patients is necessary to improve their pregnancy outcome, and we showed that aPL profile screening including aPS/PT, in addition to LA, aCL, and anti-β_2GPI, could enable better evaluation of high-risk APS patients and possibly predict further pregnancy losses. Similarly, Ulcova-Gallova et al. [39] suggested that determination of aPL only against cardiolipin in patients with reproductive failure is not sufficient for obstetric-gynecology diagnosis; therefore the investigation of aPS/PT in this group of patients could be warranted.

5. Conclusion

aPS/PT are associated with adverse pregnancy outcome irrespective of other antiphospholipid antibodies. Therefore, aPS/PT measurement might improve the evaluation of patients with early recurrent pregnancy loss, undiscovered by other aPL tests. Further studies including a larger number of patients with pregnancy complications and/or reproductive failure and apparently healthy donors are needed to determine the independent effects of various antiphospholipid antibodies, as well as aPS/PT, and confirm their potential use in clinical practice.

Abbreviations

anti-β_2GPI: Antibodies against β_2-glycoprotein I
aCL: Anticardiolipin antibodies
APS: Antiphospholipid syndrome
aPS/PT: Phosphatidylserine dependent antiprothrombin antibodies
aPT: Antibodies against prothrombin alone
AUC: Area under the curve
CI: Confidence interval
LA: Lupus anticoagulant
OR: Odds ratio
PS: Phosphatidylserine
ROC: Receiver operating characteristic.

Conflict of Interests

The authors declare they have no conflict of interests.

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Research Article

Anti-Cyclic Citrullinated Peptide Antibodies and Severity of Interstitial Lung Disease in Women with Rheumatoid Arthritis

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Objective. To evaluate whether serum titers of second-generation anticyclic citrullinated peptide antibodies (anti-CCP2) are associated with the severity and extent of interstitial lung disease in rheumatoid arthritis (RA-ILD). *Methods*. In across-sectional study, 39 RA-ILD patients confirmed by high-resolution computed tomography (HRCT) were compared with 42 RA without lung involvement (RA only). Characteristics related to RA-ILD were assessed in all of the patients and serum anti-CCP2 titers quantified. *Results*. Higher anti-CCP2 titers were found in RA-ILD compared with RA only (medians 77.9 versus 30.2 U/mL, $P < 0.001$). In the logistic regression analysis after adjustment for age, disease duration (DD), smoke exposure, disease activity, functioning, erythrocyte sedimentation rate, and methotrexate (MTX) treatment duration, the characteristics associated with RA-ILD were higher anti-CCP2 titers ($P = 0.003$) and + RF ($P = 0.002$). In multivariate linear regression, the variables associated with severity of ground-glass score were anti-CCP2 titers ($P = 0.02$) and with fibrosis score DD ($P = 0.01$), anti-CCP2 titers ($P < 0.001$), and MTX treatment duration ($P < 0.001$). *Conclusions*. Anti-CCP2 antibodies are markers of severity and extent of RA-ILD in HRCT. Further longitudinal studies are required to identify if higher anti-CCP2 titers are associated with worst prognosis in RA-ILD.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory disease that involves synovial joints and other organs and extra-articular involvements associated with impairment in physical function, higher morbidity, and premature mortality [1, 2].

Interstitial lung disease (ILD) is an infrequent but extremely relevant extra-articular manifestation that decreases the patients' health-related quality of life (QOL) and life expectancy [2]. With the development of more accurate diagnostic methods, ILD has been reported with a prevalence of up to 61% in patients with RA [3]. ILD in RA is associated with around threefold the risk for mortality as compared with RA without this entity [4].

Some hypotheses concerning RA pathogenesis suggest that major susceptibility genes, particularly HLA-DR, shared epitopes that interact with smoking to trigger RA-specific responses to citrullinated proteins, signifying a clear relationship between smoking and the development of the immune response directed against citrullinated peptides [5]. One of the consequences of these reactions is the formation of anticyclic citrullinated peptide antibodies (anti-CCP), which are observed in around 55–69% of patients with RA [6]. These autoantibodies are highly specific markers for RA and are useful for predicting RA development and progression [7, 8], although the association between anti-CCP antibodies and extra-articular manifestations was not conclusive. Currently, there are few studies evaluating the association between anti-CCP autoantibodies and ILD in RA. Inui et al., on evaluating 18 patients with RA associated with ILD, did not find an association between anti-CCP and ILD [9]. On the other hand, Nikiphorou et al. recently demonstrated, in an abstract, their results of a multicenter study in which anti-CCP antibodies were strongly associated with ILD in RA [10]. Recently, Yin et al. identified ILD in 71 from among their 285 patients with RA, observing that positivity for second-generation anti-CCP (anti-CCP2) was associated with an increase in risk of ILD [11]. Kelly et al., from a multicenter study, identified 230 patients with proven ILD in RA. These authors identified that anti-CCP antibody titers comprised the most relevant factor associated with ILD in RA on univariate analysis, and this factor remained associated with ILD in the multivariate approach [12]. Reynisdottir et al., employing a different approach, analyzed the findings of high-resolution computed tomography (HRCT) in 70 patients with early, untreated RA who were positive for anticitrullinated proteins (ACPA-positive) compared with 35 patients with early, untreated ACPA-negative RA [13]. These authors identified that 63% of patients with ACPA-positive RA had abnormalities in HRCT compared with 37% of patients with ACPA-negative RA ($P = 0.02$) [13]. ILD is a major complication in RA, where prognosis is influenced by the presence of pulmonary active disease and severity of the lung involvement. Sathi et al. described that patients with RA with findings of more extensive lung disease on HRCT have a worse prognosis compared with patients with RA with limited ILD [14]. Currently, the extent and severity of ILD on HRCT and not merely the presence of ILD are considered as factors associated with the prognosis, leading to the development of tomographically validated scales to identify the severity of the lung involvement. Nonetheless, although the majority of studies have investigated the relationship of anti-CCP and ILD in RA, these studies have not evaluated whether there is an association of anti-CCP titers with the extent and severity of ILD on HRCT utilizing a validated scale.

To date, there is a lack of information on whether higher titers of these autoantibodies are related to clinical parameters for ILD severity, including the extent of lung damage assessed by validated tomographic scores. Therefore, our aim in this study was to examine the relationship between serum levels of anti-CCP2 and severity of the extent of ILD damage in patients with RA.

2. Materials and Methods

2.1. Study Design: Comparative Cross-Sectional Study

Patients. The study included patients with RA attending an outpatient rheumatology clinic in a secondary-care center (Hospital General Regional-110 of the Mexican Institute for Social Security (IMSS)) located in Guadalajara, JAL, Mexico. Patients were eligible if they met American College of Rheumatology 1987 classification criteria for RA [15] and were 18 years of age or older. Patients were not eligible if they had history of asthma or pulmonary tuberculosis, active respiratory infection, mental or psychiatric disorders, and any overlapped syndrome or if they exhibited an obstructive pattern during spirometry. Patients with criteria of MTX pneumonitis were excluded. From a cohort of 600 patients with RA, we identified 42 patients with RA with ILD data and these were compared with 39 patients with RA only selected consecutively from the same cohort and matched by gender and range of age.

In order to assess the ascertainment presence of ILD in RA, we performed a structured assessment of ILD based on the following strategy.

2.2. Definition and Ascertainment of ILD. Classification criteria for RA-ILD were based on the following three parameters:

(a) clinical symptoms, such as cough, phlegm, wheezing, bilateral inspiratory and expiratory crackles, and breathlessness,

(b) abnormalities in pulmonary function test (PFT) characterized by a decrease in forced vital capacity (FVC) <80% according to the predicted rate,

(c) radiographic evidence of ILD on HRCT, by means of bilateral outlying reticular opacities or honeycombing with or without activity for ground-glass pattern >5%.

Instead, criteria for inclusion of patients with RA without ILD (no RA-ILD) were based on the following three parameters:

(a) absence of clinical symptoms for lung involvement, such as cough, phlegm, wheezing, bilateral inspiratory and expiratory crackles, and breathlessness,

(b) PFT characterized by FVC ≥80% (predicted rate),

(c) no radiographic evidence of ILD on HRCT, by means of bilateral outlying reticular opacities or honeycombing ≤5% without activity for ground-glass pattern.

2.3. Clinical Assessment of Disease Characteristics of RA. A structured questionnaire was applied to patients to evaluate demographical, clinical, and therapeutic variables related with RA. The patients' synovial joints were examined for swelling and tenderness by a trained examiner. Disease activity was assessed employing the disease activity score in 28 joints (DAS28) [16], and functioning was assessed using the validated Spanish version for HAQ-Di [17]. Steinbrocker et al. [18] radiological stage and global functional status were also evaluated.

2.4. Cardiopulmonary Evaluation. Assessment of cardiopulmonary function included the following indices.

(a) The 6-minute walk test (6MWT) [19] was performed according to the American Thoracic Society (ATS) guidelines. The 6MWT measures the distance a patient can walk rapidly on a hard surface in a period of 6 minutes and is thought to reflect well a person's functional activity level for daily physical activities.

(b) The modified Borg scale [20] is a subjective scale that assesses the perception of dyspnea by the patient. This was performed immediately before and after the 6MWT, placing the degree of dyspnea on a scale of 0–100 mm, where 0 is none (no dyspnea) and 100 is the maximal dyspnea observed.

(c) The validated Mexican-Spanish version of the Saint George Respiratory Questionnaire (SGRQ) [21] consists of a self-administered questionnaire for measuring the impairment of patient-perceived health-related QOL in lung diseases in three domains, including symptoms, activity, and impact. Scores can range from 0 (no impairment) to 100 (worst impairment) for each domain; higher scores connote greater distress and, thus, worse health-related QOL. The questionnaire was administered and scored according to the instruction manual prior to the execution of 6MWT and PFT.

2.5. Screening with Pulmonary Function Tests (PFT). A screening spirometry was performed with a SpiroPro, Sensormedics ver. 2.0, according to the recommendations published in 2005 by ATS and European Respiratory Society (ERS) [22]. The spirometries were performed to assess forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), and the FEV_1/FVC ratio. Observed values were expressed as a percentage of the predicted value compared with individuals of similar gender, age, weight, and height. A restrictive pattern was defined as an FVC of <80% of that predicted in the absence of concomitant obstructive abnormality.

2.6. High-Resolution Computed Tomography (HRCT). HRCT was performed using a single tomographer (4th generation equipment, Siemens SOMATOM AR.T. equipment). Following a standardized protocol, the HRCT was performed with the patient in prone position, using sections about 1-2 mm thick (at 10-mm intervals). Images were reconstructed with a high spatial algorithm and filmed using standard lung window settings (WL-700, [WW] 1000–1500 [HU]). HRCT scans were obtained at the suspended end-inspiratory volume with the patient in the supine position, and additional scans were obtained with the patient in the prone position, when necessary, to demonstrate the reversibility of high attenuation in dependent lung.

All images were evaluated independently and in random order by two observers (one experienced thoracic radiologist and an experienced pulmonologist) who were blinded to the clinical and pathological data. The final assessment was achieved by consensus with an adjudicator if there were disagreements in interpretation. Distribution patterns were visually assessed in three defined regions (upper, middle, or lower regions) of both lungs. The upper zone is from the superior aspect of the transverse aortic arch to the lung apices, the middle region from the top of the transverse aortic arch to the inferior pulmonary vein, and the lower zone from the inferior veins to the diaphragm. According to Kazarooni et al. [23], standardized sheet was used to tabulate the presence or absence of two features: (1) ground-glass opacity, defined as an area of increased attenuation, and (2) honeycombing, defined as subpleural clustered cystic air spaces with distinct walls of 3–25 mm in diameter, on a scale of 0–5 in the three lobes of both lungs as follows: 0: no alveolar disease; 1: ground-glass pattern involving <5% of the lobe; 2: involving >25%; 3: involving 25–45%; 4: involving 50–75%; 5: involving >75% of the lobe for the alveolar score and 0: nonfibrosis; 1: septal thickening without honeycombing; 2: honeycombing involving >25% of the lobe; 3: involving 25 to 49%; 4: involving 50–75%; 5: involving >75% of the lobe for the interstitial score. The sum of each pattern derives from the score of the three evaluated sections.

2.7. Anti-CCP and Other Laboratory Measurements. Erythrocyte sedimentation rate (ESR, mm/h) was measured using the Wintrobe method. Fasting sera were stored at −70°C until the determination of anti-CCP2 antibodies by enzyme-linked immunosorbent assay (ELISA), using a commercial sandwich ELISA kit (EUROIMMUN, Medizinische Labordiagnostika, AG, Lubeck, Germany), with cut-off values for seropositivity for anti-CCP2 of >20 U/mL.

2.8. Statistical Analysis. Quantitative variables were expressed as medians and ranges and qualitative variables as numbers and percentages. According to the distribution of anti-CCP2, we used nonparametric statistics. For comparisons of quantitative variables between patients with RA-ILD and RA only, we used Mann-Whitney *U* test, and for comparisons of qualitative variables between these groups, we utilized the chi-square test (or the Fisher exact test, if required). A correlation between anti-CCP2 titers with parameters of physical examination, disease activity indices, FVC, cardiopulmonary assessment, SGRQ domains, and HRCT scores was performed with Spearman's coefficient (*rho*).

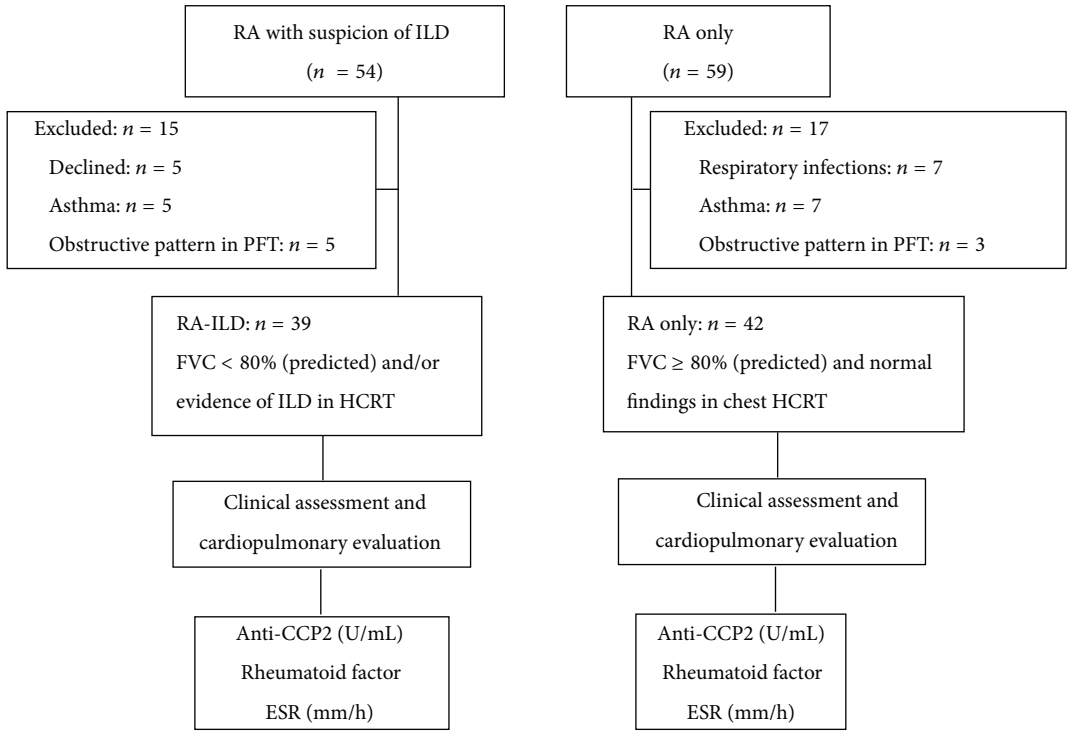

Figure 1: Study flow chart. RA: rheumatoid arthritis; RA-ILD: rheumatoid arthritis with interstitial lung disease; PFT: pulmonary function tests; HRCT: high-resolution computed tomography; FVC: forced vital capacity; anti-CCP: anti-cyclic citrullinated peptide antibodies; ESR: erythrocyte sedimentation rate.

Significance was set at the 0.05 level. We built a multivariate logistic regression model with stepwise selection variables to identify risk factors for interstitial lung disease in RA. Thereafter, we performed a linear regression analysis in order to identify the variables associated with higher ground-glass and interstitial fibrosis scores in the HRCT. Those variables with a P value of <0.20 on univariate analysis or those with biologic plausibility to influence the development of RA-ILD were included in these multivariate models. All of the analyses were performed with SPSS ver. 8.0 statistical software.

2.9. Ethics. The study was approved by the Institutional Review Board of the Mexican Institute for Social Security (IMSS) of the participating hospital (approval number IMSS R-2010-1303-29), with all subjects providing written informed consent.

3. Results

Figure 1 presents the study's flow chart of the patients meeting the inclusion criteria. Of 54 RA patients with a suspicion of ILD, 15 were excluded from the study because they declined to participate ($n = 5$) or had exclusion criteria for the study ($n = 10$), whereas, of 59 RA patients without ILD, 17 were excluded because they met one of the exclusion criteria (see Figure 1).

Table 1 compares the characteristics of patients with RA-ILD ($n = 39$) versus RA only ($n = 42$). Patients with RA-ILD had higher scores for DAS28 (3.9 versus 2.5, $P < 0.001$) and HAQ-Di (0.8 versus 0.4, $P < 0.001$). Higher anti-CCP2 titers were found in patients with RA-ILD compared to RA only (77.9 versus 30.2 U/mL, $P < 0.001$); the frequency of positive rheumatoid factor (RF) was also higher in RA-ILD (97.4% versus 35.7%, $P < 0.001$), and levels of ESR were higher in ILD (32 versus 19.5, $P < 0.001$). A higher frequency of rheumatoid nodules history was found to be associated with the occurrence of RA-ILD (74.4 versus 14.7%, $P < 0.001$). Other findings associated with RA-ILD were higher frequency of higher MTX doses at the time of the study ($P < 0.001$), longer MTX duration ($P = 0.002$), and higher accumulated dose of MTX ($P < 0.001$). No statistical associations were observed between RA-ILD and age, the DD of RA, and smoking history.

Figure 2 illustrates a comparison between anti-CCP2 titers in patients with RA only, compared with RA-ILD. Higher anti-CCP2 titers were observed in RA-ILD (77.9 versus 30.2, $P < 0.001$), whereas none of the patients with RA only had anti-CCP2 titers above 100 U/mL.

Table 2 compares the scores of cardiopulmonary scales and SGRQ between patients with RA only and patients with RA-ILD. Higher scores on the SGRQ and modified Borg scales following exercise were observed in RA-ILD ($P < 0.001$). Patients with RA-ILD had also lower distance in the 6MWT compared with RA only (310.0 versus 410.0, $P < 0.001$).

In data not shown in the tables, a correlation among anti-CCP2 titers was observed with DAS28 (rho = 0.420, $P < 0.001$), HAQ-Di (rho = 0.46, $P < 0.001$), SGRQ symptoms

Table 1: Comparison in selected clinical variables between patients with RA and interstitial lung disease (RA-ILD) and patients with RA without interstitial lung disease (RA only).

Variable	RA patients groups		P
	RA-ILD $n = 39$	RA only $n = 42$	
Age, years	51.0 (36.0–72.0)	49.0 (24.0–73.0)	0.21
Smoking history, n (%)	9 (23.1)	13 (31.0)	0.46
Current smokers, n (%)	1 (2.6)	6 (14.3)	0.11
RA characteristics			
Disease duration, years	7.0 (1.0–35.0)	6.5 (0.75–25.0)	0.26
DAS28 (units)	3.9 (1.7–5.3)	2.5 (1.7–5.1)	<0.001
Inactive (<2.8)	13 (33.3)	29 (69.0)	0.002
Active (≥2.8)	26 (66.7)	13 (31.0)	
HAQ-Di (units)	0.8 (0.2–3.0)	0.4 (0.2–2.4)	<0.001
Impairment in HAQ-Di ≥0.6, n (%)	29 (74.4)	14 (33.3)	<0.001
Global functional status III-IV, n (%)	21 (44.7)	0 (0%)	—
Steinbrocker stage-hands, III or IV, n (%)	6 (12.8)	0 (0%)	—
Rheumatoid nodules history, n (%)	29 (74.4)	5 (14.7)	<0.001
ESR, mm/h	32.0 (14.0–62.0)	19.5 (8.0–45.0)	<0.001
Positive RF, n (%)	38 (97.4)	15 (35.7)	<0.001
Anti-CCP titers	77.9 (25.2–200.0)	30.2 (7.0–70.14)	<0.001
Positive anti-CCP	39 (100)	27 (64.3)	<0.001
DMARDs			
Methotrexate, n (%)	39 (100)	41 (97.6)	1.00
At the time of the study, mg/week	15.0 (10.0–22.5)	7.5 (0–12.5)	<0.001
MTX treatment duration, years	7.0 (2.5–30.0)	5.0 (0.8–13.0)	0.002
MTX accumulated doses, grams	6.8 (3.1–129.6)	1.1 (0–2.1)	<0.001
Azathioprine, n (%)	27 (69.2)	21 (50.0)	0.11
Chloroquine, n (%)	10 (25.6)	19 (45.2)	0.10
Corticosteroids utilization, n (%)	37 (94.9)	37 (88.1)	0.43

Qualitative variables were expressed in frequency (%); quantitative variables were expressed in medians (ranges); RA: rheumatoid arthritis; ILD: interstitial lung disease; ESR: erythrocyte sedimentation rate; VAS: visual analogue scale; HAQ-Di: Health Assessment Questionnaire-Disability Index; RF: rheumatoid factor; anti-CCP2: anti-cyclic citrullinated peptide antibodies (second generation); DMARDs: disease-modifying antirheumatic drugs. P values for comparisons between medians were computed with Mann-Whitney U test and for comparison between proportions were computed with chi-square (or Fisher exact test if applicable).

(rho = 0.547, $P < 0.001$), SGRQ activity (rho = 0.498, $P < 0.001$), SGRQ impact (rho = 0.518, $P < 0.001$), 6MWT (rho = −0.632, $P < 0.001$), pre-6MWT VAS modified Borg scale (rho = 0.637, $P < 0.001$), post-6MWT VAS modified Borg scale (rho = 0.619, $P < 0.001$), MTX treatment duration (rho = 0.293, $P = 0.008$), as well as FVC% (rho = −0.632, $P < 0.001$), and all the HRCT scores: ground-glass (rho = 0.566, $P < 0.001$) and interstitial fibrosis (rho = 0.70, $P < 0.001$).

In data that are not shown in tables, we performed a multivariable logistic regression analysis to identify variables associated with restrictive pattern in lung function tests. In the final model, the higher anti-CCP2 antibodies titers (OR 1.08 95% IC 1.02–1.14, $P = 0.004$) were associated with restrictive pattern in FVC, whereas factors that did not have statistical significance with FVC% were age, disease duration, RF, and years of treatment with MTX.

Table 3 shows the results of the multivariate logistic regression analysis to identify associated variables with RA-ILD. In the final model, after the adjustment for age, disease duration, smoke exposure, DAS28, HAQ-Di, ESR, and MTX treatment duration, two variables were associated with an increase of risk for RA-ILD: the higher anti-CCP2 antibodies titers (OR, 1.06; 95% CI, 1.02–1.10, $P = 0.003$) and positive RF (OR, 28.58; 95% CI 3.31–246.95, $P = 0.002$).

Table 4 presents the results of multiple linear regression analysis evaluating factors associated with higher severity of ILD according to the HCRT scores. After the adjustment for age, disease duration, DAS28, and MTX duration, we observed that the anti-CCP2 titers were significantly associated ($P = 0.02$) with higher severity of the extension in the ground-glass score. Similarly, after adjustment for age, DAS28 for the higher fibrosis score was significantly associated with higher disease duration ($P = 0.01$), anti-CCP2 titers ($P < 0.001$), and duration of treatment with MTX ($P < 0.001$).

4. Discussion

In the present study, anti-CCP2 titers were associated with the presence and severity of ILD in RA. These anti-CCP2 titers were correlated with impairment in several parameters

Table 2: Comparison of cardiopulmonary scales and Saint George Respiratory Questionnaire between patients with rheumatoid arthritis and interstitial lung disease (RA-ILD) and patients with RA only.

Variable	RA-ILD $n = 39$	RA only $n = 42$	P
Pulmonary symptoms			
Cough, n (%)*	31 (66.0)	0 (0%)	—
Phlegm, n (%)*	10 (21.3)	0 (0%)	—
Wheezing, n (%)*	3 (6.4)	0 (0%)	—
Bilateral inspiratory/expiratory crackles, n (%)*	33 (70.2)	0 (0%)	—
Breathlessness, n (%)*	19 (40.4)	0 (0%)	—
Cardiopulmonary scales			
6MWT, meters	310.0 (170.0–549.0)	410.0 (270.0–549.0)	<0.001
Pre-6MWT VAS modified Borg scale	1.0 (0–3.1)	0 (0–2.0)	—
Post-6MWT VAS modified Borg scale	2.0 (0.5–8.0)	1.0 (0–5.0)	<0.001
Development of dyspnea, n (%)	19 (23.8)	7 (16.7)	0.002
SGRQ, %			
Symptoms	14.0 (0–30.0)	3.0 (0–24.0)	<0.001
Activity	10.0 (0–38.0)	4.0 (0–27.0)	<0.001
Impact	10.0 (0–38.0)	3.5 (0–13.0)	<0.001
Total	13.0 (0–37.0)	5.0 (0–25.0)	<0.001
Lung function			
FVC (% of predicted)	71.0 (52.0–91.0)	86.0 (80.0–99.0)	<0.001
Restrictive patterns, n (%)*	32 (68.1)	0 (0%)	—

Qualitative variables were expressed in frequency (%); quantitative variables were expressed in medians (ranges); FVC: forced vital capacity; 6MWT: six-minute walk test; VAS: visual analogue scale; SGRQ: Saint George Respiratory Questionnaire. *This variable is not accepted for evaluation by the program. P values were computed as follows: for quantitative variables with Mann-Whitney U test and for qualitative variables with chi-square (or Fisher exact test if required).

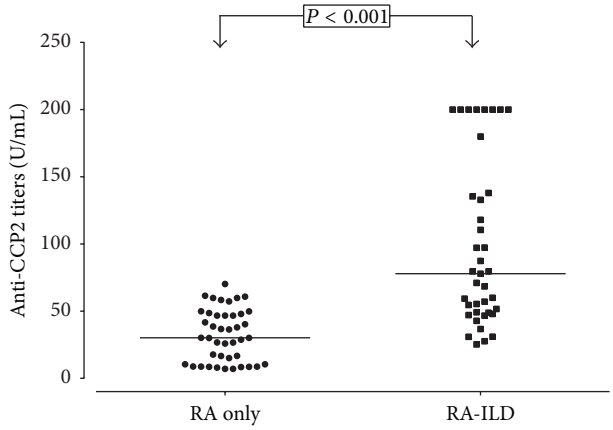

Figure 2: Anti-cyclic citrullinated peptide (anti-CCP2) titers in patients with rheumatoid arthritis without interstitial lung disease (RA-only), compared with patients with rheumatoid arthritis and interstitial lung disease (RA-ILD) group. The cut-off value of anti-CCP2 for positivity was 20 U/mL. Horizontal bars indicate the median. P values for the comparison of anti-CCP2 titers between groups were obtained by Mann-Whitney U test.

for ILD severity, including the SGRQ, 6MWT, Borg scales, decrease in FVC%, and higher scores for ILD involvement and severity identified in the HRCT severity. An association between ILD and anti-CCP2 titers remained after adjustment for age, disease duration, and exposure to smoke, in the multivariate model and duration of treatment with MTX, whereas positive RF was also a factor associated with ILD. Additionally, we observed that the only factors that predicted in the multivariate linear regression the higher scores for fibrosis score were anti-CCP2 titers and longer MTX treatment duration; instead, higher fibrosis scores were inversely associated with disease duration.

Our results, regarding the association between anti-CCP2 and ILD, are similar to the findings by Nikiphorou et al. [10], who observed that anti-CCP2 titers are significantly higher in patients with RA who had ILD. On the other side, Inui et al. [9] did not observe an association between presence or levels of anti-CCP2 and ILD. One possible explanation for these differences between studies was that Inui et al. included only 18 patients with ILD associated with RA, and, therefore, because of the small sample evaluated, it is likely that this lack of differences can be explained by a type II error.

Recently, Yin et al. identified ILD in 71 from their 285 patients with RA, observing that positivity for second-generation anti-CCP (anti-CCP2) was associated with an increase in risk of ILD [11]. Kelly et al., from a multicenter study, identified 230 patients with proven ILD in RA. These authors identified that anti-CCP antibody titers were comprised of the most relevant factor associated with ILD in RA on univariate analysis and this factor remained associated with ILD in the multivariate approach [12]. Reynisdottir et al., using a different approach, analyzed the findings of HRCT in 70 patients with early untreated RA who were positive for anticitrullinated proteins (ACPA-positive) compared with 35 ACPA-negative RA [13]. These authors identified that 63% of ACPA-positive patients had abnormalities on HRCT,

Table 3: Logistic regression analysis performed to assess the risk factors associated with the RA-ILD.

Criterion predictor	Method *Enter*			Method *Forward Stepwise*		
	OR	95% CI	*P*	OR	95% CI	*P*
Age, years	1.01	0.89–1.15	0.86	Not in the model	—	—
Disease duration >5 years	10.79	0.68–170.99	0.09	Not in the model	—	—
Smoke exposure	1.19	0.20–7.13	0.84	Not in the model	—	—
DAS28	0.29	0.03–1.48	0.12	Not in the model	—	—
HAQ-Di	1.17	0.12–11.16	0.89	Not in the model	—	—
ESR, mm/h	1.18	0.98–1.42	0.08	Not in the model		
Anti-CCP2 titers	1.05	1.01–1.10	0.01	1.06	1.02–1.10	0.003
+Rheumatoid factor	26.84	2.31–311.58	0.009	28.58	3.31–246.95	0.002
MTX treatment duration	1.60	1.00–2.56	0.05	Not in the model	—	—

DAS28: disease activity score; HAQ-Di: Health Assessment Questionnaire-Disability Index; ESR: erythrocyte sedimentation rate; MTX: methotrexate; anti-CCP2 titers: anti-cyclic citrullinated peptide antibodies titers (second generation); OR: odds ratios; 95% CI: 95% confidence interval. Variables were adjusted using logistic regression analysis. Dependent variable: presence or absence of interstitial lung disease. Covariates: age (quantitative), disease duration >5 years (qualitative), smoke exposure (qualitative), DAS28 (quantitative), HAQ-Di (quantitative), anti-CCP2 titers (quantitative), +rheumatoid factor (qualitative), and MTX duration in treatment (quantitative).

Table 4: Multiple linear regression analysis assessing the association of anti-CCP2 titers with the ground-glass and fibrosis scores observed in HRCT adjusting by selected variables.

Independent variables	HRCT			
	Enter		*Forward Stepwise*	
	β	*P* value	β	*P* value
Ground-glass score				
Age, years	0.026	0.85	—	Not in the model
Disease duration, years	0.068	0.90	—	Not in the model
DAS28	0.851	0.53	—	Not in the model
Anti-CCP2 titers	0.048	0.03	0.053	0.02
MTX treatment duration, years	−0.299	0.85	—	Not in the model
Fibrosis score				
Age, years	−0.069	0.19	—	Not in the model
Disease duration, years	−0.510	0.01	−0.506	0.01
DAS28	0.430	0.41	—	Not in the model
Anti-CCP2 titers	0.065	<0.001	0.070	<0.001
MTX treatment duration, years	0.879	<0.001	1.035	<0001

Anti-CCP2 titers: anti-cyclic citrullinated peptide antibodies titers (second generation); DAS28: disease activity score; HAQ-Di: Health Assessment Questionnaire; MTX: methotrexate. Dependent variables: first model: ground-glass score, second model: fibrosis score. Covariates included in this analysis were those quantitative variables that had statistical significance in the univariate analysis or were considered with biologic plausibility to explain the severity of ILD in HRCT.

compared with 37% of ACPA-negative patients ($P = 0.02$) [13]. To date, to the best of our knowledge, there are no studies evaluating if the anti-CCP2 titers are associated with the extent and patterns of severity of lung involvement in ILD-RA. We reported that high titers of these autoantibodies are associated with a higher extent of lung involvement in our patients even after adjustment for other variables.

Reynisdottir et al. [13] observed increased staining for citrullinated proteins on bronchial biopsies obtained from patients with RA and positive anti-CCP. Rangel-Moreno et al. reported [24] higher levels of anti-CCP in serum and bronchoalveolar lavages in patients with RA, and these antibodies are increased in the patients with RA who had well-developed inducible bronchus-associated lymphoid tissue, suggesting that these antibodies are produced locally in the lungs. Citrullinated proteins in the lungs are currently considered as autoantigens that may trigger an immune response associated with the development of anti-CCP2 and other antibodies that may act as markers associated with the tissue damage [25]. In addition to anti-CCP2 levels, RF and elevated ESR were biomarkers associated with ILD in RA on univariate analysis. An association between positive RF and ILD has been previously identified by several studies [11–13]; our findings are consistent with their results, whereas our finding of an elevated ESR in patients with ILD in RA is inconsistent with the majority of the reported data. Yin et al. [11] did not observe differences in ESR between the group with ILD and the group without ILD in their study. On the other hand, Inui et al. [9] observed a nonsignificant trend for elevated ESR in patients with RA-ILD.

Instead, the association observed in the present study between MTX treatment and ILD is consistent with data reported in the literature. Roubille and Haraoui [26] examined evidence regarding the association between ILD and synthetic or biological DMARDs; these authors concluded, in their systematic review, that the incidence of MTX-associated pneumonitis has been estimated as ranging from 0.3 to 8% of patients with rheumatic disorders. Conway et al., in a meta-analysis, identified an increase in the risk of pneumonitis in patients receiving MTX [27]. Although in our study nearly all of the patients with RA received MTX at the time of the study, we were unable to identify if the patients not treated with this drug had lower risk for ILD.

Related to the association observed between anti-CCP2 and RA-ILD, ACPAs are specific for RA and correspond to a subset of RA that is distinct from RA ACPA-negative in terms of pathogenesis, disease prognosis, and response to therapy. This information about ACPAs suggests that the presence of autoimmunity to citrullinated peptides and the developmental may be initiated within the respiratory system. Not only are citrullinated proteins limited to synovial tissue, but they have also been identified at extra-articular sites in patients with RA. Bongartz et al. [28] found that citrullination is developed inside mononuclear cells in lung tissue in open-lung biopsy specimens from patients with RA-associated interstitial pneumonia. These authors also observed that, despite the high specificity of anti-CCP for RA, citrullination was also found in lung tissue from patients with idiopathic interstitial pneumonia. Posttranslational modification of citrullination is developed in an environment of inflammation. This protein citrullination is a phenomenon that is produced early in the disease course and that might be involved in the development of the disease. Zhu et al., in a meta-analysis, observed that ACPA-positive serum indicated a higher risk for ILD and interstitial pulmonary fibrosis (IPF) among patients with RA (OR, 4.679, 95% CI 2.071–10.572, $P < 0.001$) [29]. Giles et al. observed high serum ACPA titer associated with RA-ILD, after adjustment for confounding factors (age, sex, current or former smoking, and FR) [30].

A diagnosis of ILD in RA identifies a patient with higher risk for the worst prognosis considering that the median survival in RA-ILD is around 10 years shorter than that observed in the general population, lung disease being directly responsible of around 10 to 20% of all RA-associated mortalities [31]. Kelly et al. [12] identified that the subtype of usual interstitial pneumonia/overlap syndromes has around 3.9-fold risk for death in comparison with the subtype of nonspecific interstitial pneumonia/cryptogenic organizing pneumonia. An extensive disease had around 2-fold increase in the risk for death from any cause versus those limited diseases. Assayag et al. [31] identified in a systematic review that the extent of fibrosis and usual interstitial pneumonia is a predictor of mortality in RA-ILD.

Several studies observed that the positivity of anti-CCP2 and RF is associated with RA-ILD. Our study also observed in the multivariate logistic regression analysis that anti-CCP2 and RF were associated with RA-ILD. Our data are in agreement with the most recent studies. Yin et al. observed that positive rates of anti-CCP2 and RF in patients with RA-ILD were significantly higher than those of the patients with RA only [11]. However, Reynisdottir et al. found no significant difference in RF positivity in RA-ILD [13].

In our results, we are surprised that a shorter duration of disease was associated with the fibrosis score in the tomographic findings; several studies have associated the presence of ACPA-positive with the presence of pulmonary damage, mainly interstitial pulmonary and fibrosis pulmonary pattern; however, to our knowledge, no study has linked shorter duration of disease with RA-ILD and positive ACPA. Follow-up studies are required especially in patients with early RA, in which lung function comprises the value.

Our study possesses several limitations. This a cross-sectional design; therefore, it is unable to demonstrate the causality of any variable for the development of ILD in RA; however, our findings of the association between anti-CCP2 titers and the presence and severity of this complication are relevant for further studies in experimental models or longitudinal studies. In addition, none of our patients had a pulmonary biopsy. Thus, we have no information concerning the histological pattern exhibited by patients with ILD, and it would be interesting to evaluate whether these high anti-CCP2 titers may correlate with the histologic patterns in lung tissue involvement. On the other hand, to the best of our knowledge, there are no previous studies assessing whether the severity of ILD in RA is associated with higher titers of anti-CCP2. These findings of higher anti-CCP2 titers and ILD severity are not only limited to the HCRT score; they are also associated with other characteristics of impairment in ILD, such as decreased 6MWT, increases in the score for symptoms or impact in SGRQ, and decreases in FVC%. Another strength of the study was the utilization of a multivariate model to adjust the association of anti-CCP2 antibodies with ILD by other confounders; only two studies have previously used this statistical approach [10, 13], obtaining similar results to those observed in our study. In this respect, after adjustment for different factors, we observed that the relationship between the titers of these autoantibodies and the severity of ILD remained significant on multivariate analysis.

In conclusion, we identified that anti-CCP2 titers constitute an independent factor associated not only with the presence but also with the severity of RA-ILD; the relevance of these markers in patients with established ILD for future outcomes, such as progression of lung involvement and mortality, remains to be established.

Disclosure

Partial results of this work were presented at the 77th American College of Rheumatology Annual Meeting and these results were published as an Abstract of this Meeting in Arthritis Rheum.2013; S175:S176 (Abstract Suppl).

Conflict of Interests

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

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Research Article

Induction of Apoptosis Coupled to Endoplasmic Reticulum Stress through Regulation of CHOP and JNK in Bone Marrow Mesenchymal Stem Cells from Patients with Systemic Lupus Erythematosus

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Previous studies indicated that bone marrow mesenchymal stem cells (BM-MSCs) from patients with systemic lupus erythematosus (SLE) exhibited the phenomenon of apoptosis. In this study, we aimed to investigate whether apoptosis of BM-MSCs from SLE patients were dysregulated. In this paper, endoplasmic reticulum stress (ERS) was evidenced by increased expression of phosphorylated protein kinase RNA-like ER kinase (PERK) and inositol-requiring protein-1 (IRE-1). We also found the activation of downstream target eukaryotic translation initiator factor 2α (eIF 2α) and CCAAT/enhancer-binding protein- (C/EBP-) homologous protein (CHOP) in BM-MSCs from SLE patients. Interestingly, we discovered that 4-phenylbutyric acid (4-PBA), a selective inhibitor of ERS, blocked the apoptosis of BM-MSCs from SLE patients and alleviated the level of Jun N-terminal kinase1/2 (JNK1/2) and CHOP. Furthermore, blockage of PERK signaling expression by siRNA not only significantly reduced the expression of CHOP, but also activated the anti-apoptotic regulator B-cell lymphoma-2 (Bcl-2). Blockage of IRE-1 or JNK1/2 by siRNA resulted in the decreased expression of JNK1/2 and proapoptosis protein Bcl-2 associated protein X (BAX). These results implicated that ERS-mediated apoptosis was a critical determinant of BM-MSCs from SLE patients.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune inflammatory disease characterized by multiorgan involvements and various clinical manifestations [1]. Current treatments have achieved successes, but some patients with refractory SLE are still suffering from poor prognosis. Allogenic mesenchymal stem cells transplantation (MSCT) has shown to be a powerful strategy in the treatment of refractory SLE patients [2, 3]. However, syngeneic bone marrow MSC transplantation (BM-MSCT) was ineffective [4, 5]. These studies suggested that bone marrow mesenchymal stem cells (BM-MSCs) might be somewhat functionally deficient and participated in the pathological process of SLE. Previous studies found that hematopoietic stem cells (HSC) from SLE patients were prone to apoptosis with defects in their function [6]. The paper of Li et al. showed that there were increased frequencies of apoptosis and aging in BM-MSCs from SLE patients in comparison with control groups [7]. Our data revealed that BM-MSCs from SLE patients exhibited some abnormalities of the cytoskeleton and ultrastructure [8–11]. All these studies suggested that researches in the apoptosis of BM-MSCs from SLE had a profound and historical significance in the evolution process of the disease. In this study, we aimed to discover the molecular mechanism contributing to the pathogenesis of the disease.

Protein folding in the endoplasmic reticulum (ER) is impaired under various physical and pathological conditions,

termed endoplasmic reticulum stress (ERS). ERS is buffered by the activation of the unfolded protein response (UPR), a homeostatic signaling network that orchestrates the recovery of ER function, and failure to adapt to ERS results in apoptosis [12, 13]. The UPR signals through three distinct stress sensors located at the ER membrane: protein kinase RNA-like ER kinase (PERK), inositol-requiring protein-1 (IRE-1), and activating transcription factor-6 (ATF-6) [14]. Among them, PERK, whose intrinsic kinase activity is induced by oligomerization, regulates the phosphorylation of the eukaryotic translation initiation factor 2α (eIF 2α), which induces the suppression of global mRNA translation to protect cells against ERS [15]. Previous studies showed that the transcription factor CCAAT/enhancer-binding protein-(C/EBP-) homologous protein (CHOP) operates at the ERS pathways to introduce the apoptosis of cells [16, 17]. Over expression of CHOP plays an important role in apoptosis [18, 19]. IRE-1-mediated activation of Jun N-terminal kinase (JNK) contributes to cell death [20]. These results suggested that the induction of ERS was closely related with apoptosis. However, it was still not confirmed whether ERS was involved in the pathological process of BM-MSCs from SLE patients.

In the present paper, we observed dilated, distorted, and swollen ER in apoptosis BM-MSCs from SLE patients and upregulated expression of phosphorylated IRE-1 and PERK. We then found that, in BM-MSCs from SLE patients, 4-phenylbutyric acid (4-PBA), the inhibitor of ERS [21], could partly reduce the apoptosis situation. Further, ERS suppressed the expression of B-cell lymphoma 2 (Bcl-2) via introduction of CHOP and led to the activation of Bcl-2 associated protein X (BAX) through activation of JNK, which contributed to ERS-induced apoptosis in BM-MSCs from SLE patients.

2. Methods

2.1. Recruitment of SLE Patients. Twelve SLE patients between 16 and 43 years of age (mean 27.3 ± 7.81 years) were enrolled in this study (Table 1). Our previous study had indicated that the BM-MSCs from treated- and untreated-SLE patients were senescent [8], so we did not group in this study. The SLE diagnosis was made based on the criteria proposed by the American College of Rheumatology [22]. The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) was used to measure the disease activity. All patients were categorized as active using a cutoff SLEDAI score of eight. Ten healthy subjects were used as the normal group (Table 1). All research subjects were females with a similar age distributions (mean 26.8 ± 7.45 years). All participators gave consent to the study, which was approved by the Ethics Committee of the Affiliated Hospital of Nantong University.

2.2. Isolation, Cell Culture, and Identification of BM-MSCs from SLE and Normal Subjects. BM-MSCs were generated from bone marrow aspirate according to a technique described previously [7]. Five milliliters of heparinized BM were 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 ×g for 25 min at room temperature. The mononuclear cells were collected at the interface and resuspended in low-glucose Dulbecco-Modified Eagle Medium (L-DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS). Then, the cells were plated at a density of 2×10^7 cells per 25 cm^2 dish and cultured at 37°C in a 5% CO_2 incubator. The medium was replaced, and nonadherent cells were removed after 5 days and every 3 days thereafter. When the BM-MSCs became nearly confluent, the adherent cells were released from the dishes with 0.25% trypsin–EDTA (Gibco, USA) and were then replated 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. After 3 passages (p3), cells were used for the following

Table 1: Details of 10 controls and 12 SLE patients.

Control/ patient	Age	Disease duration	Current treated	SLEDAI
P1	16	1 year	Pred 20 mg/day HCQ 0.2/day	11
P2	24	8 months	Pred 15 mg/day HCQ 0.2/day LEF 0.2/day	14
P3	19	2 years	Pred 15 mg/day HCQ 0.2/day LEF 0.2/day	9
P4	37	1 year	Pred 17.5 mg/day HCQ 0.2/day	13
P5	22	16 months	Pred 12.5 mg/day HCQ 0.2/day CTX 0.4/2 weeks	16
P6	32	2 years	None	16
P7	33	3 years	Pred 15 mg/day HCQ 0.2/day	11
P8	41	5 years	Pred 7.5 mg/day HCQ 0.2/day	8
P9	18	3 days	None	9
P10	27	2 days	Pred 12.5 mg/day HCQ 0.2/day	12
P11	28	2 days	None	14
P12	31	4 days	None	21
C1	18			
C2	31			
C3	21			
C4	25			
C5	24			
C6	22			
C7	37			
C8	43			
C9	25			
C10	22			

Pred: prednisolone; HCQ: hydroxychloroquine.
LEF: leflunomide; CTX: cyclophosphamide.

studies. After 4 passages (p4), the cells were treated with or without 4-phenylbutyric acid (4-PBA) (P21005) (100 μM) for 24 hours. Transfection of cells with various mammalian expression conducted by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was in accordance with the methods provided by manufacturer's specification.

2.3. FCM Analysis for Cell Apoptosis. The Annexin V-FITC/PI Apoptosis Detection Kit (Becton Dickinson, USA) was employed to evaluate the apoptosis of P4 BM-MSCs from 12 SLE patients and 12 normal controls by using FCM analysis. After being washed with iced cold PBS, 1×10^5cells were resuspended in 100 μL of binding buffer. Five microliter Annexin V and 5 μL propidium iodide (PI) were added to the cells. After incubation for 15 min (25°C) in the dark, 400 μL of 1×binding buffer was added to each tube and FCM analysis was performed immediately. Data acquisition and analysis were assayed by BD LSRII analyzer (Becton Dickinson, USA) using "CellQuest" software. Annexin V (+) and PI (−)/PI (+) cells were considered as apoptotic cells.

2.4. Transmission Electron Microscopic Examination. Electron microscopy was performed by using the methods described as previous [23]. Briefly, BM-MSCs were washed twice with PBS and fixed in 4% glutaraldehyde. They were then postfixed with 1% OsO4, dehydrated stepwise in increasing concentrations of ethanol, and embedded in Epon 812 epoxy resin. Ultrathin sections were then localized and viewed with a transmission electron microscope (JEM-1230, JEOL Ltd., Japan).

2.5. Western Blot Analysis. After being subjected to the indicated treatments, approximate 5×10^6 BM-MSCs were washed with cold PBS three times and lysed with cell lysis solution. Sample buffer was added to cytosolic extracts, and after boiling for 10 min, equal amounts of supernatant from each sample were fractionated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electro transferred to polyvinylidenedifluoride (PVDF) membranes (Millipore, USA). The membranes were blocked with 5% skim milk for 2 h at room temperature. Then the membranes were incubated with antibody for p-PERK, PERK, p-IRE1, IRE1, BAX, Bcl-2 (1 : 800; Cell Signaling), caspase-3, eIF 2α, CHOP, p-JNK1/2, JNK1/2, p-extracellular signal-regulated kinase (ERK), ERK, p-p38, p38 (1 : 800; Santa Cruz) at 4°C overnight. At last, immunoreactive bands were then detected by incubation with conjugates of anti-rabbit IgG with horseradish peroxidase (1 : 2,000; Southern-Biotech) for 2 h and visualized by using enhanced chemiluminescence system (ECL; Pierce Company, USA).

2.6. Immunofluorescent Staining. BM-MSCs grown on coverslips were fixed with 4% paraformaldehyde at 4°C for 40 min, followed by several rinses in phosphate-buffered saline (PBS). Nonspecific binding sites were blocked at room temperature for 2 h with 5% normal horse serum (Sigma-Aldrich, St. Louis, MO, USA) or normal donkey serum (Sigma-Aldrich, St. Louis, MO, USA) diluted in 0.1% Triton X-100-PBS. BM-MSCs were then incubated overnight at 4°C with primary antibodies: CHOP (Cell signaling, MA, USA), JNK1/2 and caspase-3 (green; Santa Cruz, CA, USA) at 1 : 300 dilution with blocking buffer, followed by a mixture of fluorescein isothiocyanate and tetramethylrhodamineisothiocyanate-conjugated secondary antibodies (BD Biosciences) for 2 h at 4°C. BM-MSCs were finally incubated with DAPI (blue; Sigma, MO, USA), which was used to stain the nucleus for 30 min at 37°C. The slides were visualized by using a fluorescent microscope (Leica, Microsystems, Germany). All photomicrographs shown in this study were representative of multiple experiments.

2.7. Transfection with siRNA. CHOP and IRE-1 siRNA were designed by siGENOME ON-TARGET plus SMARTpool siRNA purchased from Dhamarcon RNAi Technologies. CHOP (DDIT3) target sequences are GGUAUGAGGACCUGCAAGA, CACCAAGCAUGAACAAUUG, GGAAACAGAGUGGUCAUUC, CAGCUGAGUCAUUGCCUUU. IRE-1 (ERN1) target sequences are CUACCCAAACAUCGGGAAA, CUCCAGAGAUGCUGAGCGA, AUAAUGAAGGCCUGACGAA, GUCCAGCUGUUGCGAGAAU. Nontargeting control sequences were not provided. JNK1/2 and PERK siRNA (1, # 6232, 2, # 6233) was purchased from Cell Signaling Technology, Inc. (Danvers, MA) and sequences were not provided. Cells at 50 to 60% confluence were transfect with siRNA (20–50 nM) using the RNAifect Transfection Regaent (QIAGEN) according to the manufacturer's protocol. Cells were cultured for 48 h, and then treated with vehicle for an additional 48 h. Proteins were then isolated for western blotting.

2.8. Statistical Analysis. Statistical analysis was performed by one-way ANOVA. The intergroup comparisons (post-hoc analysis) among the data with equal variances were performed with the least significant difference (LSD) method, while Tamhane's T2 method was used for the data with unequal variances. $P < 0.05$ was considered to be significant.

3. Results

3.1. Apoptosis of BMSCs from SLE Patients. The primary culture of BM-MSCs was successful in 12 cases of SLE patients and 10 cases of healthy donors. We sought to assess apoptotic cells using FCM analysis by Annexin V-FITC/PI staining at the fourth passage. A significant increase was discovered in Annexin V-positive cells among BM-MSCs from SLE patients (32.3 ± 12.0%) compared to normal controls (4.1 ± 3.7%) ($P < 0.05$) (Figure 1(a)). To investigate the functions of intrinsic pathway during the apoptosis of BM-MSCs from SLE patients, the expressions of Bcl-2 and BAX were measured by western blot. Results revealed that the expression of Bcl-2 significantly decreased in BM-MSCs from SLE patients compared to normal controls. The level of BAX and cleaved caspase-3 significantly increased in BM-MSCs from SLE patients compared to normal controls (Figure 1(b)). We also discovered that the immunoreactivity of caspase-3 was enhanced in BM-MSCs from SLE patients (Figure 1(c)). All these results showed that BM-MSCs were apoptotic in SLE patients group.

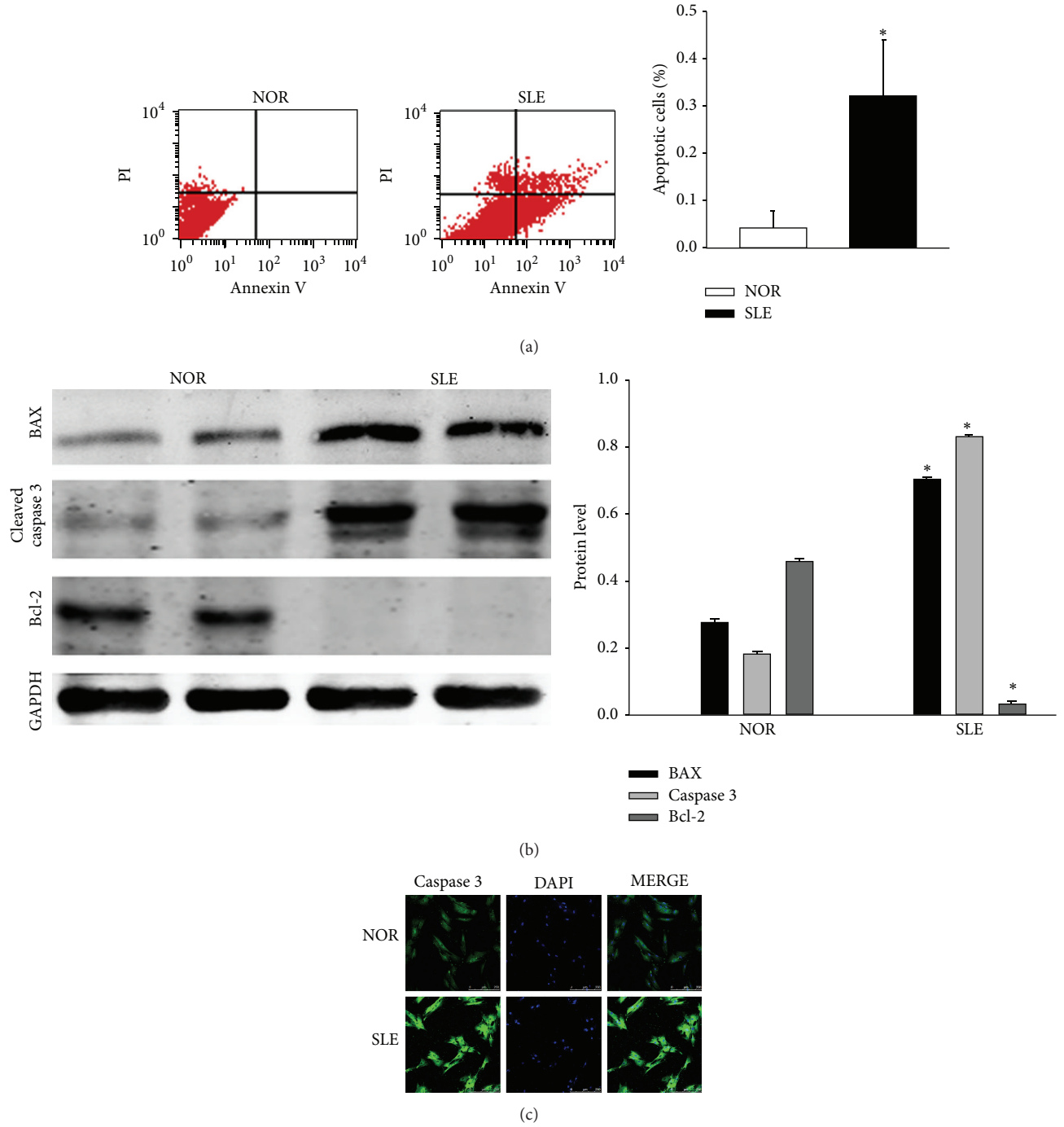


Figure 1: Apoptosis of BMSCs from SLE patients. (a) BM-MSCs at passage 4 were isolated and harvested from SLE patients and normal controls, and analyzed by Annexin V-FITC/PI staining. Annexin V-positive cells were 4.1 ± 3.7% in normal controls, 32.3 ± 12.0% in SLE patients ($P < 0.05$). (b) Western blot analysis of BAX, active caspase-3 and Bcl-2 was performed in BM-MSCs from normal controls and SLE patients. (c) BM-MSCs from normal controls and SLE patients were fixed and stained with antiactive caspase-3. FITC-labeled secondary antibody was used (green fluorescence) Nuclei were stained with DAPI (blue fluorescence). Images were captured by a confocal laser microscope. (Bar represents mean ± SD, $^*P < 0.05$ compared with the normal group).

3.2. ERS Was Involved with BM-MSCs from SLE Patients. Electron microscopy was used to evaluate the ultrastructural changes of ER in the BM-MSCs. Cells in the control appeared to be normal with relatively healthy-looking ER, mitochondria and nuclei. Dilated and distorted ER, swollen mitochondria, and condensation of chromatin were found in the BM-MSCs from SLE patients; more protein aggregates were seen within the ER lumen than that observed in control groups, indicating severe ERS in BM-MSCs from SLE patients. In addition, we also found a mixed

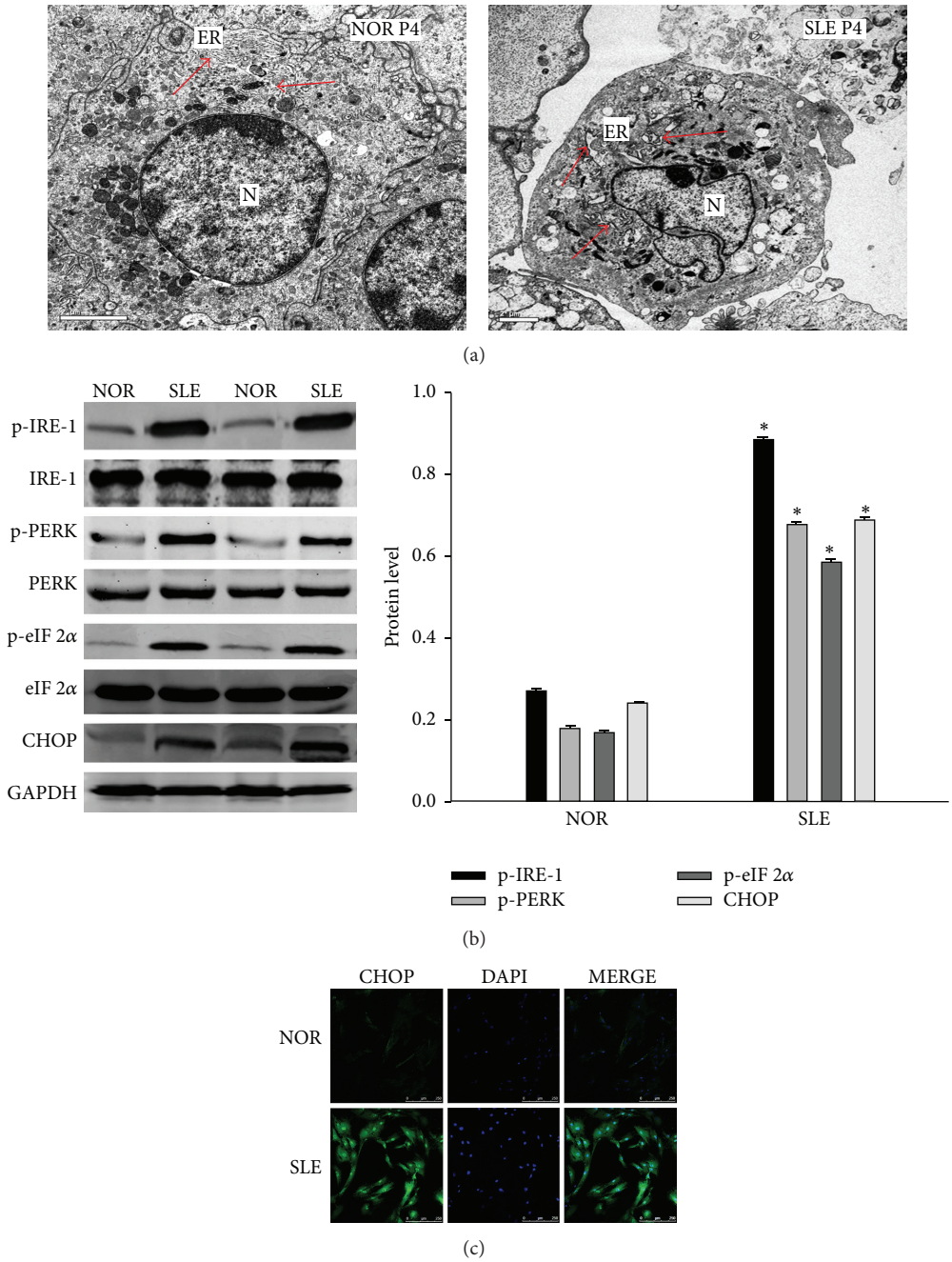


Figure 2: ERS was involved with BM-MSCs from SLE patients. (a) ER ultrastructural changes in the BM-MSCs from SLE patients and normal controls. The BM-MSCs were processed and examined under a transmission electron microscope. Compared with the control, there was an extensive increase in the size of the ER in BM-MSCs from SLE patients, in which arrows indicate protein aggregates within ER lumen. ER, endoplasmic reticulum; N, nucleus. Arrow indicates the normal ER or the dilatation of ER. (b) Upregulated expression of genes involved in ERS in BM-MSCs of SLE patients and normal controls. The levels of p-IRE-1, p-PERK, p-eIF 2α and CHOP were analyzed by using Western Blot analysis. Total IRE-1, PERK, eIF 2α and GAPDH were used as control for protein loading. (c) Immunofluorescence analysis of CHOP expression in the control group or SLE group. (Bar represents mean ± SD, $^{*}P < 0.05$ compared with the normal group).

feature of apoptosis in BM-MSCs from SLE patients, such as shrunken nuclei, disrupted cell membranes, damaged organelles and formation of numerous vacuoles in the cytoplasm (Figure 2(a)). Upregulation of ERS transducer phosphorylated IRE-1 and PERK were observed but not ATF-6. The similar results were found in western blot analysis that eIF 2α and CHOP protein expression were significantly upregulated in BM-MSCs from SLE patients, respectively (Figure 2(b)). To study the involvement of CHOP in the apoptosis of BM-MSCs from SLE patients, we first investigated whether the translocation of CHOP protein turned into the nucleus. CHOP nuclear translocation represented

the carrying of stress signal into the nucleus and therefore a functional activation. In BM-MSCs from SLE patients, CHOP was apparently more abundant in the nucleus than in that of controls (Figure 2(c)). These results suggest that ER stress accompanied apoptotic BM-MSCs from SLE patients.

3.3. Intervention to ERS Partly Rescued the Apoptosis of BM-MSCs from SLE Patients. To determine whether ERS was involved with the apoptosis in BM-MSCs from SLE patients, we treated BM-MSCs from SLE patients with 100 μM 4-PBA as the previous study described [24]. Interestingly, when treated with 4-PBA, the frequency of apoptotic cells measured by FCM analysis decreased distinctly in SLE group (Figure 3(a)). The relatively lower level of phosphorylated IRE-1 and PERK in 4-PBA-treated cells indicated the alleviation of ERS in BM-MSCs from SLE patients. Additionally, the relative level of Bcl-2 could be partly restored in 4-PBA-treated BM-MSCs from SLE patients, and the expression of BAX and cleaved caspase-3 was lower than that in BM-MSCs from SLE patients (Figure 3(b)). We also discovered the same result with the immunofluorescence analysis of cleaved caspase-3 (Figure 3(c)). Taken together, these data suggested that ERS played an important role in the apoptosis of BM-MSCs from SLE patients.

3.4. The Role of ERS in Apoptosis of BM-MSCs from SLE Patients. We further used a siRNA approach to determine the role of ERS in the apoptosis of BM-MSCs from SLE patients. Cells were transfected with 40 nM siRNAs for CHOP and PERK for 48 h, respectively. Western blot analysis demonstrated that transfection of si-CHOP resulted in a suppression of CHOP expression induced by ERS in BM-MSCs from SLE patients, as compared with cells transfected with control scrambled siRNA. Bcl-2 increased in BM-MSCs from SLE patients transfected with CHOP siRNA as compared to scrambled siRNA-transfected cells (Figure 4(a)). Since CHOP gene promoter contained binding sites for PERK induced by ERS, we further characterized the role of PERK in ERS-induced cell death. We silenced PERK expression by 50 nM si-PERK transfection for 48 h. PERK siRNA significantly blocked phosphorylated PERK protein expression induced by ERS. In addition, CHOP was also suppressed by PERK siRNA transfection (Figure 4(b)). The immunofluorescence of CHOP was decreased by si-PERK transfection (Figure 4(c)). Taken together, these results indicated that ERS-induced apoptosis might be involved in the apoptosis of BM-MSCs from SLE patients, at least in part, through the upregulation of PERK and CHOP proteins expression.

3.5. The Role of MAPK on ERS-Induced Apoptosis of BM-MSCs from SLE Patients. Activation of mitogen-activated protein kinase (MAPK) was implicated in the regulation of ERS-induced apoptosis. We investigated the condition of MAPK in BM-MSCs from SLE patients. Phosphorylation of JNK 1/2 and ERK 1/2 were detected in BM-MSCs from SLE patients, but not p38 MAPK. The ERK 1/2 phosphorylation downregulation occurred in BM-MSCs from SLE patients, which could be restored in 4-PBA-treated group. On the contrary, the phosphorylation of JNK1/2 increased in BM-MSCs from SLE patients and was partly reduced with 4-PBA-treated (Figure 5(a)). After the treatment with 4-PBA, we observed, by immunofluorescence analysis, that the high expression of phosphorylated JNK 1/2 in BM-MSCs from SLE was reversed, especially in the nucleus (Figure 5(b)). These findings suggested that JNK1/2 activation was involved in ERS introduced apoptosis of BM-MSCs from SLE patients. To further characterize the role of JNK 1/2 in the apoptosis of BM-MSCs from SLE patients, we silenced JNK 1/2 expression by specific siRNA transfection. JNK 1/2 expression and phosphorylation were reduced by si-JNK transfection. In addition, the ERS-induced expression of BAX was also reduced by si-JNK transfection (Figure 5(c)). Then we silenced IRE-1 expression by si-IRE-1 transfection. IRE-1 protein expression induced by ERS was significantly blocked by IRE-1 siRNA. In addition, JNK1/2 was also inhibited by IRE-1 siRNA transfection (Figure 5(d)). These findings suggested that JNK1/2 activated by IRE-1 was involved in ERS-induced apoptosis in BM-MSCs from SLE patients.

4. Discussion

Recently, SLE has been postulated by some to be a stem cell disorder disease [7, 25]. Our studies demonstrated that BM-MSCs from SLE patients appeared to show abnormity in early passages, in accordance with a previous report [8, 9]. Li et al. showed that there were increased frequencies of apoptosis in BM-MSCs from SLE patients in comparison with control groups [7]. Thus, we speculated that the apoptosis might be involved with functional abnormalities in BM-MSCs from SLE patients, which in turn participated in the pathogenesis of SLE. Our results revealed the involvement of ERS in the apoptosis of BM-MSCs from SLE patients. Activation of the UPR plays a protective role to cells under ERS [26]. Physiological processes that demand a high rate of protein synthesis and secretion must sustain activation of the UPR's adaptive programs without triggering cell death pathways. However, prolonged activation of UPR by excessive ERS can convert its role to cytotoxic by activation of multiple apoptotic pathways in mammalian cells [27]. The mechanisms initiating apoptosis under conditions of irreversible ER damage are now partially understood and may involve a series of complementary pathways [28]. The ERS transducer proteins ATF-6, IRE-1 and PERK constitute the core stress regulator of the UPR, and transducer signals from the ER to the cytoplasm and nucleus after ERS [29–31]. Previous papers reported that the induction of ERS had closely relationship with apoptosis [32, 33]. Chronic ER stress leads to BAX-dependent apoptosis through the transcriptional upregulation of BCL-2 homology 3 (BH3-) only proteins, such as BCL-2-interacting mediator of cell death (BIM) and p53 upregulated modulator of apoptosis (PUMA), which are upstream BCL-2 family members [34]. The transcription of one of the key UPR pro-apoptotic players, termed CHOP, is positively controlled by the PERK–ATF4 axis [28]. CHOP promotes both the transcription of BIM and the down regulation of BCL-2 expression, contributing to the induction of apoptosis

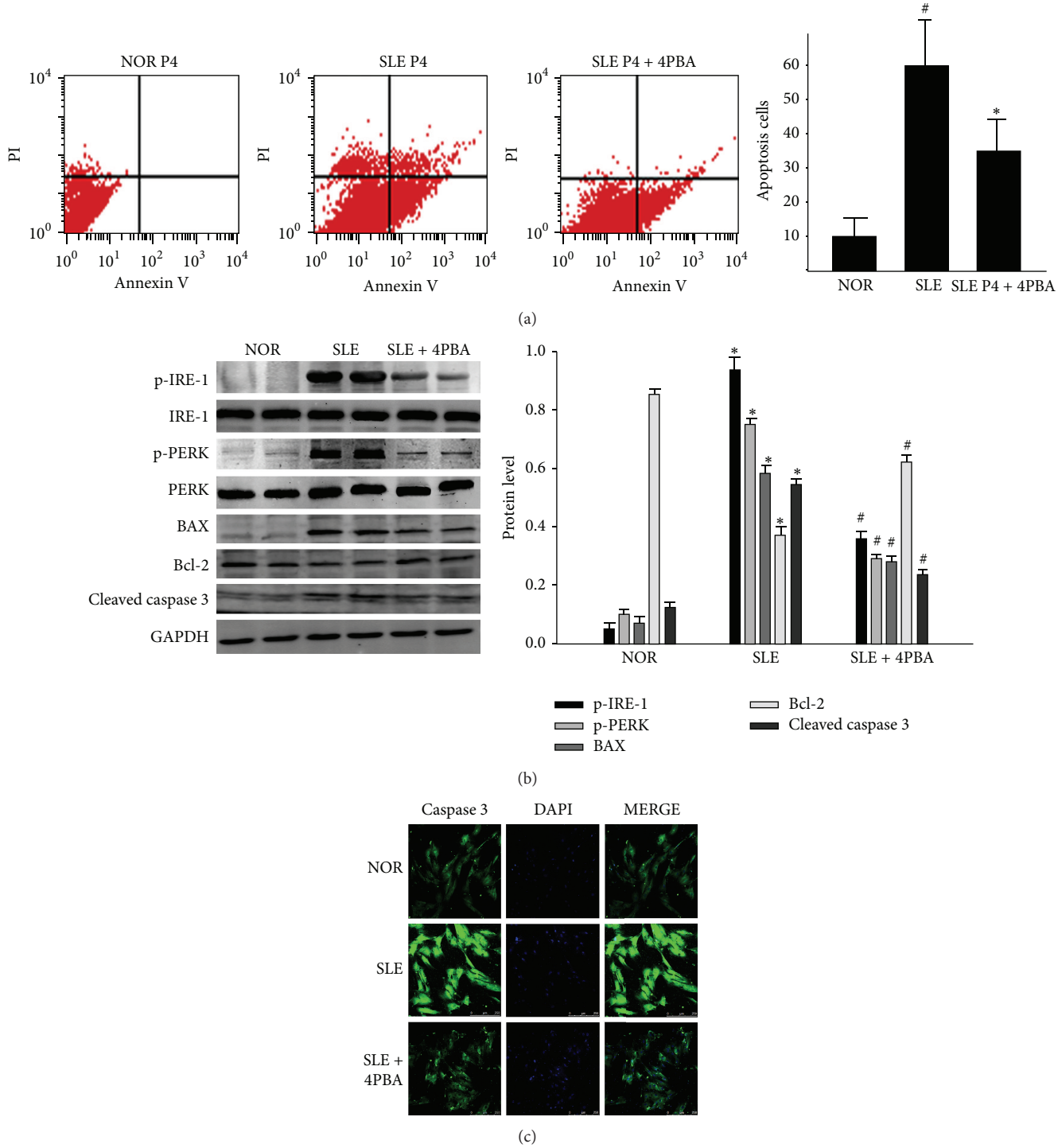
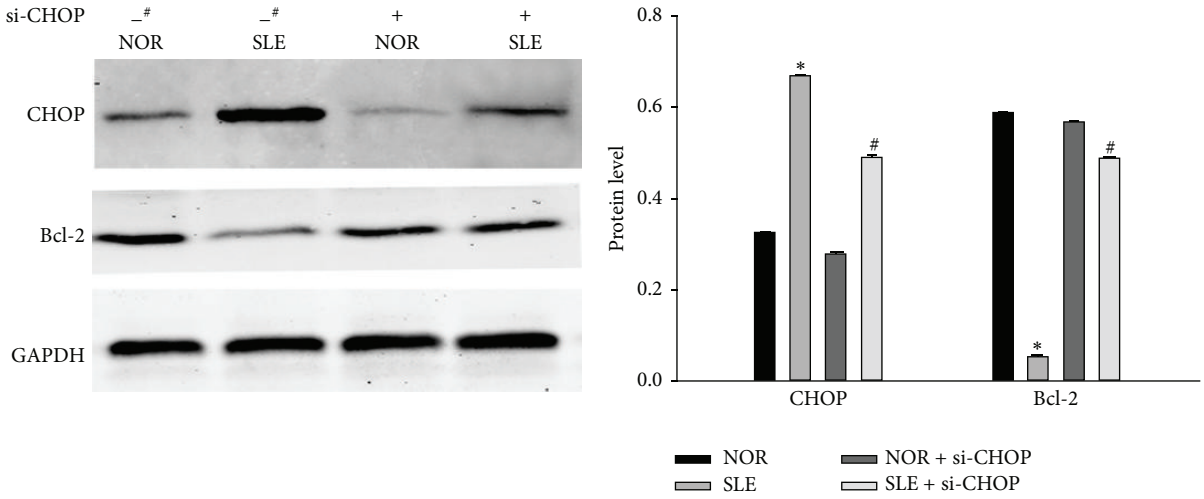


FIGURE 3: Intervention to ERS can partly rescue the apoptosis of BM-MSCs from SLE patients. BM-MSCs of SLE patients and normal controls were treated with (+) or without (−) 4-PBA (100 μM) from the same time point for the indicated time. (a) Cell viability was assessed by FCM analysis. (b) Cell lysates were analyzed for the levels of p-IRE-1, p-PERK, BAX, Bcl-2 and cleaved caspase-3 via western blot. (Bar represents mean ± SD, $^{*}P < 0.05$ compared with the normal group, $^{\#}P < 0.05$ compared with the SLE group). (c) Immunofluorescence analysis was used to analyze the level of cleaved caspase-3.



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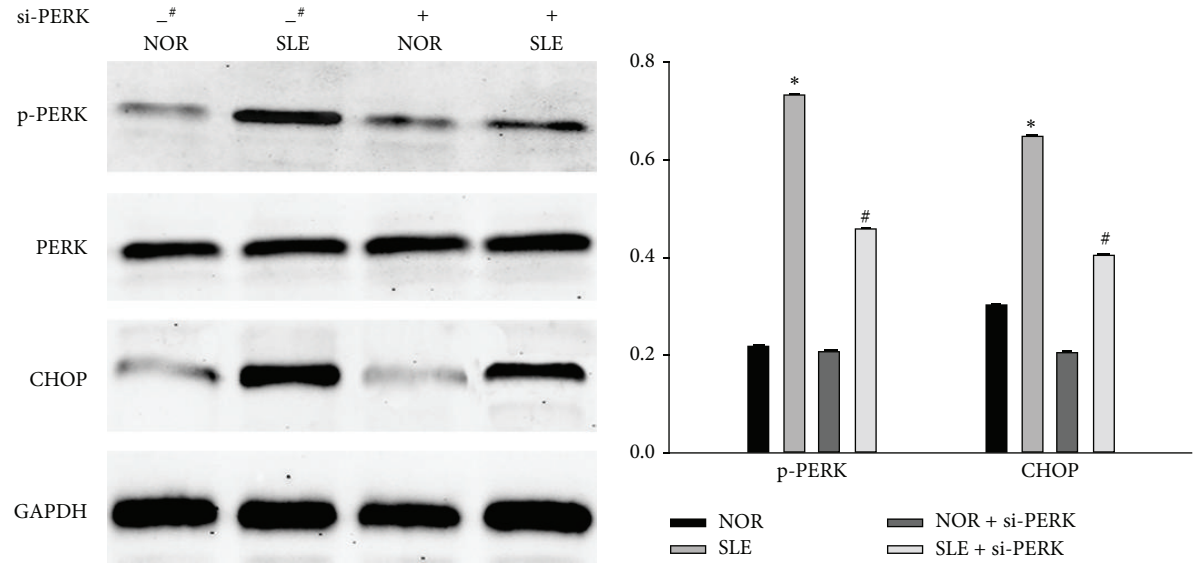

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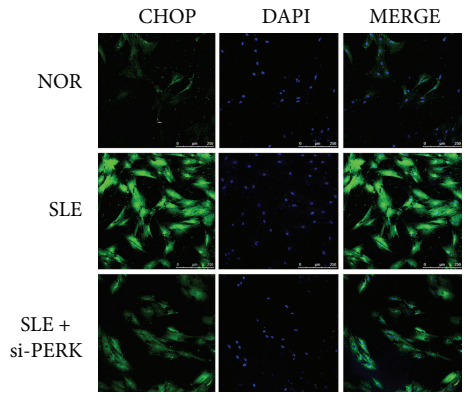

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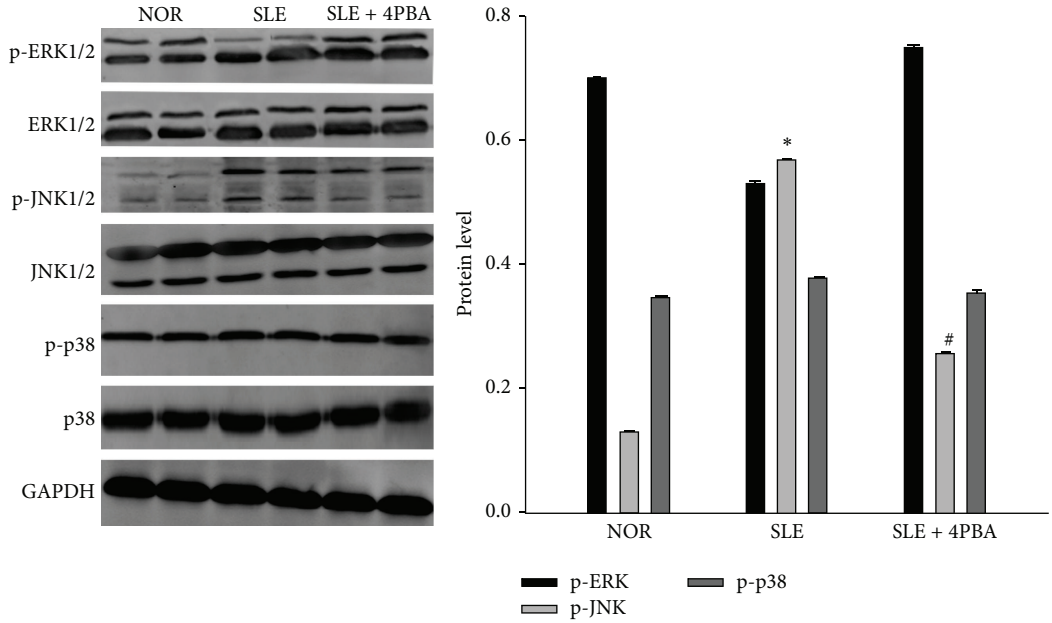
Figure 4: The role of ERS in apoptosis of BM-MSCs from SLE patients. (a) BM-MSCs of SLE patients and normal controls were transfected with scramble siRNA (#) and 40 nM CHOP siRNA respectively, for 48 h using the RNAifect transfection reagent. Western blot analysis was performed for CHOP and Bcl-2 respectively. (Bar represents mean ± SD, $^{*}P < 0.05$ compared with the normal group, $^{\#}P < 0.05$ compared with the SLE group). (b) BM-MSCs of SLE patients and normal controls were transfected with scramble siRNA (#) and 50 nM PERK siRNA, respectively, for 48 h using the RNAifect transfection reagent. Western blot analysis was used for PERK and CHOP respectively. GAPDH was used as an internal control. (Bar represents mean ± SD, $^{*}P < 0.05$ compared with the normal group, $^{\#}P < 0.05$ compared with the SLE group). (c) Immunofluorescence analysis was performed for CHOP expression.



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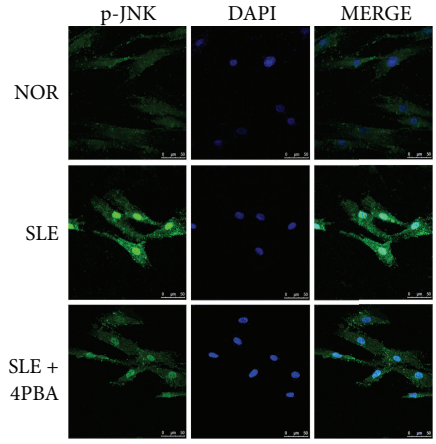

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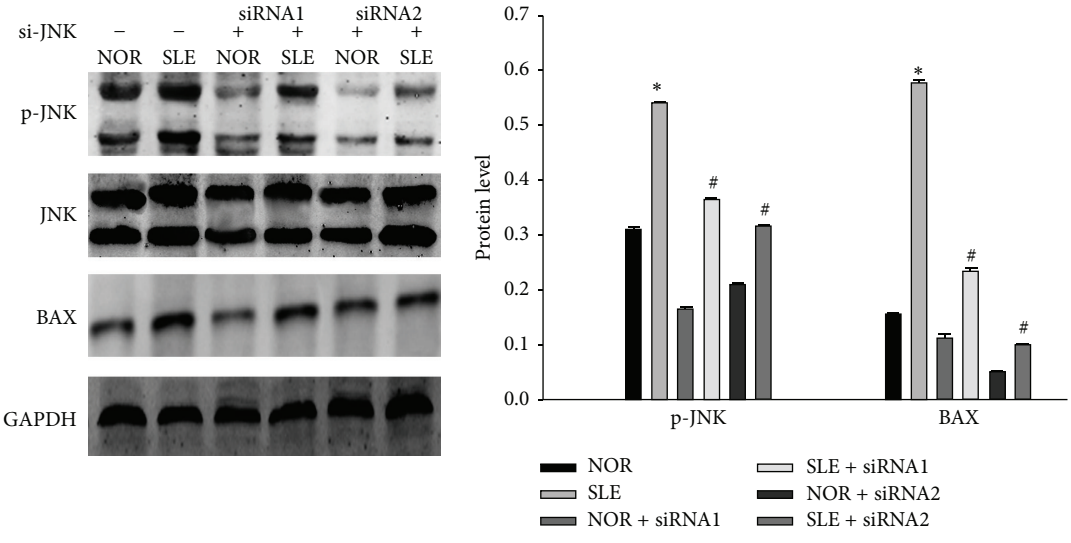

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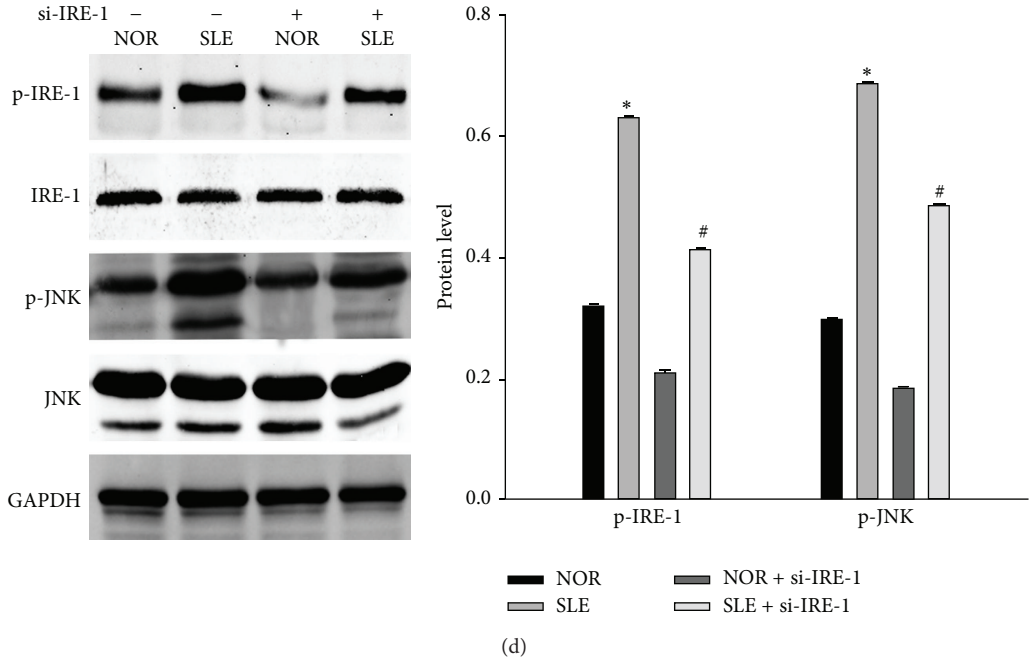

Figure 5: The role of MAPK on ERS-induced apoptosis of BM-MSCs from SLE patients. (a) BM-MSCs of SLE patients and normal controls were treated with (+) or without (−) 4-PBA (100 μM) from the same time point for the indicated time. The levels of p-ERK1/2, total ERK1/2, p-JNK1/2, total JNK1/2, p-p38, and total p38 were detected by western blotting respectively. (b) Immunofluorescence analysis of p-JNK1/2 expression in the control group and SLE group treated with or without 4-PBA. (c) BM-MSCs of SLE patients and normal controls were transfected with scramble ($^{\#}$) or 20 nM JNK1/2 siRNA for 48 h using the RNAifect transfection reagent. Western blot analysis was performed for p-JNK1/2 and BAX. (d) BM-MSCs of SLE patients and normal controls were transfected with scramble ($^{\#}$) or 50 nM IRE-1siRNA for 48 h using the RNAifect transfection reagent. Western blot analysis was performed for p-JNK1/2 and p-IRE-1. (Bar represents mean ± SD, $^{*}P < 0.05$ compared with the normal group, $^{\#}P < 0.05$ compared with the SLE group).

[28, 34]. In addition to CHOP, ATF4 and p53 are also involved in the direct transcriptional upregulation of BH3-only proteins under ER stress [34]. Many other complementary mechanisms are proposed to induce cell death under excessive ER stress, including activation of the BH3-only protein BH3-interacting domain death agonist (BID) by caspase 2, as well as ER calcium release, which may sensitize mitochondria to activate apoptosis [28, 35]. Under certain conditions, IRE1α activation is also linked to apoptosis, possibly through its ability to activate MAPKs and the subsequent downstream engagement of the BCL-2 family members, as well as the degradation of mRNAs encoding for key folding mediators through CHOP [36]. In this study, we discovered swollen ER in apoptotic BM-MSCs from SLE patients. The activation of IRE-1 and PERK significantly increased in the apoptotic BM-MSCs from SLE patients. Interestingly, the intervention to ERS could recover the phenomenon of apoptosis in BM-MSCs from SLE patients. This suggested that ERS played an important role in the process of apoptosis in BM-MSCs from SLE patients.

One of the characteristic features of ERS was the increased expression of CHOP. It was a member of the C/EBP family of transcription factors. Although CHOP was originally identified as part of the DNA damage response pathway, its induction was probably the most sensitive to ERS conditions where it played a key role in the ERS-induced apoptosis, through the mechanisms of that are not entirely delineated. The PERK-eIF2α-ATF4 arm of the UPR is required to induce CHOP protein expression [17]. CHOP-induced cell death has been associated with downregulation of BCL-2 levels [19]. In this study, we found that the expression of CHOP increased in BM-MSCs from SLE patients but decreased when treated with 4-PBA. Cells were transfected with siRNA for CHOP. Western blot analysis demonstrated that transfection of si-CHOP resulted in a suppression of CHOP expression induced by ERS in BM-MSCs from SLE patients, as compared with cells transfected with control scrambled siRNA. Bcl-2 increased in BM-MSCs from SLE patients transfected with CHOP siRNA as compared to scrambled siRNA-transfected cells. Since CHOP gene promoter contained binding sites for PERK induced by ERS, we further characterized the role of PERK in the ERS-induced apoptosis. We silenced PERK expression by si-PERK transfection. PERK siRNA significantly blocked PERK protein expression induced by ERS. In addition, CHOP was also suppressed by PERK siRNA transfection. These results indicated that ERS-induced cell death through the upregulation of PERK and CHOP proteins expression.

The greater MAPK signaling cascade consists of a sequence of successively acting kinases that result in the dual phosphorylation and activation of terminal kinases p38, JNKs, and extracellular signal-regulated kinases (ERKs) [37]. Furthermore, activation of MAPKs has been implicated in the regulation of gene expression in the ERS signaling cascade

and is involved in many aspects of the control of cellular proliferation and apoptosis. The JNK pathway has been shown to be a positive regulator of ERS induced apoptosis [38]. ERS do so indirectly through the activation of downstream molecules JNK1/2, which regulate the expression and activity of various pro- and antiapoptotic proteins such as BCL-2 family members and further push the cell down the path of apoptosis [39]. Previous reports showed activation of p38 and JNK were responsible for oxidative stress induced apoptosis [32]. But their respective roles in apoptosis of BM-MSCs from SLE patients were unknown. In our study, phosphorylation of JNK1/2 was observed in BM-MSCs from SLE patients. Increasing and sustaining expression of IRE-1 and its downstream target JNK1/2 were reduced when treated with 4-PBA in BM-MSCs from SLE patients. Inhibition of IRE-1 expression by siRNA led to downregulation of JNK1/2, and partially reduced the expression of proapoptotic protein BAX. These findings implicated that ERS mediated the apoptosis via the cooperation with the JNK pathway.

5. Conclusions

In conclusion, we provided evidence that ERS participated in the process of apoptosis in BM-MSCs from SLE patients. Our study also found that ERS induced the apoptosis through upregulating expression of JNK1/2 and CHOP. All of these findings suggested that SLE BM-MSCs were involved with ERS, which might implicate the possible involvement of BM-MSCs in the pathogenesis of SLE. These findings would suggest a novel strategy for the potential therapies of SLE patients.

Abbreviations

SLE:	Systemic lupus erythematosus
MSC:	Mesenchymal stem cells transplantation
BM-MSCT:	Bone marrow MSC transplantation
BM-MSCs:	Bone marrow mesenchymal stem cells
HSC:	Hematopoietic stem cells
ER:	Endoplasmic reticulum
ERS:	Endoplasmic reticulum stress
UPR:	Unfolded protein response
IRE-1:	Inositol-requiring protein-1
PERK:	Protein kinase RNA-like ER kinase
ATF-6:	Activating transcription factor-6
eIF 2α:	Eukaryotic translation initiation factor 2α
CHOP:	CCAAT/enhancer-binding protein (C/EBP)-homologous protein
JNK:	Jun N-terminal kinase
BAX:	Associated protein X
Bcl-2:	B-cell lymphoma 2
4-PBA:	4-phenylbutyric acid
MAPK:	Mitogen-activated protein kinase
ERK:	Extracellular signal-regulated kinase
SLEDAI:	Systemic Lupus Erythematosus Disease Activity Index
L-DMEM:	Low-glucose Dulbecco-Modified Eagle Medium
FBS:	Fetal bovine serum
PI:	Propidium iodide
PBS:	Phosphate-buffered saline
PVDF:	polyvinylidenedifluoride
SDS-PAGE:	SDS-polyacrylamide gel electrophoresis
BH3:	BCL-2 homology 3
BIM:	BCL-2-interacting mediator of cell death
PUMA:	p53 upregulated modulator of apoptosis
BID:	BH3-interacting domain death agonist.

Conflict of Interests

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

Authors' Contribution

Yan Meng and Genkai Guo have contributed equally to this work.

Acknowledgments

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Research Article

Characterization of CD30/CD30L$^+$ Cells in Peripheral Blood and Synovial Fluid of Patients with Rheumatoid Arthritis

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The CD30/CD30L signalling system has been implicated in the pathogenesis of several autoimmune and inflammatory conditions. In rheumatoid arthritis (RA), soluble CD30 (sCD30) levels reflect the recruitment of CD30$^+$ T cells into the inflamed joints and correlate with a positive response to immunosuppressive therapy. The aim of our report was to clarify the role of CD30/CD30L signalling system in the pathogenesis of RA. Our analysis of the CD30L$^+$ T cell subsets in peripheral blood (PB) and synovial fluid (SF) of RA patients and of the related cytokine profiles suggests the involvement of CD30/CD30L signalling in polarization of T cells towards a Th17 phenotype with proinflammatory features. Moreover, in RA SF nearly 50% of Treg cells express CD30, probably as an attempt to downmodulate the ongoing inflammation. We also show here that the engagement of CD30L on neutrophils stimulated with CD30/Fc chimera may play a crucial role in RA inflammation since activated neutrophils release IL-8, thus potentially amplifying the local inflammatory damage. In conclusion, the results obtained suggest that the complex CD30/CD30L signalling pathway is implicated in the pathogenesis and progression of RA synovitis through a concerted action on several immune effector cells.

1. Introduction

CD30 is a member of the tumour necrosis factor receptor (TNFR) superfamily that includes, among others, TNFR, CD40, Fas (CD95), and OX-40 (CD134) [1]. Human CD30 is a type 1 glycoprotein and its cytoplasmic region is characterized by the presence of several serine/threonine phosphorylation sites which regulate cell signalling, once the receptor is engaged [2]. In nonpathological conditions, activated T- and B-lymphocytes and NK cells generally express the CD30 molecule, while lower levels of expression are present in activated monocytes and eosinophils [3]. Moreover, CD30 is found on a small percentage of CD8$^+$ T cells while only a negligible expression on naïve or resting lymphocytes has been described [4]. Several mechanisms are able to trigger CD30 expression on T cells, including mitogen activation, antigen receptor cross-linking, and viral infections [5].

CD30L belongs to the TNF family [2] and is the only known ligand for CD30. It is found, at RNA transcription level, in B cells, activated T cells, macrophages, granulocytes, eosinophils, and some HTLV-1-positive T cell lines [6–13], while at protein level, CD30L is detected on activated peripheral blood T cells, B cells, neutrophils, mast cells, monocytes, and macrophages [14]. The CD30L molecule is cleaved and released by CD30L$^+$ cells in presence of CD30$^+$ cells and the soluble form of CD30L (sCD30L) has the ability to induce CD30$^+$ cells apoptosis [15].

Interaction of CD30L with cells expressing CD30 induces signals that cause cell proliferation or cell death. Interestingly, upon binding to CD30, CD30L is also able to transduce

a signal. One of the possible consequences of this reverse signalling is cell proliferation [12].

The role of CD30/CD30L interaction in health and disease is still not totally understood, in part due to the pleiotropic nature of CD30 signals.

In vitro, CD30 expression has been mainly associated with a Th2/Th0 phenotype [16], although *in vivo* studies suggest that the relationship between $CD30^+$ T cells and Th1 or Th2 profiles is very complex. Some of us have proposed a novel regulatory mechanism for CD30 in Th1 polarized responses such as rheumatoid arthritis [17].

Indeed in autoimmune and chronic inflammatory diseases, several studies have provided evidences that CD30/CD30L signalling is involved in T helper (Th)2 cell responses and Th2-associated diseases [18, 19]. However, recent observations have shown that CD30/CD30L signalling plays a role also in Th1 and Th17 responses and in Th1-associated diseases [16, 20–22]. Furthermore, it is also involved in the regulation of memory T-cell response: in a murine transplantation model, antigen-induced T regulatory (Treg) cells, but not naïve ones, were able to suppress allograft rejection mediated by memory $CD8^+$ T cells in an Ag-specific manner [23]. This suppression was related to an enhanced apoptosis of allospecific memory $CD8^+$ T cells in the graft due to the presence of CD30 expressing regulatory T (Treg) cells and to the CD30/CD30L interaction [24–26].

As far as rheumatoid arthritis (RA) concerns, patients affected by the disease have increased levels of soluble CD30 (sCD30) in both serum and synovial fluid (SF) [27]. This feature could reflect the presence of $CD30^+$ synovial T lymphocytes, recruited at the site of tissue aggression with the possible aim to downmodulate inflammation [28].

These data may be crucial for the understanding of the cellular mechanisms underlying clinical response to classical and biological disease modifying drugs since we have shown that sCD30 levels correlate with response to treatment [29, 30], whereas high levels of sCD30L seem to predict lack of response to biological therapy [15].

The complex CD30/CD30L signalling pathway is indeed further complicated by the role played by the soluble forms of CD30 and CD30L. In animal model sCD30 is able to inhibit CD30/CD30L interaction and, at the same time, activate CD30L by reverse signalling [20]. Moreover, in a murine model, inhibition of CD30/CD30L signalling by viral encoded CD30 leads to a decrease in Th1 cytokines production, such as for IFN-γ, conferring to this mechanism a potential relevant role in the control of a Th1-response to viral infection [20].

RA is considered primarily a Th1-driven condition [31, 32] although the presence of increased levels of Th2 cytokines, such as IL-4 and IL-10, is found in the early stages of the disease [27]. It has been reported that IL-4 has the ability to induce CD30 membrane expression [33] and increased levels of IL-4 have been found in both SF and serum of RA patients [34].

CD30/CD30L signalling is also involved in Th17 induction. $CD4^+$ T cells taken from CD30L- or CD30-deficient mice showed a reduced ability to differentiate into Th17 cells [31]. *In vivo* experiments showed that transfection of $CD30L^{-/-}$ $CD4^+$ T cells in severe combined immunodeficiency (SCID) CD30L-deficient mice leads to an altered Th17 differentiation, while transferring $CD30L^{+/+}$ $CD4^+$ T cells causes a normal Th17 differentiation. The data suggest that CD30/CD30L signalling carried out by the T-T cell interaction plays a critical role in Th17 cell differentiation [21, 35, 36].

Therefore the CD30/CD30L signalling has different effects depending on the disease and on the cytokine milieu in which it takes place. Moreover it is further complicated by the functional activity of the soluble molecules.

The aim of this work was to clarify some of the controversial issues related to the role played by $CD30/CD30L^+$ cells in the pathogenesis of rheumatoid synovitis.

2. Materials and Methods

2.1. Patients. Fourteen patients (10 females and 4 males, mean age 54 ± 16) affected by RA were enrolled in the study. RA patients met the American College of Rheumatology classification criteria for RA [37]. Eight donors with posttraumatic synovitis were used as controls.

A written informed consent was obtained from all of the participants in the study. The study was approved by local Ethical Committee. All clinical investigations have been conducted according to the principles expressed in the Helsinki declaration.

2.2. Isolation of Peripheral Blood and Synovial Fluid Mononuclear Cells. Peripheral blood mononuclear cells (PBMCs) and synovial fluid mononuclear cells (SFMCs) were isolated from heparinized PB and from SF treated with hyaluronidase (Sigma, St. Louis, MO) through density gradient centrifugation using Lymphoprep Ficoll-Isopaque (Axis-Shield, Oslo, NO) according to manufacturer's instruction. Briefly, 10 mL PB or SF was diluted with 10 mL PBS and then layered over 10 mL Lymphoprep in a 50 mL centrifuge tube. Samples were centrifuged at 800 ×g for 20 minutes and cells were collected using a Pasteur pipette. Cells were washed twice with PBS at 1200 rpm for 10 minutes and then counted in a Burker chamber using acridine orange staining.

2.3. PBMCs and SFMCs Immunophenotype by FACS Analysis. The immunophenotypic analysis of PBMC and SFMC populations was carried out using flow cytometry. From each sample we obtained three different tubes containing 1×10^6 cells each that were stained with different monoclonal antibodies. Samples for CD30 detection were stained with CD4 APC-H7 (5 μL), CD8 FITC (10 μL), CD3 PerCp (10 μL), and CD30 PE antibodies (10 μL); samples for CD30L detection were stained with CD4 APC-H7 (5 μL), CD8 FITC (10 μL), CD3 PerCp (10 μL), and CD30L PE antibodies (20 μL); finally samples for Treg cells detection were stained with CD4 APC-H7 (5 μL), CD25 FITC (20 μL), CD3 PerCp (10 μL), CD127 PE-Cy7 (5 μL), and CD30 PE antibodies (10 μL). Staining of surface molecules was performed for 20 minutes at room temperature protected from the light. Treg tubes were then fixed, permeabilized, and incubated with

ALEXA647 FoxP3 antibody. Briefly, cells were washed with 2 mL wash buffer at 250 ×g for 10 minutes and then fixed with Human Foxp3 Buffer A for 10 minutes at room temperature. Cells were washed and then permeabilized incubating with 0.5 mL working solution Human Foxp3 Buffer C for 30 minutes. Cells were then washed and stained with FoxP3 ALEXA647 monoclonal antibody (20 μL) for 30 minutes. All reagents were purchased from Becton Dickinson (San Jose, CA, USA), except for CD30L monoclonal antibody (R&D System, Minneapolis, MN, USA). Samples were analysed on a FACSCanto cytometer (Becton Dickinson). Data were analysed by FlowJo 9.3.3 software (Tree Star, Ashland, OR).

2.4. Time Course of CD30 and CD30L Cell Surface Expression. PBMCs from healthy donors were activated with Dynabeads Human T-Activator CD3/CD28 (Life Technologies) in a 14 mL polypropylene round-bottom tube with a ratio of 1 : 1 and incubated for 6, 10, 15, 20, 24, and 48 hours in RPMI + 10% FCS at 5% CO_2. Cells were harvested, washed, and stained for 20 minutes with CD3 APC (Becton Dickinson) and CD30 PE (Becton Dickinson) or CD30L PE (R&D System) antibodies. Cells were acquired on a FACSCalibur cytometer (Becton Dickinson) and analysed with FlowJo 9.3.3 software (Tree Star).

2.5. PBMCs and SFMCs Stimulation by CD30/Fc Chimera. PBMCs and SFMCs were activated as described above and cultured in presence or absence of 20 μg/mL CD30/Fc chimera (R&D Systems). PBMCs were harvested after 24, 48, and 120 hours of incubation, while SFMCs were harvested after 15, 24, and 48 hours. Cells and supernatants were collected for RNA extraction and cytokine levels evaluation, respectively.

2.6. RNA Isolation and RT-PCR. Total RNA was extracted from PBMCs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following manufacturer's instructions. First-strand cDNA was generated using the SuperScript III First-Strand Synthesis System for RT-PCR Kit (Invitrogen), with random hexamers, according to the manufacturer's protocol. cDNA was aliquoted in equal volumes and stored at −20°C.

2.7. Cytokines Production Evaluated by Real Time RT-PCR. PCR was performed in a total volume of 25 μL containing 1× TaqMan Universal PCR Master mix, no AmpErase UNG, and 2.5 μL of cDNA; predesigned, gene-specific primers and probe sets for each gene (IL-17 Hs00174383_m1, IL-4 Hs00174122_m1, IL-6 Hs00985639_m1, IFN-γ Hs00989291_m1, and IL-10 Hs00961622_m1) were obtained from Assay-on-Demand Gene Expression Products (Applied Biosystems, Foster City, CA, USA). Real Time PCR reactions were carried out in a two-tube system and in singleplex. The real time amplifications included 10 minutes at 95°C (AmpliTaq Gold activation), followed by 40 cycles at 95°C for 15 seconds and at 60°C for one minute. Thermocycling and signal detection were performed with 7500 Sequence Detector (Applied Biosystems). Signals were detected according to the manufacturer's instructions. This technique allows the identification of the cycling point where PCR product is detectable by means of fluorescence emission (threshold cycle or Ct value). The Ct value correlates with the starting quantity of target mRNA. Relative expression levels were calculated for each sample after normalization against the housekeeping gene GAPDH, using the ΔΔCt method for comparing relative fold expression differences. The data are expressed as fold change. A fold change >1.5 was considered a significant increase in transcription. Ct values for each reaction were determined using TaqMan SDS analysis software. For each amount of RNA tested triplicate Ct values were averaged. Because Ct values vary linearly with the logarithm of the amount of RNA, this average represents a geometric mean.

2.8. Cytokines Secretion Evaluated by ELISA Kit. Supernatants of the previously described experiment were tested for cytokine levels using commercial ELISA kit purchased from R&D Systems (Human Quantikine ELISA). IL-6, IL-10, IL-4, IFN-γ, and IL-17 were evaluated in the supernatants following the manufacturer instructions. Plates were read at 450 nm with TECAN Sunrise III (Tecan).

2.9. Cytokines Secretion Assay by Flow Cytometry. PBMCs were activated for 24 hours with Dynabeads Human T-Activator CD3/CD28 (Life Technologies) and cultured for 3 hours with or without 20 μg/mL CD30/Fc chimera (R&D Systems) in RPMI + 10% FCS. Cells were then harvested and tested for IL-17 secretion using Cytokine Secretion Assays (Miltenyi Biotec, Bergisch Gladbach, Germany) following manufacturer's instructions. Briefly, cells were suspended in 2 mL medium, washed with 2 mL cold buffer at 300 ×g for 10 minutes, and resuspended in 90 μL cold medium. Cells were incubated 5 minutes on ice with 10 μL IL-17 catch reagent. Cells were diluted with warm medium and incubated for 45 minutes at 37°C, washed with cold buffer and then stained with CD3 PerCp (Becton Dickinson), CD30L PE (R&D Systems), and IL-17 Detection Antibody APC. Cells were acquired on a FACSCanto cytometer (Becton Dickinson). Analysis was performed with FlowJo 9.3.3 software (Tree Star).

2.10. Neutrophils Isolation. Neutrophils from fresh buffy coat of normal subjects were isolated through density gradient centrifugation using Lymphoprep Ficoll-Isopaque (Axis-Shield, Oslo, NO) according to manufacturer's instruction. Briefly, 10 mL of PB was diluted with 10 mL PBS and then layered over 10 mL Lymphoprep in a 50 mL centrifuge tube. Samples were centrifuged at 800 ×g for 20 minutes and cells were collected using a Pasteur pipette. Obtained cells were put in a new tube in a final volume of 40 mL PBS. In order to achieve erythrocytes sedimentation, 8 mL of dextran was added and after 10–15 minutes, after separation of phases, supernatant was transferred in a new tube. Cells were washed with PBS at 1200 rpm for 5 minutes and 7.5 mL of NaCl 0.2% was added. After 50 seconds, 17.5 mL of NaCl 1.2% was added; cells were mixed gently and washed again with PBS. Cells were then counted using acridine orange staining in a Burker chamber and purified using the EasySep Negative Selection Human Neutrophil Enrichment Kit (StemCell Technologies,

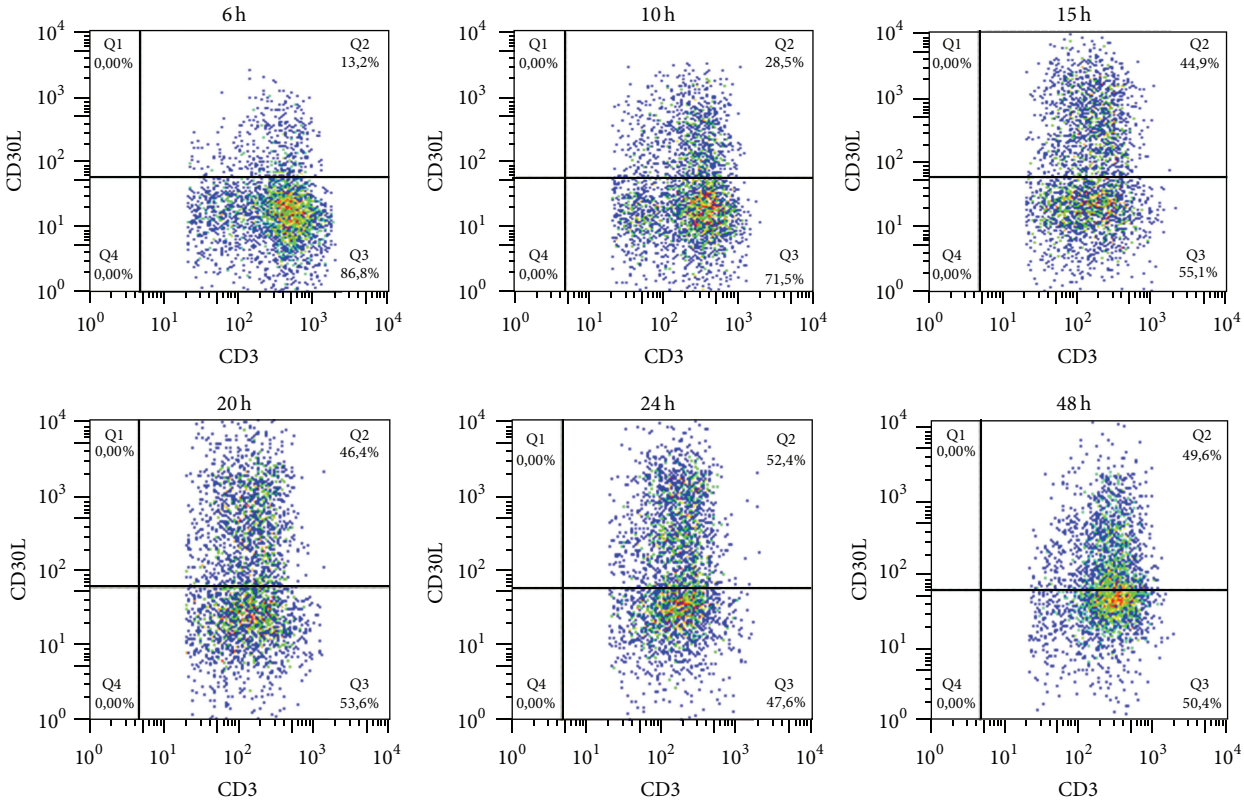

Figure 1: Surface CD30L expression upon cell activation. Percentage of $CD30L^+$ T cells rapidly increases in the first 15 hours reaching the maximum value (52.4%) after 24 hours from activation with CD3/CD28 beads.

Vancouver, Canada) according to manufacturer's instructions. Briefly, cells were placed in a 5 mL polystyrene tube at a concentration of 50×10^6 cells/mL and incubated for 10 minutes with 100 μL EasySep Human Neutrophil Enrichment Cocktail. Two hundreds microL EasySep Nanoparticles were then added into the tube and after 10 minutes the tube was placed in a magnet for 5 minutes. Magnet and tube were inverted and the desired fraction was collected in a new tube. Neutrophils were counted as described above.

2.11. CD30L Expression on Neutrophils. RNA isolation and transcription have already been described. The amplified DNA obtained after RT-PCR was run in a 1.5% agarose gel. cDNA obtained from DG75 cells (human Burkitt lymphoma, DSMZ) that constitutively express CD30L was used as positive control. In order to analyse CD30L membrane expression, purified neutrophils were also preincubated with normal human serum (Invitrogen) for 10 minutes and then incubated with CD30L PE monoclonal antibody (R&D Systems) for 20 minutes. Cells were acquired on a FACSCanto cytometer (Becton Dickinson) and analysis was performed with FlowJo 9.3.3 software (Tree Star).

2.12. Neutrophils Cytokines Secretion Evaluated by ELISA Kit. Neutrophils were seeded in a 6-well plate at a final concentration of 2×10^6 cells/mL in RPMI + 10% FCS. They were cultured alone and with 20 μg/mL CD30/Fc chimera (R&D Systems), with 10 ng/mL LPS (Invitrogen), or with both CD30/Fc chimera and LPS as a costimulus. Cells were harvested after 1, 3, and 10 hours and levels of IL-8, TNF-α, MMP-9, VEGF, IFN-γ, and IL-17 were evaluated using commercial ELISA kits purchased from R&D Systems (Human Quantikine ELISA). Plates were read at 450 nm with TECAN Sunrise III (Tecan).

2.13. Statistical Analysis. All the calculations were performed with SPSS 21.0 statistical package (SPSS Inc., Chicago, IL, USA). Quantitative data with a normal distribution are expressed as mean ± SD and were analysed with Student's t-test.

A value of $P < 0.05$ was considered significant.

3. Results and Discussion

3.1. Immunophenotypic Analysis of T Cells from Patients with RA. In order to better clarify the expression of CD30 and CD30L molecules by T cells in RA we analysed the expression of these molecules by $CD4^+$ and $CD8^+$ T cell subsets in both PB and SF of 14 RA patients and 8 control subjects. In the same samples we also evaluated the frequency of Treg cells expressing the CD30 molecule. Mean values of percentages of each subpopulation in RA patients and control subjects are reported in Table 1.

In PB samples, we did not find any significant difference in the percentages of all $CD30^+$ T cell subsets between RA patient and control groups. On the contrary, in SF samples,

Table 1: $CD30^+$ and $CD30L^+$ T cells phenotype in the peripheral blood and synovial fluid of RA patients and controls.

RA patients ($n = 14$)	Sample	$CD3^+$ $CD4^+$	$CD3^+$ $CD8^+$	$CD4^+$ $CD30^+$	$CD4^+$ $CD30L^+$	$CD8^+$ $CD30^+$	$CD8^+$ $CD30L^+$	Treg	Treg $CD30^+$
Mean ± S.D.	PB	73.9 ± 12.5	23.8 ± 12.3	0.8 ± 0.7	2.9 ± 1.8	0.1 ± 0.2	2.1 ± 0.2	5.0 ± 3.8	12.5 ± 15.2
Mean ± S.D.	SF	58.0 ± 10.7	38.8 ± 11.2	8.6 ± 5.6	13.9 ± 10.4	0.3 ± 0.5	2.3 ± 3.2	13.8 ± 4.8	42.3 ± 14.7
Controls ($n = 8$)	Sample	$CD3^+$ $CD4^+$	$CD3^+$ $CD8^+$	$CD4^+$ $CD30^+$	$CD4^+$ $CD30L^+$	$CD8^+$ $CD30^+$	$CD8^+$ $CD30L^+$	Treg	Treg $CD30^+$
Mean ± S.D.	PB	72.3 ± 9.5	22.3 ± 10.2	0.3 ± 0.5	3.2 ± 0.9	0.3 ± 0.5	1.7 ± 1.7	4.5 ± 1.8	4.1 ± 4.3
Mean ± S.D.	SF	62.0 ± 7.1	33.0 ± 4.2	0.5 ± 0.7	10.0 ± 1.4	0.5 ± 0.7	6.9 ± 5.8	4.0 ± 2.8	19.0 ± 11.3

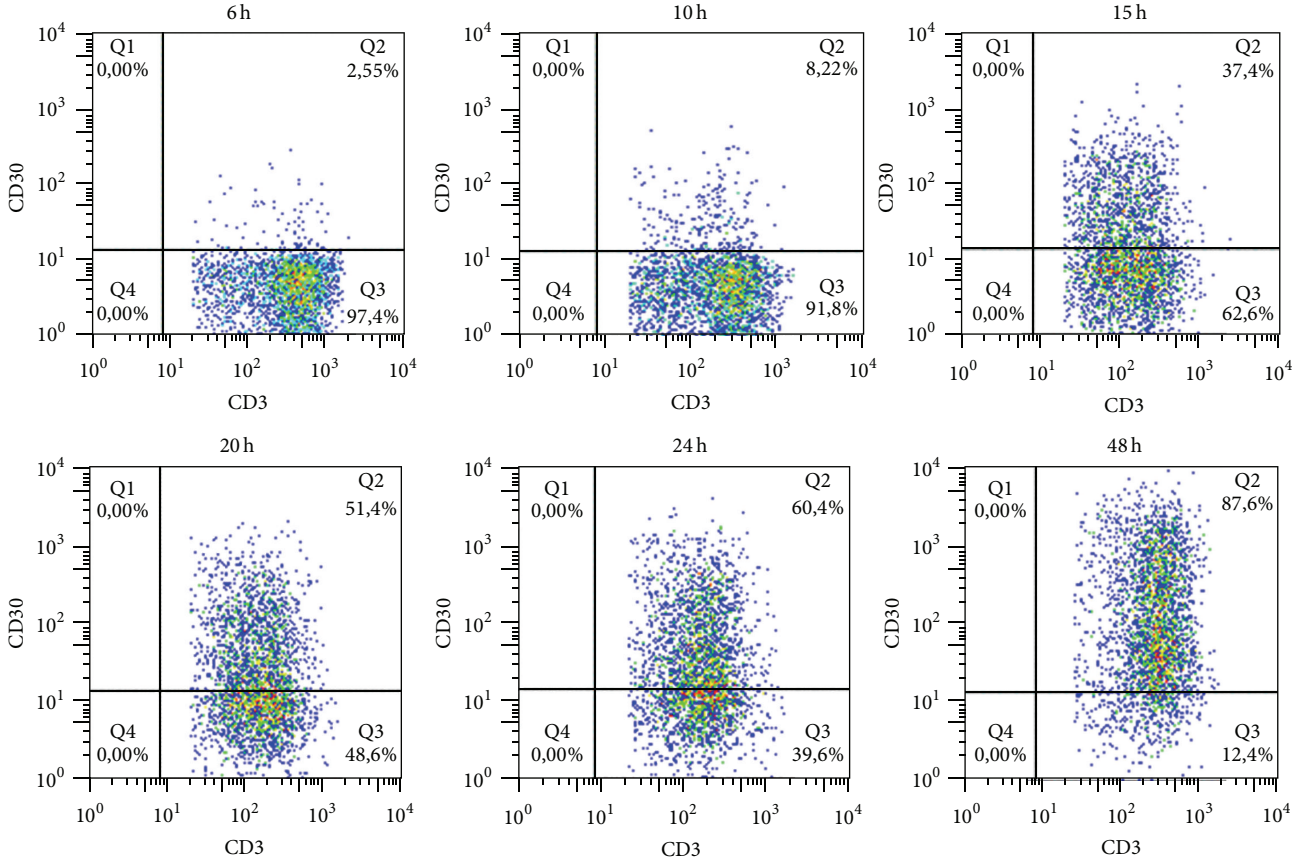

Figure 2: Surface CD30 expression upon cell activation. Percentage of $CD30^+$ T cells increases until it reaches the maximum value (87.6%) after 48 hours from activation with CD3/CD28 beads.

we found that RA patients had a percentage of $CD4^+$ T cells expressing the CD30 molecule much higher than in controls (8.6% ± 5.6 versus 0.5% ± 0.7).

The percentage of Treg population among total $CD4^+$ cells in the SF of RA patients was higher than the percentage found in the SF of control subjects (13.8% ± 4.8 versus 4.0% ± 2.8). Remarkably in RA patients 42.3% ± 14.7 of the total Treg cells also express the CD30 molecule whereas only 19.0% ± 11.3 of the total Treg subset are $CD30^+$ in the control subjects (Table 1).

Such a high frequency of Treg cells may indicate that in patients affected by RA synovitis there is an attempt to downmodulate inflammation in order to control disease progression. Moreover, the high percentage of Treg expressing the CD30 molecule strengthens the knowledge that CD30 is expressed by cells that have an anti-inflammatory behaviour.

Finally, we did not observe differences in $CD30L^+$ T cell percentages in the different T-cell subsets between RA patients and the control group. The only significant difference is represented by the percentage of $CD4^+CD30L^+$ T cell subset that is higher in SF than in PB samples in both patients and controls, suggesting that CD30L molecule is preferentially expressed by cells present at sites of inflammation.

3.2. CD30 and CD30L Expression over Time upon Cell Activation. The expression of surface CD30 and CD30L molecules is induced on T cells following activation with CD3/CD28 beads. In order to study the kinetics of expression of surface

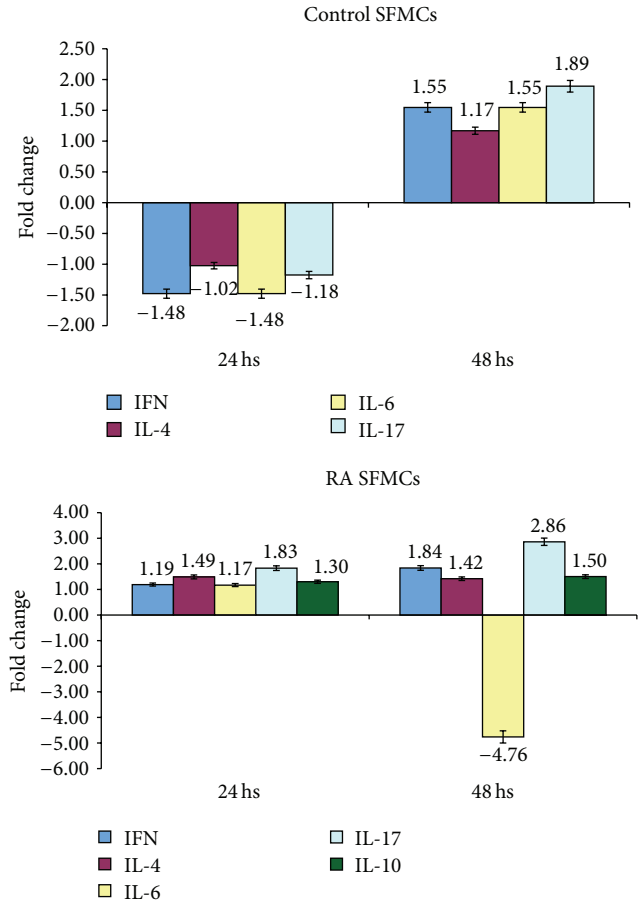

Figure 3: Modulation of expression of genes encoding for cytokines in activated SFMCs following incubation with CD30/Fc chimera in controls and patients with RA.

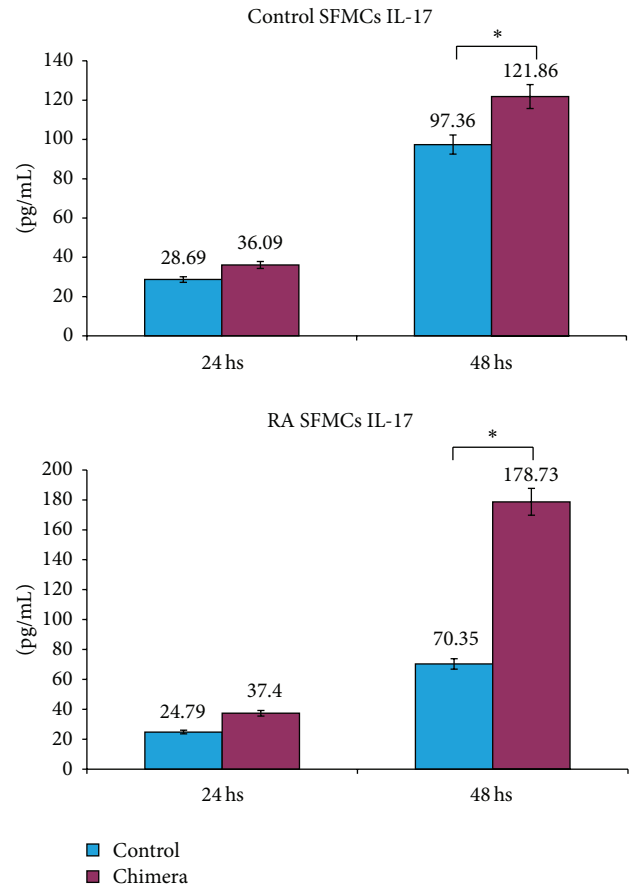

Figure 4: Levels of IL-17 released in the medium by activated SFMCs in presence or absence of CD30/Fc chimera in control subjects and in patients with RA. CD30/Fc chimera stimulus induces the production of IL-17 at 48 hours. * indicates a P value < 0.05.

CD30 or CD30L molecule, we performed a time course experiment evaluating the percentage of positive activated T cells from healthy donors.

We observed an increase in percentage of $CD30L^+$ cell population after 15 hours of stimulation with a peak at 24 hours when 52.4% of T cells expressed CD30L on their surface (Figure 1). CD30 expression appears later, since 87.6% of T cells are $CD30^+$ 48 hours after stimulation (Figure 2).

Since CD30 is mainly expressed by T cells with a Th2 [16, 17] phenotype and CD30L by T cells with a Th1 phenotype [18–21], the results of this experiment suggest that, upon activation, T cells expressing the CD30L molecule with a proinflammatory profile are mainly present at the first stages of inflammation, whereas cells expressing the CD30 molecule are present later on with the aim to downmodulate and possibly switch off inflammation.

3.3. Cytokines Expression Induced by CD30/Fc Chimera. In order to evaluate the effects of CD30/CD30L signalling in $CD30L^+$ T cells we studied the expression of gene transcripts of different cytokines by activated SFMCs from 4 control subjects and from 5 RA patients at 24 and 48 hours. SFMCs from control subjects showed an overexpression of transcripts encoding for IFN-γ, IL-6, and IL-17 at 48 hours, whereas SFMCs from patients with RA had an increase of transcripts encoding for IL-17 at 24 and 48 hours and for IFN-γ and IL-10 at 48 hours after incubation with CD30/Fc chimera. On the contrary we observed a decrease of gene transcripts for IL-6 after 48 hours (Figure 3).

We then evaluated the levels of IL-17 in the supernatants of SFMCs obtained from control subjects and RA patients 24 and 48 hours after stimulation with the CD30/Fc chimera and found a statistically increased level of the cytokine in the supernatant of SFMCs from both groups at 48 hours after stimulation with the CD30/Fc chimera versus unstimulated samples (Figure 4). On the contrary we did not observe any difference in the detection of the other cytokines studied in the supernatant: IL-6, IL-10, IL-4, and IFN-γ.

These data are consistent with the increased transcripts for IL-17 detected by quantitative PCR and indicate the stimulation of CD30L induces the production of a proinflammatory cytokine.

Finally, we have evaluated the percentage of IL-17 producing T cells by FACS analysis of cytokine secreting cells using SFMCs obtained from RA patients. We observed that

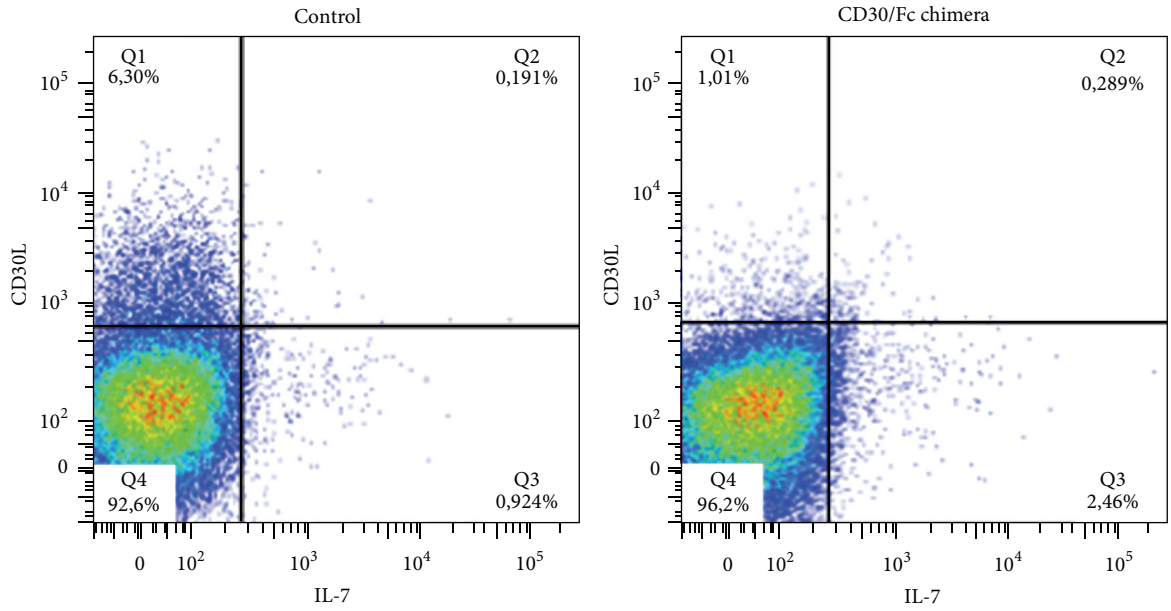

Figure 5: FACS analysis of IL-17 producing cells among SFMCs from RA patients, stimulated with CD30/Fc chimera. After 3 hours of CD30/Fc chimera stimulation, the percentage of IL-17 producing T cell is 2.749%, while it is 1.115% in the unstimulated cells.

samples cultured in presence of CD30/Fc chimera doubled the percentage of IL-17 producing cells (Figure 5).

Also these data suggest that the stimulation of $CD30L^+$ T cells via CD30/Fc chimera promotes the production of IL-17.

The results obtained so far are consistent with an increase of IL-17 (at both transcript and protein levels) suggesting that the CD30/CD30L signalling is involved in polarizing the Th17 response with proinflammatory effects, as already observed in the murine model [20, 35, 36].

It is interesting to note that IL-17 transcripts and supernatant levels are higher in patients with RA compared to controls indicating that SFMCs from patients are more susceptible to IL-17 production.

3.4. Neutrophils Express CD30L on Their Surface. Neutrophils are thought to play a pivotal role in the pathogenesis of RA inflammation and are also involved in bone erosion, a typical feature of the disease. The presence of CD30L on neutrophils has been investigated long time ago only in one report using a monoclonal antibody which is no more commercially available [12]. For this reason we wanted to confirm this observation at both transcript and protein levels. RT-PCR showed a band at 463 bp for both DG75 (positive control) and neutrophils (Figure 6(a)) and FACS analysis revealed the expression of CD30L on neutrophils cell surface (Figure 6(b)).

3.5. Molecules Secreted by Neutrophils Stimulated by CD30/Fc Chimera. Neutrophils incubated with LPS plus CD30/Fc chimera showed a statistically significant increased level of IL-8 in the culture medium compared with the level detected in the supernatant of neutrophils incubated with CD30/Fc chimera or with LPS alone (Figure 7). The other analysed molecules (TNF-α, MMP-9, VEGF, IFN-γ, and IL-17) did not show any significant difference with or without CD30/Fc chimera (data not shown).

In conclusion, neutrophils express CD30L and once activated by LPS, in the presence of CD30/Fc chimera, they increase the production of the proinflammatory cytokine IL-8, able to recruit neutrophils at site of inflammation and to stimulate angiogenesis.

4. Conclusion

The aim of this study was to clarify the role of CD30/CD30L signalling in the pathogenesis of rheumatoid arthritis. First of all we observed an increased percentage of $CD4^+CD30^+$ T cells in the SF of patients with RA compared with controls suggesting an attempt to control inflammation. This result is in accordance with our previously reported hypothesis on the role of $CD30^+$ T cells in RA [18].

Moreover the high percentage of regulatory T cells that display CD30 molecule in the SF of patients affected by RA strengthens the idea that CD30 molecule is expressed by T cells aiming at downmodulating the inflammatory process.

The finding of a higher percentage of $CD30L^+$ T cells in SF than in PB suggests that these cells may be involved in favouring local inflammation.

The kinetic of surface expression of CD30 and CD30L molecules seems to further confirm the observations reported above, since we observed a larger population of $CD30L^+$ T cells with proinflammatory properties in the first 24 hours after cell activation, whereas a larger $CD30^+$ T cell population is present after 48 hours of cell activation suggesting an attempt to switch the inflammatory process off.

We have demonstrated that stimulation of CD30L with the CD30/Fc chimera, a molecule that behaves as sCD30,

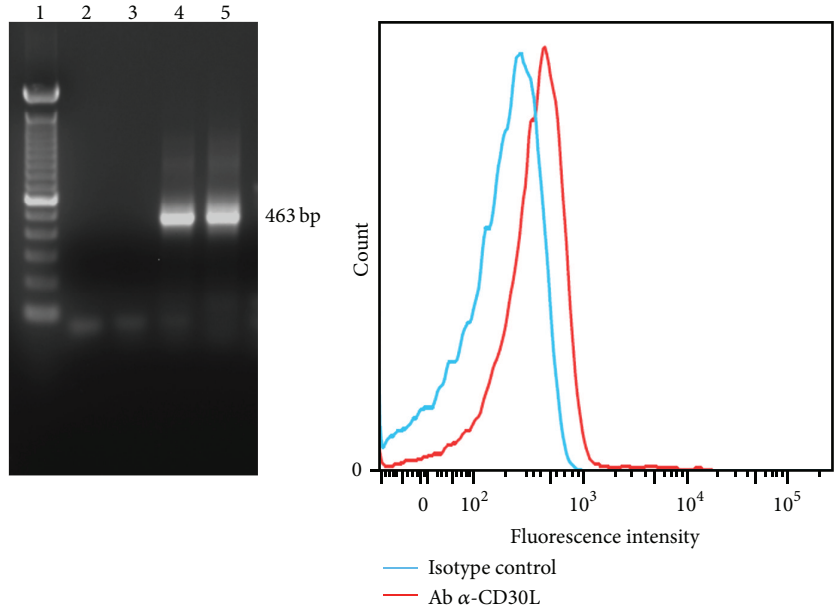

Figure 6: Neutrophils express CD30L. (a) Gel electrophoresis of RT-PCR products obtained from CD30L transcripts in neutrophils (lane 4) and DG75, used as positive control (lane 5) and negative controls (lanes 2 and 3, resp.). (b) CD30L surface expression obtained with FACS analysis.

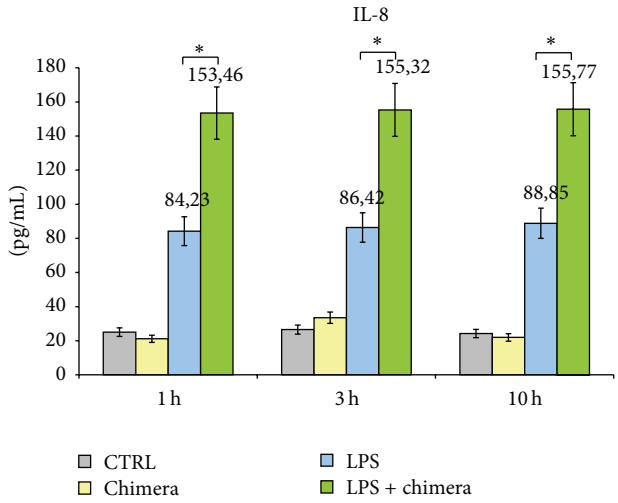

Figure 7: Levels of IL-8 released in the medium by healthy donor's neutrophils in presence or absence of CD30/Fc chimera and activated or not with LPS. Neutrophils incubated with LPS and with CD30/Fc chimera displayed a statistical significant increase in IL-8 production. * indicates a P value < 0.05.

is able to favor the polarization of T cells towards a Th17 phenotype with proinflammatory features. Indeed this has been shown firstly at gene transcripts level by real time PCR, secondly by evaluating the IL-17 secretion in the supernatant, and finally by analysing the cytokine secretion cells by FACS analysis.

These findings are in accordance with data obtained in animal models [21, 35, 36].

In the last part of this work we have confirmed the presence of surface CD30L on neutrophils and that activated neutrophils release IL-8 once stimulated with CD30/Fc chimera, thus amplifying the inflammatory response.

In conclusion the study of $CD30^+$ and $CD30L^+$ cells helps in clarifying the complex pro- and anti-inflammatory mechanisms that take place in RA and possibly pave new avenues for the understanding of the response to therapy and envisage the possibility of novel treatments directed against the CD30L molecule.

Conflict of Interests

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

Authors' Contribution

Alessandro Barbieri and Marzia Dolcino contributed equally to the work.

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Clinical Study

Clinical Characteristics of Cerebral Venous Sinus Thrombosis in Patients with Systemic Lupus Erythematosus: A Single-Centre Experience in China

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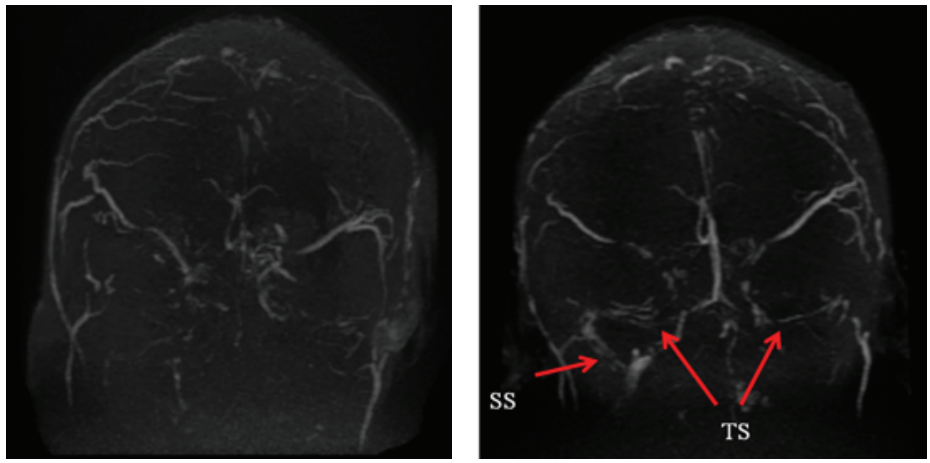
Clinical characteristics of systemic lupus erythematosus (SLE) patients complicated with cerebral venous sinus thrombosis (CVST) between 2000 and 2013 were analyzed through this retrospective, single-centre study. Of 4747 hospitalized SLE patients, 17 (0.36%, 12 females, average age 30) had CVST. Headache (88.2%) was the most common neurological symptom followed by nausea or vomiting (47.1%), conscious disturbance (41.2%), edema of eyelids or conjunctiva (35.3%), blurred vision or diplopia (35.3%), and seizure (35.3%). Increased intracranial pressure (ICP) occurred in 13 cases (76.5%). Magnetic resonance venography (MRV) detected thrombosis in the transverse (82.4%), sigmoid (52.9%), and sagittal (35.3%) sinuses, with frequent (70.6%) multiple sinus occlusions. Compared to SLE patients without CVST, SLE patients with CVST had a higher prevalence of thrombocytopenia and positive antiphospholipid antibodies and a higher SLE disease activity index (SLEDAI) score. 13 patients achieved improvement following glucocorticoids and immunosuppressants treatment, as well as anticoagulants, while 3 patients died at the hospital. CVST is relatively rare in SLE and tends to occur in active lupus patients. Intracranial hypertension is common but nonspecific clinical feature, so MRV evaluation is necessary to establish a diagnosis. Aggressive treatment for the rapid control of SLE activity combined with anticoagulants can improve the prognosis.

1. Introduction

Systemic lupus erythematosus (SLE) is a heterogeneous, multisystem autoimmune disease associated with the production of antibodies against self-antigens. Its pathologic basis is vasculitis induced by immune complex, which suggests that blood vessels might be an important target of autoimmunity overactivity. As a result, there is a wide variation in vessel involvement in SLE, including arteriostenosis and deep venous thrombosis, while glomerulonephritis and defused alveolar hemorrhage can also occur to a certain extent. Cerebral venous sinus thrombosis (CVST) is an uncommon disorder in SLE that is a manifestation of overlapping neuropsychiatric lupus (NPLE) [1] and vessel disease. CVST has been rarely reported and is potentially *fatal* to the patient due to the acute neurological deterioration that can occur. As such, practitioners should be familiar with this condition, so the correct diagnosis can be made as soon as possible to improve patient prognosis.

The etiology of CVST is complicated [2]. In SLE, the main culprits are vascular injuries caused by vasculitis. Antiphospholipid antibodies (APLs) and prothrombotic tendencies might also be responsible for CVST [3].

By investigating the clinical features of CVST in SLE and analyzing the differences between SLE patients with and without CVST, this study seeks to explore the characteristics and risk factors for CVST in SLE patients.



(a) (b)

Figure 1: MRV of a SLE patient with CVST (Case number 5). (a) *Onset of CVST (June 2012)*. Occlusion of superior sagittal sinus (SSS), bilateral transverse sinus (TS), and bilateral sigmoid sinus (SS). (b) *Follow-up after treatment (November 2013)*. Recanalization of bilateral transverse sinus (TS) and right sigmoid sinus (SS) (arrows).

2. Patients and Methods

2.1. Patients. 4747 SLE patients admitted to the Peking Union Medical College Hospital from January 2000 to December 2013 were reviewed. All patients were diagnosed as having SLE according to 1997 American College of Rheumatology (ACR) revised classification criteria [4]. In the SLE cohort, after exclusion of patients with infectious and traumatic CVST or incomplete data, we identified 17 cases (0.36%) complicated with CVST as indicated by clinical features, lumbar puncture, imaging materials (magnetic resonance venography (MRV)), and at least one neurologist's confirmation. For each case ($n = 17$), three age- and sex-matched controls ($n = 51$) were randomly selected from the contemporaneously admitted SLE patients without CVST. The local institutional review board approved the study. Because the study was based on a review of medical records that had been obtained for clinical purposes, the requirement for written informed consent was waived.

2.2. Statistical Analyses. SPSS version 16.0 (SPSS Inc., Chicago, USA) was used to statistically analyze the data. Numerical data and categorical data were expressed as mean ± SD (range) and percentage, respectively. The significance was estimated by Student's t-test, Pearson's chi-square, or Fisher's exact test (when expected frequencies were <5). P values < 0.05 were considered to be statistically significant.

3. Results

3.1. Demographic Characteristics. Among 17 SLE patients with CVST, 12 (70.6%) were women. The median age was 30 years old (range 12–52) and the SLE disease duration was 12 months (range 2–120). The mode of onset was acute (<48 h) in 12 patients (70.6%) and progressive (subacute or chronic) for the other 5 patients. In one case, CVST occurred as an initial manifestation. All patients came from North China.

3.2. Neurological Features of CVST in Lupus. Headache was the most common neurological manifestation (15/17, 88.2%), and among these cases, 8 patients suffered from nausea/vomiting (47.1%). Seven (41.2%) cases had conscious disturbance, 6 (35.3%) had edema of eyelids or conjunctiva, and 6 (35.3%) had blurred vision or diplopia. Hearing loss occurred in 2 cases (11.8%) while 6 cases had seizures (35.3%). As complications, subarachnoid hemorrhage (SAH, 4/17, 23.5%), sinusitis (3/17, 17.6%), and cerebral ischemia and infarction (2/17, 11.8%) were also found in SLE patients with CVST (Table 1).

All patients underwent lumbar puncture, which showed that the intracranial pressure (ICP) was higher than 330 mm H_2O in 7 patients (41.2%) and 6 patients' ICP ranged from 180 to 320 mm H_2O, while the remaining 4 were lower than 180 mm H_2O. Biochemical analysis of cerebrospinal fluid (CSF) indicated that the protein level was higher than normal in 9 patients (52.9%) and glucose and chloride levels were normal. Myelin basic protein (MBP) was elevated in 5 patients. From examination of MRV images, we found that thrombosis in the transverse sinus (TS) was the most common site (14/17, 82.4%), followed by sigmoid sinus (SS, 9/17, 52.9%). Thrombosis in the superior sagittal sinus (SSS, 6/17, 35.3%) and inferior sagittal sinus (ISS, 1/17, 5.9%) was also seen. Twelve patients suffered from more than one sinus thrombosis (12/17, 70.6%) (Figure 1(a)).

3.3. Systemic Manifestations of SLE. Most SLE patients with CVST (16/17, 94.1%) also had involvement of other organs, with lupus nephritis (12/17, 70.6%), hemocytopenia (10/17, 58.8%), arthritis (6/17, 35.3%), fever (6/17, 35.3%), rash (5/17, 29.4%), and serositis (5/17, 29.4%) being the most common conditions. The mean SLE disease activity index (SLEDAI)

Table 1: Neurologic features of the 17 patients with SLE and CVST.

Parameter	Value*
Age at CVST diagnosis, mean ± SD years	28.4 ± 11.3
CVST as initial feature of SLE	1 (5.9%)
Type of onset	
Acute	12 (70.6%)
Progressive	5 (29.4%)
Clinical features of CVST	
Persistent headache	15 (88.2%)
Nausea/vomiting	8 (47.1%)
Conscious disturbance	7 (41.2%)
Blurred vision/diplopia	6 (35.3%)
Edema of eyelids or conjunctiva	6 (35.3%)
Seizure	6 (35.3%)
Hearing loss	2 (11.8%)
Site of CVST occlusion	
Superior sagittal sinus	6 (35.3%)
Transverse sinuses	14 (82.4%)
Sigmoid sinuses	9 (52.9%)
Inferior sagittal sinus	1 (5.9%)
Elevated CSF pressure	13 (76.5%)
Subarachnoid hemorrhage	4 (23.5%)
Sinusitis	3 (17.6%)
Cerebral ischemia or infarction	2 (11.8%)

*Values are the number (percentage); CVST = cerebral venous sinus thrombosis; CSF = cerebrospinal fluid.

score (8 points of CVST excluded) of these patients was 12.1 ± 3.7. Twelve cases had hypoalbuminemia (12/17, 70.6%) and the mean serum albumin level of all 17 cases was (30.4 ± 7.5) g/L.

The prothrombin time and activated partial thromboplastin time were normal in most cases. Three patients had positive antiribosomal RNA-protein (rRNP) antibody. APLs, including anticardiolipin (5/17, 29.4%) antibodies, anti-β_2GP_1 antibodies (4/17, 23.5%), and lupus anticoagulants (6/17, 35.3%), were detected in 7 cases (7/17, 41.2%) in this cohort. Thrombophilia factors (protein S and protein C) were detected in 12 patients, and no protein C or S abnormality was found. Only one patient accompanied other thromboses in the veins (Table 2).

3.4. Comparison with SLE Cases without CVST. Compared with SLE patients without CVST, the incidence of thrombocytopenia (58.8% versus 23.5%, $P = 0.007$) and prevalence of APLs (41.2% versus 15.7%, $P = 0.043$) were significantly higher in SLE patients with CVST. SLE patients with CVST also had much higher disease activity (SLEDAI scores) (20.1 ± 3.7) when compared with SLE patients without CVST (12.8 ± 5.3, $P = 0.001$) (Table 3).

3.5. Treatments and Prognosis. All patients received glucocorticoid (GC) combined with immunosuppressant treatment. Thirteen patients were treated by pulse therapy with GC (methylprednisolone 0.5~1 g/d × 3~5 day). Cyclophosphamide (CTX) was selected as the preferred immunosuppressant for 17 patients, and FK506 or mycophenolate mofetil was added for 2 cases. Moreover, 1 patient had plasmapheresis.

Anticoagulation was utilized in 11 patients, with heparin or low molecular weight heparin (LMWH) being used with preference. Some patients did not receive anticoagulation because of severe SAH or thrombocytopenia. Seven patients had been intrathecally injected about 3 to 5 times with dexamethasone (10 mg), after which their CSF pressure decreased from over 330 mm H_2O to 115~250 mm H_2O.

Thirteen patients clinically improved, and one patient was lost to follow-up. Three patients died during hospitalization. Specifically, one patient died of cerebral hernia, the other one died of pneumonia, and the third one committed suicide. During the follow-up period (mean 36.8 ± 21.7 months), repeat of cerebral MRV was performed in 8 patients and 5 of them had partial or total recanalization of CVST (Figure 1(b)). No patient experienced a relapse of CVST.

4. Discussion

NPLE is one of the most important manifestations of SLE, which occurs in 14%~80% of adult [5] and 22%~95% of pediatric [6, 7] SLE patients. In 1999, ACR named NPLE into 19 categories [8]. CVST is one of the rare and severe cerebrovascular diseases, and its clinical features lack specificity. Thus, rheumatologists should be aware of this rare but potentially fatal complication of SLE.

As an uncommon disorder that accounts for about 0.5% of all strokes [9], CVST presents as a variety of neurologic symptoms and can lead to severe morbidity and mortality [10]. The causes of CVST include infection, dehydration, local trauma, rheumatologic diseases such as SLE and Behçet's disease (BD) [11] as well as thrombophilia including nephritic syndrome, antithrombin III deficiency, pregnancy, cancer, and use of oral contraceptives [2]. The current study showed that the incidence of CVST was about 0.36% in SLE, which is much lower than that of CVST in BD (7.8% [11]), making this condition quite rare. It is not clear when and why CVST could happen and whether this condition is due to high SLE activity.

Several mechanisms might contribute to the formation of CVST in SLE patients. Endothelial cell injury caused by immune complex-induced vasculitis was thought to play an important role in NPLE, which includes CVST. The existence of APLs may be another cause of CVST in SLE [12]. Thrombophilia is related to some SLE complications, such as nephritic syndrome [13] and hyperfibrinogenemia caused by chronic inflammatory status and could represent another etiologic factor. Infections of the middle ear, facial skin, or intracalvarium, which were known as the most common causes of CVST [14], were secondary consequences of GC and immunosuppressant treatment and are sometimes minor factors associated with CVST in SLE. Based on our data, no related craniofacial infections were found in the SLE patients complicated with CVST. Renal involvement was documented

Table 2: Clinical manifestations of SLE patients with CVST.

Number	Gender/age (y)	Organ involvement	SLEDAI	Site of CVST occlusion	Treatment	Outcome
1	F/16	F, K, H	21	SSS, (B) TS, (B) SS	GC (pulse) + CTX	Lost
2	F/18	F, K, H, C	25	(L) TS	GC (pulse) + CTX	Died
3	F/17	F, R, A, K	22	(R) TS, (R) SS	GC + CTX	Died
4	F/20	F, H	15	SSS	GC (pulse) + CTX	Survived
5	M/38	H, K	20	SSS, (B) TS, (B) SS	GC (pulse) + CTX + FK506	Survived
6	F/36	R, A, H, C, P	17	(L) TS, (L) SS	GC + CTX	Survived
7	F/43	R, A, S	18	SSS, (L) TS	GC + CTX	Survived
8	F/52	A, K, S	19	(R) ISS, (L) TS	GC (pulse) + CTX	Survived
9	F/30	F, K, P	24	(R) SS, (R) TS	GC (pulse) + CTX	Survived
10	F/20	S, K, H, TTP	30	SSS, (L) TS	GC (pulse) + CTX + MMF + plasmapheresis	Survived
11	M/30	None	16	SSS, (B) TS	GC (pulse) + CTX	Survived
12	M/14	F, GI, K	20	(R) SS	GC (pulse) + CTX	Survived
13	F/41	R, A, K	18	(B) TS, (B) SS	GC (pulse) + CTX	Died
14	M/28	K, A	20	(L) SS, (L) TS	GC (pulse) + CTX	Survived
15	F/30	R, H	17	(R) TS	GC + CTX	Survived
16	M/34	K	20	(L) SS	GC (pulse) + CTX	Survived
17	F/15	K, CAPS	19	SSS	GC (pulse) + CTX	Survived

Organ Involvement. F: fever, R: rash, H: hemocytopenia, C: cardiac involvement, K: kidney disease, A: arthritis, P: pulmonary involvement, S: serositis, GI: gastrointestinal involvement, and CAPS: catastrophic antiphospholipid syndrome.
Site of CVST Occlusion. B: bilateral, L: left, R: right, SSS: superior sagittal sinus, ISS: inferior sagittal sinus, SS: sigmoid sinus, and TS: transverse sinus.
Treatment. GC: glucocorticoid, CTX: cyclophosphamide, and MMF: mycophenolate mofetil.

in 12 cases, but few of them fulfilled the criteria of nephritic syndrome. APLs were found much more frequently (41.2%) in this group compared to SLE patients without CVST (15.7%), and it hints that these molecules are involved in CVST etiology in SLE. Moreover, most cases had high disease activities and good responses to GC (systemic administration or intrathecal injection) and immunosuppressants, which supports that cerebral vasculitis might be another possible CVST mechanism.

Headaches, which can be persistent and severe and the only neurological sign, are the most frequent symptom of CVST and occur in about 90 percent of CVST patients [15]. In the current study, 88.2% patients suffered from headache, most of which increased gradually over days or weeks, while some had acute-onset mimicking or were accompanied by or even initially presenting as a SAH [16, 17]. Four cases (23.5%) coexisted with SAH in this cohort. The cause of headaches is likely intracranial hypertension (ICH), which can also cause vomiting, papilledema, and diplopia due to involvement of the cranial nerves. Thirteen patients (76.5%) were confirmed as ICH and the pressures in 7 cases were higher than 330 mm H_2O in the current study. Headache is also a common manifestation of NPLE [18]. Most lupus headaches are chronic and are not accompanied by ICH or other neurological signs, while headaches in the majority of CVST cases are acute or subacute. Besides headaches, about half of patients develop other neurological signs to indicate their location to practitioners [19]. SSS involvement frequently leads to paralysis and seizure, while thrombosis of the TS can be an underlying cause of acute aphasia. Isolated or multiple cranial nerve palsies are usually caused by blockade of TS or SS. Cavernous sinus syndrome, manifesting as oculomotor nerve palsies, facial pain, sensory loss in the distribution of the trigeminal nerve, or proptosis and chemosis, is often caused by infections and is very rare in SLE.

Analysis of the cerebrospinal fluid through lumbar puncture is almost always nonspecific but is necessary to differentiate infections. In this study, slightly elevated CSF protein was found in more than half of patients (9/17, 52.9%). MRV is now the most sensitive tool for detecting CVST [19], but its specificity is based on meticulous knowledge of the anatomy and common variations of the cerebral sinus such as left transverse sinus hypoplasia or atresia could be seen in some patients [20]. Therefore, the diagnosis of CVST should be determined not only by imaging but also by clinical manifestations and CSF measures. SSS was reported to be the most frequently involved site (62.5%) and TS was the second (41.2%~44.7%) [15]. Infections always cause thrombosis of the cavernous sinus, TS, or SS. When thrombophilia exists, the SSS is frequently affected. Previously, there were no data concerning CVST associated with SLE. In the current study, TS was the most common site of thrombosis occurrence (14/17, 82.4%) followed by SS (9/17, 52.9%), SSS (6/17, 35.3%), and the inferior sagittal sinus (ISS, 1/17, 5.9%). Twelve CVST patients had been affected by more than one sinus (70.6%).

The current study provided information about the systemic involvement of SLE cases with CVST. The data represented that most cases were accompanied by renal and

Table 3: Clinical comparison of SLE patients with CVST and without CVST.

Characteristics	SLE with CVST ($N = 17$)	SLE without CVST ($N = 51$)	P value
Age (years, mean ± SD)	28.4 ± 11.3	32.7 ± 11.1	0.168
Gender (F/M)	12/5	43/8	0.620
Disease duration (months, mean ± SD)	30.0 ± 39.6	38.2 ± 33.0	0.400
Fever	6/17 (35.3%)	24/51 (47.1%)	0.398
Rash	5/17 (41.2%)	21/51 (41.2%)	0.300
Musculoskeletal involvement	6/17 (35.3%)	27/51 (52.9%)	0.207
Hemocytopenia	10/17 (58.8%)	33/51 (64.7%)	0.516
Lymphopenia	4/17 (23.5%)	18/51 (35.2%)	0.320
Anemia	9/17 (52.9%)	19/51 (37.3%)	0.255
Thrombocytopenia	10/17 (58.8%)	12/51 (23.5%)	0.007*
Serositis	5/17 (29.4%)	11/51 (21.6%)	0.523
Kidney involvement	12/17 (70.6%)	30/51 (58.8%)	0.387
Gastrointestinal involvement	1/17 (5.9%)	7/51 (13.7%)	0.669
Other neurological manifestations	6/17 (35.3%)	11/51 (21.6%)	0.334
Cardiovascular involvement	2/17 (11.8%)	7/51 (13.7%)	1.000
Serum albumin (g/L, mean ± SD)	29.8 ± 6.9	30.7 ± 8.6	0.715
ESR (mm/1 h, mean ± SD)	42.2 ± 24.6	38.0 ± 24.5	0.537
Hypocomplementemia	12/17 (70.6%)	37/51 (72.5%)	1.000
Anti-dsDNA	8/17 (47.1%)	35/51 (68.6%)	0.110
Anti-Sm	6/17 (35.3%)	19/51 (37.3%)	0.885
Anti-RNP	5/17 (29.4%)	24/51 (47.1%)	0.203
Anti-SSA	12/17 (70.6%)	28/51 (54.9%)	0.198
Anti-SSB	1/17 (5.9%)	6/51 (11.8%)	0.670
Anti-rRNP	3/17 (17.6%)	12/51 (23.5%)	0.745
APL	7/17 (41.2%)	8/51 (15.7%)	0.043*
SLEDAI (CVST included, mean ± SD)	20.1 ± 3.7	12.8 ± 5.3	0.001*

* $P < 0.05$.
Anti-dsDNA: antidouble stranded DNA antibody, Anti-Sm: anti-Smith antibody, Anti-SSA: anti-SSA antibody, Anti-SSB: anti-SSB antibody, Anti-RNP: anti-u1 small-nuclear RNA-protein antibody, Anti-rRNP: antiribosomal RNA-protein antibody, APL: antiphospholipid antibody, and SLEDAI: SLE disease activity index.

hematological involvement or arthritis, fever, and rash. The higher prevalence of thrombocytopenia in SLE patients with CVST might partly result from damage caused by autoantibodies and depletion of platelets when thrombosis occurs. The meaning of APLs was apparent and confirmed that these antibodies were involved in CVST in SLE. Concerning SLE activity, the SLEDAI of the CVST group was higher than the controls, indicating that the occurrence of CVST might parallel the systemic active situation of SLE.

The treatment of patients with CVST should include four aspects: removal of precipitating factors, administration of antithrombotic therapy, lowering of ICH, and relieving of neurological symptoms [15, 21, 22]. For SLE patients with CVST, the underlying cause of CVST is SLE. The current study showed that these cases had high SLE activity and rapid disease progression so that aggressive treatments to control SLE are necessary and important. In order to achieve clinical remission as soon as possible, most cases needed GC pulse therapy combined with CTX, which is one of the most powerful immunosuppressants. Anticoagulation was considered as the cornerstone of CVST treatment [9, 21]. Heparin [23] or LMWH was utilized by 11 cases and followed by warfarin in the current study, while SAH [24] and severe thrombocytopenia were contraindications to anticoagulation in other patients. Patients had also been treated with dehydration drugs and intrathecal injections of dexamethasone (10 mg) to normalize their ICP.

In the past, the mortality rate of CVST in SLE reached 30%~50% [25]. In part due to modern neuroimaging and LMWH administration, the mortality rate was reduced to 8%~14% and the outcomes continued to improve [26, 27]. In the current study, the mortality was 11.8% (2/17, 1 case died as the result of suicide rather than CVST). Complete or partial recovery was observed in most patients who survived (13/17, 76.5%), while no patient experienced a relapse of CVST or had a poor outcome with permanent neurological deficits during the mean 3-year follow-up.

5. Conclusions

As one of the rare and severe complications of SLE, CVST usually occurs in active SLE patients and is accompanied by systemic involvement (especially thrombocytopenia). CVST in SLE may be caused by a variety of pathogenic factors,

such as vasculitis and APLs. The clinical picture of CVST is nonspecific and may vary significantly due to different venous sinus involvements, which can make the diagnosis quite difficult. Lumbar puncture and MRV are necessary for establishing a diagnosis of CVST. Treatment should include controlling SLE activity, anticoagulation administration, and ICH management. Early diagnosis and prompt management of CVST and the underlying disease could significantly improve the prognosis of lupus with CVST.

Conflict of Interests

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

Authors' Contribution

Li Wang and Hua Chen contributed equally to this study. Dr. Li Wang, Dr. Hua Chen, and Dr. Wenjie Zheng designed the study. Dr. Li Wang and Dr. Hua Chen analyzed the data and wrote the paper. Dr. Wenjie Zheng revised the paper. Professor Wanli Liu, Dr. Xuan Zhang, and Dr. Fengchun Zhang critically reviewed the paper and provided valuable input. Dr. Yao Zhang assessed and confirmed the neurologic data and images. All authors read and approved the final paper.

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Review Article

Lessons from Microglia Aging for the Link between Inflammatory Bone Disorders and Alzheimer's Disease

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Bone is sensitive to overactive immune responses, which initiate the onset of inflammatory bone disorders, such as rheumatoid arthritis and periodontitis, resulting in a significant systemic inflammatory response. On the other hand, neuroinflammation is strongly implicated in Alzheimer's disease (AD), which can be enhanced by systemic inflammation, such as that due to cardiovascular disease and diabetes. There is growing clinical evidence supporting the concept that rheumatoid arthritis and periodontitis are positively linked to AD, suggesting that inflammatory bone disorders are risk factors for this condition. Recent studies have suggested that leptomeningeal cells play an important role in transducing systemic inflammatory signals to brain-resident microglia. More importantly, senescent-type, but not juvenile-type, microglia provoke neuroinflammation in response to systemic inflammation. Because the prevalence of rheumatoid arthritis and periodontitis increases with age, inflammatory bone disorders may be significant sources of covert systemic inflammation among elderly people. The present review article highlights our current understanding of the link between inflammatory bone disorders and AD with a special focus on microglia aging.

1. Introduction

Bone is a major site of immune responses, termed "osteoimmunology" [1]. An overactive immune response may initiate the onset of inflammatory bone disorders, such as rheumatoid arthritis (RA) and periodontitis, which can be significant sources of covert systemic inflammation. Because the prevalence of RA and periodontitis increases with age, it is important to recognize the contribution of these inflammatory bone disorders in the increasing elderly population worldwide. On the other hand, Alzheimer's disease (AD), the most common form of dementia, is known to be the most common cause of disability in elderly subjects. Although the molecular mechanisms involved in the etiology and pathogenesis of AD have not been completely elucidated, the accumulation of β-amyloid (Aβ) and hyperphosphorylation of tau in the brain is the hallmarks of AD, and microglia-mediated neuroinflammation is well known to be related to the onset and progression of AD due to the release of proinflammatory mediators [2, 3]. Interleukin-1β (IL-1β) is the key molecule involved in the neuroinflammation observed in cases of AD, as IL-1β drives the release of multiple inflammatory mediators by activated microglia, leading to a self-propagating cycle of neuroinflammation, which results in direct neurotoxicity and contributes to promoting the formation of dystrophic neurites [4].

It is well known that chronic systemic inflammation can alter the degree of neuroinflammation in the brain [5, 6]. RA and periodontitis, both chronic systemic inflammatory diseases, not only are associated with other systemic inflammatory diseases, such as atherosclerosis and diabetes, but also directly initiate or hasten the rate of progression of AD [7]. An increasing number of clinical studies have demonstrated the impact of RA and periodontitis on AD [8], and recent experimental studies have clarified the route of transduction of inflammatory signals from RA and periodontitis to the brain. We herein review the current understanding of the link between inflammatory bone disorders and AD.

2. Clinical Evidence for Inflammatory Bone Disorders as a Potential Risk Factor for AD

2.1. Rheumatoid Arthritis and AD. An inverse relationship between RA and AD has been reported since the early 1990s.

A reduced prevalence of AD was described in RA patients who were long-term users of nonsteroidal anti-inflammatory agents (NSAIDs) analyzed in a postmortem survey [9], and a meta-analysis including 17 epidemiological studies demonstrated that NSAID use is a protective factor for AD onset [10]. Furthermore, a prospective study of 7,000 healthy subjects using NSAIDs for joint symptoms, including RA, showed that the long-term use of NSAIDs protects against AD [11]. Another systematic review of multiple prospective and nonprospective studies further showed that NSAID exposure is associated with a decreased risk of AD [12]. More recently, an increased risk of cognitive impairment in patients with midlife RA was confirmed based on a 21-year follow-up of the association between RA or arthritis and dementia/AD in several case-control and hospital- and register-based studies, which indicates that the presence of joint disorders, especially RA, in midlife appears to be associated with a worse cognitive status later in life [13].

2.2. Periodontitis and AD. The first hypothesis of a positive link between periodontitis and AD was raised in 2008. Kamer et al. proposed that periodontitis induces systemic inflammation, which stimulates the production of Aβ and tau protein in the brain, leading to Alzheimer's neuropathology [14]. In addition to the effects of low-grade chronic inflammation itself, periodontitis causes or promotes other chronic systemic inflammatory diseases, including atherosclerosis, cardiovascular disease, and diabetes, indicating that periodontitis is a significant source of systemic inflammatory molecules [15]. Based on the contribution of periodontitis to systemic inflammation, and the potential role of systemic inflammation in the onset of neuroinflammation, it is reasonable to consider that chronic periodontitis is a risk factor for the incidence and progression of AD.

There is growing clinical evidence that chronic periodontitis is closely linked to the initiation and progression of AD. Noble et al. identified a cross-sectional association between a serologic marker of a common periodontitis pathogen, *Porphyromonas gingivalis* (*P. gingivalis*), and poor cognitive test performance among patients older than 60 years in the Third National Health and Nutrition Examination Survey (NHANES-III) [16]. This preliminary study suggested that periodontitis is associated with cognitive impairment, especially in the elderly. Furthermore, Stein et al. examined the serum levels of antibodies against periodontal bacteria in participants who were eventually diagnosed with AD in comparison with that observed in non-AD controls [17] and found elevated levels of antibodies to periodontal bacteria years before the onset of cognitive impairment, suggesting that periodontitis potentially carries a risk of AD development and/or progression.

More recently, Poole et al. assessed the presence of the major three periodontal bacteria, the so-called "red complex" including *Treponema denticola*, *Tannerella forsythia,* and *P. gingivalis* and/or bacterial components in the brain tissue of individuals with and without dementia [18]. The authors obtained statistically significant evidence of the presence of lipopolysaccharide (LPS) from *P. gingivalis* in the AD cases, thus confirming that LPS from periodontal bacteria can access the AD brain during life. Moreover, Riviere et al. detected oral *Treponema* in the trigeminal ganglia, brain stem, and cortex and hippocampus of AD patients using molecular and immunological techniques [19].

Taken together, clinical evidence suggests that the chronic inflammation associated with inflammatory bone disorders may have an important role in increasing the risk of cognitive impairment in cases of AD (Figure 1). However, the exact route by which these disorders transduce systemic inflammatory signals into the brain remains unclear.

3. Oxidative Damages and Cellular Stress Responses in the Inflammatory Bone Disorders and AD

3.1. Oxidative Damage in Inflammatory Bone Disorders. Receptor activator of nuclear factor-κB ligand (RANKL) is a critical factor for the pathogenesis of inflammatory bone disorders due to its requirement for both the formation and activation of osteoclasts [20, 21]. For this reason, inhibition of the RANKL expression represents an innovative therapeutic target for controlling osteoclast formation in inflammatory bone disorders [20]. The nuclear factor-κB (NF-κB) pathway is central for regulating the RANKL-dependent osteoclast formation, function, and survival [22–24], which explains the inhibitory effects on osteoclast formation induced by the prevention of NF-κB activation [22, 25]. Furthermore, reactive oxygen species (ROS) act as intracellular signaling molecules involved not only in the regulation of RANKL-dependent osteoclast formation but also in the peroxidation of lipids and oxidative damage to proteins and DNA [26, 27] as well as RANKL in turn to increase the ROS levels. It has been identified that a decrease in the ROS levels results in a reduction in osteoclast formation and attenuation of bone destruction, as found in both *in vitro* and *in vivo* models [27]. This raises the possibility that antioxidants may be therapeutic targets for the treatment of inflammatory bone disorders.

3.2. Oxidative Damage in AD. Oxidative stress-induced cell damage is the major component of harmful cascades activated in the development of aging-related neurodegenerative disorders, including AD [28–30]. Numerous reports have provided the direct morphological and biochemical evidence indicating a connection between oxidative stress and cell death in the brain of patients with AD [31–33]. Furthermore, ROS accumulate unfolded or misfolded proteins in the AD brain [34], and increasing evidence supports the role of ROS in the pathogenesis of AD, as Aβ oligomers directly generate hydrogen peroxide [35]. Moreover, the overproduction of ROS under condition of oxidative stress acts as a second messenger in signal transduction cascades leading to the activation of mitogen-activated protein kinases (MAPK), as the intracellular redox state of cells regulates cellular signaling pathways [36, 37]. The major MAPK subfamilies, c-Jun N-terminal kinase, p38, and extracellular signal-regulated kinase 1/2 are known to be cell death factors produced in response to oxidative stress [38, 39]. Indeed, the levels of

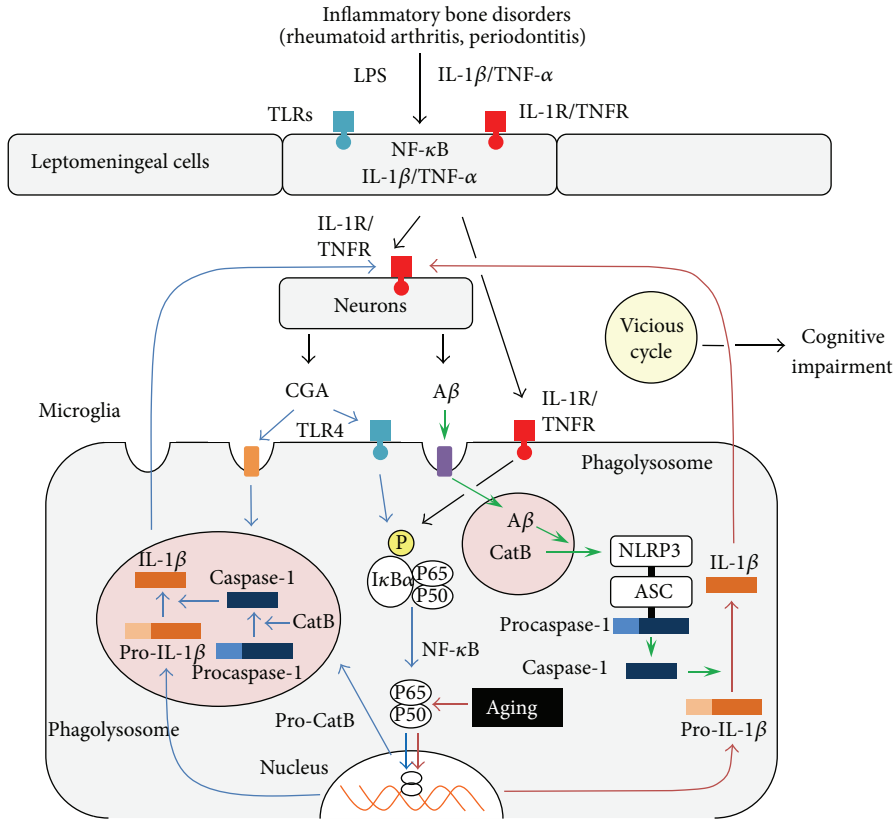

Figure 1: A schematic illustration of the transduction of signals from inflammatory bone diseases to brain-resident microglia through the leptomeninges and two different pathways for the IL-1β production activated by CGA and Aβ in microglia. In individuals with inflammatory bone diseases, IL-1β and TNF-α secreted by macrophages and periodontal bacterial components, including LPS, activate IL-1R/TNFR and TLRs localized on the surface of leptomeningeal cells to secrete IL-1β and TNF-α. IL-1β and TNF-α then stimulate both brain-resident microglia and neurons. After stimulation, the neuronal production and secretion of CGA and Aβ is increased. CGA and Aβ subsequently activate two different pathways for the IL-1β production in microglia, the NLRP3 inflammasome-CatB pathway via Aβ (open arrows), and the phagosome-CatB pathway via CGA (blue arrows). The NF-κB pathway activated during aging (red arrows) supports the production and secretion of IL-1β by Aβ. The leptomeningeal cell-neuron-microglia interactions form a vicious cycle of IL-1β production, culminating in the onset of cognitive impairment in AD.

phosphorylated MAPKs are increased in the postmortem brains of AD patients [40–42]. Therefore, antioxidant therapy is considered to be a promising approach for the prevention and clinical management of AD [43, 44].

3.3. Redox-Dependent Control of Oxidative Damage and Cellular Stress Responses: Relevance to Antioxidant Strategies. The modulation of endogenous cellular defense mechanisms represents an innovative approach to providing therapeutic interventions for diseases causing chronic tissue damage, including inflammatory bone disorders as well as AD. Cellular stress response consists of prosurvival pathways controlled by cytoprotective genes called vitagenes [45] that stimulate the production of molecules endowed with antioxidant and antiapoptotic potential [46]. Vitagenes include members of the heat shock protein (Hsp) family, such as heme oxygenase-1 and Hsp72, sirtuins, and the thioredoxin/thioredoxin reductase system [47, 48]. Increasing evidence suggests that the Hsps promotes cytoprotective conditions in the human disease states, such as chronic inflammation, bone diseases, and AD, as thoroughly reviewed by Calabrese et al. [46–48]. Furthermore, previous studies have indicated that sirtuin 1 is a potent inhibitor of NF-κB transcription [49]. Therefore, vitagenes are emerging candidates as pharmacological agents for treating antioxidative damages [50].

4. Inflammatory Bone Disorders and Neuroinflammation

4.1. IL-1β Processing in Inflammatory Bone Disorders. Bone is sensitive to an overactive immune responses, which are mediated by macrophages, dendritic cells, and T cells, for diverting proinflammatory mediators in the bone microenvironment. In particular, the dramatic impact of proinflammatory mediators on bone cells, including osteoclasts and osteoblasts, is a key component of the pathogenesis of inflammatory bone disorders.

IL-1β is considered to be a critical inducer of the pathogenesis and tissue damage observed in cases of inflammatory bone disorders, including RA [51] and periodontitis [52], as IL-1β impairs the migration of osteoblasts [53] and upregulates the RANKL expression induced by osteocytes [54]. Our

recent studies suggest that osteoclast precursors are capable of producing multiple mediators [20]. Furthermore, IL-1β is used as a biomarker to assess the therapeutic outcomes of patients with chronic periodontitis [55]. Therefore, IL-1β has become a focus of research due its potential as an attractive therapeutic target for inflammatory bone disorders [20].

The processing and secretion of IL-1β are tightly regulated by a two-step mechanism. The first step consists of the transcription and production of pro-IL-1β which depends on the activation of NF-κB by Toll-like receptors (TLRs), and the second step consists of the activation of inflammasomes, which in turn activate procaspase-1. Inflammasomes are multiprotein oligomers consisting of the NOD-like receptor (NLR) family, including the pyrin domain-containing 3 (NLRP3), and the non-NLR family proteins, melanoma 2 receptor, and procaspase-1. Upon activation, the NLRP3 inflammasome binds to an adaptor protein, ASC, which in turn recruits procaspase-1 for autoactivation. Finally, caspase-1 cleaves pro-IL-1β, which is then secreted in a mature form [56]. Recent studies have provided evidence that the NLRP3 inflammasome is involved in the pathogenesis of both RA and chronic periodontitis. For example, Walle et al. showed that the negative regulation of NLRP3 inflammasome activation induced by A20 markedly protects against the onset of RA-associated inflammation and cartilage destruction, highlighting the contribution of the NLRP3 inflammasome to the pathology of RA [57]. Similarly, Park et al. found significantly higher levels of the NLRP3 inflammasome and caspase-1 in the gingival tissues of patients with chronic periodontitis compared to that observed in healthy controls. Furthermore, the activation of both the NLRP3 and AIM2 inflammasomes is necessary for IL-1β secretion after stimulation with *P. gingivalis*, the main bacteria that induces periodontitis [58].

4.2. Peripheral Fiber Sprouting in Inflammatory Bone Disorders. Peripheral nervous fiber sprouting is observed in inflammatory bone disorders and considered to be associated with pain, the major symptom of inflammatory bone disorders. However, our previous studies using an animal model of RA, adjuvant arthritic (AA) rats, showed that sprouting of peripheral sensory fibers is closely linked with bone remodeling and local inflammatory processes [59]. Infiltrated macrophages and $CD4^+$ T cells produce nerve growth factor (NGF) and express its high-affinity receptor, TrkA, in the inflamed synovial tissues. However, a dense network of its low-affinity receptor, p75-positive nerve fibers, with numerous terminal varicosity, is also observed. These findings suggest that infiltrating mononuclear cells secrete NGF in an autocrine or paracrine manner in the inflamed synovium to promote sensory nerve fiber sprouting, as most of new sprouting GAP-43-positive fibers are calcitonin gene related peptide (CGRP)-positive sensory fibers located around osteophytes. However, the surgical reduction of CGRP-positive sensory fibers via resection of the sciatic nerves prior to adjuvant injection suppresses the size of osteophytes and delays the recovery of inflammation due to the effects of prolonged infiltration of Th1 cells in the synovial tissues in AA rats. These observations suggest the involvement of sporting sensory fibers in the bone remodeling and local inflammatory processes observed in inflammatory bone disorders.

More recently, the function of sprouting sympathetic fibers in inflammatory bone disorders has also been noted. Longo et al. showed that the sprouting of sympathetic fibers into the upper dermis of the skin, which results from increases in the mature NGF levels in the skin following joint and bone damage, is present in AA rats. The pharmacological suppression of the sympathetic fiber function with systemic guanethidine significantly decreases the pain-related behavior associated with arthritis in these animals, thus suggesting that sprouting sympathetic fibers contribute to the pain-related behavior associated with inflammatory bone disorders [60]. Furthermore, the dense sympathetic innervation of joints suggests the importance of this phenomenon in the onset of inflammatory bone disorders, as the sympathetic system controls the blood flow in the joint as well as the degree of vascular permeability and thus influences the extent of inflammation, as immune cells exhibit adrenergic signaling pathways. Therefore, the sprouting of sympathetic fibers may have local effects on osseous tissues in addition to effects on the local and systemic immune functions in cases of inflammatory bone disorders [61]. Taken together, these findings indicate that inflammatory bone disorders are a source of chronic systemic inflammation.

4.3. Inflammatory Bone Disorders and Neuroinflammation. It is well known that chronic systemic inflammation has causal links to neuroinflammation via the actons of systemically released proinflammatory mediators including IL-1β and that microglia are the primary brain cells responding to systemic inflammation [5, 6]. Repeated LPS-induced chronic systemic inflammation in mice results in prolonged IL-1β production as well as microglial activation in the brain [62]. Furthermore, mice subjected to systemic inflammatory challenges in late gestation are predisposed to develop AD-like neuropathology during the course of aging. These mice also display chronic elevation of IL-1β, an increased expression of hippocampal amyloid precursor protein and its proteolytic fragments and enhanced tau phosphorylation, thus resulting in a significant impairment of working memory in old age. More importantly, this phenotype is strongly exacerbated when the prenatal infection is followed by a second systemic inflammatory challenge in adulthood, further suggesting that systemic inflammation represents a major risk factor for the development of AD [63].

Several routes by which systemic immune signals may be transmitted to the brain have been studied intensively [5, 6]. First, the direct pathway involves the circumventricular organs, specialized regions lacking a contiguous blood-brain barrier. In circumventricular organs, pathogen-associated molecular patterns induce the production and release of proinflammatory mediators stimulated by macrophage-like cells expressing TLRs. These cytokines are able to enter the brain by volume diffusion. A second route involves the IL-1 receptors located on the perivascular macrophages and

endothelial cells of brain capillaries. The activation of IL-1 receptors by circulating mediators initiates the release of cytokines into the brain, without the physical entry of constituents across the blood-brain barrier. A third route comprises the overflow of cytokine transporters into the systemic circulation, which then allows cytokines to gain access to the brain through these transport systems. A fourth route involves the transmission of systemic immune signals via the autonomic nervous system. Systemic cytokines directly stimulate primary afferent nerves, such as the vagus nerve, which in turn activate central pathways involved in sickness behavior.

In addition to these four "classical" routes, we further found that a leptomeningeal pathway may be involved. The leptomeninges covering the surface of the brain parenchyma provide a physical boundary at the cerebrospinal fluid-blood barrier. The activation of leptomeningeal cells by circulating cytokines induces the production and release of proinflammatory cytokines into the brain [64, 65]. Therefore, leptomeningeal cells are able to transmit signals from systemic immune cells into the brain-resident microglia (Figure 1).

5. Age-Dependent Differences in the Microglial Responses Acting via Leptomeninges in Inflammatory Bone Disorders

5.1. Age-Dependent Differential Microglial Responses in AA. It is interesting to note that inflammatory bone disorders induce age-dependent differential responses in microglia. Using a model of RA, rat AA, we found that activated microglia in the proximity of the leptomeninges produce anti-inflammatory cytokines, such as IL-10 and transforming growth factor-β1 (TGF-β1), in young adult AA rats [6, 66, 67]. In contrast, activated microglia in close proximity to the leptomeninges in middle-aged AA rats produce IL-1β and exhibited an increased expression of prostaglandin E_2 (PGE_2) synthesizing enzymes, such as cyclooxygenase-2 and microsomal prostaglandin synthase-1 [68]. In cultured leptomeningeal cells, IL-1β and PGE_2, respectively, caused the marked loss of occludin and ZO-1, two major tight junction proteins: pretreatment with IL-10 and TGF-β1 significantly antagonizes these effects. Therefore, chronic systemic inflammation induces age-dependent phenotypic changes in microglia, yielding an anti-inflammatory cell phenotype in young rats and a proinflammatory cell phenotype in middle-aged rats. Furthermore, age strongly influences the barrier functions of the leptomeninges as a result of the age-dependent differential microglial responses in the setting of inflammatory bone disorders [68].

5.2. Functional Outcomes of Differential Microglial Phenotypic Changes in AA. These observations prompted further investigation of the functional outcomes of the resultant differential microglial phenotypic changes noted under condition of chronic systemic inflammation. We therefore examined the effects of chronic systemic inflammation on long-term potentiation (LTP) in the hippocampus in young adult and middle-aged rats, as LTP is the cellular substrate for learning and memory [69]. Consequently, the incidence of LTP in the Schaffer collateral-CA1 synapses was not affected in the young adult AA rats, whereas the formation of hippocampal LTP was significantly impaired in the middle-aged AA rats. The systemic administration of minocycline, a known inhibitor of microglial activation, significantly restored the magnitude of LTP in middle-aged AA rats. These observations suggest that chronic systemic inflammation induces deficits in the hippocampal LTP in middle-aged rats via the effects of neuroinflammation, which is primarily induced by brain-resident microglia.

Therefore, it is considered that microglia may be primed during aging, even by middle age. Primed microglia are hyperresponsive to secondary stimuli and can thus produce an exaggerated inflammatory response in the brain. It is also considered that age-dependent autophagic and lysosomal dysfunction allows for the dominance of ROS-hypergenerating older mitochondria in microglia. The increased levels of intracellular ROS, in turn, activate redox-sensitive transcription factors, such as NF-κB, to provoke an exaggerated inflammatory response [70]. Therefore, increased oxidative stress and the resultant activation of redox-sensitive transcription factors observed during aging may drive the emergence of senescent-type microglia (microglia aging). This may explain why Aβ, which is not capable of sufficiently activating NF-κB, is able to induce the secretion of IL-1β by activated microglia isolated from the aged mouse brain but cannot induce IL-1β secretion from the young adult mouse brain [71]. These observations may partly explain why senescence is an important causative factor for AD. It is important to note that the inflammatory bone disorders RA and periodontitis are generally found in the middle-aged and older populations, thus further indicting the risk association between inflammatory bone disorders and AD.

6. Aβ and Chromogranin A (CGA): Key Players in the Neuroinflammation in the AD Brain

6.1. Activation of the NLRP3 Inflammasome-Cathepsin B (CatB) Pathway by Aβ. Latz's group has proposed a model for the activation of the NLR family, NLRP3 inflammasomes. According to this model, the phagocytosis of various molecules, including fibrillar Aβ42 and silica crystals, by LPS-primed microglia/macrophages causes phagosomal destabilization and lysosomal rupture. The subsequent secretion of CatB into the cytoplasm triggers the activation of the NLRP3 inflammasome directly or indirectly, thereby leading to the production and secretion of mature IL-1β [66, 67]. Recently, CatB was found to directly interact with the leucine-rich-repeat domain of NLRP3 [72]. After activation, the NLRP3 inflammasome mediates procaspase-1 activation to promote the processing and secretion of IL-1β. Therefore, caspase-1 is an essential enzyme for IL-1β production, as it is required for the processing of inactive precursors into mature, active forms that can then be secreted

from cells (Figure 1). Latz's group also recently reported that NLRP3-deficient mice carrying mutations associated with familial AD show improvements in spatial memory deficits, reductions in the expression of caspase-1 and IL-1β in the brain, and enhanced Aβ clearance [73]. These observations suggest that Aβ activates microglia surrounding plaques to induce IL-1β production via the NLRP3-CatB pathway.

6.2. Activation of the Phagolysosome-CatB Pathway by CGA. In addition to Aβ, CGA, a neurosecretory acidic glycoprotein, is found in 30–40% of AD neuritic plaques [74], and CGA-positive plaques are surrounded more frequently by hyperactivated microglia in comparison to that observed in the case of Aβ-positive neuritic plaques [72]. More importantly, CGA alone is capable of inducing IL-1β production by microglia, whereas Aβ induces the IL-1β production only by LPS-primed or senescent-type microglia [71].

There is evidence suggesting that CatB is associated with the maturation of pro-IL-1β in the endosomal/lysosomal system, as CatB can effectively cleave procaspase-1 in a cell-free system only at an acidic pH [75, 76]. We recently demonstrated that CatB and caspase-1 are colocalized and that CA-074Me markedly inhibits the caspase-1 expression in the CGA-induced proteolytic processing of procaspase-1 to its mature form in the lysosome-related vesicles of microglia, which contain inactive forms of IL-1β [71, 77]. Furthermore, there are no signs of any leakage of CatB in microglia following treatment with CGA. The typical size of primary lysosomes is less than 1 μm in diameter, whereas the mean diameter of CatB-containing enlarged lysosomes in CGA-stimulated microglia is 4.2 μm. These findings are consistent with previous observations showing that IL-1β and CatB are colocalized in phagolysosomes and that the secretion of IL-1β involves the exocytosis of phagolysosomes in LPS-activated human monocytes [77, 78] (Figure 1). However, the possibility that CatB is indirectly involved in the activation of caspase-1 via the proteolytic maturation of caspase-11 cannot be totally ruled out, as caspase-11 can activate procaspase-1 [79]. Furthermore, CGA leaked from damaged neurons also activates microglia surrounding plaque to induce IL-1β production at an earlier age than fibrillar Aβ via the phagolysosome-CatB pathway [71].

6.3. Increase in Aβ and CGA following the Onset of Inflammatory Bone Disorders. Our preliminary experiments showed that AA and chronic systemic treatment with *P. gingivalis* LPS induces the intracellular accumulation of Aβ and CGA in hippocampal pyramidal neurons, leading to memory impairment in middle-aged animals (Wu et al., unpublished observations, Figure 1). However, further studies are needed to clarify the mechanism underlying the accumulation of Aβ and CGA in presence of inflammatory bone disorders. It is noteworthy that the IL-1β secreted from activated microglia further accelerates tangle formation in cortical neurons via tau hyperphosphorylation, thus indicating that activated microglia may also play important roles in tau pathology in AD [80].

7. Conclusion

Recent evidence indicates that inflammatory bone disorders are potential risk factors for AD. In individuals with chronic inflammatory bone disorders, proinflammatory blood cells and bacterial components, including LPS, activate the receptors localized on the surface of leptomeningeal cells, which in turn activate brain-resident microglia to evoke neuroinflammation (Figure 1). The intense neuroinflammation evoked by senescent-type microglia may contribute to the initiation and progression of AD, resulting in cognitive impairment. Therefore, providing early treatment of inflammatory bone disorders may delay the onset and limit the severity and/or progression of AD. More importantly, microglia undergo several morphological and functional changes involving the induction of exaggerated neuroinflammation in response to systemic inflammation during normal aging. Therefore, pharmacological approaches aimed at rejuvenating senescent-type microglia may also constitute a promising avenue for future research to reduce the risk of AD.

Conflict of Interests

The authors declare no competing financial interests.

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Review Article

The Novel PKCθ from Benchtop to Clinic

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The protein kinases C (PKCs) are a family of serine/threonine kinases involved in regulating multiple essential cellular processes such as survival, proliferation, and differentiation. Of particular interest is the novel, calcium-independent PKCθ which plays a central role in immune responses. PKCθ shares structural similarities with other PKC family members, mainly consisting of an N-terminal regulatory domain and a C-terminal catalytic domain tethered by a hinge region. This isozyme, however, is unique in that it translocates to the immunological synapse between a T cell and an antigen-presenting cell (APC) upon T cell receptor-peptide MHC recognition. Thereafter, PKCθ interacts physically and functionally with downstream effectors to mediate T cell activation and differentiation, subsequently leading to inflammation. PKCθ-specific perturbations have been identified in several diseases, most notably autoimmune disorders, and hence the modulation of its activity presents an attractive therapeutic intervention. To that end, many inhibitors of PKCs and PKCθ have been developed and tested in preclinical and clinical studies. And although selectivity remains a challenge, results are promising for the future development of effective PKCθ inhibitors that would greatly advance the treatment of several T-cell mediated diseases.

1. Introduction

Cells respond to environmental stimuli through complex signal transduction pathways. Among key players are the protein kinase C (PKC) family highlighted by numerous studies in regulation of the cell cycle, cancer development, and the stress response [1]. The particular PKC isozyme activated, its cellular localization, and the ensuing protein-protein interactions differentially affect cell survival [2]. Primarily expressed in lymphoid tissues, hematopoietic cells, and muscle cells [3], the novel isozyme PKCθ shares its regulatory N-terminal domain and C-terminal catalytic domain with other PKC family members [1]. PKCθ, however, plays a unique role in immune responses by modulating multiple molecules such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein 1 (AP-1), mitogen-activated protein kinase (MAPK), and c-Jun N-terminal kinases (JNK). Interestingly, it is the only member of the PKC family known to translocate to the immunological synapse between a T cell and an antigen-presenting cell (APC) upon T cell receptor-peptide MHC recognition [4, 5]. PKCθ interacts physically and functionally with downstream effectors to mediate T cell activation, differentiation, and migration. In addition to its role in inflammation, PKCθ is implicated in certain disorders ranging from autoimmunity, neuroinflammatory diseases, muscular dystrophy, cancer, and diabetes. Here we review experimental studies done on PKCθ and their contribution to the development of new therapeutic agents, targeting PKCθ, particularly in inflammatory contexts.

2. The Structural and Functional Features of PKC Family Members

The PKC family consists of 12 serine/threonine kinases that are divided into three groups based on their corresponding activators/cofactors, conventional (cPKCs), novel (nPKCs), and atypical (aPKCs). The cPKCs include the α, β, and γ isozymes which are activated by Ca^{2+}, diacylglycerol (DAG) and tumor-promoting phorbol esters (PMA), in the presence of phosphatidylserine (PS) [6, 7]. The nPKCs (ε, η, δ and θ) are activated by DAG and PMA only. The aPKC group includes ι, ζ, and μ kinases which are not activated by Ca^{2+}, DAG, or PMA but depend on protein-protein interaction for activation such as p62 in the case of PKC ζ [8–10]. An additional group in the PKC family named PKC-related-kinases (PRKs) was also described [11]. This group is also considered the fourth of the PKC family and consists of three members PRK1–3. Like aPKCs, PRKs do not bind Ca^{2+}, DAG, or phorbol esters. They are similar in structure to PKCs except for the C1 domain. In addition, PRKs have HR1 motifs which are not present in other PKCs and are presumably responsible for the RhoA binding property of the PRKs.

The structure of protein kinases consists of a regulatory N-terminal domain and a catalytic C-terminal domain held together by a hinge region [12]. Cleavage of the hinge liberates the catalytic domain leading to constitutive activation of PKC. The catalytic domain includes phosphorylation and autophosphorylation sites (discussed later) and, hence, is referred to as the kinase domain. It also contains two highly conserved regions among all PKC isozymes; the C3 element consists of an ATP-binding site and the C4 region is dedicated for substrate binding [12]. On the other hand, the regulatory moiety contains three domains, the pseudosubstrate domain (autoinhibitory sequence), the C1 domain that binds DAG and phorbol esters, and the C2 domain that binds Ca^{2+} [1]. All protein kinases possess the pseudosubstrate domain, but not all isozymes have functional C1 and C2 cofactor binding domains [13]. For instance, cPKCs contain pseudosubstrate, C1 and C2 domains. The nPKCs have pseudosubstrate, C1 and a variant form of C2 domain making it insensitive to Ca^{2+} activation. The atypical PKCs possess a variant form of C1 with the absence of C2 domain [13].

2.1. Characteristics of Kinase Catalytic Domain and Pseudosubstrate Domain. The structure of the kinase domain was brought to light when the crystal structure of protein kinase A was first resolved by Knighton and colleagues in 1991 [14]. The ATP and protein substrate bind in the kinase cleft situated between two lobes, consisting of β-sheets at the N-terminus and α helix on the C-terminus [15, 16]. Before it becomes catalytically competent, yet still inactive, nascent PKCs undergo conformational changes. Such changes occur at three phosphorylation sites that are conserved, among PKC isozymes as well as protein kinases A and G [9]. These sites are located at the activation loop (also referred to as T-loop) positioned at the tip of the kinase domain, at the turn-motif named after the "apex of a turn" structure of the PKA, and at the hydrophobic motif in the C-terminal end of the kinase domain. The order by importance of the phosphorylation starts with the foremost and the rate-limiting phosphorylation at the activation loop by phosphoinositide-dependent kinase (PDK-1) [13, 17–19]. PDK-1 requires phosphatidylinositol-3,4,5-triphosphate for PKC ζ phosphorylation [20–22]. In absence of PDK-1, PKC isozymes become prone to rapid degradation before turning into catalytically competent enzymes [23]. The second step of phosphorylation continues with the phosphorylation of the turn-motif (T638 in PKCα and S643 in PKCδ) followed by phosphorylation of the hydrophobic motif (S657 in PKCα and S662 in PKCδ) [24–26]. In cPKCs, the turn motif and the hydrophobic motif are autophosphorylated, whereas in nPKCs autophosphorylation occurs only on the turn motif; phosphorylation on the hydrophobic motif is carried out by other kinases. Unlike other PKCs, phosphorylation of the activation loop in PKCθ is sufficient for NF-κB stimulation [27]. Studies have shown that mutation at the hydrophobic motif replacing the phosphorylated residue serine by alanine contributes to PKCs thermo-instability [28, 29]. Therefore, the hydrophobic motif, but not the activation loop, is considered a direct mediator for PKC stability [23]. It appears that the hydrophobic motif actually functions as a docking-site for PDK-1 enzyme through its repetitive negatively charged aspartate sequence called PDK-1 interacting fragment (PIF) [16, 30]. This interaction allows PDK-1 to access the activation loop. The atypical PKCs possess an acidic phosphomimetic aspartic acid or glutamic acid in the hydrophobic motif that enhances binding of PDK-1 and phosphorylation of the activation loop [17, 18]. In addition to PDK-1, rapamycin (mTOR) complex 2 (mTORC2) regulates the phosphorylation of the turn motif rather than the hydrophobic motif in cPKC isozymes and novel PKCε [31]. How such phosphorylation protects PKCs from degradation is still not fully understood. However, it is well established that the acidic residues surrounding the activation loop and the binding of the pseudosubstrate post-phosphorylation are essential for enzyme stability [32].

The pseudosubstrate domain is located at the extremity of the regulatory site. It was first described by Kemp and colleagues as a stretch of amino acids that resembles the substrate, except that it contains an alanine residue instead of serine/threonine [33]. A pseudosubstrate has a dual function; it controls both maturation and activation prior to cofactor binding [34]. As mentioned earlier, nascent PKCs need to be phosphorylated to become mature or catalytically competent. Binding of the pseudosubstrate shields the catalytic loop from PDK-1 and prevents its phosphorylation as shown in *in vitro* experiments [35]. Therefore, for PDK-1 to phosphorylate the kinase domain, PKC kinase domain should be in an "open" position devoid of any pseudosubstrate [35]. Once PDK-1 phosphorylates the activation loop, the kinase domain PKC becomes catalytically competent; it undergoes a conformational change indulging the pseudosubstrate to bind at the substrate-binding site. At that point, PKC is said to be "mature" and resistant to phosphatases [35]. For PKC to become catalytically active, upon cofactor binding (DAG,

Ca^{2+} and PS), another conformational change displaces the pseudosubstrate from the substrate-binding site giving access to the substrate and allowing subsequent phosphorylation [35].

2.2. The Topological Properties of PKCs. The localization of PKC family members in the cell dictates their respective functions. Compartmentalization of PKCs to the membrane is mediated by scaffold/adaptor proteins [34]. Scaffold proteins interact with nascent/competent, mature and active PKC isozymes regulating the kinases' activities either positively or negatively. Examples of scaffold proteins are: receptor for activated C kinase (RACK), substrates that interacts with C kinase (STICK), receptor for inactive C kinase (RICK), and A-kinase activating protein (AKAP) [34]. RACKs and STICKs bind to active PKCs whereas AKAPs and RICKs interact with inactive PKCs. Binding of RACK increases the phosphorylation capacity of PKCs several-folds independently from the substrate identity [36]. However, STICK itself acts as a substrate for PKC in addition to its function as an anchoring protein [36, 37]. Caveolins represent another group of scaffold that helps PKCα and PKCζ translocate to the caveolar microdomains where they are subsequently activated [38]. AKAP79 recruits PKC isozymes to the postsynaptic dendritic fraction rendering them inactive [39]. Several other scaffold proteins such as CARMA1 (CARD-containing MAGUK protein 1), 14-3-3τ, and Vav1 are particularly involved in regulating PKCθ's translocation and activation and will be discussed later in the manuscript.

2.3. Termination of the Kinase Activity in PKCs. The kinase activity of PKCs is terminated by dephosphorylation [40]. However, this process takes place when protein kinases are in an "open" conformation, in other words, when the kinase domain is unbound by the pseudosubstrate or when a particular PKC is constitutively active [41]. For cPKCs and nPKCs, dephosphorylation is carried out by the PP2C member PHLPP (pleckstrin homology domain leucine-rich repeat protein phosphatase) at the hydrophobic motif and by PP1/PP2A protein phosphatases at the turn motif [40, 42–44]. In other contexts, the effect of phosphatases on PKCs is indirect. For instance, the dephosphorylation of PKCθ downstream molecules, CARMA1, by PP2A leads to PKCθ deactivation [45]. Hence, dephosphorylation predisposes "naked" protein kinases to ubiquitination and degradation [46, 47]. There are two types of ubiquitination, proteasomal and lysosomal ubiquitination. The former requires multiple ubiquitin tags while the lysosomal pathway involves a monoubiquitination [48]. Many PKC isozymes, including α, δ, and ε, undergo proteasomal ubiquitination in response to tumor-promoting phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Other protein kinases undergo lysosomal ubiquitination such as PKCθ (discussed in the next section) and PKCε. Importantly, ubiquitination not only mediates protein degradation but can also modify the kinase activity. Indeed, monoubiquitinated PKCε promotes IKKβ phosphorylation, thereby triggering tumorigenesis [49].

3. A Novel PKC Isoenzyme, PKCθ

3.1. Structural Domains of PKCθ. Primarily expressed in lymphoid tissues and hematopoietic cells [50], PKCθ is a single polypeptide kinase composed of 706 amino acids that typically phosphorylates serine or threonine residues. PKCθ shares its structure with other PKC family members; it contains a regulatory N-terminal domain and a C-terminal catalytic domain tethered together by a hinge region as seen in Figure 1 [1]. The regulatory domain of PKCθ consists of the C2-like domain sequence, similar to the Ca^{2+}-binding C2 domain of other PKCs, except that it does not really bind Ca^{2+}. The C2-like domain allows PKCθ to interact with a receptor for activated C kinase (RACK) which regulates its translocation to the membrane [49]. In addition to the C2-like domain, the regulatory domain of PKCθ includes C1a and C1b domains that have diacylglycerol (DAG) and phorbol esters binding sites [51]. The C1b domain has much higher affinity for diacylglycerol than the C1a domain [52]. The pseudosubstrate region in the C1a domain consists of a small sequence of amino acids that mimics a substrate and binds to the substrate-binding cavity in the catalytic domain [33]. However, this sequence lacks phosphorylatable serine and threonine so it prevents access of substrates to the catalytic domain and keeps the enzyme inactive. Moreover, regulatory domains include the variable V1, V2 and V3 domains. The V3 domain, with its proline-rich motif, is unique to PKCθ, essential and sufficient for its translocation to immunological synapses by linking it to CD28 receptor via the kinase Lck [53, 54].

The crystal structure of PKCθ catalytic domain has been published in 2004 [55] revealing an N-terminal lobe and a C-terminal lobe. The catalytic C-terminal domain consists of an ATP binding site, V4, substrate binding site, and V5. ATP binds to a glycine-rich loop (GXGXXG) at the interface of the two lobes while the substrate binds to an αC helix. Additionally, important elements of the conserved catalytic domain include a kinase activation loop with phosphorylatable threonine 538 (pT538), a hydrophobic motif containing phosphorylatable serine 695 (pS695), and a turn motif containing conserved phosphorylatable serine 676 (pS676) and phosphorylatable serine 685 (pS685) [55]. The catalytic domains of PKCs are highly conserved, with the exception of the variable V5 region consisting of 60–70 amino acids. This variable domain highly contributes to the regulation of PKCα activity through multiple mechanisms; by stabilizing the kinase through direct interactions with its N-lobe, by interacting with the pseudosubstrate in the N-terminal regulatory domain and by mediating subcellular localization through interaction with RACK [56]. Nothing has been published yet on the role of the V5 domain in PKCθ isozyme.

3.2. Physical and Functional Interactions of PKCθ with Substrates and Regulators. PKCθ can interact either physically or functionally, activating or synergizing with the activity of other proteins. Many examples will be summarized in this section starting with T cells proteins. The 14-3-3 family

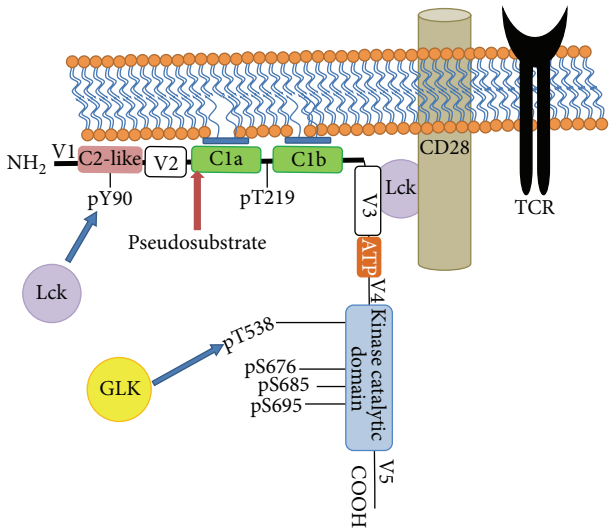

Figure 1: A schematic model of membrane-associated PKCθ in TCR/CD28 stimulated T cells. Regulatory N-terminal domain consists of V1, C2-like, V2, C1a, C1b, and V3 domains. Catalytic C-terminal domain consists of ATP binding site, V4, kinase catalytic domain (substrate binding site), and V5. PKCθ binds to the membrane through diacylglycerol by its C1a and C1b domains. It interacts with CD28 via Lck through its V3 domain. Blue arrows represent phosphorylation by respective enzymes on specific amino acid residues. V: variable domain; C: constant domain; GLK: germinal center kinase- (GSK-) like kinase; Lck: Lymphoid cell kinase; TCR: T cell receptor.

proteins were described as potential regulators of PKCs [57]. These proteins associate with several protooncogene and oncogene products modulating their activity. 14-3-3τ isoform is highly expressed in T cells and associates with PKCθ *in vitro* and in intact T cells. 14-3-3τ binds directly to PKCθ in the cytosol, preventing its activation and translocation to the membrane [57]. When overexpressed, it can also inhibit the enzymatic activity of PKCθ by blocking its association with substrate and/or ATP. A direct interaction between PKCθ and SAP (SLAM-Associated Protein) was also described in T cell activation signaling [58, 59]. SAP mediates the recruitment and activation of the protein kinase Fyn that, in turn, phosphorylates SLAM (Signaling Lymphocyte Activation Molecule). Phosphorylation of SLAM creates docking sites for many proteins and enzymes such as PKCθ, leading to NF-κB activation [58, 59]. It was also shown that SAP constitutively associates with PKCθ in T cells via arginine 78 of SAP, independently of Fyn, but via the formation of a ternary SLAM/SAP/PKCθ complex following T cell activation [60].

Interestingly, an E3 ubiquitin ligase, Casitas B-lineage lymphoma (Cbl-b) was described to suppress T cell activation when mediated by TCR signaling alone without CD28 costimulatory signals [61]. Upon costimulation with CD28, however, the suppression of T cell activation is removed since Cbl-b gets degraded in a mechanism that depends on the activity of PKCθ [62]. Furthermore in T cell context, the protooncogene Vav, a GDP/GTP exchange factor (GEF), was also described to associate with PKCθ in thymocytes in response to TCR-mediated apoptosis [63]. PKCθ was found to synergize with Vav for the activation of NF-κB [64]. It is likely that Vav helps in the translocation of PKCθ to synaptonemal microdomains leading to their colocalization and T cell activation [65]. It remains to be proved whether Vav translocates to the membrane following PKCθ phosphorylation or by direct contact with PKCθ [66], especially since the interaction between Vav and PKCθ appears to be a functional rather than a physical association [65]. In addition to SAP and Vav, CARMA1 is inducibly phosphorylated on S552 of its linker region by PKCθ upon TCR-CD28 costimulation. This phosphorylation mediates TCR-induced NF-κB activation [67]. Furthermore, it was shown that CARMA1 acts to contribute to the upregulation of the protein mucin in response to the bacterium *Haemophilus influenzae* and phorbol ester PMA in respiratory epithelial cells via a PKCθ-MEK-ERK pathway [68]. Other interaction mechanisms remain unclear such as the potential interaction between PKCθ and interleukin-2-inducible T-cell kinase (Itk) in T lymphocyte signal transmission [69].

In addition to its roles in regulating the activation and proliferation of lymphocytes, PKCθ appears to have an important role during muscle histogenesis [70]. Recent studies showed that PKCθ is essential for cardiomyocytes survival and cardiac tissue remodeling by preventing cardiomyocytes' death upon extensive work [71]. In skeletal muscle models, it was not understood why embryonic myoblasts differentiate in the presence of transforming growth factor beta (TGF beta) while fetal myoblasts do not. It was found that PKCθ is selectively expressed in fetal skeletal myoblasts but not in embryonic skeletal myoblasts [70]. Embryonic myoblasts lacking PKCθ did not respond to TGF beta or differentiate in its presence. However, the sensitivity of fetal myoblasts to the inhibition of differentiation exerted by TGF beta is mediated by the expression of PKCθ in these cells [70]. Recently, PKCθ was found to regulate profusion genes caveolin-3 and β1D integrin and induce focal adhesion kinase phosphorylation resulting in mononucleated myoblasts fusion and formation of multinucleated myofibers [72]. In this context, RACK1 acts as an adapter between PKCθ and integrins [73]. Another study shed light on the involvement of PKCθ in endothelial cell migration via integrins [74]. It described a novel 20 kD protein, theta-associated protein or TAP20 whose transcription depends enzymatically on active PKCθ [74]. TAP20 directly interacts with the cytoplasmic tail of the β5 integrin subunit, thus interfering with the integrin-cytoskeleton interaction required for focal adhesion formation [74]. Furthermore, PKCθ was shown to mediate the binding of leukocyte function-associated antigen 1 (LFA-1) on T cells to immunoglobulin-like cell adhesion molecule 1 (ICAM-1) on APCs following T cell activation [75]. In this context, PKCθ associates with RapGEF2 which facilitates Rap1 activation and subsequent surface distribution of LFA-1 [76]. The relocation of LFA-1 and its conformational change increase its binding affinity to ICAM-1 [77]. Moreover, the clusters of LFA-1 on the surface induce actin polymerization and remodeling, thereby enhancing T cell adhesion [78]. Cytoskeletal remodeling also involves the

microtubule cytoskeleton where the microtubule-organizing center (MTOC) becomes oriented towards the APC to enable efficient cargo trafficking toward the APC [79]. Interestingly, it was shown that PKCθ was required for MTOC reorientation [80]. In another context, PKCθ was found to be involved in spectrin-based cytoskeleton remodeling during apoptosis. Spectrin, which is known to link the cell membrane to the actin cytoskeleton, aggregates with PKCθ in the early stages of apoptosis [81]. Notably, a unique role of PKCθ was revealed in intestinal epithelial monolayers where active PKCθ directly phosphorylates tubulin monomers promoting their assembly into microtubules and increasing microtubule stability [82]. Hence, it was shown that loss of PKCθ affects the cytoskeletal integrity leading to an increase in epithelial barrier permeability, a symptom of intestinal inflammation.

3.3. PKCθ in the Immunological Synapses and Lipid Rafts. PKCθ is highly expressed in leukemic Jurkat T cells [83]. It is the only member of PKC family to be recruited to the immunological synapse in effector T cells [4]. Immunological synapses form between a T cell and an antigen-presenting cell (APC) following T cell receptor-peptide MHC recognition [4, 5]. It is composed of a central supramolecular activation cluster (cSMAC) surrounded by a peripheral supramolecular activation cluster (pSMAC). It was found that accumulation of lipid rafts in immunological synapses does not increase upon TCR/CD28 stimulation; they rather reorganize preferentially in the cSMAC instead of pSMAC [84]. PKCθ appears to be recruited to the junction between the cSMAC and pSMAC in a CD28 costimulatory-dependent manner [85, 86], more specifically by physical association with the cytoplasmic tail of CD28 [54]. Many studies investigated the mechanism by which PKCθ translocates to the immunological synapses and revealed that it partially depends on phospholipase C activity and DAG production but also on a novel signaling pathway [85, 87]. It was proposed that such translocation is mediated by the PKCθ regulatory V3 domain and requires Lck [88]. In addition to Lck, all of Vav1, phosphatidylinositol 3-kinase (PI3-K), the small GTPase Rac, and actin cytoskeleton reorganization participate in regulating the membrane localization and consequent activation of PKCθ [87, 89]. In addition to the regulatory domain, the kinase domain is of great importance with respect to the immunological synapse localization of PKCθ. An active kinase domain permits the retention of PKCθ in the immunological synapse, likely via autophosphorylated sites that are still undefined [90].

3.4. Role of PKCθ in Interleukin-2 Production during T Cell Activation. Upon TCR and CD28 costimulation, fully activated PKCθ plays an important role in mediating signaling events that lead to the activation of transcription factors such NF-κB, AP-1 and NF-AT. The NF-κB signaling pathway is the major target of PKCθ in T cell activation that leads to interleukin-2 (IL-2) production. NF-κB is usually present in the cytosol in an inactive form whereby its nuclear localization sequence is shielded by inhibitors such as IκBs [91, 92]. These inhibitors, when phosphorylated by PKCθ-activated IKKs, undergo degradation resulting in NF-κB translocation to the nucleus where it regulates gene transcription of IL-2. The activation of IKKs by PKCθ mediated by multiple effectors such as CARMA1 [67], discussed above. Another study revealed a direct interaction between PKCθ and IKKβ that shed light on a different potential pathway linking PKCθ to NF-κB [93]. AP-1, a dimer of Jun and/or Fos proteins is also a transcription factor that regulates IL-2 production. PKCθ activates SEK1, a MAP kinase that phosphorylates and activates JNK, which then activates Jun [94]. A third pathway involving NF-AT is also thought to be essential for full T cell activation, although cross-talk exists between the different PKCθ-dependent IL-2 production pathways [95]. Activation of T cells promotes activation of phospholipase C, which triggers the formation of the two second messengers, inositol triphosphate (IP3) and DAG. IP3 causes the elevation of cytosolic Ca^{2+}, which activates the Ca^{2+}-dependent serine/threonine phosphatase, calcineurin [95]. It was initially thought that PKCθ regulates IL-2 through TCR downstream effectors; however, later studies revealed that in PKCθ-deficient mice, IP3 production was reduced thereby leading to defective Ca^{2+} response and NF-AT transactivation [95, 96]. Such defect in Ca^{2+} mobilization is likely due to the lack of enzymatic activation and subsequent membrane association of PLC [95]. These findings suggest an unforeseen role of PKCθ as an upstream regulator of phospholipase C (PLCgamma1) via tyrosine kinase Tec [96].

3.5. Regulation of PKCθ Translocation to Lipid Rafts and Activation. PKCθ acts as a kinase receptor for phorbol esters and DAG to mediate many cellular responses. Hence, PKCθ is regulated by certain lipids, phosphorylation, and ubiquitination. First, lipids modulate PKCθ activity by cofactors such as DAG. The binding of DAG enhances the interaction between PKCθ and the acidic phosphatidylserine [97] which decreases the binding affinity of the pseudosubstrate inhibitor and leads to PKCθ activation as discussed earlier. Second, PKCθ activity is regulated by phosphorylation and autophosphorylation mechanisms in which many kinases participate to allow the translocation of PKCθ to the membrane. Lck directly phosphorylates PKCθ at Y90, which stimulates NF-AT and NF-κB activation in T cells [88, 98]. Lck binding regulates membrane translocation of PKCθ by forming of PKCθ/Lck/CD28 complex [53, 99, 100]. It is still unknown whether or not the Y90 phosphorylation has a direct influence on both the formation of the above complex and PKCθ catalytic activity. Furthermore, it appears that germinal center kinase-like kinase (GLK) phosphorylates PKCθ on T219, a novel S/T residue, and thereafter regulates its translocation to the lipid rafts upon TCR stimulation [101]. Indeed, T219 phosphorylation induces localization of PKCθ to lipid rafts and the immunological synapse, allowing it to activate downstream effectors in TCR signaling, independent from its kinase activity [102].

As for the role of autophosphorylation, T538, S676, S685 and S695 are important regulation sites at the catalytic domain of PKCθ [27, 103]. The PKCθ autophosphorylation sites are interdependent in that when T538 phosphorylation site is lost, the remaining sites S676 an S695 become more

susceptible to dephosphorylation by phosphatases [27]. T538 is a critical site that regulates PKCθ kinase activity and T cell activation [27] but does not seem to influence PKCθ translocation to lipid rafts [102]. Constitutive autophosphorylation of T538 occurs at the activation loop where substrates and cofactors bind near the active site of the kinase domain [104]; this step helps retain the active conformation of PKCθ [55]. Additionally, GLK directly associates with PKCθ in T cells upon anti-CD3 stimulation and phosphorylates the T538 residue [101]. Such phosphorylation at the turn motif contributes to the regulation of the enzyme's catalytic activity by stabilizing its active conformation [105, 106]. PKCθ's S676 site is constitutively autophosphorylated and its phosphorylation is moderately increased upon anti-CD3/CD28 costimulation [107]. How this phosphorylation affects the activity of PKCθ and downstream NF-κB activation remains controversial. Conversely, autophosphorylation of PKCθ on S685 appears to regulate the function of PKCθ and T cell activation during TCR signaling [103]. S695 is a constitutive autophosphorylation site in the C-terminal hydrophobic motif of PKCθ is likely induced by CD3 stimulation [89, 107, 108]. Interestingly, PKCθ S695A mutant results in great loss of T538 phosphorylation status [98, 103]. Hence, S695 phosphorylation is required for optimal PKCθ activation and T cell activation during TCR signaling [27, 102, 103] but its role in the regulation of translocation of PKCθ to the membrane is still controversial [89, 108].

As mentioned earlier, PKCs are regulated by degradation following ubiquitination. Upon sustained Ca^{2+} and calcineurin signaling, a state of anergy or antigen unresponsiveness is induced in T cells mediated by proteolytic degradation of PKCs [109]. Indeed, it was shown that PKCθ goes through lysosomal ubiquitination by activation of myriad proteins. Among these proteins is Itch, the endosome-associated E3 ligase, which catalyzes the ubiquitination and ligation of monoubiquitinated PKCθ to Tsg101 receptor, a component of ESCRT-1 complex located on lysosomal vesicles [110].

4. PKCθ Mechanisms of Action in Various Pathologies

Perturbations of PKCθ activity can result in a variety of diseases and disorders including immunological disorders such as autoimmune and inflammatory diseases, cancer, and diabetes. In the following section, we will summarize PKCθ mechanisms of action in various pathologies.

4.1. Autoimmune Responses and Inflammation. PKθ is highly expressed in some immunological disorders and conditions with inflammation. Indeed, PKCθ plays a dual role in inflammation through its differential regulation of effector T cells (T^{effs}) and regulatory T cells (T^{regs}) [5, 111]. The renowned translocation and function of PKCθ at the immunological synapse actually occurs in T^{effs}, either $CD4^+$ or $CD8^+$ T cells, as it promotes their proliferation to mediate inflammation [5]. In T^{regs}, however, PKCθ is sequestered away from the immunological synapse and this allows T^{regs} to suppress the activity of T^{effs} in order to maintain balance of immune reactions, provide tolerance to self-antigens, and prevent autoimmunity [111–113]. Hence, increased PKCθ activity has become a hallmark of autoimmune disorders, which result from activation of self-reactive T cells that differentiate into effectors and attack self-tissues [114]. Additionally, overexpression of the PKCθ-activator GLK enhances PKCθ activity and subsequent stimulation of IKK leading to autoimmunity in systemic lupus erythematosus [101]. This is also true in patients with rheumatoid arthritis where GLK expression was significantly higher in their peripheral blood T cells compared to healthy subjects, and it colocalized with phosphorylated PKCθ in T cells [115].

Therapeutically, the inhibition or suppression of PKCθ helps protect cells from autoimmune disorders. For instance, PKCθ-deficient mice show diminished severity, articular cartilage damage, and bone destruction from Th1-dependent antigen-induced arthritis compared to wild-type mice [116]. This could be due to the reduced expression of the cytokines IFN-γ, IL-2, and IL-4 in their $CD4^+$ T cells [116]. Moreover, PKCθ−/− mice immunized with myelin oligodendrocyte glycoprotein are also resistant to development of autoimmune encephalomyelitis, a model for multiple sclerosis. $CD4^+$ T cells from these mice became primed and accumulated in secondary lymphoid organs in the absence of PKCθ, with severely diminished IFN-γ, TNF, and IL-17 production [117–119]. PKCθ is also required for autoimmune hepatitis induced by concanavalin A, which normally activates CD1d-positive NK cells, rapidly resulting in the generation of the cytokines IFN-γ, IL-6, and TNF-α in large amounts that induce liver damage [120, 121]. In another model, immunization of PKCθ-deficient mice with myosin peptide revealed that these animals fail to develop autoimmune myocarditis as well as the IL-17-producing $CD4^+$ cells (Th17) which mediate the disease [122]. In fact, PKCθ promotes differentiation of T helper 17 (Th17) cells through up-regulation of transcription factor Stat3 through NF-κB and AP-1 upon TCR signaling [123].

Moreover, PKCθ is crucial for *in vivo* development and harmful immune responses of Th2 cells including pulmonary hyperresponsiveness and allergic reactions to inhaled allergen in a model of asthma [124, 125]. However, PKCθ is somewhat dispensable for Th1-mediated responses as it only affects Th1 initial development, but its deficiency does not impair their activation or cytokine production, especially under conditions that involve strong Th1-inducing stimuli [125]. In allogeneic bone marrow transplantation, PKCθ promotes graft-versus-host-disease (GVHD), which is a potentially lethal complication caused by alloreactive donor T cells that recognize mismatched major histocompatibility molecules [126]. However, in the absence of PKCθ, T cell responses triggered in mice by viral infection or administration of an antigen were relatively normal, and the graft-versus-leukemia effect was preserved [126]. PKCθ is also necessary for survival of alloreactive T cells responsible for allograft rejection through up-regulation of the anti-apoptotic protein, Bcl-xL [114, 127]. Taken together, this evidence suggests that inhibition of PKCθ under such conditions may result in more successful transplants due to long-term tolerance of grafts [121, 128].

In addition to its role in regulating autoimmune and immunosuppressive responses, PKCθ is involved in many inflammatory diseases such as nervous and muscular inflammatory diseases. First, PKCθ is involved in inflammatory brain conditions that result in blood-brain barrier dysfunction [129, 130]. The central molecule in such diseases is the proinflammatory interleukin-1beta (IL-1β) which induces activation of PKCθ and subsequent phosphorylation of the tight junction protein *zona occludens* (ZO)-1 thereby reducing transendothelial electrical resistance as is seen in barrier leakage [130]. Second, inflammation is also a major detrimental factor in muscle dystrophy that promotes muscle degeneration thereby obstructing healing. In this context, PKCθ is the suspected player though its pro-inflammatory role [131, 132]. Knockdown of PKCθ in a mouse model of Duchenne muscular dystrophy indeed prevented muscle wasting and enhanced regeneration and performance of muscle tissue [132].

4.2. Cancer. As previously mentioned, PKCθ is essential for T cell proliferation as it induces expression of IL-2 through NF-κB and AP-1. In addition, PKCθ mediates one of the mechanisms by which leukemic T cells are protected from Fas-induced apoptosis by phosphorylating the bcl-2 family protein BAD [83, 133]. PKCθ is also involved in tumor development. For example, it is a downstream player in pre-TCR-Notch3 signaling where its activation of NF-κB is responsible for the development of Notch3-dependent T-cell lymphoma [134].

Moreover, upon pre-TCR activation, PKCθ prevents Notch3 degradation by regulating the phosphorylation and localization of E3 ubiquitin ligase c-Cbl [135]. PKCθ is positively associated with breast cancer cell proliferation and invasion [136, 137]. PKCθ activates Akt, which in turn reduces activity of forkhead box O protein 3a (FOXO3a) and expression of its target genes estrogen receptor α (ERα) and p27 [136]. This pathway results in depression of the transcription factors NF-κB and c-Rel, which are highly implicated in mammary tumorigenesis [136]. In such ER-negative cells, enhanced PKCθ signaling also leads to the activation of ERK1/2 and Ste20-related proline-alanine-rich kinase (SPAK) as well as the phosphorylation of the Fos family protein Fra-1, thereby stabilizing it and regulating its role in the progression and maintenance of invasive breast cancer cell lines [137]. In addition to leukemia and breast cancer, gastrointestinal stromal tumors (GISTs), the most common mesenchymal tumors, are characterized by high expression and activation of PKCθ [138–140]. PKCθ is used as a marker for diagnosis of KIT protein-negative GIST [138, 141]. Knockdown of PKCθ inhibits cyclin A expression but causes the overexpression of the tumor suppressors p21, p27, and p53 resulting in cell-cycle arrest and apoptosis of GIST48 cells [140].

PKCθ plays a central function in the resistance to tumor development through its role in promoting T cell survival [142, 143]. It was found that up-regulation of sarco/endoplasmic reticulum Ca^{2+}-ATPase 3 (SERCA3) by tumor environment inhibits PKCθ in human $CD4^+$ T and causes retention of NF-κB in the cytosol, leading to apoptosis of these T cells [143]. Studies in PKCθ-deficient mice demonstrated the importance of PKCθ in the immune response to leukemia as these mice had higher incidence and faster onset of the disease than wild-type mice [144]. PKCθ is also expressed in natural killer (NK) cells and is considered critical for NK-cell mediated anti-tumor surveillance [145, 146]. Development of MHC-I-deficient tumor *in vivo* is more likely in PKCθ−/− mice than in wild-type mice; such phenotype was associated with reduced NK recruitment and activation [145]. In fact, PKCθ phosphorylates WASp-interacting protein (WIP), which is central for the formation of the protein complex required for NK cytotoxic activity [147]. NK cell-activating receptors also require PKCθ for intracellular signaling that leads to generation of IFN-γ [148].

4.3. Diabetes and Insulin Resistance. PKCθ is the mediator between lipid metabolism and insulin resistance, which is a leading cause of type 2 diabetes mellitus [149, 150]. Elevation in plasma free fatty acids levels increases intracellular fatty acyl-CoA and DAG which in turn activates PKCθ in skeletal muscle which phosphorylates S307 on insulin-stimulated insulin receptor substrate 1 (IRS-1) resulting in reduced tyrosine phosphorylation and IRS-1-associated PI3-kinase activity [151–153]. This event leads to insulin resistance by alleviating insulin-stimulated muscle glycogen synthesis. Similar effects of PKCθ were observed in adipose tissue and the liver [154–156]. A more recent study has actually proposed PDK-1 as a direct target of PKCθ in insulin resistance, in a pathway independent from IRS-1/2 [157]. PKCθ negatively regulates insulin receptor activation of PDK-1 by S504/332 phosphorylation, thereby inhibiting PDK-1-mediated Akt phosphorylation and subsequent PI-3K signaling. Up-regulation of PKCθ that is inversely proportional to insulin sensitivity has also been reported in type 2 diabetic subjects [158]. Furthermore, PKCθ expression in critical regions of the amygdala and hypothalamus is linked to diet-induced obesity and reduced insulin signaling at the level of the central nervous system response [159–162].

5. PKCθ as Target in Clinic

Activation of T cells presents the initiating event in immunological disorders and plays an important role in regulating the immune response. Isozyme-specific perturbations in PKC activity have been identified in numerous human diseases [163]. Therefore, the modulation of PKC activity presents an attractive approach for clinical drug development. Accordingly, agents that inhibit PKCs could contribute to the suppression of immune responses to achieve successful transplants and to prevent many immunological disorders resulting from autoimmune and inflammatory diseases. Many hurdles challenge the development of kinase-specific inhibitors including potency, and selectivity. Most of the PKC domains show high sequence and structural similarity among the isoforms, making it difficult to design molecules that selectively target each isoform. Furthermore, the high degree of homology in the kinase region among the more

than 500 kinases in the human genome makes the design of a PKC inhibitor targeting the kinase domain of interest a major challenge [164, 165]. Moreover, PKCs isoforms have revealed many complex interrelationships and interactions. For example, one particular isoform may be involved in different diseases. Several isoforms may be involved in one particular disease, while for a particular disease two PKC isoforms may produce contrary effects. For instance, PKCα and PKCδ play opposite roles in the proliferation and apoptosis of glioma cells [166].

5.1. PKC Inhibitors and the Clinical Trials. Inhibitors of PKC can be classified according to their sites of interaction within the PKC protein structure [163]. Inhibitors of the catalytic domain are directed to either the substrate site or ATP-binding site whereas inhibitors of the regulatory domain may target the phospholipid or phorbol ester binding site by mimicking diacylglycerol [167]. Moreover, inhibitors that disrupt protein-protein interactions at a specific subcellular location or with a specific substrate may provide a new approach to selectively inhibit the phosphorylation of substrates between unique regions in each PKC and its corresponding interacting protein or substrate [163]. Although a wealth of inhibitory compounds is available, few demonstrate specificity for either PKC alone or individual PKC isoforms. Many research efforts are underway to develop PKC-based drugs with several compounds currently in clinical trials.

The best characterized ATP-competitive small molecules are the bisindolylmaleimides [168]. These water-soluble compounds bind to the ATP-binding pocket and limit phosphorylation. The classic example, staurosporine, has pan-PKC activity, binding to all isozymes as well as several other serine/threonine kinases [169]. The experimental and docking interactions of staurosporine with PKCθ displayed important hydrogen bonding with different amino acid residues of the PKCθ active site [163]. In fact, staurosporine is one of the most powerful PKC inhibitors in *in vitro* models [163]. However, its poor kinase selectivity hampered its further development, prompting efforts to synthesize more PKC-selective analogues. Among these are 7-hydroxystaurosporine or UCN-01 [170] and N-benzoyl-staurosporine [171], which have less PKC-inhibitory activity than the parent compound, but a higher degree of PKC selectivity when assayed for inhibition of different kinases [172]. However, these agents display specificity against conventional isoforms of PKC over novel Ca^{2+} independent isoforms. Sotrastaurin (AEB071) is a PKC inhibitor that has strong and specific activity against PKCθ, PKCα, and PKCβ and lesser effect on PKCδ, PKCε, and PKCη, suggesting that sotrastaurin would inhibit not only T cells, but also a variety of other cells. It inhibits more than 200 other kinases, including those important for early T cell activation, such as Lck. Sotrastaurin acts through PKC to inhibit T-cell activation that is initiated by the binding of peptide-MHC complexes and CD28 costimulation [173, 174]. *In vivo* data from rodents and nonhuman primates confirmed the potential of sotrastaurin in preventing allograft rejection and reducing the inflammatory response [175, 176]. Results from an initial clinical trial in patients with psoriasis showed improvements in clinical and histological assessments [177]; however, data from early trials in kidney transplant recipients were less encouraging. Sotrastaurin is currently used as an immunosuppressant in phase I trials for liver transplantation [178], and phase II trials for renal transplantation [179]. Although sotrastaurin appears to be well-tolerated based on published clinical trial data, long-term data is needed to confirm the safety and efficacy profile of this novel compound. Efforts to develop a more selective inhibitor led to the discovery of enzastaurin [180–183] and ruboxistaurin [184], which are more selective for PKCβ over other isozymes. Furthermore, Midostaurin (also known as PKC412 or n-benzoylstaurosporine) exhibits improved selectivity for PKC-ATP binding sites, but shows modest isozyme specificity [185, 186]. These inhibitors are undergoing clinical trials. As for enzastaurin, phase I studies showed prolonged disease stabilization in patients with lung cancer, colorectal carcinoma and renal carcinoma [187]. Ongoing clinical trials of enzastaurin alone or in combination with conventional chemotherapies are being investigated in recurrent brain tumor (Phase I), advanced or metastatic malignancies (Phase II), prostate cancer (Phase II), breast cancer, ovarian cancer, and peritoneal cavity cancer [188]. Concerning Ruboxistaurin, it has shown efficacy in the treatment of diabetic retinal and renal abnormalities both in preclinical and human studies [189]. Midostaurin was well-tolerated in phase I study in patients with malignant melanoma but unfortunately phase II trial failed to demonstrate significant clinical activity [185].

The best characterized compound targeting the activator binding C1 domain is bryostatin-1 [190]. Bryostatin-1 is a partial agonist of several members of the PKC family [191]. The binding of bryostatin-1 to PKC results in PKC activation, autophosphorylation, and translocation to the cell membrane [190]. Bryostatin-1-bound PKC is then downregulated by ubiquitination and degradation in proteasomes [190]. Bryostatin-1 is expected to modulate classical PKC isoforms associated with Ca^{2+} signaling as well as novel isoforms independent of Ca^{2+} [190]. Bryostatin-1 has been investigated for anticancer activity in phase I and II clinical trials using a wide range of tumor types [192, 193] and showed promising activity in the treatment of refractory acute leukemia and indolent hematologic malignancies [194–196]. However, several phase II studies were disappointing in melanoma [197], colorectal cancer [198], and gastric carcinoma [199]. Moreover, bryostatin-1 has demonstrated significant chemosensitizing activity when combined with conventional therapeutics including arabinofuranosylcytosine [200], tamoxifen [201], fludarabine [202], taxol [203] in leukemia cells. Protection of PKC from being downregulated by the strong ligand, phorbol ester, led to the design of selective PKC-binding bryostatin analogues. These molecules show selectivity in binding to the C1 domain of various PKC isozymes and may represent a novel class of PKC regulators [204].

5.2. PKCθ Inhibitors in Preclinical Studies. A large number of PKCθ inhibitors have been reported. These can be classified

on the basis of their parent scaffolds, such as aminopyrimidine, pyridine carbonitrile (phenyl, furan, benzofuran, benzothiophene and vinyl phenyl analogs) and thieno (2,3-b) pyridine-5-carbonitriles (2-alkenyl and 2-phenyl) derivatives (2-phenyl and 4-amino indole modification) (for chemical structures, refer to review [194]). Compounds belonging to the amino pyrimidine class are the first discovered inhibitors of PKCθ and are considered more selective than members of any other category [205].

Different derivatives have been developed by making appropriate modifications in groups R1, R2 and R3 [206]. For instance, R1 may be substituted by NO_2 and CF_3 groups; R2 may be substituted by cyclohexane ring whereas R3 by some bulkier groups like 2-bromo benzylamine, 2-chloro benzylamine. The group substitution of amino pyrimidine derivatives can affect its inhibitory activity. For example, the replacement of some groups such as nitro ($-NO_2$) with CF_3 group decreases the activity of molecules by ten times; whereas the presence of the nitro ($-NO_2$) group at the 5th position and substitution of hydrogen atom of amino group at the 2nd position with 2-bromobenzylamine, 2-SCH_3 benzylamine and 2-SCF_3 benzylamine group increases the potency of molecules in comparison with other substitution groups [207]. Moreover, the stereoisomerism and the geometric isomerism (cis, trans) can affect the biological activity of inhibitors. The pyridine carbonitrile category of PKCθ inhibitors consists of C-5 substituted 3-carbonitrile pyridine derivatives. In the derivative inhibitors, C-4 and C-5 positions are substituted with amino indole and different kinds of heteroaryl/aryl groups, respectively [207, 208]. On the basis of substituents at C-5 position, different derivatives have been developed like phenyl, furan, benzofuran, benzothiophene and phenyl vinyl analogues of pyridine carbonitrile. A series of 5-phenyl-3-pyridinecarbonitriles [209], 5-vinyl-3-pyridinecarbonitriles [210], 5-vinyl phenyl sulfonamide-3-pyridinecarbonitriles [211], 5-vinylaryl-3-pyridinecarbonitriles [212] were synthesized.

Preclinical studies have assessed the best analogs among each series by assaying their IC50 values for the inhibition of PKCθ along with their metabolic stability in rat liver microsomes and their ability to block the production of interleukin-2 in stimulated human whole blood [213]. These compounds showed improved microsomal half-lives as well as decrease of interleukin-2 production. Molecules belonging to the category of thieno[2,3b]pyridine-5-carbonitriles are highly selective in nature. They are classified into two categories on the basis of substitution at their 2nd position, that is, 2- alkenyl, phenyl and 2-aryl derivatives [213]. A series of 2-alkenyl thieno[2,3b]pyridine-5-carbonitriles [214] and 4-(indol-5-ylamino)thieno[2,3-b]pyridine-5-carbonitriles were synthesized [215]. These compounds showed a decrease in interleukin-2 production by anti-CD3 and anti-CD28 activated T-cells derived from wild-type mice, with a reduced effect on activated T-cells from PKCθ knockout mice.

The experience with PKCθ inhibitors highlights several challenges for the future. PKCθ is an attractive therapeutic target, but clinically available inhibitors need to be more specific and selective against different PKC isoforms.

6. Conclusion

PKCθ is involved in many signaling pathways that control immune responses and other cellular activities, in normal physiology as well as certain disease states. Particularly, evidence highlights the T-cell activating role of PKCθ as an initiating event in many immunological disorders. Hence, the modulation of PKC activity becomes a challenge that, once overcome, will be useful in medical applications such as the regulation of autoimmune diseases and graft rejection. Accordingly, inhibitors of PKCs and PKCθ have been developed and tested in preclinical and clinical studies. Results are promising for the future development of more specific and selective inhibitors that can greatly enhance the treatment of several T-cell mediated diseases like asthma, arthritis, multiple sclerosis, autoimmunity, and organ transplantation.

Conflict of Interests

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

Authors' Contribution

Ghassan Dbaibo, Rouba Hage-Sleiman, and Asmaa B. Hamze designed the review; Rouba Hage-Sleiman, Asmaa B. Hamze, Lina Reslan, and Hadile Kobeissy wrote the paper and revised it; Ghassan Dbaibo edited and approved the final version of the paper.

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Research Article

HMGB1 Promotes Systemic Lupus Erythematosus by Enhancing Macrophage Inflammatory Response

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Background/Purpose. HMGB1, which may act as a proinflammatory mediator, has been proposed to contribute to the pathogenesis of multiple chronic inflammatory and autoimmune diseases including systemic lupus erythematosus (SLE); however, the precise mechanism of HMGB1 in the pathogenic process of SLE remains obscure. *Method*. The expression of HMGB1 was measured by ELISA and western blot. The ELISA was also applied to detect proinflammatory cytokines levels. Furthermore, nephritic pathology was evaluated by H&E staining of renal tissues. *Results*. In this study, we found that HMGB1 levels were significantly increased and correlated with SLE disease activity in both clinical patients and murine model. Furthermore, gain- and loss-of-function analysis showed that HMGB1 exacerbated the severity of SLE. Of note, the HMGB1 levels were found to be associated with the levels of proinflammatory cytokines such as TNF-α and IL-6 in SLE patients. Further study demonstrated that increased HMGB1 expression deteriorated the severity of SLE via enhancing macrophage inflammatory response. Moreover, we found that receptor of advanced glycation end products played a critical role in HMGB1-mediated macrophage inflammatory response. *Conclusion*. These findings suggested that HMGB1 might be a risk factor for SLE, and manipulation of HMGB1 signaling might provide a therapeutic strategy for SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by chronic inflammation in multiple organs such as kidney, lung, heart, joint, and so forth [1–5]. One of the most severe manifestations of SLE is lupus nephritis, which is a potentially fatal complication [6–8]. Many researchers have reported the morbidity and mortality of SLE extensively; however, the pathogenic mechanism of SLE remains still elusive. Reports have indicated that autoantibody-mediated immune response can trigger tissue damage, and thus contributes to the pathogenesis of SLE [1, 9, 10]. In recent years, accumulating evidence indicates that deregulated production of proinflammatory cytokines such as TNF-α and IL-6 may play a critical role in immune dysfunction and mediate tissue inflammation and organ damage in SLE [1, 10]. It is reported that TNF-α levels are significantly increased and correlated with SLE disease activity, and blocking TNF-α function has been found to decrease disease activity in clinical patients [11–19]. Additionally, reports also indicate that IL-6 promotes autoantibody production in humans and mice with lupus nephritis [5, 20]. Therefore, the understanding of the detailed mechanism of inflammatory response would facilitate the advance of efficient therapies toward SLE.

Recent evidence indicates that HMGB1, a well-established damage associated molecular pattern (DAMP), is responsible for the production of proinflammatory cytokines [21–23]. HMGB1 is likely to be released from activated immune cells such as macrophages in the area of inflammation or injure [24–29]. When released, HMGB1 participates in the secretion of downstream proinflammatory cytokines via binding to cell surface receptors such as receptor of advanced glycation end products (RAGE), TLR2 and TLR4, thus contributing to the occurrence and development of diverse inflammatory diseases and autoimmune diseases [25–30]. Proinflammatory

and immune-stimulatory function of HMGB1 indicate its association with autoimmune diseases including rheumatoid arthritis and SLE [29, 31]. Furthermore, HMGB1 has been found to be significantly elevated in lupus sera and identified as one of the components in DNA-containing immune complexes that enhance proinflammatory cytokine production [32]. All these data indicate that HMGB1 might act as a new inflammation-related factor in SLE; however, the precise role of HMGB1 in the inflammatory response during the pathogenesis of SLE still remains unclear.

Murine lupus model provides a good tool to investigate the pathogenesis of SLE. Our previous study has demonstrated that activated lymphocyte derived-DNA (ALD-DNA) could induce SLE syndrome including high levels of anti-dsDNA antibody, glomerulonephritis, and proteinuria in healthy mice with conventional genetic background [33–39]. In this study we investigated the potential role of HMGB1 in the pathogenesis of SLE and its underlying mechanism. We found that HMGB1 levels were elevated and correlated with SLE disease activity both in clinical patients and murine model. Gain- and loss-of-function analysis revealed that HMGB1 aggravated the severity of SLE, which might be due to its effect on macrophage inflammatory response. Furthermore, our findings showed that HMGB1-enhanced macrophage inflammatory response was dependent on RAGE.

2. Materials and Methods

2.1. Patients and Healthy Controls. The case-control study was approved by the Ethics Committee of Fudan University. A total of 32 SLE patients were recruited, and all of the peripheral blood samples were collected from these SLE patients after obtaining informed consent. The diagnosis of SLE was established according to the four of the American College of Rheumatology (ACR) revised criteria for the diagnosis of SLE. Disease activity was evaluated using SLEDAI. Lupus nephritis was diagnosed with renal biopsy. Patients who had other autoimmune diseases were excluded. Disease activity at the time of blood sampling was assessed by the SLEDAI. Further characteristics of the patients are summarized in Table 1. The mean age of the patients was 32 (range 19 to 54) years (y), and 24 healthy individuals matched for gender and age were recruited as controls.

2.2. Mice and Cell Culture. Six-week-old female BALB/c mice were purchased from the Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). Mice were housed in a specific pathogen free room under controlled temperature and humidity. This study was strictly carried out according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, China, 1998) and with the ethical approval of the Shanghai Medical Laboratory Animal Care and Use Committee as well as the Ethical Committee of Fudan University. All surgery was performed under sodium pentobarbital anesthesia, and all animal procedures in this study were strictly performed in a manner to minimize suffering of laboratory mice. RAW264.7 cells were maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% FBS (Invitrogen Life Technologies) in a 5% CO_2 incubator at 37°C.

Table 1: Characteristics of systemic lupus erythematosus (SLE) patients and control subjects.

	SLE	Control
Number	32	24
Sex (female/male)	32/0	24/0
SLEDAI score mean ± s.d.	9.3 ± 3.9	n.a
Anti-dsDNA titre mean ± s.d. (IU/mL)	121.2 ± 86.1	n.a
Patients with nephritis (%)	(17/32) 53%	n.a
Treatment with prednisolone		
Patients, number (%)	93.75%	n.a
Treatment with hydroxychloroquine		
Patients, no. (%)	87.5%	n.a
Treatment with azathioprine		
Patients, no. (%)	0%	n.a

Values are in mean ± standard deviation (s.d.); n.a.: not applicable.

2.3. Reagents and Antibodies. pCAGGS-HMGB1 (pHMGB1) and pCAGGS (empty vector) were kindly provided by Professor Tadatsugu Taniguchi (University of Tokyo, Tokyo, Japan) [40]. HMGB1 blocker glycyrrhizin was purchased from Sigma. Glycyrrhizin was dissolved with PBS. TLR2/4 inhibitor OxPAPC was purchased from invivogen. RAGE-Fc was purchased from R&D Systems. The RAGE, HMGB1, and control siRNA were purchased from Santa Cruz Biotechnology. Macrophages were transfected with 200 nM of indicated siRNAs by Mouse Macrophage Nucleofector Kit (Lonza) according to the manufacturer's instructions. HMGB1 and RAGE antibody were obtained from Cell Signaling Technology and GAPDH antibody from Santa Cruz Biotechnology.

2.4. DNA Preparation and Generation of Murine Model of SLE. The extraction and purification of activated lymphocyte-derived DNA (ALD-DNA) and unactivated lymphocyte-derived DNA (UnALD-DNA) were performed according to our previously described methods [33–39]. To generate the murine model of SLE, six-week-old female BALB/c mice were immunized s.c. with ALD-DNA (50 μg/mouse) plus CFA (Sigma-Aldrich) on day 1, followed by s.c. injection of ALD-DNA (50 μg/mouse) emulsified with CFA (Sigma-Aldrich) on days 14 and 28 for total of three times as described previously [33–39]. Mice in each group received an equal volume of PBS plus CFA or IFA, or UnALD-DNA (50 mg/mouse) plus CFA or IFA were used as controls. To investigate the effect of HMGB1 in the pathogenic process of SLE, mice were injected intramuscularly with pHMGB1 or vector every two weeks. Mice were divided into six groups as follows: PBS plus vector, Un-ALD-DNA plus vector, ALD-DNA plus vector, PBS plus pHMGB1, Un-ALD-DNA plus pHMGB1 and ALD-DNA plus pHMGB1. To further confirm the significance of HMGB1, mice were treated with HMGB1 inhibitor glycyrrhizin (0.5 mg/mice) every day. Mice were divided into six groups as follows: PBS, Un-ALD-DNA plus

PBS, ALD-DNA plus PBS, PBS plus glycyrrhizin, Un-ALD-DNA plus glycyrrhizin, and ALD-DNA plus glycyrrhizin. Serum and urine samples were collected every 2 weeks for further experiments. Eight weeks later, mice were sacrificed, and surgically resected kidneys were collected for further cellular function and tissue histology analysis.

2.5. pHMGB1 and Glycyrrhizin Treatment in Mice. To examine the potential role of HMGB1 in SLE, 8 mice in each group were intramuscularly injected with 100 μg of pHMGB1 or empty vector per mouse 72 h earlier before injection with ALD-DNA. Mice were then injected with pHMGB1 every 2 weeks for total 6 times [35, 36]. To block the function of HMGB1 in SLE mice, mice were randomized to intramuscularly injection 0.5 mg per mouse glycyrrhizin every day for 2 months. Twenty-four hours after the initial glycyrrhizin treatment, the mice were immunized with ALD-DNA (50 μg/mouse) three times in 4 week as previously described.

2.6. Anti-dsDNA Antibody and Proteinuria Examination. Serum anti-dsDNA antibody levels in the mice were determined by ELISA analysis as described previously [33–39]. Proteinuria of the mice was measured with the BCA method (Thermo Fisher Scientific) as previously described [33–39].

2.7. Cell Sorting. Murine renal tissues were surgically resected and dispersed in RPMI 1640 containing 5% FBS and 0.1% collagenase (Sigma-Aldrich) at 37°C for 30 min, followed by progressive sieving to obtain single-cell suspensions. To analyze the inflammatory response of renal macrophages, $CD11b^+/F4/80^{high}$ renal macrophages were sorted from nephritic single-cell suspensions using a FACSAria (BD Biosciences) with FITC-labeled anti-F4/80 and PE-labeled anti-CD11b (BD Biosciences).

2.8. Pathological Analysis. For histology analysis, murine renal tissues were surgically resected and fixed in 4% paraformaldehyde (Sigma-Aldrich), processed, and embedded in paraffin. H&E staining of renal tissue sections were performed according to the manufacturer's instructions and assessed by two pathologists blinded to treatment group as previously described [33–39]. The kidney score of glomerulonephritis was determined by using the ISN/RPS2003 classification. Pictures were acquired with Nikon SCLIPSS TE2000-S microscope (Nikon) equipped with ACT-1 software (Nikon).

2.9. ELISA. Plasma was collected by EDTA as an anticoagulant, aliquoted, and stored at −80°C. To assess the levels of HMGB1, anti-dsDNA antibody, TNF-α, and IL-6 in the plasma and supernatant of cell culture, ELISA (Shino-Test, Sagamihara-shi, Kanagawa, Japan for HMGB1; ebioscience for TNF-α and IL-6; Alpha Diagnostic International for anti-dsDNA) was performed according to the manufacturer's instructions.

2.10. Western Blot. Western blot was performed as described previously [33–39]. Antibodies used here were anti-GAPDH (Santa Cruz Biotechnology), HMGB1 (Cell Signal Technology), goat anti-mouse IgG-HRP (Santa Cruz Biotechnology), and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology).

2.11. Statistical Methods. Data was represented as the mean ± standard deviation (SD). Comparisons between SLE patients and HC were analyzed by Student's t test. Correlation analysis was performed by Pearson correlation test. All analyses were performed by GraphPad Prism 5 (GraphPad Software, La Jolla, CA). A two-tailed P value < 0.05 was considered as statistically significant unless otherwise noted.

3. Results

3.1. Serum HMGB1 Levels Were Elevated and Correlated with SLE Disease Activity Both in Clinical Patients and Murine Model. To investigate whether HMGB1 was involved in the pathogenesis of SLE, we first examined the levels of HMGB1 in SLE patients. A total of 32 SLE patients were recruited to our research and the general characteristics of patients were shown in Table 1. We determined the serum concentrations of HMGB1 in SLE patients and healthy controls (HC) by ELISA. The results showed that HMGB1 concentrations in SLE patients (30.1356 ± 21.0236 ng/mL) were significantly higher than those in HC (5.0877 ± 2.7921 ng/mL, $P < 0.05$) (Figure 1(a)). Furthermore, serum HMGB1 levels in SLE patients with active renal disease (42.5672 ± 21.0052 ng/mL) were significantly higher than those in patients without active renal disease (15.7279 ± 8.8412 ng/mL, $P < 0.05$, Figure 1(b)). Moreover, we found that serum HMGB1 concentrations showed a highly significant correlation with SLE disease activity index score (SLEDAI) ($r = 0.4715$, $P = 0.0064$, Figure 1(c)) and anti-dsDNA antibody levels ($r = 0.6257$, $P = 0.0001$, Figure 1(d)). We also analyzed the expression of HMGB1 in peripheral blood mononuclear cells (PBMCs) from SLE patients (S1, S2, S3, and S4) and HC (H1, H2, H3, and H4) using western blot. As shown in Figure 1(e), the expression of HMGB1 was increased in PBMCs from SLE patient compared to HC.

We further analyzed the serum levels of HMGB1 in murine model of SLE which were generated according to our previously reported procedures [33–39]. ALD-DNA could successfully induce the SLE syndrome manifested by high levels of anti-dsDNA antibody (see Figure S1(a) in Supplementary Material available online at http://dx.doi.org/10.1155/2015/946748), proteinuria (Figure S1(b)) as well as glomerulonephritis (Figures S1(c) and S1(d)). Of interest, we found that serum HMGB1 levels were significantly increased in murine model of SLE compared with those in control mice (Figure 1(f)). Pearson correlation analysis showed that the serum HMGB1 levels were positively correlated with kidney score ($r = 0.6583$, $P = 0.0022$, Figure 1(g)), indicating that HMGB1 levels were significantly associated with the severity of lupus nephritis. Similarly, we observed that serum HMGB1 levels were related with the levels of anti-dsDNA antibody ($r = 0.7278$, $P = 0.0004$, Figure 1(h)) and urine protein ($r = 0.6652$, $P = 0.0019$, Figure 1(i)) in ALD-DNA-induced murine model of SLE. Taken together, these data indicated that HMGB1 expression

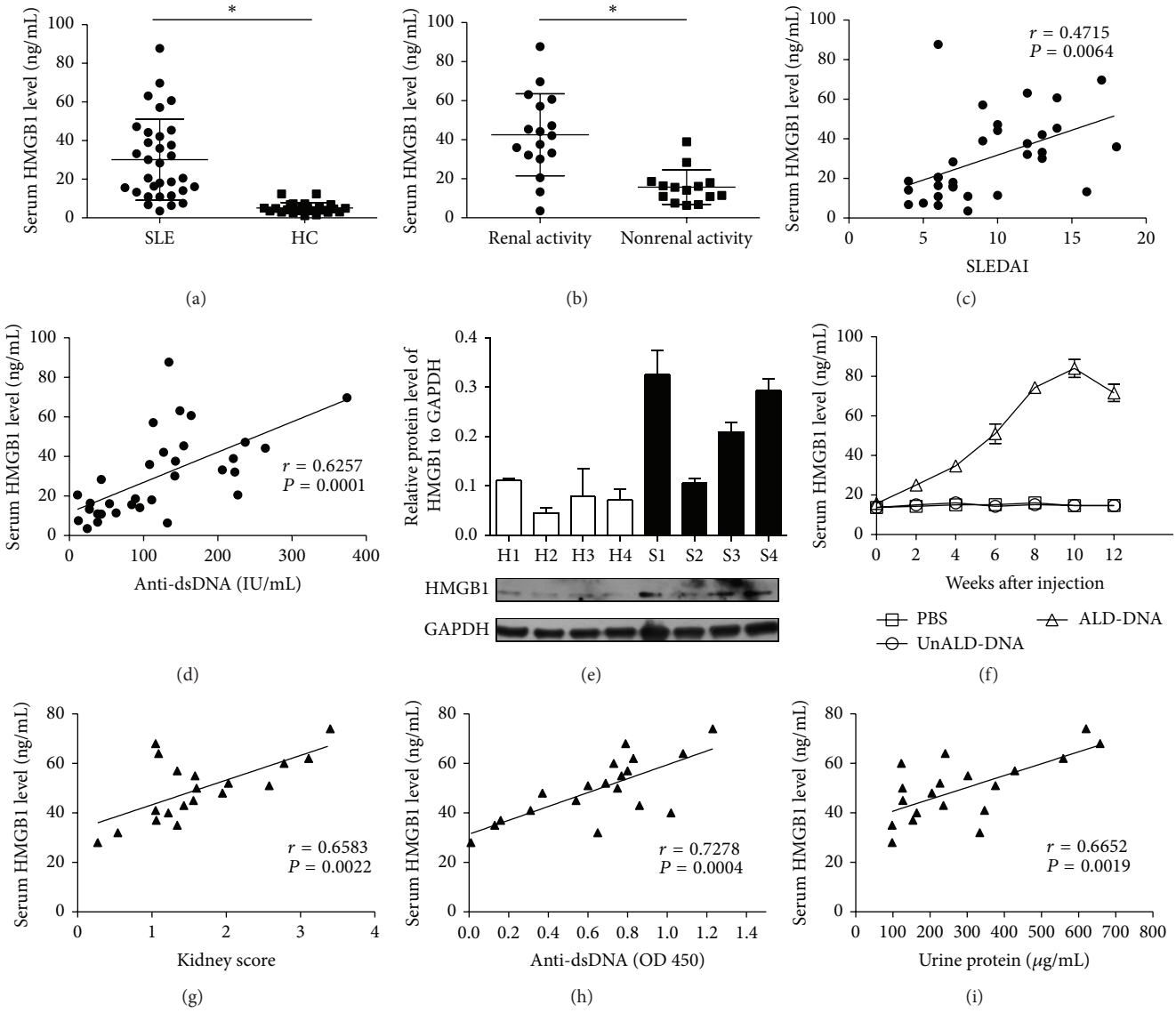

Figure 1: HMGB1 levels were elevated and correlated with SLE disease activity in both clinical patients and murine model. (a) Serum HMGB1 levels were detected by ELISA in SLE patients and HC. (b) HMGB1 levels were measured by ELISA in renal active or inactive patients. The scatter-plot represented the HMGB1 levels by ELISA. Each symbol represents one SLE patient. Horizontal lines represent the median. Data represent the average from experiments performed in triplicates for each patient. (c) Correlation analysis was performed between HMGB1 levels and SLEDAI. (d) Correlation analysis was performed between HMGB1 and anti-dsDNA antibody levels. Pearson correlation analysis was used in the correlation analysis. (e) The expression of HMGB1 was analyzed by western blot in PBMCs from SLE patients (S) and healthy controls (H). Representative western blot bands from 4 patients with SLE and 4 HC were presented. Data were representative of results obtained in three independent experiments. (f) Serum HMGB1 levels were measured by ELISA every 2 weeks after initial injection. Data are means ± SD from 8 mice in each group. (g) The correlation between serum HMGB1 levels and kidney score was carried out in SLE mice. (h) The correlation between serum HMGB1 and anti-dsDNA antibody levels was carried out in SLE mice. (i) The correlation between serum HMGB1 and urine protein levels was carried out in SLE mice. Pearson correlation analysis was used to carry out the correlation study. Each symbol indicates an individual mouse ($n = 19$). $^*P < 0.05$.

was upregulated and correlated with the severity of SLE both in clinical patients and murine model.

3.2. Forced/Inhibited HMGB1 Expression Modulated the Severity of SLE. Above data showed that HMGB1 levels were elevated and correlated with the severity of SLE. To further evaluate whether HMGB1 was involved in the pathogenesis of SLE, we upregulated the expression of HMGB1 by injecting BALB/c mice intramuscularly with a HMGB1 overexpression plasmid (pHMGB1). Results showed that the injection of pHMGB1 led to the elevation of serum HMGB1 levels in SLE mice (Figure 2(a)). To investigate the effect of increased HMGB1 levels on the progression of SLE, we analyzed anti-dsDNA antibody, proteinuria, renal pathology,

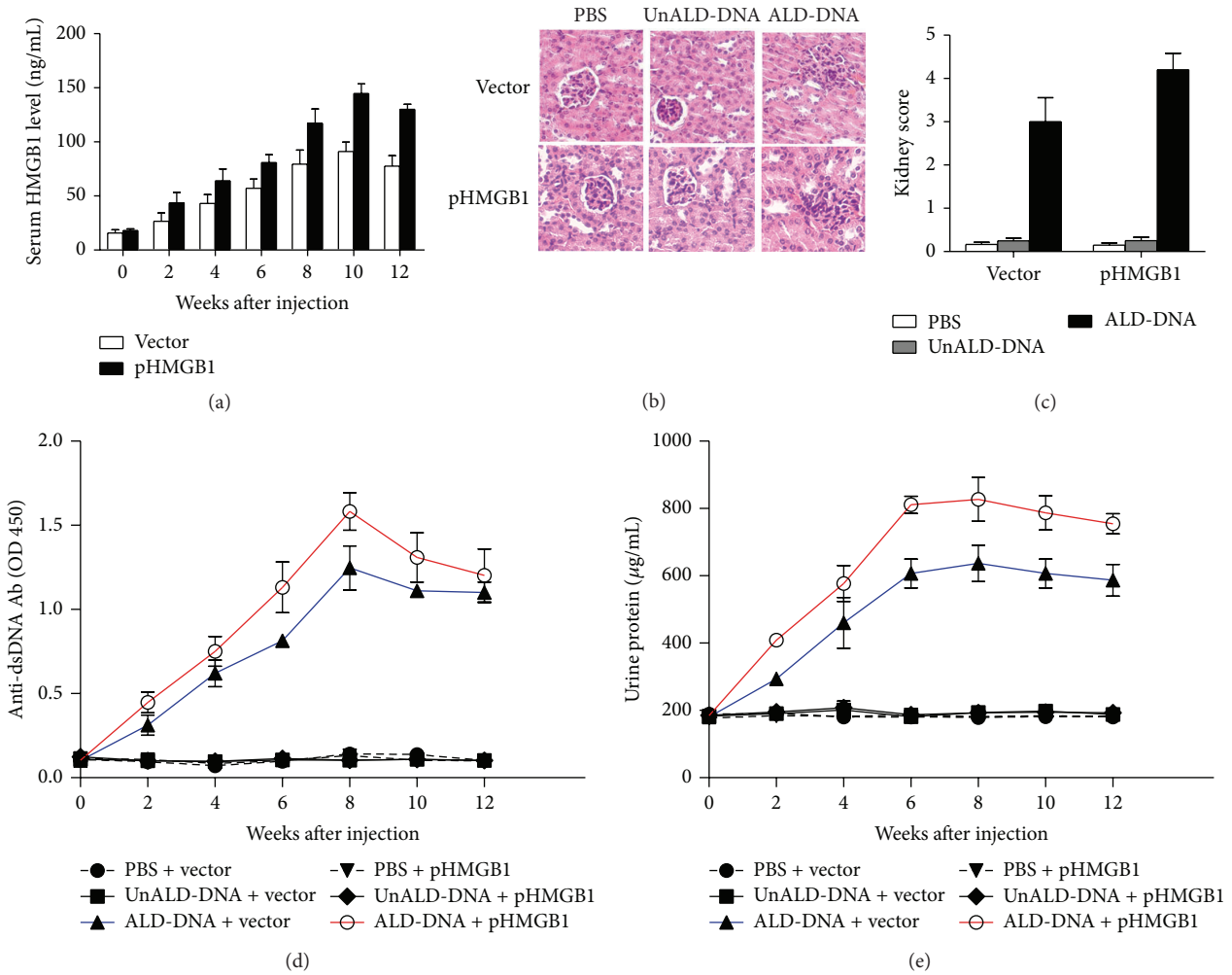

FIGURE 2: HMGB1 overexpression could promote the severity of SLE. BALB/c mice were administrated intramuscularly with 100 μg pHMGB1 or empty-vector per mouse. 72 h following injection, mice were then injected subcutaneously with ALD-DNA (50 μg/mouse) for total 3 times in 4 weeks. (a) The dynamics of serum HMGB1 levels were determined by ELISA every 2 weeks after initial injection. Data are means ± SD from 8 mice in each group. (b) Nephritic pathology was evaluated by H&E staining of renal tissues. Images (magnification ×200) are representative of at least 8 mice in each group. (c) The kidney score was assessed using paraffin sections stained with H&E in (b). (n = 8). (d) Serum anti-dsDNA antibody levels were measured by ELISA every 2 weeks after initial injection. Data are means ± SD from 8 mice in each group. (e) Urine protein levels of the mice were assessed by BCA method every 2 weeks. Data are means ± SD from 8 mice in each group. $^*P < 0.05$.

and kidney score in pHMGB1- or empty vector-treated mice. Results showed that HMGB1 overexpression exacerbated renal pathology as revealed by the increased infiltration of proinflammatory cells into glomerular mesanguim and thickened basement membrane, as well as the atrophy of glomerular mesanguim (Figure 2(b)), and upregulated the kidney score of SLE mice (Figure 2(c)). We also found that the levels of anti-dsDNA antibody (Figure 2(d)) and urine protein (Figure 2(e)) in SLE mice treated with pHMGB1 were notably elevated compared with that in empty vector-treated SLE mice.

To further confirm the effect of HMGB1 on the progression of SLE, we inhibited the function of HMGB1 *in vivo* by injecting BALB/c mice intramuscularly with glycyrrhizin which has been demonstrated to be the blocker of HMGB1 [41–43]. As shown in Figure 3(a), glycyrrhizin administration significantly decreased the serum HMGB1 levels in SLE mice. Of note, glycyrrhizin treatment was found to efficiently ameliorate renal pathology as demonstrated by decreased infiltration of proinflammatory cells into glomerular mesanguim, recovery from thicken basement membrane and the atrophic glomerular mesanguim (Figure 3(b)), and decrease the kidney score in SLE mice (Figure 3(c)). Furthermore, we revealed that glycyrrhizin treatment reduced levels of anti-dsDNA antibody (Figure 3(d)) and urine protein (Figure 3(e)) in SLE mice.

Taken together, these data demonstrated that HMGB1 played a crucial role in modulating the severity of SLE.

3.3. HMGB1 Enhanced Macrophage Inflammatory Response and Corresponded to the Proinflammatory Cytokines in SLE. It is well established that macrophages are prominent

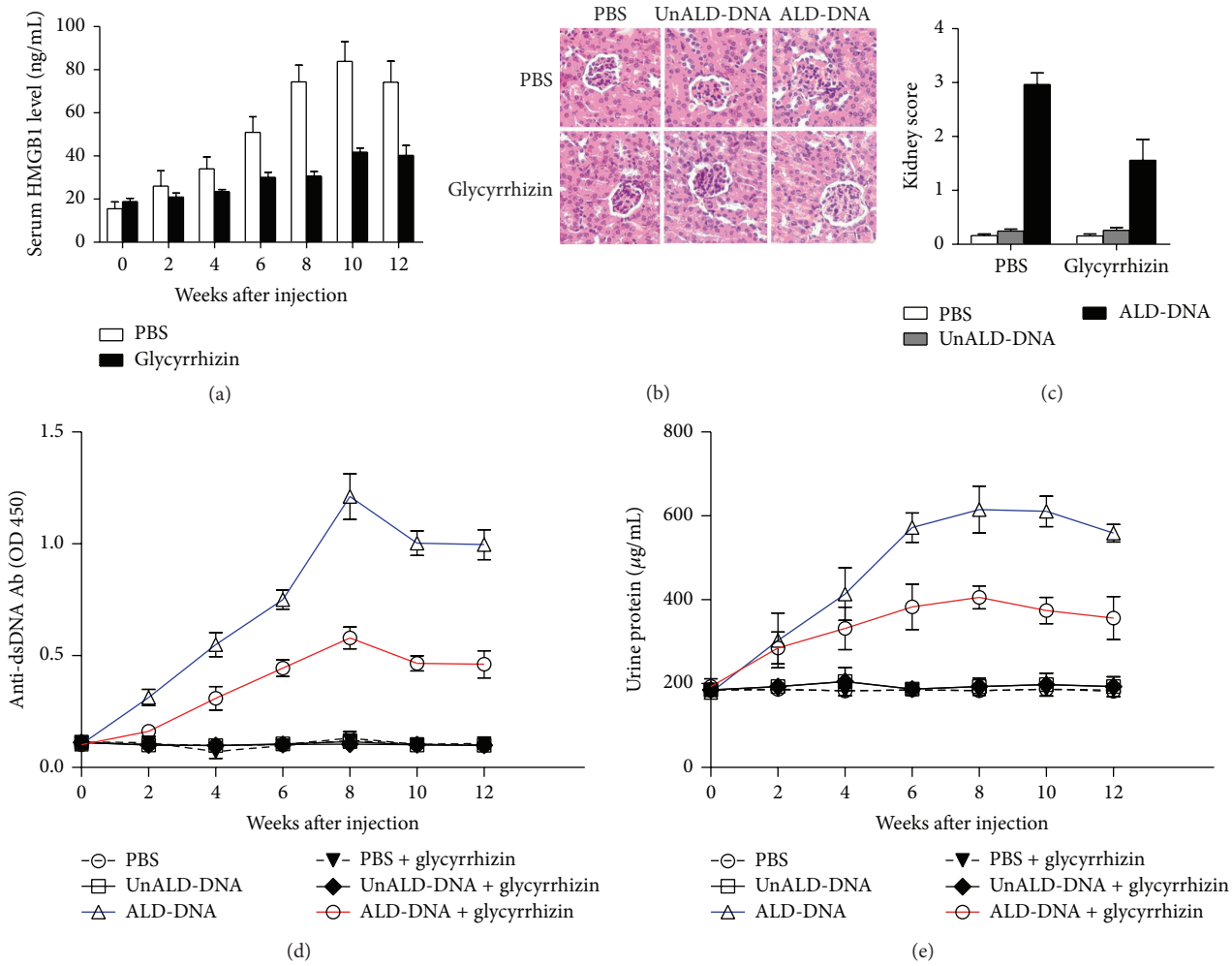

FIGURE 3: Inhibition of HMGB1 function could ameliorate the severity of SLE. BALB/c mice were administrated intramuscularly injected with glycyrrhizin (0.5 mg/mouse) or PBS to inhibit HMGB1 function. 72 h after injection, mice were then injected subcutaneously with ALD-DNA (50 μg/mouse) for total 3 times in 4 weeks. (a) The dynamics of serum HMGB1 levels were determined by ELISA every 2 weeks after initial injection. Data are means ± SD from 8 mice in each group. (b) Nephritic pathology was evaluated by H&E staining of renal tissues. Images (magnification ×200) are representative of at least 8 mice in each group. (c) The kidney score was assessed using paraffin sections stained with H&E in (b). ($n = 8$). (d) Serum anti-dsDNA antibody levels were measured by ELISA every 2 weeks after initial injection. Data are means ± SD from 8 mice in each group. (e) Urine protein levels of the mice were assessed by BCA method every 2 weeks. Data are means ± SD from 8 mice in each group. $^{*}P < 0.05$.

within the inflamed kidneys and are key mediators in lupus nephritis [44–49]. Our previous studies have also confirmed that macrophage is the central mediator in ALD-DNA-induced SLE [33–36]. To study whether HMGB1 was involved in macrophage inflammatory response, we detected the production of HMGB1 in RAW264.7 cells stimulated with ALD-DNA. The results showed that the ALD-DNA administration led to the upregulation of HMGB1 levels in RAW264.7 cells (Figures 4(a) and 4(b)). We then investigated the role of HMGB1 in ALD-DNA-induced macrophage inflammatory response by transfecting pHMGB1 into RAW264.7 cells. Results showed that transfection of pHMGB1 notably increased HMGB1 levels in RAW264.7 cells (Figure 4(c)). Of importance, HMGB1 overexpression in RAW264.7 cells was found to aggravate the secretion of TNF-α (Figure 4(e)) and IL-6 (Figure 4(f)) induced by ALD-DNA. We further downregulated HMGB1 expression by specific siRNA against HMGB1 (siHMGB1) in RAW264.7 cells. Western blot showed that transfection of siHMGB1 efficiently decreased HMGB1 levels in RAW264.7 cells (Figure 4(d)). We stimulated siHMGB1-treated RAW264.7 cells with ALD-DNA, followed by detecting the concentrations of proinflammatory cytokines in the supernatants. Results showed that the siHMGB1-mediated downregulation of HMGB1 levels significantly inhibited the secretion of TNF-α (Figure 4(g)) and IL-6 (Figure 4(h)) in ALD-DNA-stimulated macrophages. Moreover, we isolated $CD11b^{+}/F4/80^{high}$ renal macrophages from pHMGB1- or empty vector-treated SLE mice, glycyrrhizin- or PBS-treated SLE mice and stimulated these cells with ALD-DNA, followed by detecting the production of TNF-α and

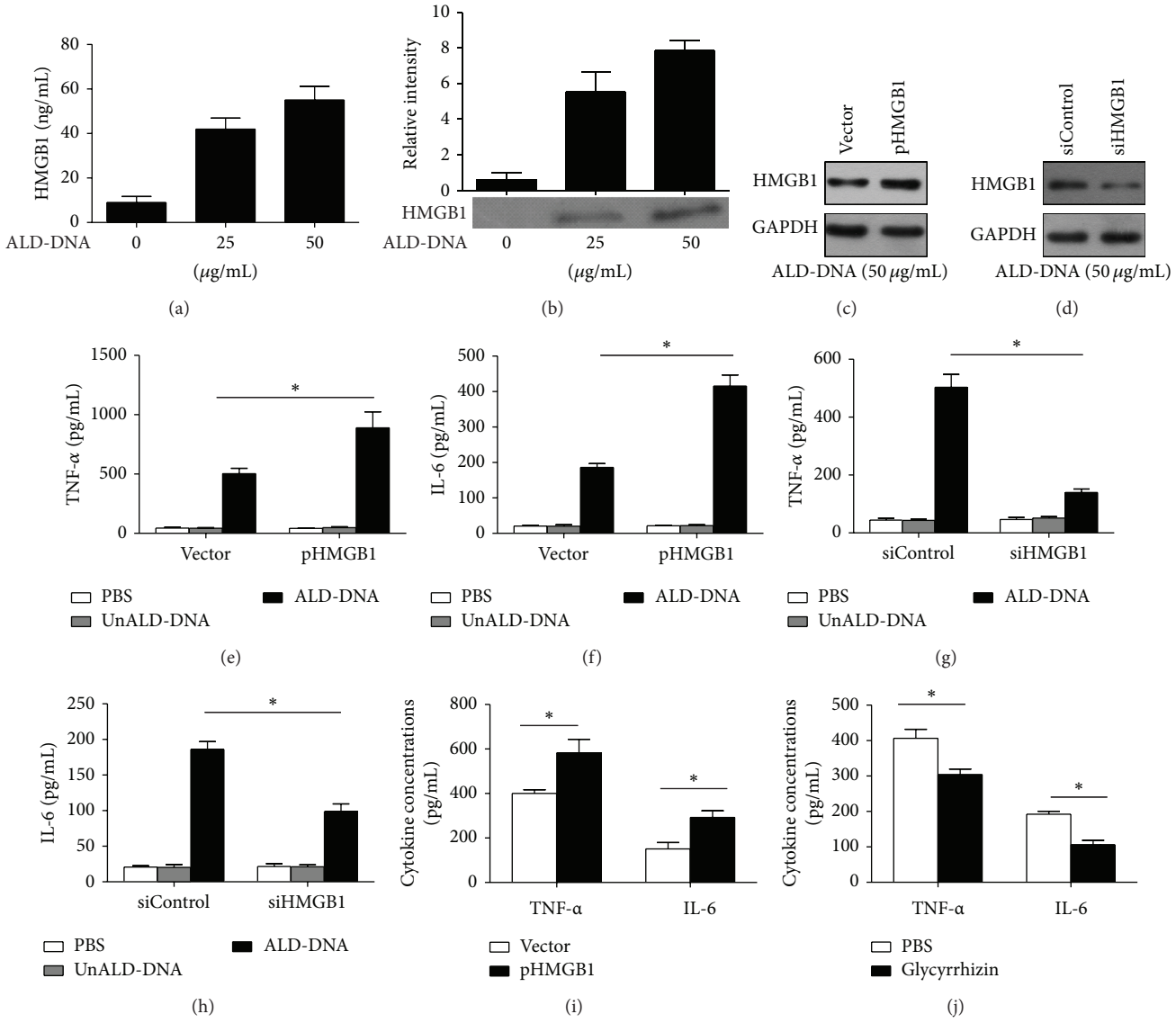

Figure 4: HMGB1 aggravated macrophage inflammatory response. (a-b) RAW264.7 cells were stimulated with ALD-DNA (0, 25, 50 μg/mL) for 24 h, levels of HMGB1 in the supernatants of RAW264.7 cells were analyzed by ELISA (a) and western blot analysis (b). Data are means ± SD of three independent experiments. (c-d) The efficiency of HMGB1 overexpression (c) and knockdown (d) was monitored by representative immunoblot of three independent experiments in ALD-DNA-stimulated RAW264.7 cells. (e-f) RAW264.7 cells were transfected with pHMGB1 or vector. 72 h after transfection, RAW264.7 cells were stimulated with PBS, UnALD-DNA or ALD-DNA (50 μg/mL) followed by analyzing the levels of TNF-α (e) and IL-6 (f) in the culture supernatants of RAW264.7 cells. Data are means ± SD of three independent experiments. (g-h) RAW264.7 cells were transfected with control siRNA (200 nM) or HMGB1 siRNA (siHMGB1, 200 nM). After 72 h RAW264.7 cells were stimulated with PBS, UnALD-DNA or ALD-DNA (50 μg/mL). ELISA assay was used to analyze the levels of TNF-α (g) and IL-6 (h) in the culture supernatants of RAW264.7 cells. Data are means ± SD of three independent experiments. (i-j) $CD11b^+/F4/80^{high}$ renal macrophages were sorted from nephritic single-cell suspensions from (i) pHMGB1- or empty vector-treated, (j) glycyrrhizin- or PBS-treated SLE mice by flow cytometry. Macrophages (2×10^5/mL) were stimulated with ALD-DNA (50 μg/mL) for 24 h. The supernatants were collected and assayed for the TNF-α and IL-6 concentrations using ELISA. Data are means ± SD from 8 mice in each group. $^*P < 0.05$.

IL-6. As shown in Figure 4(i), renal macrophages secreted much higher levels of TNF-α and IL-6 in pHMGB1-treated SLE mice than those from empty vector-treated SLE mice, whereas renal macrophages from glycyrrhizin-treated SLE mice secreted lower levels of TNF-α and IL-6 than those PBS-treated SLE mice (Figure 4(j)).

To study the relationship between HMGB1 and proinflammatory cytokines, we analyzed the correlation between HMGB1 and proinflammatory cytokines (TNF-α and IL-6) in SLE patients. We first detected the levels of serum TNF-α and IL-6 in SLE patients, and the results demonstrated that the concentrations of TNF-α (Figure 5(a)) and IL-6 (Figure 5(b))

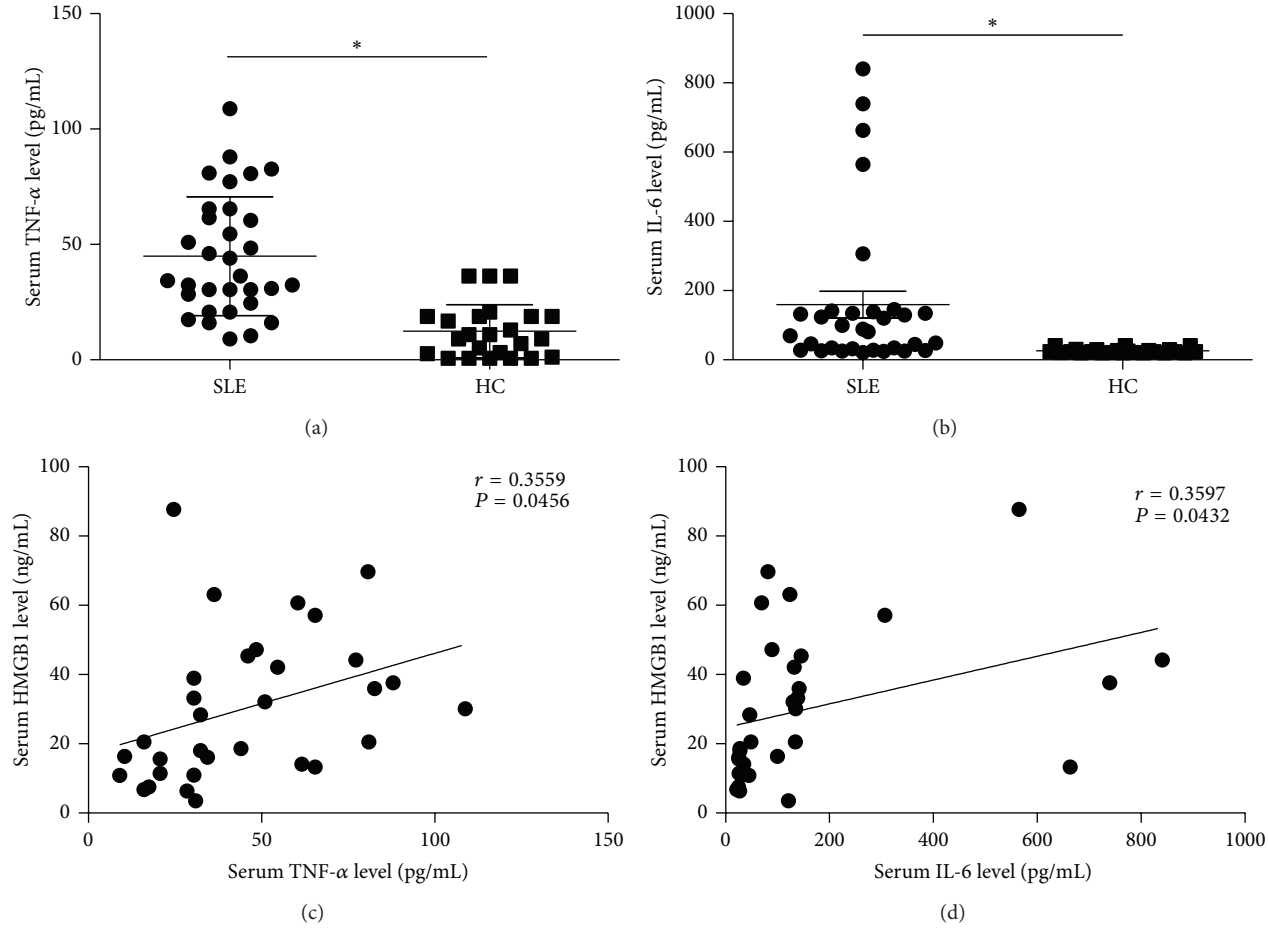

Figure 5: The HMGB1 levels were associated with proinflammatory cytokines in SLE patients. (a-b) TNF-α (a) and IL-6 (b) concentrations in sera from SLE patients and HC were detected by ELISA. The scatter-plot represented the TNF-α and IL-6 levels by ELISA analysis. Each symbol represents one SLE patient. Horizontal lines represent the median. Data represent the average from experiments performed in triplicates for each patient. (c-d) Correlation analyses were presented between HMGB1 and TNF-α levels (c), HMGB1 and IL-6 levels (d). Pearson correlation analysis was used in the correlation analysis. $^{*}P < 0.05$.

in SLE patients were significantly higher than those in HC ($P < 0.05$). Further study was conducted to analyze the correlation between HMGB1 and proinflammatory cytokines (TNF-α and IL-6). We observed that HMGB1 levels were associated with TNF-α ($r = 0.3559$, $P = 0.0456$, Figure 5(c)) and IL-6 levels ($r = 0.3597$, $P = 0.0432$, Figure 5(d)) in SLE patients.

Taken together, these data indicated that HMGB1 was pivotal for ALD-DNA-induced macrophage inflammatory response and correlated with the levels of TNF-α and IL-6 in SLE.

3.4. HMGB1-Enhanced Macrophage Inflammatory Response Was Dependent on RAGE but Not on TLR2 and TLR4. Previous studies indicate that HMGB1 is an endogenous ligand of RAGE, TLR2, and TLR4 [50–52]. To evaluate which receptor might be involved in the HMGB1-mediated inflammatory effect in ALD-DNA-stimulated macrophages, we first upregulated HMGB1 expression in RAW264.7 cells and then stimulated these cells with ALD-DNA in the presence of TLR2/4 inhibitor (OxPAPC) or RAGE inhibitor (RAGE-Fc). The results showed that the production of TNF-α (Figure 6(a)) and IL-6 (Figure 6(b)) from RAW264.7 cells exposed to TLR2/4 inhibitor was not impaired in the process of HMGB1-enhanced macrophage inflammatory response, whereas blocking the function of RAGE with RAGE-Fc effectively reduced the secretion of TNF-α (Figure 6(a)) and IL-6 (Figure 6(b)). To further validate the importance of RAGE receptor, we downregulated the expression of RAGE by siRNA and then evaluated the effect of HMGB1 on the production of proinflammatory cytokines in ALD-DNA-stimulated RAW264.7 cells. Western blot analysis confirmed that transfection of siRAGE could notably decrease RAGE levels in RAW264.7 cells (Figure 6(c)). Of importance, it was found that siRNA-mediated downregulation of RAGE significantly inhibited HMGB1-enhanced release of TNF-α (Figure 6(d)) and IL-6 (Figure 6(e)) in ALD-DNA-stimulated macrophages. Collectively, these data indicated that RAGE might be critical for proinflammatory signaling during the process of HMGB1-aggravated macrophages inflammatory

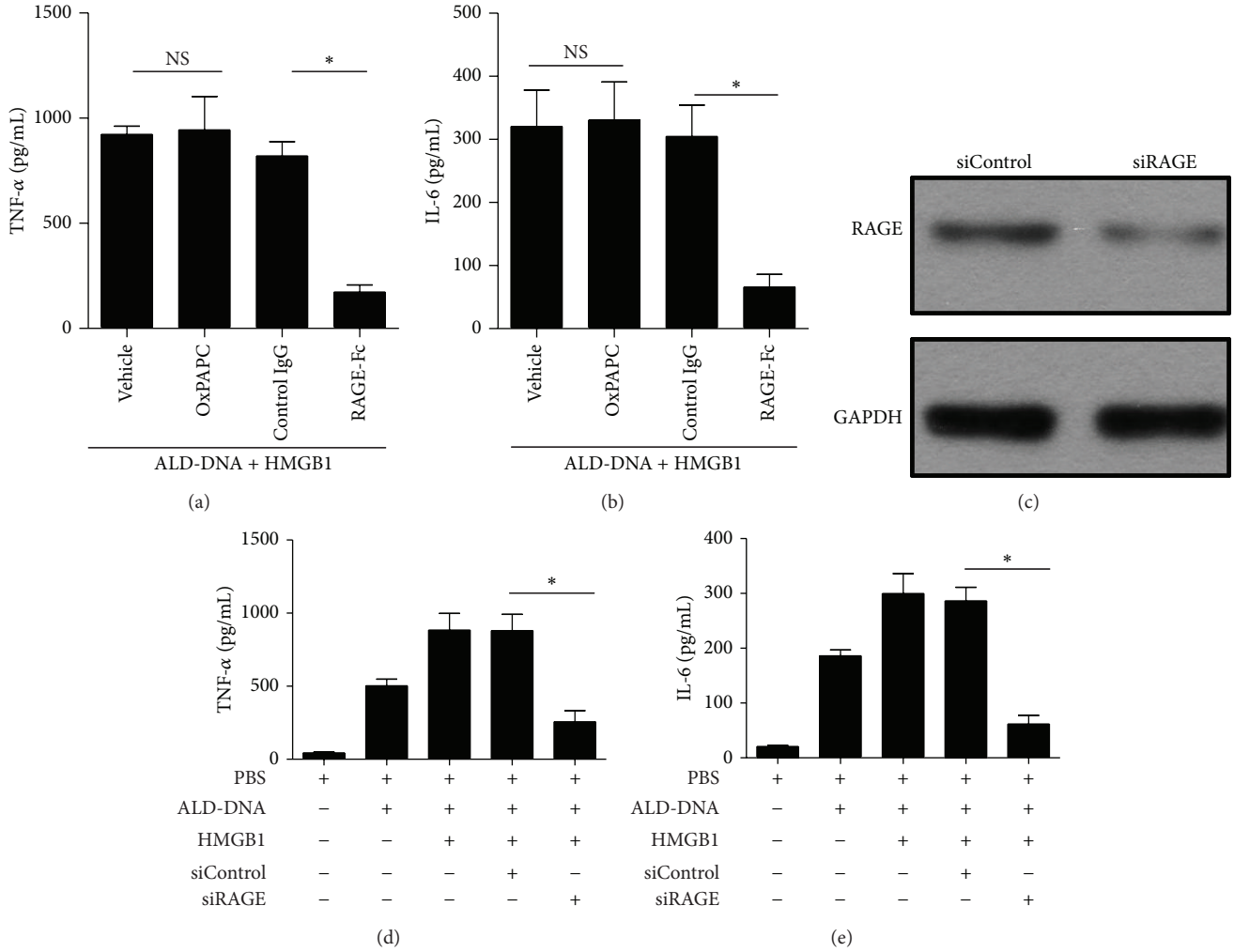

Figure 6: HMGB1-enhanced macrophage inflammatory response induced by ALD-DNA might be dependent on RAGE but not on TLR2 and TLR4. (a-b) RAW264.7 cells were transfected with pHMGB1, and then stimulated with ALD-DNA (50 μg/mL) in the presence of OxPAPC (30 μg/mL) or RAGE-Fc (10 μg/mL) for 24 h. The supernatants were collected and assayed for the concentrations of TNF-α (a) and IL-6 (b) using ELISA. (c) Representative immunoblot of three independent experiments has shown the efficiency of RAGE knockdown. (d-e) RAW264.7 cells transfected with siRAGE and pHMGB1 were stimulated with 50 μg/mL of ALD-DNA for 24 h. The supernatants were collected and assayed for the concentrations TNF-α (d) and IL-6 (e) using ELISA.* $P < 0.05$.

response induced by ALD-DNA, whereas TLR2 and TLR4 appeared to be dispensable.

4. Discussion

SLE, a chronic inflammatory autoimmune disorder, is a potentially fatal disease characterized by immune complex deposition and the subsequent inflammation that contributes to severe tissue damage [3, 5]. Recent reports show that some multifunctional proteins such as HMGB1 might be involved in autoimmune and inflammatory diseases including SLE [31, 52–55]. Our previous study revealed that HMGB1 was required for autoantibody induction by DNA-containing immune complexes in SLE patients [56], implying that the critical role of HMGB1 in the pathogenesis of SLE. In the present investigation, we further explored the role and mechanism of HMGB1 in the pathogenesis of SLE.

Our results demonstrated that HMGB1 levels were increased and correlated with the severity of SLE in both clinical patients and murine model, consistent with previous findings [57–60]. To evaluate whether HMGB1 was involved in the pathogenesis of SLE, we overexpressed HMGB1 in ALD-DNA-immunized mice and found that HMGB1 overexpression significantly enhanced the severity of SLE. To further confirm the role of HMGB1 in SLE, we broke its function with glycyrrhizin. Glycyrrhizin is commonly used in clinical application for its anti-inflammatory activity, and it binds to HMGB1 directly, thereby inhibiting the extracellular HMGB1 secretion [41]. Evidence also shows that glycyrrhizin administration could suppress HMGB1 function resulting in

the amelioration of ischemic spinal cord injury and damage caused by cerebral hemorrhage [42, 43]. Our results showed that blockade of HMGB1 function by glycyrrhizin led to dramatic downregulation of serum HMGB1 levels, and thus decreased the severity of SLE. These data suggested that HMGB1 played a crucial role in the pathogenesis of SLE, implicating a promising HMGB1-based therapeutic strategy against SLE. However, the mechanisms of HMGB1 elevation in SLE still deserved further studies.

The increasing number of evidence has emerged to suggest the crucial role of proinflammatory cytokines in the pathogenesis of SLE. The consequence of disorder of proinflammatory cytokines would be an immune dysregulation followed by local inflammatory processes and tissue damage [61, 62]. Circumstantial data suggests that TNF-α may serve as an important autocrine and paracrine factor in glomerular injury [11–13]. In addition, IL-6 is produced in many cell types like monocytes, fibroblasts, endothelial cells, and also T and B lymphocytes and has a range of biological activities on various target cells [63]. Considering the importance of HMGB1 in the regulation of inflammatory response, we analyzed the correlation between HMGB1 and proinflammatory cytokines expression in SLE. Our studies found that the concentrations of TNF-α and IL-6 in SLE patients were significantly higher than those in HC, consistent with previous study [64]. Further studies showed that HMGB1 levels were correlated with the levels of TNF-α and IL-6 in SLE patients. Moreover, our data found that HMGB1 promoted inflammatory response of renal macrophages in SLE mice. These results suggested that HMGB1 might be involved in the pathogenesis of SLE via regulating macrophage inflammatory response, however, the definite relationship between HMGB1 and macrophage inflammatory response needs further investigation.

Accumulating data demonstrate that activated macrophages that infiltrate kidneys mediate the onset of an aggressive adaptive immune response leading to the pathogenesis of SLE in mice [65–70]. These reports give a clue that macrophages play a crucial pathogenic role in the development of SLE. Our previous study has also indicated that ALD-DNA immunization lead to macrophage infiltration and aberrant activation, which mediate the onset and aggravation of SLE, indicating that aberrant activation of macrophage plays a crucial pathogenic role in ALD-DNA-induced SLE [33–39]. Here, we found that HMGB1 enhanced ALD-DNA-induced macrophage inflammatory response both *in vivo* and *in vitro*. The HMGB1 levels were closely correlated with macrophage inflammatory response. HMGB1 is a ubiquitously expressed, abundant architectural chromosomal protein of 215 amino acids, with a highly conserved sequence across species [29]. At least three receptors are reported to mediate the proinflammatory and immune-activate effects of extracellular HMGB1: RAGE, TLR2, and TLR4 [25–28]. Our results suggested that RAGE might be critical for proinflammatory signaling during the process of HMGB1-aggravated macrophage inflammatory response induced by ALD-DNA, whereas TLR2 and TLR4 seemed to be dispensable. Collectively, it seemed that HMGB1 was a crucial cofactor that could modify the stimulatory activity of macrophage.

5. Conclusion

In summary, our research reported that HMGB1 levels were significantly increased and correlated with SLE disease activity in both clinical patients and murine model. Further study suggested that HMGB1 aggravated the severity of SLE via facilitating macrophage inflammatory response. Moreover, RAGE might be critical for proinflammatory signaling during the process of HMGB1-aggravated macrophages inflammatory response. These findings may help to develop anti-inflammatory therapeutics which blunted macrophage activation by blocking HMGB1 function in SLE.

Conflict of Interests

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

Acknowledgments

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Clinical Study

Influence of Anti-TNF and Disease Modifying Antirheumatic Drugs Therapy on Pulmonary Forced Vital Capacity Associated to Ankylosing Spondylitis: A 2-Year Follow-Up Observational Study

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Objective. To evaluate the effect of anti-TNF agents plus synthetic disease modifying antirheumatic drugs (DMARDs) versus DMARDs alone for ankylosing spondylitis (AS) with reduced pulmonary function vital capacity (FVC%). *Methods*. In an observational study, we included AS who had FVC% <80% at baseline. Twenty patients were taking DMARDs and 16 received anti-TNF + DMARDs. Outcome measures: changes in FVC%, BASDAI, BASFI, 6-minute walk test (6MWT), Borg scale after 6MWT, and St. George's Respiratory Questionnaire at 24 months. *Results*. Both DMARDs and anti-TNF + DMARDs groups had

similar baseline values in FVC%. Significant improvement was achieved with anti-TNF + DMARDs in FVC%, at 24 months, when compared to DMARDs alone ($P = 0.04$). Similarly, patients in anti-TNF + DMARDs group had greater improvement in BASDAI, BASFI, Borg scale, and 6MWT when compared to DMARDs alone. After 2 years of follow-up, 14/16 (87.5%) in the anti-TNF + DMARDs group achieved the primary outcome: FVC% ≥80%, compared with 11/20 (55%) in the DMARDs group ($P = 0.04$). *Conclusions*. Patients with anti-TNF + DMARDs had a greater improvement in FVC% and cardiopulmonary scales at 24 months compared with DMARDs. This preliminary study supports the fact that anti-TNF agents may offer additional benefits compared to DMARDs in patients with AS who have reduced FVC%.

1. Introduction

Patients with ankylosing spondylitis (AS) present a higher proportion of pleuropulmonary and lung functional abnormalities when compared to healthy controls [1]. Mainly, a prevalence of 18–57% in restrictive respiratory pattern on pulmonary function tests (PFT) has been related to spinal and chest wall mobility limitation and in some extent to disease activity indices [2–5], suggesting that disease activity could play a role. Other research groups have suggested the existence of a subjacent inflammatory process in the lung parenchyma of some patients with AS that leads to a reduction of ventilatory function with repercussion in the forced vital capacity. Şenocak et al. [6], for example, observed abnormalities in 85% of their patients with AS in the high resolution computed tomography. Our research group has recently described that around 57% of AS patients had abnormalities in the lung function tests suggesting a restrictive pattern [7]. A systematic review [8] has identified that, in patients with AS, abnormalities in the HRCT can be found in up to 61%. Different findings include radiographic features of nonspecific interstitial abnormalities in 33%, interlobular septal thickening in 30%, ground glass attenuation in 11.2%, and upper lobe fibrosis in 6.9% [8]. Despite these findings, the information available evaluating an association between treatment and improvement in PFT is scarce. So far, only one prospective clinical trial related to PFT has been published, where a significant improvement on PFT was shown after 12 weeks of treatment with etanercept when compared to placebo [9]. With respect to clinical trials, in 2011, Braun et al. demonstrated superiority in clinical efficacy achieved with anti-TNF agents versus synthetic disease modifying antirheumatic drugs (DMARDs) in AS [10].

Until now, the long-term effect of synthetic DMARD and anti-TNF agents on PFT has not been systematically compared in AS patients. Therefore, the objective of the present study was to evaluate, in a two-year observational study, modifications on pulmonary FVC% as well as other cardiopulmonary indices in patients with AS who had reduced FVC% receiving DMARDs or anti-TNF agents + DMARDs.

2. Patients and Methods

2.1. Study Design. The study is designed as observational prospective cohort with two-year follow-up.

2.2. Patients Selection. We enrolled patients attending an outpatient rheumatology clinic, in a secondary-care center (Hospital General Regional No. 110 of the IMSS) in Guadalajara, Jal., Mexico. To be eligible for the study patients met the following criteria:

(a) to be ≥18 years old, (b) to meet the modified New York criteria [11], and (c) to have pulmonary function tests with a reduced FVC% <80% [12, 13].

2.3. Methodology to Evaluate Patients. One-hundred and twenty consecutive AS patients were screened with PFT, seeking abnormalities in the FVC%. Spirometry was performed according to the ATS/ERS Task Force considerations [14, 15]. Other parameters evaluated besides the FVC% were forced expiratory volume in one second (FEV_1) and the FEV_1/FVC ratio. Observed values were expressed as a percentage of the predicted value compared with individuals of similar sex, age, weight, and height. Restrictive abnormalities were defined with FVC < 80%, FEV_1/FVC ≥ 70%, and decreased or normal FEV_1%. A normal PFT was considered when patients had FVC% > 80%, FEV_1 > 80%, and FEV_1/FVC > 80%. Furthermore, we arbitrarily classified the reduced FVC% as follows: mild decrement (≥70–79% of predicted value), moderate decrement (51–69% of predicted value), and severe decrement (50% or less of predicted value). We excluded patients with overlapping syndrome (defined as those patients with a suspected or confirmed connective tissue disease associated to AS such as rheumatoid arthritis, systemic lupus erythematosus, or scleroderma), pregnant patients, and pateints with presence of active infections, cardiac failure grade III or IV, chronic obstructive pulmonary disease, asthma, or pulmonary tuberculosis.

2.4. Cohort Assembly. The decision to prescribe DMARDs or the addition of anti-TNF agent + DMARDs was left to attending rheumatologist discretion, which was independent of the study. In our hospital except in well-defined cases where synthetic DMARD may have intolerable side effects, all the patients with a diagnosis of AS initiate with NSAIDs combined with a DMARD (usually sulfasalazine or methotrexate) and anti-TNF agents are prescribed only after failure to this combination. Nevertheless, the rheumatologist can be free to prescribe a second combined synthetic DMARD before the initiation of anti-TNF agents in those patients with reasonable suspicion that they may achieve a satisfactory response. Patients with AS and the presence of uveitis received azathioprine. According to the therapeutic schema for treatment, patients that were escalated to receive anti-TNF therapy continued taking the synthetic DMARD, which was originally prescribed. All the patients that received an anti-TNF agent were tested with a PPD and chest X-rays in order to discard latent tuberculosis. If a patient presented a positive PPD test (>5 mm), or suggestive images on X-rays,

they were excluded from initiation of anti-TNF therapy and referred to the specialist in infectious diseases.

Two groups were assembled: (a) DMARDs ($n = 20$): patients receiving a DMARD, including one or more of the following drugs: oral methotrexate: 7.5–15 mg/week and oral sulfasalazine 1–1.5 g/day during the entire study period, and (b) patients who escalated to anti-TNF agent + their previous DMARD (anti-TNF + DMARDs) ($n = 16$). Briefly, as described above, this cohort of escalation to anti-TNF agent was constituted by these patients with AS DMARD treatment who fail to achieve improvement with DMARDs alone; therefore, an anti-TNF treatment was added by the rheumatologist, either etanercept administered as a subcutaneous injection 25 mg twice a week; infliximab 3–5 mg/kg given intravenously at zero, two, and four weeks and thereafter every eight weeks; or subcutaneous adalimumab 40 mg every 2 weeks, during the entire study period.

2.5. Cointerventions and Dropouts. During the study period all patients received oral nonsteroidal anti-inflammatory drugs (NSAIDs) and in case of being required one or more shot of intramuscular diclofenac were used as adjuvant treatment for spinal or inflammatory joint pain. Additionally, they could receive oral acetaminophen 500 mg to 2 gr daily for pain. Discontinuation or changes in originally assigned therapy were identified and reported.

2.6. Baseline Evaluations. PFT including FVC%, FEV, and FEV_1/FVC were performed as described previously. A structured questionnaire was used to evaluate demographic and clinical variables including disease duration, smoking, and comorbidities. Patients were assessed by the same trained researcher at baseline (time of the initial prescription by attending rheumatologist) and at 6, 12, 18, and 24 months for the following variables: (a) disease activity according to Bath Ankylosing Spondylitis Activity Index (BASDAI) [16] and (b) functioning according to Bath Ankylosing Spondylitis Functional Index (BASFI) [17]. In order to evaluate repercussions secondary to lung affection (deteriorated FVC%), we used the following indices: (a) Saint George Respiratory Questionnaire (SGRQ) [18], a specific health-related quality of life index (HRQOL) for patients with pulmonary disease that consists of a 50-item questionnaire, evaluating 3 domains: symptoms, activity, and disease impact with 10 multiple choice questions and 40 true or false answers, (b) 6-Minute Walk Test (6MWT) [19], used to evaluate one-time cardiopulmonary functional status, and (c) Modified Borg Scale that provides an individual measurement of dyspnea intensity before and after the 6MWT; this test was used to assess the severity of dyspnea [20].

2.7. Follow-Up Evaluations. All patients were followed up with the similar strategy. Follow-up took place at 6-month intervals during a period of 2 years. Throughout each visit, the same researchers completed a questionnaire detailing any change in antirheumatic therapy, adverse events associated with the therapy, and evaluated FVC%, FEV_1, FEV_1/FVC, BASDAI, BASFI, SGRQ, 6MWT and the Modified Borg Scale.

2.8. Primary Outcome Measure. Response was defined as increment in FVC% based on the statistical difference between the evaluations during the follow-up compared with baseline and with the immediate previous measurement. Additionally, improvement in disease activity, functioning indices, and cardiopulmonary scales were also evaluated as secondary outcome measures.

2.9. Discontinuation. Reasons for discontinuation were identified.

2.10. Statistical Analysis. Due to the nonparametric distribution of the data and/or small sample size, we used medians and ranges in order to describe quantitative variables and for qualitative variables, frequencies, and percentages. Mann-Whitney U test was used to compare quantitative variables including medians of FVC% and clinical characteristics between the two groups: (a) DMARDs and (b) anti-TNF + DMARDs. Chi-square (or Fisher exact test when appropriated) were used to compare proportions of qualitative variables between groups of treatment and McNemar test was used to compare differences in intragroup proportions at 2 different time points. For comparison between FVC% at follow-up regarding the baseline values and at 2 different time points, we used Wilcoxon test, and when the comparisons included 3 or more time points we used Friedman test. Statistical significance was considered as $P \leq 0.05$. All statistical analyses were performed using SPSS, version 10.0.

2.11. Ethics. The study was approved by the Institutional Review Board of the Mexican Institute for Social Security (IMSS) of the participating hospital (approval number IMSS R-2009-1301-63); all patients were informed about the study objectives and signed a voluntary consent prior to inclusion. The study was performed following the guidelines of the Declaration of Helsinki.

3. Results

Figure 1 shows the cohort flowchart. We screened 120 patients with AS. Sixty-five patients (54.17%) were excluded because they had normal PFT, 11 (9.17%) with restrictive ventilatory pattern were excluded because they had coexisting asthma, and 8 patients (6.67%) were excluded because they had active infection. Therefore, 36 patients with AS and FVC < 80% were included; from them, 20 were receiving DMARDs and 16 received anti-TNF agents + DMARDs.

There were no significant differences between age, gender, disease activity parameters, lung function test results, cardiopulmonary scales, and SGRQ at baseline (Table 1). A small difference, yet not significant, was observed between DMARDs and anti-TNF + DMARDs groups in disease duration (11 years versus 15 years, resp., $P = 0.07$). In the DMARDs group, 8 patients (40%) were taking methotrexate + sulfasalazine, 5 (25%) sulfasalazine alone, 4 (20%) methotrexate alone, and 2 (10%) methotrexate + azathioprine and 1 patient (5%) received azathioprine alone. In anti-TNF + DMARDs group, 10 patients (62.4%) received etanercept, 5 (31.3%) infliximab, and 1 (6.3%) adalimumab; DMARDs prescribed

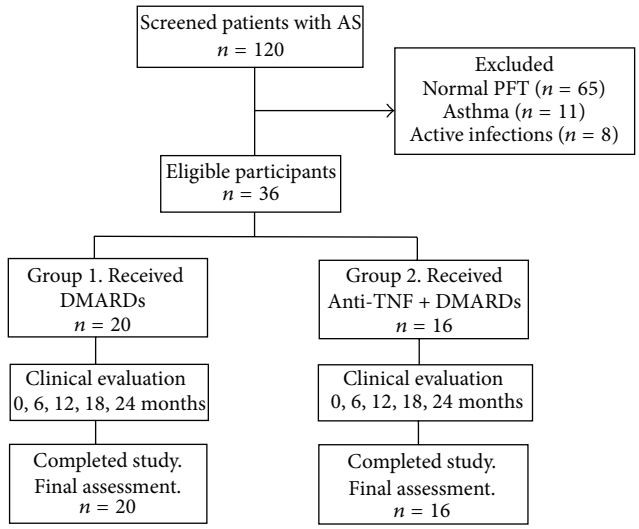

Figure 1: Flowchart of the patients during the cohort. AS, ankylosing spondylitis; LFT, lung function test; DMARDs, disease modifying antirheumatic drugs.

in combination with anti-TNF were methotrexate alone in 4 patients, azathioprine alone in 2, sulfasalazine in 3, and methotrexate + sulfasalazine in 4 patients, methotrexate + azathioprine in 1, sulfasalazine + azathioprine in 1 and methotrexate + sulfasalazine + azathioprine in 1 patient. All patients continued receiving this medication during the entire study.

Table 2 shows the secondary outcomes observed in both groups. In the intragroup comparisons at 12 months or 24 months versus baseline there was a significant improvement in all secondary variables in the DMARDs group including BASDAI, BASFI, Post-6MWT Borg scale, 6MWT, and total STGRQ%. Similarly in the intragroup comparison between 12 and 24 months versus baseline in the anti-TNF + DMARDs group a significant improvement was observed in all these variables.

3.1. Comparison of Changes in FVC%. Figure 2 shows the increment observed in FVC% from baseline to the end of the follow-up in the group with anti-TNF + DMARDs versus the group with DMARDs alone. A persistent increment in FVC% was observed in both groups, with this improvement in FVC% being higher in the anti-TNF + DMARDs group and statistical significance being observed for the difference (Δ) of this increment in favor of the anti-TNF + DMARDs since the 6 months ($P < 0.001$) and persisting with significant differences during all the length of follow-up.

3.2. Primary Outcomes: Comparison between Groups. Table 3 shows the comparison between groups on the primary outcomes: there was a clear difference in the increase of FVC% in the group receiving anti-TNF + DMARDs at 24 months with respect to DMARDs alone group and there was also a higher increase in FEV_1/FVC in patients receiving anti-TNF + DMARDs at 24 months with respect to DMARDs alone group ($P = 0.03$). Only 2 patients (12.5%) remained as a restrictive pattern in LFT in the anti-TNF + DMARDs at 24 months compared with 9 (45%) of the patients in the DMARDs alone group. Finally a 87.5% of patients in the anti-TNF + DMARDs group achieved normal FVC% at 24 months (≥80%) compared with only 55% achieving normal FVC% in the DMARDs alone group ($P = 0.04$).

3.3. Side Effects. Most frequent adverse events in DMARDs alone group were gastritis and other gastrointestinal side effects (30%), anorexia (25%), and upper airways infections (25%), while in anti-TNF + DMARDs group they were local reactions (44%), upper airways infections (38%), and gastritis and vomiting (13% each). There were no differences in total adverse events between groups of treatment.

4. Discussion

In this study we observed that AS patients receiving anti-TNF + DMARDs had a greater significant improvement in FVC% and other pulmonary test parameters at 24 months compared with patients who were taking only DMARDs. To date, the information about the effect of DMARDs in PFT associated to AS is scarce. In our study, the quality of life associated with pulmonary affection (SGRQ) and cardiopulmonary tests including Borg and 6MWT improved significantly in both groups during the 2 years of follow-up.

Our findings are in accordance with the study by Dougados et al. [9] in their 12-week controlled trial, since they found a significant improvement in FVC% and other parameters of lung function tests with etanercept in comparison to placebo. This remarkable study has some differences in comparison with our study: first the study performed by Dougados et al, was a randomised controlled double-blind trial providing high quality information; nevertheless our study has 3 main interesting differences with the previous study. First we had a comparison group with DMARDs and this provides a comparison with drugs that may modify the response in our outcomes; even when compared with DMARDs the addition

Table 1: Comparison of selected variables at baseline between treatment groups.

Variable	Treatment groups		P
	DMARDs $n = 20$	Anti-TNF + DMARDs $n = 16$	
Males, n (%)	14 (70.0)	14 (87.5)	0.26
Age, years	41 (26–67)	45 (27–59)	0.40
AS characteristics			
Disease duration, years	11 (1–27)	15 (7–27)	0.07
Past history or present history, n (%)			
Enthesopathy	7 (35.0)	11 (68.8)	0.09
Peripheral arthritis	3 (15.0)	6 (37.5)	0.15
Uveitis	3 (15.0)	5 (31.3)	0.42
BASDAI, units	5 (3–8)	5 (4–8)	0.28
BASFI, units	6 (3–7)	5 (3–8)	0.42
Erythrocyte sedimentation rate, mm/h	29.50 (21.0–62.0)	21.0 (12.0–48.0)	0.001
Presence of cervical syndesmophytes, n (%)	15 (75.0)	12 (75.0)	1.00
Presence of lumbar syndesmophytes, n (%)	12 (60.0)	9 (56.3)	0.20
Cervical or lumbar syndesmophytes bridging, n (%)	1 (5.0)	6 (37.5)	0.02
Cardiopulmonary scales			
6MWT, mt	282 (235–386)	322 (230–380)	0.89
Pre-6MWT VAS Borg scale	0 (0-1)	0 (0–0.5)	0.35
Post-6MWT VAS Borg scale	2 (0–4.6)	2.2 (0–4.1)	0.37
Development of dyspnea, n (%)	9 (45)	7 (44)	0.60
Health-related quality of life score			
SGRQ, %			
Symptoms	30 (2–57)	23 (3–43)	0.77
Activity	28 (1–59)	25 (2–57)	0.32
Impact	14 (1–43)	16 (0–45)	0.40
Total	37 (3–58)	29 (3–58)	0.29
Lung function test			
FVC (% of predicted)	69 (52–79)	69 (57–77)	0.37
FEV_1 (% of predicted)	82 (80–90)	82 (81–85)	0.60
Restrictive pattern, n (%)	20 (100)	16 (100)	1.00
Severity of restrictive pattern			
Mild (70–79%), n (%)	9 (45.0)	8 (50.0)	0.70
Moderate (50–69%), n (%)	7 (35.0)	2 (12.5)	
Severe (50% or less), n (%)	4 (20.0)	6 (37.5)	
Treatment			
Methotrexate, n (%)	14 (70.0)	10 (62.5)	1.00
Sulfasalazine, n (%)	13 (65.0)	9 (56.3)	0.73
Azathioprine, n (%)	3 (15.0)	4 (25)	0.42
Etanercept, n (%)	0	10 (62.4)	—
Infliximab, n (%)	0	5 (31.3)	—
Adalimumab, n (%)	0	1 (6.3)	—
Corticosteroids utilization, n (%)	4 (20.0)	2 (12.5)	0.67

AS, ankylosing spondylitis; DMARDs, disease modifying anti-rheumatic drugs; anti-TNF, Tumor necrosis factor-alpha; BASDAI, Bath AS Disease Activity Index; BASFI, Bath AS Functional Index; 6MWT, six-minute walk test; VAS, visual analogue scale; SGRQ, St. George's Respiratory Questionnaire; FVC, forced vital capacity; FEV_1%, forced expiratory volume in 1 second.

Quantitative variables are presented as median and range; qualitative variables are presented in number (%). Comparisons between proportions were compared with Fisher exact test; comparisons between medians were evaluated with Mann-Whitney U test.

Table 2: Secondary outcomes. Intragroup changes of selected clinical variables.

Variable	Baseline	12 months	P	24 months	P
DMARDs group (n = 20)					
BASDAI, units*	5 (3–8.0)	3 (1–6)	<0.001	1 (0–3)	<0.001
BASFI, units*	6 (3–7)	3 (1–5)	<0.001	1 (0–3)	<0.001
Post-6MWT Borg scale*	2.2 (0–4.6)	1.0 (0–3.8)	0.001	0.5 (0–2.1)	0.003
6MWT, m*	282 (235–386)	308 (280–425)	<0.001	334 (307–440)	<0.001
Total SGRQ%*	37 (3–58)	9 (0–53)	<0.001	0 (0–20)	0.003
anti-TNF + DMARDs group (n = 16)					
BASDAI, units*	5 (4–8)	2 (1–5)	<0.001	0 (0-1)	<0.001
BASFI, units*	5 (3–8)	2 (1.0–4.3)	<0.001	1 (0-1)	<0.001
Post-6MWT Borg scale*	2.2 (0–4.1)	1.3 (0–3)	0.001	0.5 (0–1.1)	0.002
6MWT, m*	322 (230–380)	368 (280–440)	<0.001	400 (315–460)	<0.001
Total SGRQ%*	29 (3–58)	7 (0–34)	0.001	0 (0–4)	0.011

DMARDs group, group receiving disease modifying antirheumatic drugs; anti-TNF + DMARDs group, group receiving antitumor necrosis factor agents + DMARDs; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functioning Index; 6MWT, six-minute walk test; SGRQ, St. George's Respiratory Questionnaire. Quantitative variables are presented as medians (and ranges); qualitative variables are presented in number (%). P values were obtained using Wilcoxon test comparing responses at 12 and 24 months with the baseline. *Significant difference with two-factor ANOVA Friedman test $P < 0.001$.

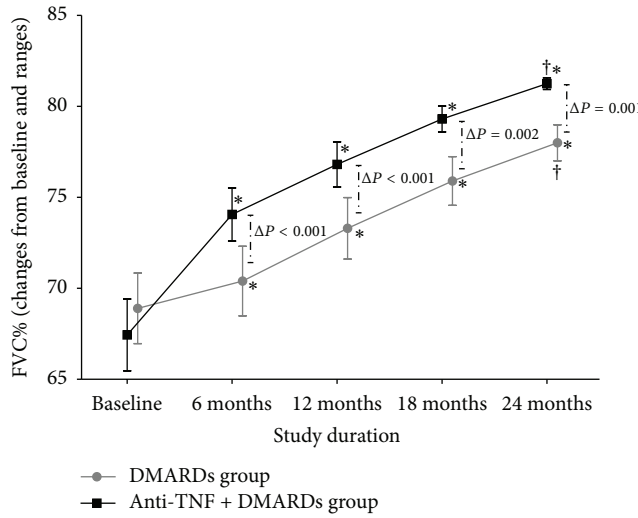

Figure 2: Changes in FVC% during 24 months of the study by treatment group. Modification of FVC (% of predicted value) from baseline to 2 years in the DMARDs (n = 20) and anti-TNF + DMARDS group (n = 16). Gray circle is DMARDs group, and black square is anti-TNF + DMARDs group. Values are represented as mean and standard error of the mean. *Comparison in means of each evaluation versus baseline using paired Student t-test ($P < 0.001$). ΔComparison of the absolute change at 6, 12, 18, and 24 months between groups using unpaired Student t-test. †Two-factor ANOVA Friedman test $P < 0.001$.

of anti-TNF agents + DMARDs shows a higher significant improvement in the pulmonary parameters. Second we had a 2 years of follow-up; during the entire study we observed a persistent increment in the FVC% corresponding to the clinical improvement not only in traditional disease activity indices but also in cardiopulmonary variables such as Borg or 6MWT, and finally this cohort represents patients with a wide spectrum of treatments and severity of the disease that is more related to the usual clinical scenario. Nevertheless it is relevant the recognition of the limitations of our exploratory study: the small sample size could reduce the representativeness of the total AS population with a restrictive ventilatory pattern and the accuracy of the observed effects in treatments; likewise, a lack of blindness might induce an expectancy bias. Only patients refractory to DMARDs were included in the anti-TNF + DMARDs group, which could affect the results observed. The use of different combinations of anti-TNF and DMARD therapies diminishes the representativeness of a specific therapy and the results could differ if each specific treatment combination were evaluated separately. Another limitation in this study is the lack of other pulmonary studies such as high resolution computed tomography (HCRT) to evaluate parenchymal damage. Future studies should address whether our observations of a sustained improvement with

Table 3: Primary outcomes. Comparison in absolute changes on pulmonary function tests between baseline and 24 months in DMARDs group versus anti-TNF+DMARDs group.

Variable	DMARDs $n = 20$			Anti-TNF + DMARDs $n = 16$			Comparison between groups at 24 months (Δ absolute change)
	Baseline	24 months	Absolute change	Baseline	24 months	Absolute change	P
FVC %, median (ranges)	69 (52–79)	80 (70–82)	11 (2–18)	69 (57–77)	82 (79–83)	13 (5–23)	0.04
FEV_1%, median (ranges)	82 (80–90)	86 (82–95)	3 (−2–8)	82 (81–85)	85 (81–90)	3 (−4–6)	0.60
Ratio FEV_1/FVC, median (ranges)	94.5 (87–112)	97 (90–107)	2 (−15-14)	84 (82–99)	90 (86–99)	4 (0–13)	0.03
Patients with improvement	% achieving improvement					% achieving improvement	Comparison of % achieving improvement
Restrictive pattern, n (%)	20 (100)	9 (45)	11 (55.0)	16 (100)	2 (12.5)	14 (87.5)	0.04
Changes of cut-off point in FVC%							
Normal (≥80%), n (%)	0	11 (55.0)	—	0	14 (87.5)	—	<0.001
FVC% 70–79%, n (%)	9 (45.0)	7 (35.0)	—	8 (50.0)	2 (12.5)	—	
FVC% 50–69%, n (%)	7 (35.0)	2 (10.0)	—	2 (12.5)	0	—	
FVC% <50%, n (%)	4 (20.0)	0	—	6 (37.5)	0	—	

DMARDs, disease modifying antirheumatic drugs; anti-TNF + DMARDs, antitumor necrosis factor + DMARDs; FVC%, forced vital capacity; FEV_1%, forced expiratory volume in 1 second. Quantitative variables are shown as median and ranges; qualitative variables are shown in frequencies (%). Absolute change is obtained from the differences at 24 months versus baseline. P values for Δ absolute change: comparison between groups of differences in absolute changes at baseline and 24 months. Comparison of the absolute change between groups at 24 month was performed with Mann-Whitney U test. Fisher exact test was used to compare the proportion of patients who achieve changes of the cut-off point in FVC%.

anti-TNF + DMARDs in FVC% and other cardiopulmonary parameters is correlated with the findings observed in HRCT in these patients.

In conclusion, we found that anti-TNF + DMARDs treatment is superior to DMARDs alone in improving PFT and functional capacity parameters in AS in long-term treatment. Further long-term double-blind, randomized, controlled trials or multicenter cohorts are required in order to corroborate the long-term effects on lung function tests of anti-TNF + DMARDs observed in the present cohort.

Conflict of Interests

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

Authors' Contribution

Alberto Daniel Rocha-Muñoz and Aniel Jessica Leticia Brambila-Tapia contributed equally to this study.

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Review Article

The Influence and Role of Microbial Factors in Autoimmune Kidney Diseases: A Systematic Review

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A better understanding of the pathophysiology of autoimmune disorders is desired to allow tailored interventions. Despite increased scientific interest a direct pathogenic factor in autoimmune renal disease has been described only in a minority like membranous nephropathy or ANCA-associated vasculitis. Nonetheless the initial step leading to the formation of these antibodies is still obscure. In this review we will focus on the possible role of microbial factors in this context. *Staphylococcus aureus* may be a direct pathogenetic factor in granulomatosis with polyangiitis (GPA). Chronic bacterial colonization or chronic infections of the upper respiratory tract have been proposed as trigger of IgA vasculitis and IgA nephropathy. Interventions to remove major lymphoid organs, such as tonsillectomy, have shown conflicting results but may be an option in IgA vasculitis. Interestingly no clear clinical benefit despite similar local colonization with bacterial strains has been detected in patients with IgA nephropathy. In systemic lupus erythematosus injection of bacterial lipopolysaccharide induced progressive lupus nephritis in mouse models. The aim of this review is to discuss and summarize the knowledge of microbial antigens in autoimmune renal disease. Novel methods may provide insight into the involvement of microbial antigens in the onset, progression, and prognosis of autoimmune kidney disorders.

1. Introduction

Over the last decades several investigations have focused on the pathogenesis of immunologically mediated kidney diseases. Besides the detection of novel aspects responsible for the onset of particular diseases, such as the phospholipase A2 (PLA2) receptor [1] and more recently thrombospondin type-1 domain-containing 7A antibodies [2] in membranous nephropathy, there has also been particular interest in the role of microbial agents.

Abnormalities in the immune reactions can induce kidney damage, either by provoking an autoimmune phenomenon or induction of molecular mimicry. The immune response comprises the innate as well as the more orchestrated adaptive immunity. The former acts as the first line of defence and is mediated by monocytes/macrophages, neutrophils, natural killer cells, complement activation, and cytokines. Pathogen-associated small molecules recognized by pattern recognition receptors such as toll-like receptors (TLR), C-type lectin receptors, and complement activation via the mannose-binding lectin pathway initiate innate recognition of bacteria and thus activation of inflammation and coagulation. Once persistent, the antigenic stimulus leads to the activation of the adaptive immunity triggering a T-cell and B-cell response. An autoimmune response occurs whenever the immune system activation leads to a response against self-antigens. Several factors are implicated in the loss of immune tolerance including genetic, hormonal, and environmental factors as well as a defective clearance of apoptotic cells [3–5].

Characterization of the human microbiome [6] offers novel opportunities for a better understanding of these complex phenomena as some commensal bacteria may provoke inflammation while others control it via engagement of TLRs and pathogen-related receptors [7]. Alterations of the microbiota have already been identified in several autoimmune diseases, including inflammatory bowel diseases, type-1 diabetes, and experimental autoimmune encephalomyelitis [8]. However, a negative impact of the microbiota on inflammation and autoimmunity in genetically susceptible subjects

has been reported as well. In particular, T-cell modulation as shown by induction of either Th17 or regulatory T-cell responses triggered development or protection from autoimmune/inflammatory disease [7, 9].

Bacterial infection-related glomerulonephritis has attracted attention for a long period of time, for example, with streptococcal infections as leading cause of postinfectious glomerulonephritis in children and staphylococcal infection in adults [10]. In this review, we focus on the microbial influence on the onset and progression of autoimmune kidney disorders.

2. Materials and Methods

2.1. Search Strategy. A systematic literature search of the MEDLINE database was conducted, using the key words: "(bacteria OR bacter* OR microbiology OR microbio* OR microbiome OR microbial OR microorganism OR microbiota) AND (glomerulonephritis OR nephrotic syndrome)." Additional studies were identified by examining the bibliography of the retrieved articles by forward search. The search was limited to articles published in English and reports on postinfectious and IgA-dominant acute staphylococcal associated glomerulonephritis forms were excluded from further analysis.

3. Results

3.1. Search Results. The systematic search (performed on July 1, 2014) overall resulted in 2508 records. A large number of articles ($n = 2196$) was excluded, since these records reported on treatment regimens, antibody findings (such as anti-neutrophil cytoplasmic antibodies), and patients with virus-related diseases (such as hepatitis C-associated membranoproliferative glomerulonephritis or human immunodeficiency virus-associated kidney disease) or were single case reports/case series. After access to full text articles we excluded another 244 articles, which were not reporting on bacterial antigens or bacteria leading to autoimmune kidney diseases but rather again reported on poststreptococcal or poststaphylococcal glomerulonephritis or viral antigens, leading to a number of 68 articles for further full text analysis. Through forward search of the retrieved bibliography we identified another 34 records, which were also analyzed. Of these, one article was not indexed in common electronic databases. After exclusion of one article reporting the same results a total of 100 records were finally included (see Figure 1).

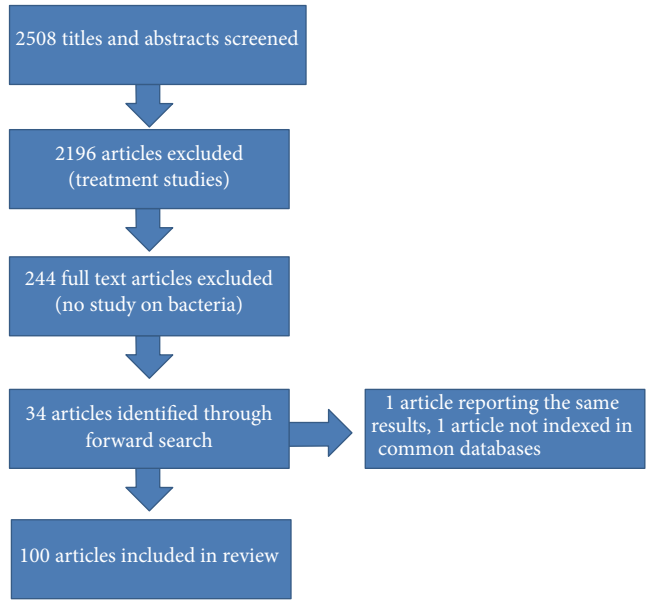

Figure 1: The search strategy is shown. Of initial 2508 titles screened, the majority (2196 articles) were excluded due to not reporting on bacterial influence in primary glomerulonephritis. 244 reports were accessed in full text but could be excluded for not reporting on bacterial agents in primary glomerular diseases (i.e., postinfectious glomerulonephritis or virus-related kidney diseases). The bibliography of the retrieved articles revealed another 34 reports. One record reporting the same results and another one not indexed in common databases were excluded from final analysis, leading to a total number of 100 articles.

3.2. Anti-Neutrophil Cytoplasmic Antibody-Associated Vasculitis. The group of anti-neutrophil cytoplasmic antibody- (ANCA-) associated vasculitis (AAV) consists of four distinct diseases, namely, granulomatosis with polyangiitis (GPA, former Wegener's granulomatosis), microscopic polyangiitis (MPA), eosinophilic granulomatosis with polyangiitis (EGPA, former Churg-Strauss syndrome), and renal limited vasculitis. The extent of kidney involvement varies between and within each clinical entity being around 90–100% in MPA, 50–80% in GPA, and, depending on the ANCA status, 4–51% in EGPA [11]. Several genetic traits, environmental factors, and the use of specific medications have been identified as potential triggers to develop AAV.

3.2.1. Granulomatosis with Polyangiitis: A Role for Staphylococcus aureus? Bacterial antigens potentially implicated in the pathogenesis of AAV have been studied for three decades. In a prospective longitudinal cohort the chronic presence of *Staphylococcus aureus* obtained from nasal swab cultures was identified as an independent risk factor for relapse of GPA when compared to noncarriers. Chronic *Staphylococcus aureus* carriers were defined as a positive culture in ≥75% of the specimen (mean of 22.6 cultures per patient, follow-up for at least one year) examined [12]. A subsequent prospective, randomized, placebo-controlled trial with a majority of GPA patients suffering from renal involvement evaluated the efficacy of trimethoprim-sulfamethoxazole twice daily for 24 months and revealed a significant reduction of disease relapses. It was postulated that the elimination or reduction of *Staphylococcus aureus* may be responsible for this beneficial effect, although nasal swabs were not performed. In addition, a significant reduction of respiratory and nonrespiratory tract infections was reported in actively treated patients [13]. Chronic nasal colonization of *Staphylococcus aureus* (positivity in ≥80% of samples obtained) was furthermore confirmed as independent risk factor for relapse in biopsy-proven GPA

in another cohort [14]. A retrospective longitudinal cohort study revealed that a high proportion of GPA patients (>70%) carried at least one *Staphylococcus aureus* superantigen, which can lead to an antigen-independent T-cell activation. In general, the presence of *Staphylococcus aureus* was accompanied by a higher risk for relapse, which however was modulated by the type of superantigen present, with mostly tsst-1 being associated with disease relapse [15]. Further investigations to address whether *Staphylococcus aureus* or related superantigens are capable of T-cell expansion were performed. T-cell expansion was present at a significantly higher rate in patients with GPA than in healthy individuals but was neither associated with the presence of *Staphylococcus aureus* nor associated with its superantigens [16]. In order to elucidate further pathogenetic consequences of *Staphylococcus aureus* presence in vasculitis, staphylococcal acid phosphatase (SAcP) and its binding ability were studied in human umbilical vein endothelial cells. SAcP was capable of binding to endothelial cells in a concentration-dependent manner and endothelial cell-bound SAcP was recognized by sera of patients with GPA [17]. Immunization of rats with *Staphylococcus aureus* ($n = 7$) led to the development of pauci-immune glomerulonephritis in one rat [18].

3.2.2. Molecular Mimicry in Pauci-Immune Glomerulonephritis: A Role for Gram Negative Bacteria? In patients with pauci-immune glomerulonephritis, gp130 was detected in about 90% of patients in their active phase and frequently coappeared with myeloperoxidase and proteinase 3 [19]. Gp 130 is identical with human lysosomal associated membrane protein (hLAMP-2) which again shares a 100% homology of the epitope $P_{41–49}$ to the amino acids 72–80 of mature bacterial adhesin FimH, which is present on the tip of fimbriae crucial for attachment to host epithelia of Gram negative bacteria such as *Escherichia coli*, *Klebsiella pneumonia*, and *Proteus mirabilis*. A retrospective survey revealed a high proportion of Gram negative infections prior to the onset of disease activity and autoantibodies to hLAMP-2 were detected in nearly all patients with renal involvement and underlying AAV in one study. The same authors reported that hLAMP-2 autoantibodies, when injected into rats, were causative of developing focal necrotizing glomerulonephritis and a monoclonal antibody directed against hLAMP-2 induced apoptosis of human microvascular endothelium *in vitro* [20]. However, another group of investigators was not able to confirm these interesting findings when injection of anti-hLAMP-2 antibodies into rats did not provoke onset of glomerulonephritis [21]. Nonetheless *Escherichia coli* immunization of rats led to induction of pauci-immune glomerulonephritis and detectable ANCA in one out of eight animals [18] and another experimental study revealed that bacterial lipopolysaccharide (LPS) dose-dependently increased renal injury induced by anti-MPO directed immunoglobulin G (IgG) as demonstrated by the formation of glomerular crescents [22] (Table 1).

3.2.3. Conclusion. In summary in GPA nasal carriage of *Staphylococcus aureus* seems to play a pivotal role in the disease onset and relapse. As far as Gram negative bacteria are concerned there is some evidence that cross reactivity of antibodies directed against surface adhesin FimH, which has structural homology with hLAMP-2 against endothelial cells, can cause vasculitis. However this evidence comes out of one single group and still needs confirmation [19, 20, 23].

3.3. IgA Vasculitis. Immunoglobulin A vasculitis (IgA vasculitis, formerly known as Henoch-Schönlein purpura) is a small vessel vasculitis with predominantly deposition of IgA1 within the capillaries, venules, or arterioles of affected tissues. The disease affects the skin and the gastrointestinal tract in a majority of patients and glomerulonephritis, indistinguishable from IgA nephropathy on kidney biopsy, is present [24] in approximately half of the cases. The risk of developing chronic kidney disease (CKD) is higher in adults when compared to children and varies between 35% and 69% [25].

Evidence for a potential microbial influence on the pathogenesis stems from the observation that onset of vasculitis is frequently preceded by an upper respiratory tract infection. It was proposed that the tonsils play an especially important role and chronic infections of the oropharynx and the middle ear can be detected in a majority of children with IgA vasculitis with apical periodontitis, rhinosinusitis, and otitis media being present in 53%, 48%, and 10%, respectively. In one report eradication of the infectious focus (administration of antibiotics and root canal therapy) resulted in a permanent remission without recurrence of disease in 31 out of 40 patients [26]. Nephritis-associated plasmin receptor (NAPI-r), a group A streptococcal antigen, was detected in ten out of 33 patients within the glomerular mesangium of patients with IgA vasculitis, whereas NAPI-r staining was present in only four out of 120 patients with other renal disorders. Moreover, patients with IgA vasculitis showed significantly higher antistreptolysin O (ASLO) titers than patients with other renal diseases and IgA vasculitis patients with positive intraglomerular NAPI-r staining exhibited significant higher ASLO titers than those without [27]. In another prospective study ASLO titer positivity was associated with a 10-fold increase in the risk of developing IgA vasculitis [28] and examination of kidney biopsies revealed presence of IgA-binding region of three different streptococcal M proteins, namely, M4, M22, and M60, in seven out of 13 subjects. On electron microscopy these were present within the glomerular basement membrane (GBM) and the mesangial matrix. Analysis of skin biopsies obtained from IgA vasculitis patients indicated the presence of the same M proteins in the majority of patients investigated but these were absent in kidney biopsy specimens of patients with anti-GBM disease, postinfectious glomerulonephritis, lupus nephritis, and membranous nephropathy [29]. Additionally, a role of *Staphylococcus aureus* has been proposed in IgA vasculitis when it was shown that the percentage of T-cell receptor-(TCR-) Vβ-(5.2 + 5.3) and Vβ 8-positive cells, members of the T-cell repertoire with marked expression after stimulation with staphylococcal enterotoxin, was significantly increased in patients with active disease when compared to healthy individuals whereas specific TCR-Vβ was not present when infection was controlled [30].

Table 1: Study characteristics of relevant studies in ANCA-associated vasculitis are shown. Most reports investigated the role of *Staphylococcus aureus* in GPA, but more recently a role of molecular mimicry with Gram negative bacteria was proposed as investigated by Kain et al. as well as by Peschel et al. [19, 20, 23] and Roth et al. [21].

Reference	Design	Disease	Subjects	Biological agents
Stegeman et al. [12]	Cohort study	GPA	71 humans	*Staphylococcus aureus*
Stegeman et al. [13]	RCT	GPA	81 humans	*Staphylococcus aureus*
Zycinska et al. [14]	Cohort study	GPA	28 humans	*Staphylococcus aureus*
Popa et al. [15]	Cohort study	GPA	62 humans	*Staphylococcus aureus*
Popa et al. [16]	(1) Cross sectional (2) Cohort study	GPA	(1) 36 humans (2) 10 humans	*Staphylococcus aureus*
Brons et al. [17]	Cell study	GPA	Human umbilical vein endothelial cells (HUVECs), glomerular endothelial cells	*Staphylococcus aureus*
Savige et al. [18]	Animal study	AAV	(1) 7 adult male Wistar rats (2) 8 adult male Wistar rats	(1) *Staphylococcus aureus* (2) *Escherichia coli*
Kain et al. [19]	Cross sectional	Pauci-immune glomerulonephritis	16 humans	Molecular mimicry with Gram negative bacteria
Kain et al. [20]	(1) Cross sectional (2) Animal study (3) Cell study (4) Cross sectional (5) Cross sectional (6) Animal study (7) Retrospective study	Pauci-immune glomerulonephritis	(1) 246 humans (2) 15 Wistar Kyoto rats (3) Human neutrophils (4) 11 humans (5) 15 humans (6) 10 Wistar Kyoto rats (7) 13 humans	Molecular mimicry with Gram negative bacteria
Roth et al. [21]	(1) Cross sectional (2) Animal study	ANCA glomerulonephritis	(1) 680 patients (2) 10 Wistar Kyoto rats	Molecular mimicry with Gram negative bacteria
Huugen et al. [22]	Animal study	Anti-MPO IgG-induced vasculitis	$Mpo^{-/-}$ mice and C57BL/6 mice	Bacterial lipopolysaccharide
Peschel et al. [23]	Cross sectional	ANCA glomerulonephritis	(1) 11 humans (2) 5 humans (controls)	Molecular mimicry with Gram negative bacteria

Additional microbes have been alleged to contribute to the pathogenesis of IgA vasculitis. Immunohistochemistry of kidney specimen revealed presence of *Helicobacter pylori* antigen in 100% of samples investigated and the positive staining was restricted to glomerular structures [31]. Mesangial staining against *Haemophilus parainfluenzae* antigen was detected in 35% of kidney biopsy samples but only in 4% in other kidney diseases. Moreover, significantly higher plasma IgA1 antibody levels against *Haemophilus parainfluenzae* were present in IgA vasculitis than in patients with other renal diseases [32] (Table 2).

3.3.1. Conclusion. In summary there might be a role of bacterial infections in IgA vasculitis with the tonsils as a mediator of ongoing inflammation. Streptococcal M proteins along with NAPI-r can be detected in renal biopsies, while ASLO titers were significantly elevated when compared to other renal disorders [27–29]. Other papers revealed presence of *Helicobacter pylori* and *Haemophilus influenzae* in respective kidney biopsies, while IgA1 antibodies were more abundantly found in the latter case than in patients with other kidney diseases [30, 31]. IgA vasculitis associated with *Staphylococcus aureus* infections has also been described [30]. Tonsillectomy as a therapeutic option yielded excellent remission rates in refractory IgA vasculitis patients in small cohorts, further supporting a critical role of the tonsils in the pathogenesis [33].

3.4. IgA Nephropathy. IgA nephropathy is the most common glomerulonephritis and is characterized by presence of mesangial deposition of IgA1 subclass, which is deficient in galactose. A majority of children or adolescents affected present with macroscopic hematuria (AE) shortly after an upper respiratory tract or gastrointestinal infection, whereas in contrast adult patients commonly present with microscopic hematuria, proteinuria, and hypertension. Microbes have been implicated in etiopathogenesis for a long time as it was assumed that an infection may facilitate synthesis of anti-glycan antibodies cross-reacting with galactose-deficient IgA1. For the nephritogenicity of galactose-deficient IgA1 formation of immune complexes is critical [34].

3.4.1. Bacteria Implicated in IgA Nephropathy Compared to IgA Vasculitis. In line with this idea, a pivotal role of the tonsils has been proposed. Bacterial strains in patients with chronic tonsillitis and IgA nephropathy showed a similar distribution and alpha streptococci were most abundantly present in both groups [35]. When compared to IgA vasculitis periodontal disease and rhinosinusitis (55% and 18% of patients) were present at a similar frequency in patients with

Table 2: Study characteristics of relevant studies in IgA vasculitis are shown. Influence of streptococcal proteins and ASO titers has been investigated most intensely. However, there might also be a role for *Staphylococcus aureus, Helicobacter pylori*, or *Haemophilus parainfluenzae* antigens in the pathogenesis of IgA vasculitis.

Reference	Design	Disease	Subjects	Biological agents
Masuda et al. [27]	Cross sectional	IgA vasculitis	33 patients	Group A streptococci and their antigen Nephritis associated plasmin receptor (NAPl-r)
Al-Sheyyab et al. [28]	Prospective case control	IgA vasculitis	48 children 48 controls	Antistreptolysin O titer
Schmitt et al. [29]	Cross sectional	IgA nephropathy/IgA vasculitis	16 humans (IgA nephropathy) 17 humans (IgA vasculitis)	IgA-binding M proteins of group A streptococci
Hirayama et al. [30]	Cross sectional	IgA vasculitis	6 patients 45 controls	*Staphylococcus aureus*
Li et al. [31]	Cross sectional	IgA vasculitis/membranous nephropathy/lupus nephritis	IgA vasculitis ($n = 10$), membranous nephropathy ($n = 9$), and lupus nephritis ($n = 27$)	*Helicobacter pylori*
Ogura et al. [32]	Cross sectional	IgA nephropathy/IgA vasculitis	32 children	*Haemophilus parainfluenzae*

IgA nephropathy, whereas otitis media was only seen in the systemic disease [26].

3.4.2. Bacteria Implicated in IgA Nephropathy Compared to Controls. Analysis of tonsil specimens obtained from 68 patients with IgA nephropathy revealed presence of periodontal bacteria like *Haemophilus segnis* and *Campylobacter rectus* in about half of the cases examined, significantly more frequent when compared to controls with chronic tonsillitis, while *Treponema* sp. could be detected in 24% of patients with IgA nephropathy and in 7% of controls (no significant difference). Remission of proteinuria in IgA nephropathy subjects was achieved more often when *Campylobacter rectus* and *Treponema* sp. were present [36]. Examination of 32 patients revealed *Helicobacter pylori* in their palatine tonsils and half of them had coexistence with *Actinomyces israelii* even though the latter finding did not differ for patients with recurrent pharyngotonsillitis only [37]. In another study, tonsillectomy specimens from 14 patients with IgA nephropathy revealed presence of coccoid form *Helicobacter pylori* in all samples and cytotoxin-associated antigen A (CagA) was produced by a majority of the strains [38]. In both studies the authors noted a higher prevalence of *Helicobacter pylori* in palatine glands in patients with IgA nephropathy compared to those with recurrent pharyngotonsillitis only [37, 38].

3.4.3. In Vivo Alterations Associated with Microbial Factors in IgA Nephropathy. Blood samples of 21 IgA nephropathy patients indicated significantly higher antibody titers to the IgA-binding region (BR) of streptococcal M proteins M4 and M60 when compared to healthy controls [39]. Recognition of outer membrane of *Haemophilus parainfluenzae* antigens (OMHP) by sera obtained from patients with IgA nephropathy revealed homology with amino acid sequences from outer membrane protein P6 precursor, P5, and P2 porin protein of *Haemophilus influenzae* [40].

Analysis of 35 patients with IgA nephropathy showed presence of IgA antibodies against four of five investigated pneumococcal polysaccharides (7F, 9N, and 14 and 23F), the titers of which were significantly increased when compared to patients with other glomerulopathies, whereas IgG antibodies did not differ between both groups [41]. Moreover, IgA-BR of streptococcal M proteins M4, M22, and M60 could be detected in 10 out of 16 kidney biopsies obtained from patients with IgA nephropathy. These proteins showed the same mesangial and glomerular deposition as observed in IgA vasculitis and colocalization with IgA deposits [29].

Moreover, patients with IgA nephropathy and *Helicobacter pylori* infection showed a greater rate of IgA anti-*Helicobacter pylori* seropositivity and a more pronounced IgA and IgG anti-*Helicobacter pylori* response compared to patients without renal disease, the latter predominantly existing of polymeric IgA1, IgG2, and IgG3 in the diseased group [42].

As already pointed out earlier significantly higher levels of IgA and IgG antibodies directed against OMHP have been observed in patients with IgA nephropathy compared to patients with other glomerular diseases [40] and the titers of IgA antibodies against *Haemophilus parainfluenzae* significantly correlated with the degree of glomerular injury especially in patients with episodes of macroscopic hematuria [43].

Reactivity of IgA eluted from kidney tissues against *Haemophilus influenzae* could be demonstrated in three out of 5 kidney biopsies but the same phenomenon was also observed in two out of 6 non-IgA nephropathy renal specimens [44]. Measurement of IgA, IgM antibody titers against *Escherichia coli* and *Haemophilus influenzae* revealed significantly higher values against both bacteria in IgA nephropathy which correlated with total serum IgA and IgM [45].

IgA class antibodies against *Staphylococcus aureus* were also increased in patients with IgA nephropathy compared

to healthy controls. Moreover, the avidity of these antibodies against *Staphylococcus aureus* was significantly lower in patients compared to controls [46]. Interestingly, 79 out of 116 renal biopsy specimens revealed presence of a *Staphylococcus aureus* cell envelope antigen in the glomeruli of patients with IgA nephropathy and colocalisation thereof with glomerular deposits of IgA [47].

3.4.4. In Vitro Alterations Associated with Microbial Factors in IgA Nephropathy. Streptococcal M5 isolated from peripheral blood mononuclear cells (PBMC) of IgA nephropathy patients increased surface IgA-positive B cells by 1.6-fold and induced transforming growth factor-β in PBMC supernatants by 3-fold compared to controls. In addition, the proliferation capacity of lymphocytes was higher when compared to the one observed in patients with nonproliferative glomerulonephritis [48] and tonsillar lymphocytes showed a significant increase in the IgA1/IgA ratio after stimulation with hemolytic streptococci [49]. An immunologic role has also been proposed for *Helicobacter pylori*-associated CagA. Stimulation of a B-cell line with CagA resulted in cell proliferation and dose-dependently increased production of IgA1 and CagA led to the stimulation of underglycosylated IgA1 *in vitro* [50].

Lymphocytes obtained from palatine tonsils of patients with IgA nephropathy showed a significantly higher stimulation index to along with a higher level of IgA and IgA1 antibodies against *Haemophilus parainfluenzae* antigens compared to lymphocytes from patients with chronic tonsillitis [51]. These results were corroborated by others [52], suggesting that *Haemophilus parainfluenzae* antigens are capable of stimulating tonsillar T and B cells in patients with IgA nephropathy. Tonsillar mononuclear cells of patients with IgA nephropathy exhibited a higher capability to produce transforming growth factor-β and interleukin-10 along with total IgA after stimulation with OMHP antigens than those from patients with chronic tonsillitis *in vitro* [53]. Moreover, it was shown that *Haemophilus parainfluenzae*-specific IgA is produced by *Haemophilus parainfluenzae* and other bacteria and viruses possessing specific CpG motifs in tonsillar mononuclear cells [54]. Stimulation of tonsillar T cells from IgA nephropathy patients with *Haemophilus parainfluenzae* is enhancing the expression of TCR-Vβ-6 *in vitro* and further experiments indicated that the TCR-Vβ-6 was used more frequently in tonsillar T cells of patients with IgA nephropathy and the proportion of these cells in peripheral blood decreased significantly following tonsillectomy compared to controls, suggesting a selective expansion of T cells in IgA nephropathy [55].

Administration of specific pneumococcal C-polysaccharide (PnC) led to deposition of IgA-PnC within the GBM and nuclear factor-kappa B transcription factor was activated early and progressively increased in response to glomerular IgA-PnC deposits in a mouse model [56]. Moreover, in the same experimental setting investigating the role of outer membrane antigen components of *Haemophilus parainfluenzae* indicated a direct nephritogenic effect. Mice either ingesting orally or receiving intraperitoneally OMHP antigens develop glomerular IgA deposition and mesangial expansion, similar to the findings in IgA nephropathy [57].

Induction of glomerulonephritis resembling an IgA nephropathy phenotype was observed in Balb/c mice after immunization with *Staphylococcus aureus* antigens [58]. Immunization with a maltose-binding protein and a 20-amino acid peptide derived from *Staphylococcus aureus* was also capable of inducing an IgA nephropathy like phenotype. Furthermore, anti-20-peptide antibodies labelled glomeruli of patients with IgA nephropathy [59] (Table 3).

3.5. Conclusion. In summary IgA nephropathy presents the most common form of glomerulonephritis. Microbial factors have been studied within the tonsils and lymphocytes as well as mononuclear cells extracted from tonsillectomy specimens. *Haemophilus segnis*, *Campylobacter rectus*, and *Treponema* sp. were more abundantly present in tonsils of IgA nephropathy patients examined. Since remission of proteinuria was more frequent in patients carrying *Campylobacter rectus* and *Treponema* sp. [36] a pathogenetic role of these strains can be discussed. Antibodies against pneumococcal polysaccharides and detection of M proteins in kidney biopsies reveal a possible role of streptococci in IgA nephropathy [29, 41]. Administration of streptococcal M5 led to increased proliferation capacity of lymphocytes [48], whereas administration of PnC increased IgA-PnC deposits within the kidney [56]. Stimulation of tonsillar lymphocytes with hemolytic (AE) streptococci led to an increase of the IgA1/IgA ratio *in vitro* [49]. Evidence from *in vivo* and *in vitro* investigations thus suggested a role of streptococci in IgA nephropathy. *Helicobacter pylori* was also present more abundantly in the palatine tonsils, and most of the strains isolated produced CagA [37, 38]. CagA stimulation of a B-cell line led to an increased production of hypoglycosylated IgA1, which is implicated in the pathogenesis of IgA nephropathy [50]. Antibodies against OMHP antigens have been observed in IgA nephropathy patients. Moreover, molecular mimicry of these antigens was shown with other membrane proteins of *Haemophilus parainfluenzae* [40]. Further investigations observed a pronounced tonsillar T and B cell response and a lymphocyte response following stimulation with *Haemophilus parainfluenzae* [51, 52]. Injection or ingestion of OMHP antigens led to the onset of kidney disease, resembling the phenotype of IgA nephropathy [57]. Taken together, there might be an important role of *Haemophilus parainfluenzae*, since OMHP antigens are more abundantly expressed and lead to an IgA nephropathy like phenotype in a mouse model. However, as these results come from one research group, one should interpret the findings with caution. *Haemophilus influenzae* could be eluted from kidney biopsies of IgA nephropathy patients and higher antibody levels against *Haemophilus influenzae* and *Escherichia coli* could be detected [44, 45]. More evidence is clearly needed to support a role of both bacterial strains in the pathogenesis of IgA nephropathy. A clear-cut role has been proposed for *Staphylococcus aureus* in IgA nephropathy, since *Staphylococcus aureus* antibodies directed against IgA were increased [46], a *Staphylococcus aureus* envelope antigen was present in glomeruli of a majority of patients [47], and

Table 3: Study characteristics of relevant studies in IgA nephropathy are shown. Several bacterial strains have been identified by *in vivo* and *in vitro* experiments. There might be a pivotal role of Streptococci, *Helicobacter pylori, Haemophilus parainfluenzae, Haemophilus influenzae*, and *Staphylococcus aureus* in the disease onset or progression of IgA nephropathy as has been depicted.

Reference	Design	Disease	Subjects	Biological agents
Huang et al. [35]	Cross sectional	IgA nephropathy	106 patients	*Streptococcus* sp., *Neisseria* sp., *Haemophilus parainfluenzae, Staphylococcus* sp., *Bacillus proteus,* and*Streptococcus pneumoniae*
Nagasawa et al. [36]	Cohort study	IgA nephropathy	68 IgA nephropathy patients and 28 controls	*Haemophilus segnis, Campylobacter rectus,* and *Treponema* sp.
Drew et al. [41]	Cross sectional	IgA nephropathy	IgA nephropathy (35), systemic lupus erythematosus (6), membranous nephropathy (8), anti-GBM disease (6), and controls (20)	Pneumococcal polysaccharides
Schmitt et al. [29]	Cross sectional	IgA nephropathy IgA vasculitis	16 humans (IgA nephropathy) 17 humans (IgA vasculitis)	IgA-binding M proteins of group A streptococci
Schmitt el al. [39]	Cross sectional	IgA nephropathy	IgA nephropathy (21) Controls (83)	Streptococci
Nishikawa et al. [48]	Cell study	IgA nephropathy	Lymphocytes from IgA nephropathy patients	Streptococci
Chao et al. [56]	Animal study	IgA nephropathy	B-cell-deficient mice	Pneumococcal C-polysaccharide
Liu et al. [49]	Cross sectional	IgA nephropathy	27 patients	Haemolytic streptococcus and lipopolysaccharide
Kusano et al. [37]	Cross sectional	IgA nephropathy	32 IgA nephropathy patients 141 controls	*Helicobacter pylori*and*Actinomyces israelii*
Kusano et al. [38]	Cohort study	IgA nephropathy	55 patients	*Helicobacter pylori*
Yang et al. [50]	Cell study	IgA nephropathy	B cell line DAKIKI cells	*Helicobacter pylori*
Barratt et al. [42]	Cross sectional	IgA nephropathy	22 IgA nephropathy patients 9 controls	*Helicobacter pylori*
Suzuki et al. [40]	Cross-sectional	IgA nephropathy	44 IgA nephropathy patients 62 controls	*Haemophilus parainfluenzae*
Suzuki et al. [43]	Cross-sectional	IgA nephropathy	44 IgA nephropathy patients 62 controls	*Haemophilus parainfluenzae*
Suzuki et al. [51]	Cell study	IgA nephropathy	Tonsillar lymphocytes	*Haemophilus parainfluenzae*
Suzuki, et al. [52]	Cell study	IgA nephropathy	Tonsillar lymphocytes	*Haemophilus parainfluenzae*
Fujieda et al. [53]	Cell study	IgA nephropathy	Tonsillar mononuclear cells	*Haemophilus parainfluenzae*
Sunaga et al. [54]	Cell study	IgA nephropathy	Tonsillar mononuclear cells	*Haemophilus parainfluenzae*
Yamamoto et al. [57]	Animal study	IgA nephropathy	120 C3H/HeN mice	*Haemophilus parainfluenzae*
Nozawa et al. [55]	Cell study	IgA nephropathy	Tonsillar T cells	*Haemophilus parainfluenzae*
Ogawa et al. [44]	Cell study	IgA nephropathy	Glomerular IgA deposits	*Haemophilus influenzae*
Hirabayashi et al. [45]	Cross sectional	IgA nephropathy	24 IgA nephropathy patients 22 controls	*Escherichia coli* and *Haemophilus influenzae*
Shimizu et al. [46]	Cross sectional	IgA nephropathy, Post-MRSA glomerulonephritis	IgA nephropathy ($n = 16$) and post-MRSA infection GN ($n = 19$) Controls ($n = 13$)	*Staphylococcus aureus*
Koyama et al. [47]	Cross sectional	IgA nephropathy	Glomeruli from 238 kidney biopsies	*Staphylococcus aureus*
Sharmin et al. [58]	Animal study	IgA nephropathy	Balb/c mice (Th2 dominant type) and C57BL/6 mice (Th1 dominant type)	*Staphylococcus aureus*
Zhang et al. [59]	Animal study	IgA nephropathy	Balb/c mice	*Staphylococcus aureus*

immunization of Balb/c mice with *Staphylococcus aureus* antigens led to a phenotype resembling IgA nephropathy [58]. A therapeutic role of performing tonsillectomy has been proposed for several years. A recent multicenter (AE) study revealed no benefit regarding clinical remission rates, although proteinuria decreased in a significant manner [60].

3.6. Recent Advantages and Future Perspectives. A recent genome wide association study revealed novel associations of IgA nephropathy and loci associated with inflammatory bowel disease or maintenance of the intestinal epithelial barrier and potential alteration in the response to mucosal pathogens, further highlighting an interplay between the intestine and the kidneys in IgA nephropathy [61]. These findings are in line with observations that enteric budesonide administered to abate intestinal inflammation significantly reduced proteinuria and improved kidney function in a small preliminary study [62]. Further insights into intestine-kidney interaction may come from studies looking into the effects of enteric budesonide on the intestinal epithelial barrier and changes in the composition of gut microbiota. A recently published study investigated patients with "progressive" versus "nonprogressing" IgA nephropathy and subjects with an impaired kidney function showed a lower diversity of intestinal microbiota [63]. However, further studies are clearly warranted since in particular stool microbiome analysis is still in its infancy and several potential flaws (i.e., contamination of DNA extraction kits) may influence results.

3.7. Nephrotic Syndrome. Nephrotic proteinuric renal diseases comprise three distinct entities, membranous nephropathy, focal segmental glomerulosclerosis (FSGS), and minimal change disease (MCD). In idiopathic membranous nephropathy the target antigen has been identified with PLA2 receptor antibody positivity in approximately 70% of patients [1], with the number increasing to over 80% in untreated patients [64]. More recently, in patients with PLA2 receptor antibody negativity, thrombospondin type-1 domain-containing 7A antibodies have been identified as a circulatory factor in 10% of the remaining patients [2]. The pathogenesis of FSGS and MCD has been studied intensively, but convincing pathogenic factors have not been elucidated so far. While membranous nephropathy and focal segmental glomerulosclerosis tend to progress to end stage renal failure in some patients [65, 66], minimal change disease exhibits a more benign disease course [67].

In membranous nephropathy, the infection rate of *Helicobacter pylori* as detected by a high-molecular-weight cell-associated protein test was found to be significantly higher (66%) when compared to an age-matched control group without history of kidney disease (44%). When eradication was achieved in four patients, remission was reported in three of them. However, the disease course may have been influenced by concomitant glucocorticoid treatment as well [68]. Examination of kidney biopsy specimens showed *Helicobacter pylori* antigen in about two-thirds of the patients with membranous nephropathy [31], a number similar to the one described in an earlier study [69].

Conflicting results regarding the frequency of urinary tract infections preceding onset of nephrotic syndrome in children have been reported. While in an Indian cohort urinary tract infections were common in patients with non-steroid responsive nephrotic syndrome or during relapse with approximately 40% [70], only one out of 32 Nigerian children with nephrotic syndrome presented with urinary tract infection at the initial diagnosis [71] (Table 4).

3.7.1. Conclusion. Microbial antigens may play a role in membranous nephropathy as was shown by an abundance of *Helicobacter pylori* antigen deposition in renal biopsy specimens and evidence of infection in blood [31, 68]. Further investigations to clarify the role of infections prior to onset of nephrotic syndrome are clearly warranted, since urinary tract infections are transiently increasing proteinuria and might be a first step in the onset of nephrotic diseases. Bacterial lipopolysaccharide was capable of inducing CD80, a proposed factor involved in the onset of proteinuria [72]. Costimulation blockade with abatacept induced complete or partial remission in five patients with severe to treat FSGS [73]. Thus, molecular mimicry or a direct role of bacterial strains may be at least partially responsible for CD80 upregulation.

3.8. Systemic Lupus Erythematosus and Lupus Nephritis. Systemic lupus erythematosus is a remarkable complex autoimmune disorder with considerable heterogeneity in the onset of symptoms, the presentation of organ involvement, and therapeutic response towards immunomodulatory as well as immunosuppressive medication. Renal involvement, present in about 50% of patients within the first year after diagnosis, is one of the most important hallmarks necessitating more intensive immunosuppression. Antibodies directed against nuclear antigens play a pivotal role in the development and monitoring of the disease [74].

Presence of *Helicobacter pylori* in kidney biopsy specimens from lupus nephritis patients (classes I–V) was observed in 18 out of 27 patients in one study [31]. However, other associations of bacterial strains with kidney disease or lupus disease activity have not been shown yet.

More evidence for an impact of microbes in SLE largely derives from animal models and *in vitro* experiments. Cross-reactivity of anti-pneumococcal antibodies obtained from a patient after vaccination has been shown with foreign- and self-antigens. Moreover, these antibodies exerted the capability to bind anti-double-stranded DNA antibodies, indicating a potential role of molecular mimicry [75]. Further investigations revealed that in total eight antibodies either reacting with pneumococcal saccharide or DNA were able to bind glomerular structures. Of these, six bound to renal protein antigens which had previously been described to be cross-reactive with DNA, whereas the remaining two bound to histones [76]. Effects of two additional bacteria could be shown more recently. *Escherichia coli* DNA and CpG-oligodeoxynucleotides increased DNA antibodies in lupus mice, accompanied by progression of mild to crescentic glomerulonephritis, interstitial fibrosis, and heavy proteinuria [77]. Cholera toxin B, a component of

Table 4: Study characteristics of relevant studies in nephrotic syndrome are shown. There are a small number of studies indicating a role of *Helicobacter pylori* infection in membranous nephropathy. Microbial agents in the other entities have not been studied yet. Moreover, conflicting results have been presented examining the role of infections prior to onset of nephrotic syndrome.

Reference	Design	Disease	Subjects	Biological agents
Moriyama et al. [68]	Cross sectional	Membranous nephropathy	32 patients	*Helicobacter pylori*
Li et al. [31]	Cross sectional	IgA vasculitis/membranous nephropathy/lupus nephritis	IgA vasculitis ($n = 10$), membranous nephropathy ($n = 9$), and lupus nephritis ($n = 27$)	*Helicobacter pylori*
Nagashima et al. [69]	Cross sectional	membranous nephropathy	16 patients	*Helicobacter pylori*
Gulati et al. [70]	Retrospective study	Primary nephrotic syndrome	37 children	Non-*Escherichia coli* organisms, urinary tract infection
Adedoyin et al. [71]	Cross sectional	Primary nephrotic syndrome and acute glomerulonephritis	67 children	Coliforms, *Klebsiella* sp., and *Staphylococcus aureus*, urinary tract infection

Vibrio cholerae, promoted autoantibody production and onset of glomerulonephritis in lupus prone mice [78]

Several investigations in mice highlighted that injection of bacterial lipopolysaccharide induced anti-double-stranded DNA antibodies [79–81]. Moreover, it was shown that glomerular pathology worsened during the time of follow-up after injection of lipopolysaccharide with progressive deposition of immune complexes [80]. These findings could further be corroborated by an increased deposition of immune complexes in kidneys and exacerbated lupus nephritis following exposure to lipopolysaccharide [82]. The effects executed by lipopolysaccharide lasted for six weeks and glomerular dysfunction progressed from nephritis to permanent chronic kidney damage [83]. After injection of lipopolysaccharide a shift in lupus nephritis, from mesangial expansion to necrosis of capillary loops, epithelial proliferation, and glomerulosclerosis with concomitant renal insufficiency and increasing proteinuria, has been observed [84] (Table 5).

3.8.1. Conclusion. In systemic lupus erythematosus, at least mouse models indicate a role of bacteria in the development and progression of nephritis, associated with an increase in immune complexes, polyclonal B-cell activation, increasing proteinuria, and irreversible kidney damage. Furthermore, *Helicobacter pylori* antigen could be detected in two-thirds of the kidney biopsies examined [31]. However, a pathogenetic role thereof is doubtful. Further investigations in humans are clearly warranted to corroborate these findings. Since pathogenic steps leading to the onset of systemic lupus erythematosus still remain to be in the dark, studying microbial agents may be one of the fields of interest.

4. Discussion and Future Perspective

Bacterial antigens are implicated in the onset and in the progression of many autoimmune kidney disorders. There is evidence that *Staphylococcus aureus* exerts a direct pathogenic role in nasal epithelia in GPA. Other bacteria may not be directly related to the development of autoimmune kidney disorders, but molecular mimicry or deposition of immune complexes secondary to infections may contribute to renal involvement or damage.

However there are several limitations when assessing data about the role of microbial agents in autoimmune kidney disorders. Some of the most interesting findings have been proposed by single research groups, like the findings of hLAMP-2 in pauci-immune crescentic glomerulonephritis or the role of *Haemophilus parainfluenzae* in IgA nephropathy. Nevertheless, both proposed implications rely on robust data. Moreover, some of the investigations have focused on detection of antigens in kidney biopsies or eluting antigens from kidney biopsy samples. There is a strong need to corroborate such associative findings in more convincing pathomechanistic experimental models. The lack of proof of causality is also a problem with studies in which an abundance of bacterial strains was detected in tonsils of patients with IgA nephropathy, as no satisfying effect of tonsillectomy to induce clinical remission was demonstrated in a recent multicenter (AE) trial even though the operation was able to mitigate proteinuria [60].

More investigations with novel methods (i.e., examination of the human microbiome) will yield a way to better define the role of bacteria in these diseases. Several sites of interest such as the sinuses in GPA, the tonsils and the intestine in IgA vasculitis, and IgA nephropathy may show significant alterations in bacterial colonization diversity, which in turn may contribute to an immunologic imbalance (i.e., altered T-cell homeostasis) leading to local inflammation and provoking the onset or the recurrence of disease. Since studies of the human microbiome already offered insights into several other autoimmune disorders, we are convinced that more profound analyses with longitudinal sample collection (onset of disease, remission, and relapse) will also clarify at least in part etiopathogenesis of some autoimmune kidney disorders. Furthermore, results obtained from

Table 5: Study characteristics of relevant studies in systemic lupus erythematosus and lupus nephritis are shown. Most experience is gathered from mouse models with induction of lupus-specific antibodies after injection of lipopolysaccharide. Lipopolysaccharide along with specific bacterial strains was capable of worsening kidney involvement in lupus mouse models, indicating a potential role of bacterial antigens in the progression of lupus nephritis.

Reference	Design	Disease	Subjects	Biological agents
Kowal et al. [75]	Cell study	*Lupus nephritis*	1 patient	Anti-bacterial antibodies which bind double-stranded DNA
Chowdhry et al. [76]	Cell study	*Lupus nephritis*	1 patient	Antibodies binding bacterial polysaccharide and glomeruli
Gilkeson et al. [79]	Animal study	*Lupus nephritis*	BALB/c and C57BL/6 mice	*Escherichia coli* dsDNA
Izui et al. [80]	Animal study	*SLE*	Mice	Lipopolysaccharides
Fournié et al. [81]	Animal study	*SLE*	Athymic C57BL/6 nude mice	Lipopolysaccharide
Granholm and Cavallo [82]	Animal study	*Lupus nephritis*	BXSB mouse	Lipopolysaccharide
Granholm and Cavallo [83]	Animal study	*Lupus nephritis*	NZB/W mice	Lipopolysaccharide
Cavallo and Granholm [84]	Animal study	*Lupus nephritis*	MRL/lpr mice	Lipopolysaccharide
Anders et al. [77]	Animal study	*Lupus nephritis*	MRL/lpr mice	*Escherichia coli*
Deng and Tsokos [78]	Animal study	*SLE*	MRL/*lpr*/*2J* mice, F_1 (NZB/W F_1) mice, MRL/MpJ mice, B6.MRL/*lpr* mice, CD40 ligand knockout mice, and C57BL/6 (B6) mice	Cholera toxin B

microbiome analysis may enable us to prescribe tailored therapeutic measures aiming to eliminate abundant strains, such as trimethoprim-sulfamethoxazole treatment in limited GPA or eradication of infectious foci in IgA vasculitis, potentially restoring microbial and epithelial barrier imbalance.

Conflict of Interests

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

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Research Article

Effect of Leflunomide on the Abnormal Expression of Lipid Rafts and F-Actin in B Lymphocytes from Patients with Systemic Lupus Erythematosus

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Purposes. To investigate the possible changes in B cell subsets and in B cell expression patterns of lipid rafts (LRs) and F-actin in patients with SLE and whether leflunomide treatment may have effect on these changes. *Methods*. The B cell subsets and LRs expression were determined by flow cytometry and confocal microscopy, and F-actin expression was examined by confocal microscopy. *Results*. $CD27^+IgD^+$ B cell subsets were significantly decreased while $CD38^+CD95^+$ B cell subsets increased in SLE patients. The LRs levels of B cells were remarkably increased and positively correlated with SLEDAI and anti-dsDNA titer in SLE patients. The expression level of LRs was significantly higher in $CD38^+$ B cells than $CD38^-$ B cells and negatively correlated with C3 levels. The increased expression of LRs was associated with reduced expression of F-actin in the B cells from active SLE patients. Furthermore, in vitro treatment of the cells with A771726 reduced the expression level of LRs, attenuated the overaggregation of LRs, and normalized the distribution of F-actin. *Conclusions*. There were abnormalities in B cell subsets and LRs and F-actin expression of B cell from SLE patients. Modulation of B cell expression of LRs and F-actin by LEF could be a potential therapeutic target for SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that is known to be associated with polyclonal B cell hyperreactivity [1]. The pathogenesis of SLE remains not well understood. A combination of genetic factors, environmental influences, and sex hormones may contribute to the susceptibility to the disease [2]. While T cells have been considered to play a central role in the pathogenesis of SLE before, emerging evidences indicate that B cells may be a more important player than T cells in the pathogenesis of SLE [1, 3–5].

Lipid rafts (LRs), the specialized cholesterol- and glycosphingolipid-rich microdomains in the cellular membrane, have been reported to play an important role in the activation of B cells, including effects on B cell antigen receptor- (BCR-) initiated signal transduction, endocytosis of BCR-antigen complexes, loading of antigenic peptides onto MHC class II molecules, and receipt of helper signals via the CD40 receptor [6, 7]. Lipid rafts are also linked to the actin cytoskeleton which is important for lymphocyte antigen presenting [7].

Actin is a multifunctional protein that forms microfilaments presented either as a free monomer called G-actin or as a polymer microfilament called F-actin. Via polymerization and depolymerization, the actin cytoskeleton regulates a number of cellular functions. Dynamic regulation of F-actin cytoskeleton is critical to numerous physical cellular processes, including cell motility, cell division and cytokinesis, cell signaling, and the establishment and maintenance of cell junctions and cell shape. Many of these processes are mediated by extensive and intimate interactions of actin with cellular membranes. The actin cytoskeleton has also been

reported to be involved in B cell activation by participating in the processing of the maturation of MHC class II molecules, internalization of BCR, and interaction between FcεRI and lipid raft components [8, 9].

Abnormal expression of lipid rafts and F-actin on T cells in SLE patients has been studied well [10–13]. The expression pattern of LRs and F-actin on B cells in SLE patients remains not quite clear [14].

Leflunomide (LEF) is one of the most important immunosuppressive drugs and has been extensively used in the therapy of rheumatoid arthritis in recent years. It also showed great success in treatment of SLE in both animal models and patients, though it was still not recommended as one choice of therapeutic drugs for SLE until now [15]. Following intestinal absorption, LEF is rapidly converted into its active metabolite A771726 that has been known to inhibit dihydroorotate-dehydrogenase (DHODH) and tyrosine kinases, leading to decreased proliferation of T and B cells [16]. However, the specific mechanism of how LEF regulates lymphocyte activation remained unclear. Whether LEF is involved in modulation of LRs and F-actin expression to exert its therapeutic effect on SLE is not fully understood. A better understanding about the role of LEF in SLE will help us in using it appropriately.

To clarify the changes in B cell subsets and LRs and F-action expression pattern on B cells and determine the possible role of LEF in these changes in the patients with SLE, we measured CD19, CD38, CD95, CD27, IgD, GM1, and F-actin expression levels and their patterns on B cells from SLE patients. Our results suggest that there were abnormalities at B cell subsets and expression of LRs and F-actin on B cells, which could be modified by A771726 in SLE.

2. Patients and Methods

2.1. Patients and Healthy Controls. 54 SLE patients (51 females, 3 males; average age: 32.06 ± 11.38), 15 sex-matched (all females) primary Sjögren's syndrome (pSS) patients, and 20 age- and sex-matched healthy volunteers (19 females, 1 male; average age: 30.60 ± 10.78) who served as controls were enrolled for this study. The diagnosis of SLE was according to the 1997 revised American College of Rheumatology (ACR) criteria for the classification of SLE [17]. The diagnosis of pSS was according to the 2002 American-European Consensus Group criteria [18]. Disease activity in the SLE patients was assessed using the SLE Disease Activity Index (SLEDAI). In our cohort, SLE patients were divided into two subgroups based on SLEDAI including 37 active SLE (SLEDAI > 6) and 17 inactive SLE (SLEDAI ≤ 5) [19]. None of the patients were treated with statins which could alter lipid raft domains. All patients either were not exposed to or have discontinued for at least 2 weeks glucocorticoid and immunosuppressant drugs before recruitment into this study.

This study was performed according to the principles of the Declaration of Helsinki and each participant completed written informed consent before measurements. This study was also approved by the Local Ethics Committee (Institutional Review Board of Guangdong General Hospital).

2.2. Flow Cytometric Analysis for Peripheral Blood B Cell Subsets. 15 mL EDTA-treated blood samples were obtained after an overnight fasting. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over Ficoll-Paque Plus. For immunofluorescence staining, the following monoclonal antibodies (mAbs) were used: CD5-PE (BD Pharmingen), CD19-FITC/CY5 (eBioscience), CD27-PE (eBioscience), CD38-CY7 (BioLegend), CD95-PE (eBioscience), and IgD-FITC (Invitrogen). To define B cell subpopulations, 10^6 PBMCs were incubated with mAbs for 30 minutes on ice. The PBMCs were simultaneously stained with different fluorescein washed with PBS twice. Four-color immunofluorescence analysis was performed on the same day by flow cytometry (Bechman Coulter Inc.). The fetched cells suspension was gated for lymphocyte and at least 10,000 lymphocyte events were acquired per sample.

2.3. Isolation and Culture of B Lymphocytes. B cells were isolated from PBMCs by positive selection using MACS anti-CD19 microbeads and MS columns (Miltenyi Biotec, Gmbh, Germany) following the manufacturer's instructions. Flow cytometry consistently showed that the percentage of B cells in the isolated subpopulation was >90% as determined by anti-CD19 mAb staining. B lymphocytes were cultured in serum-free RPMI-1640 medium at 37°C for 4 hours, with or without A771726 (kindly provided by Cinkate Corporation, China) (at 0 μg/mL, 15 μg/mL, 30 μg/mL, 45 μg/mL, and 60 μg/mL).

2.4. FACS Analysis of CTB Binding to the Plasma Membrane of B Cells. The binding of cholera toxin B subunit (CTB) (Sigma) is frequently used to identify lipid rafts, and the capacity for binding CTB is upregulated during B cell activation [20]. To analyze lipid raft, we stained cells with fluorescence-labeled CTB, which binds to the raft-associated glycosphingolipid GM1, previously shown to be a reliable marker for the detection of lipid raft domains [21]. In vitro A771726-treated B cells were fixed with 2% paraformaldehyde (PFA) at room temperature (RT) for 15 min. Fixed cells were resuspended in 100 μL phosphate buffered saline (PBS) and then stained with CTB-FITC (final concentration 2 μg/mL). At least 5000 cells were analyzed. To analyze surface CD38 or CD95 coexpression with lipid rafts in B cells, PBMCs were isolated and were stained with fluorochrome-conjugated mouse monoclonal anti-CD19, anti-CD38, and anti-CD95. Cells were fixed with 2% PFA at RT for 15 min and subsequently stained with CTB-Alexa Flour 488 (Molecular Probes Inc.) (final concentration: 10 μg/mL). Four-color immunofluorescence analysis was performed.

2.5. Visualization of Lipid Rafts and F-Actin in B Cells by Confocal Microscopy. Ex vivo and in vitro A771726-treated B cells were washed by PBS and added to poly-L-lysine cover slips and mounted to slides. Cells were fixed with 2% PFA at RT for 15 min. Staining for F-actin was achieved with rhodamine phalloidin (Cytoskeleton Inc., Denver, USA) at RT for 30 min. Membrane staining for lipid rafts was achieved with CTB-FITC (final concentration: 10 μg/mL) at 4°C for

30 min. Green and red fluorescence were recorded using a 40x objective. The colocalization of lipid rafts and F-actin was quantified using the "colocalization" tool in the Leica software.

2.6. Statistical Analyses. Data are expressed as median value and the rank. Differences between two groups were compared using the nonparametric Mann-Whiney test for unpaired data and Wilcoxon test for paired data. One-way ANOVA was used for comparison between more than two groups. Correlation was analyzed by Spearman's rank correlation test. P values less than 0.05 were considered significant.

3. Results

3.1. Abnormal B Cell Subsets in SLE. Initially, an analysis of CD5 expression in peripheral B cells was carried out in 20 active SLE patients and 11 healthy subjects. The result showed that the percentage of B cells in lymphocytes was significantly increased in SLE patients compared with normal subjects (median 16.7% (range: 2.5–48.0) versus 10.6% (range: 5.7–17.2)) ($P = 0.01$). But there was no difference in the frequency of CD5 expression on B cells between SLE patients (median 24.9% (range: 6.9–68.3)) and normal individuals (median 27.1% (range: 10.8–56.7)) ($P = 0.409$).

Further analysis of the expression of surface IgD and CD27 in B cell subsets in 17 active SLE patients and 10 healthy subjects showed no difference in $CD27^+$ B cells and IgD^+ B cells between SLE and normal control ($CD27^+$ B cells: median 45.1% (range: 10.9–80.5) versus median 38.9% (range: 27.5–70.1), $P = 0.941$; IgD^+ B cells: median 52.8% (range: 15.6–88.8) versus median 64.1% (range: 47.7–72.4), $P = 0.711$). However, a decrease in $CD27^+IgD^+$ B cell subset was observed in patients with SLE (median 7.7% (range: 2.0–32.6) versus 15.1% (range: 9.1–32.6)) ($P = 0.031$). The proportion of $CD27^+IgD^+$ B cell in patients with lupus nephritis ($n = 10$, median 5.0% (range: 2.0–11.1)) was lower than those in SLE patients without renal involvement ($n = 7$, median 11.9% (range: 4.7–32.6)) ($P = 0.032$) (Figure 1(a)). But there was no difference in the percentage of $CD27^-IgD^-$ B cells between SLE patients and normal control individuals.

Finally, we analyzed CD38 and CD95 expression in B cells surface. 12 active SLE patients and 11 healthy subjects were studied. There was no difference in the percentage of $CD95^+$ B cells between SLE patients and normal controls, but an increased expression of $CD38^+$ B cells was found and the percentage of $CD38^+CD95^+$ B cells was significantly increased (Figure 1(b)) in active SLE patients.

3.2. The Expression Level of CTB-Binding Lipid Rafts in SLE B Cells. The LRs expression level in B cells was remarkably increased in SLE patients (median 57.0% (range: 29.7–87.4), $n = 38$) compared with those in healthy controls (median 34.8% (range: 8.4–50.0), $n = 11$) ($P < 0.001$). The LRs level on B cells was significantly higher in active SLE patients than in inactive SLE patients (median 59.6% (range: 35.3–87.4), $n = 18$, versus median 50.7% (range: 29.7–62.7), $n = 20$) ($P < 0.001$) (Figure 2(a)). In addition, the LRs level in B cells in inactive SLE patients was still higher than that in healthy controls ($P = 0.003$). There was a positive correlation between the LRs level in B cells and SLEDAI ($r = 0.568$, $P < 0.001$) (Figure 2(b)). Likewise, the LRs level in B cells showed a significant positive correlation with anti-dsDNA antibody titer ($r = 0.394$, $P = 0.028$). No correlation was seen between the LRs level in B cells and blood IgG level ($r = -0.203$, $P = 0.291$) or complement factor C3 level ($r = -0.036$, $P = 0.843$).

Analysis of CD38 and CD95 coexpression with LRs in PBMCs from 12 active SLE patients and 11 healthy controls revealed a significant increased LRs expression on $CD38^+$ B cells in SLE patients compared with healthy controls (Figure 1(b)). Interestingly, in active SLE patients, LRs expression in $CD38^+$ B cells (median 36.1% (range: 15.1–47.3)) was significantly higher than that in $CD38^-$ B cells (median 10.5% (range: 4.1–31.7)), $P < 0.001$. The increased percentage of LRs expression in $CD38^+$ B cell was correlated with complement factor C3 level ($r = -0.718$, $P = 0.013$).

3.3. Colocalization Pattern of F-Actin and Lipid Rafts in SLE B Cells. To analyze the colocalization of lipid rafts and F-actin, purified B cells from 7 active SLE patients, 6 pSS patients, and 4 healthy controls were doubly stained with CTB-FITC and rhodamine phalloidin. Confocal microscopy images revealed that, in B cells from healthy controls and pSS, LRs were small and uniformly distributed on the plasma membrane. F-actin was found mainly in a cortical distribution around the perimeter of the cell. This pattern was different from the pattern seen in B cells from SLE patients, which presented with stronger staining and an irregular large clustering of LRs companying with a decrease in F-actin level (Figure 2(c)).

3.4. Effects of A771726 on the Expression of Lipid Rafts and F-Actin on SLE B Cells. Our preliminary experiments found that A771726 did not obviously affect LRs level at the concentration of 15 or 30 μg/mL. To investigate the effects of A771726 on LRs and F-actin on B cells, purified B cells from 10 active SLE patients were cultured with or without A771726 (45 μg/mL or 60 μg/mL) at 37°C for 4 hours. LRs were stained with CTB-FITC and analyzed by FACS. The percentage of CTB-binding LRs was significantly decreased after A771726 treatment and does not show significant difference between the concentration of 45 μg/mL and the concentration of 60 μg/mL (Figure 3(a)). Coexpression analysis of LRs and F-actin on SLE B cells by confocal microscopy showed that treatment of A771726 reduced the cluster of LRs associated with reversed distribution of F-actin (Figure 3(b)).

4. Discussion

Several studies have documented the specific perturbations of B cell maturation and differentiation in SLE and that alteration of B cell subsets and B cell phenotypes could play critical roles in SLE diseases. [1, 22], but the possible inherent B cell abnormalities in SLE patients remain to be defined clearly. Our results showed that there was an increased frequency of $CD38^+$ B cells and decreased percentage of

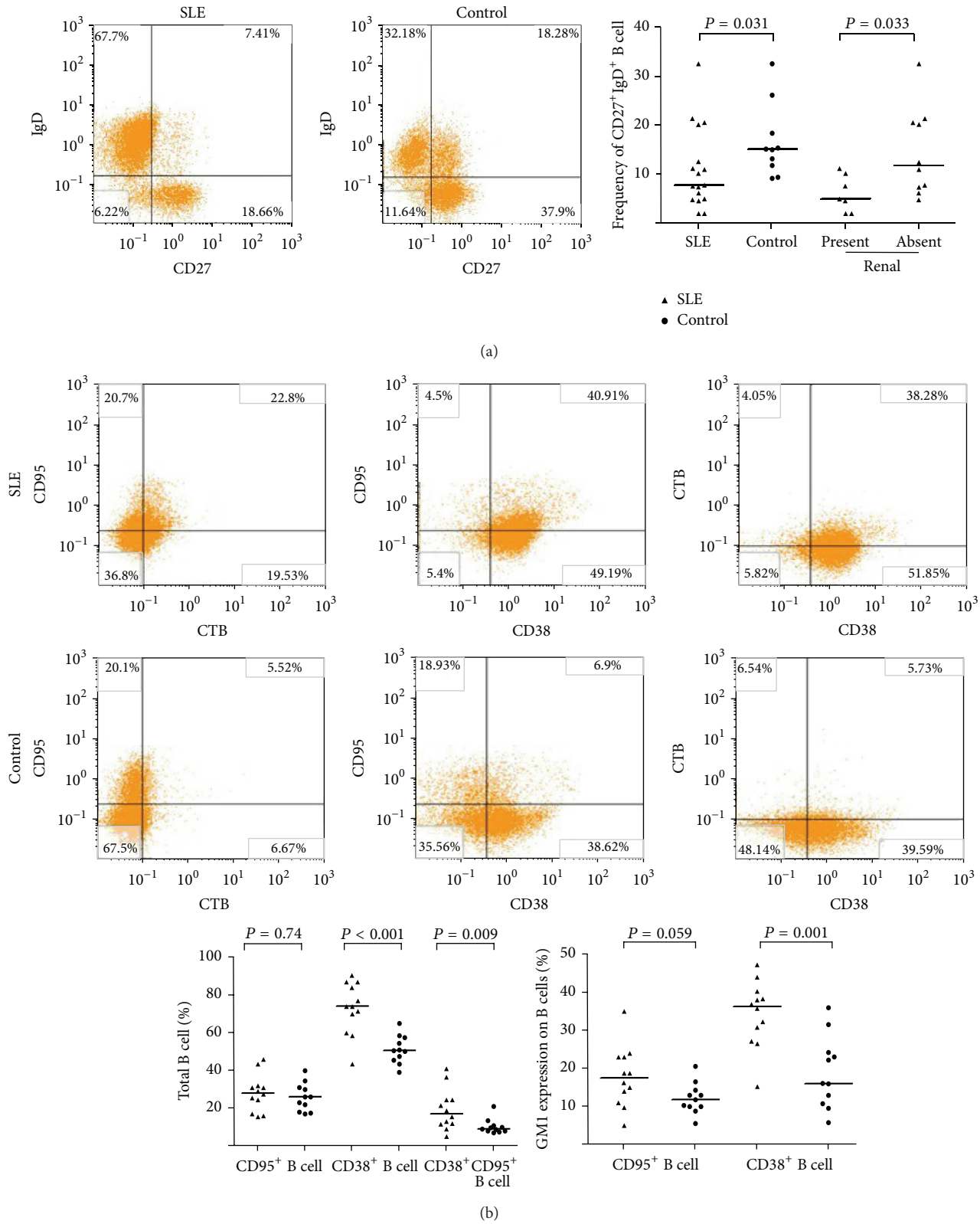

Figure 1: Surface phenotype of peripheral blood B cells was analyzed by *flow cytometry* and GM1 expression on B cells was detected by *confocal microscopy*. (a) SLE patients had a lower expression of $CD27^+IgD^+$ B cells than healthy controls, $P < 0.05$. SLE patients with renal injury had a higher expression of $CD27^+IgD^+$ B cells than SLE patients with no renal injury, $P < 0.05$. (b) The frequency of either $CD38^+CD95^+$ B cells or $CD38^+$ B cells was significantly higher in SLE patients than in healthy controls, but there was no difference in the percentage of $CD95^+$ B cells between SLE patients and healthy controls. The coexpression of CD38 and GM1 in SLE B cells was increased significantly in SLE patients. Horizontal line represents the median.

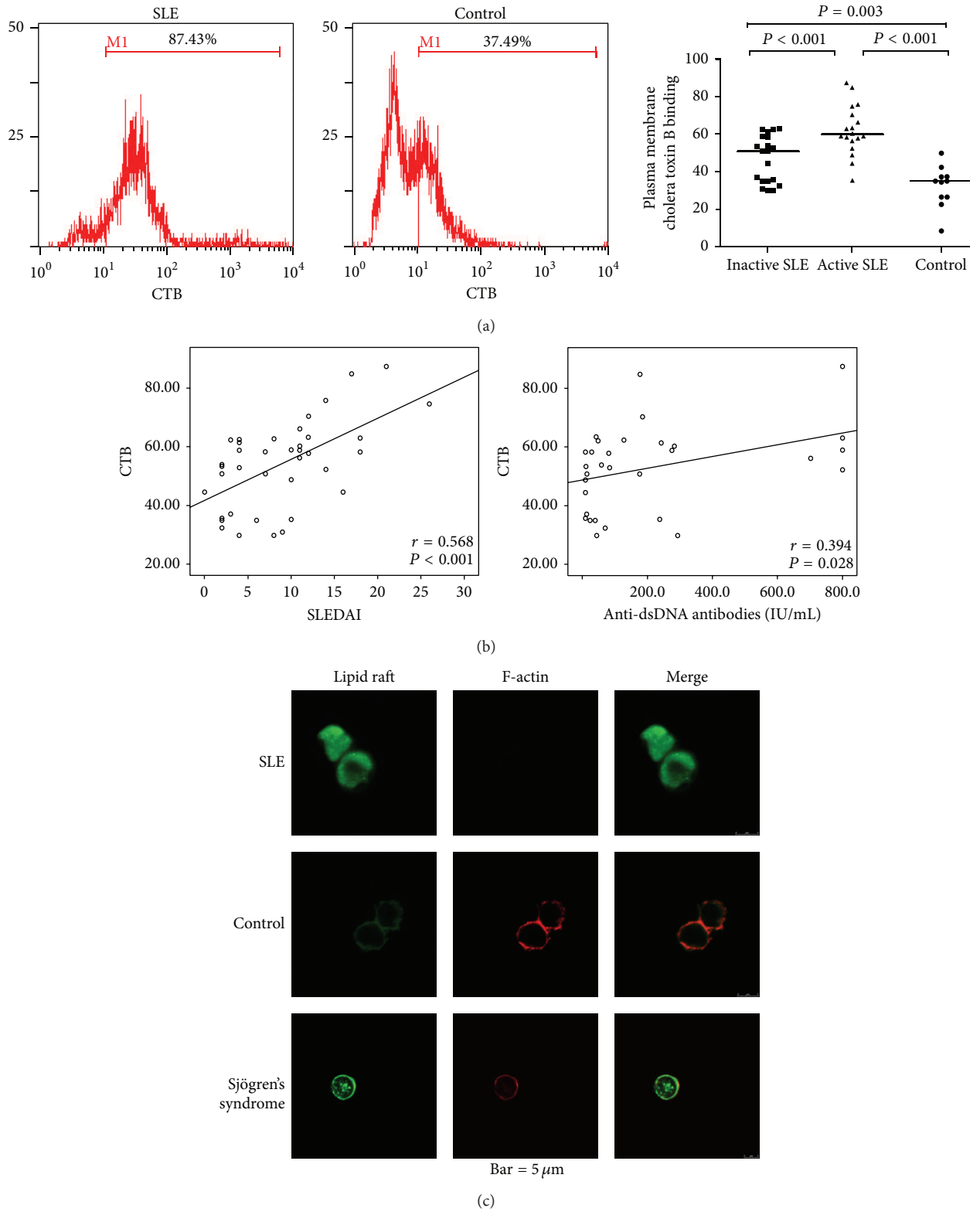

Figure 2: Altered expression of lipid rafts and F-actin in SLE B cells was analyzed by flow cytometry and confocal microscopy. (a) Expression of lipid rafts on SLE B cells was analyzed by flow cytometry. SLE B cells have more lipid rafts compartments than normal B cells. Active SLE B cells have more lipid rafts compartments than inactive SLE B cells. (b) The association between the percentage of lipid rafts (CTB) expressing B cells and the SLEDAI-score or anti-dsDNA antibody titer was analyzed by Spearman's rank correlation test. There was a significant positive correlation between the percentage of lipid rafts (CTB) expressing B cells and the SLEDAI-score or anti-dsDNA antibody titer. (c) Expression pattern of lipid rafts and F-actin in B cells was detected by confocal microscopy. There were different expression patterns of lipid rafts and F-actin in B cells from SLE patients, Sjögren's syndrome patients, and control subjects.

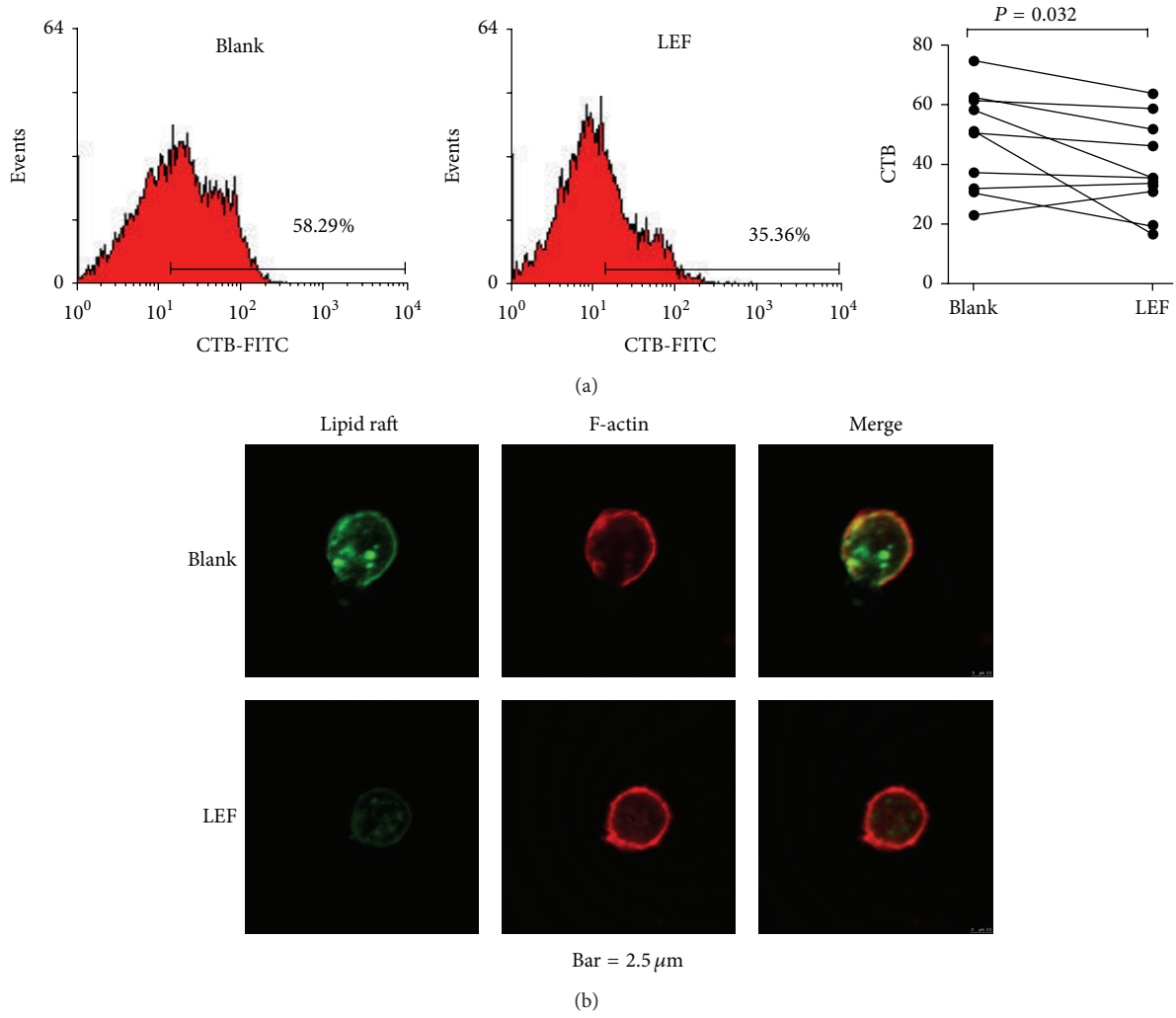

Figure 3: A771726 treatment altered the lipid rafts and F-actin expression in B cells in vitro. (a) Expression of lipid rafts on SLE B cells before and after A771726 treatment was analyzed by flow cytometry. A771726 treatment significantly reduced the lipid rafts expression level in SLE B cells. (b) Confocal microscopic profiles of B cells from 3 SLE patients show the modified lipid rafts and F-actin expression patterns after A771726 treatment. Magnification used was ×40 objective.

$CD27^+IgD^+$ B cells in SLE patients, but no difference of $CD5^+$ B cells, $CD27^+$ B cells, and IgD^+ B cells between active SLE patients and normal controls was found. Moreover, SLE patients with lupus nephritis had lower level of $CD27^+IgD^+$ B cells than SLE patients without renal involvement. Previous studies had described a new population of memory B cells lacking expression of both CD27 and IgD, and increased frequency of this subset of B cells was associated with higher disease activity index [23]. In our study, we observed no significant difference in the percentage of $CD27^-IgD^-$ B cells between SLE patients and normal controls. However, our study showed that the percentages of $CD38^+$ B cells and $CD38^+CD95^+$ B cells were significantly increased in active SLE patients, although there was no difference in the percentage of $CD95^+$ B cells between SLE patients and normal controls. These different results may be attributed to SLE hyperheterogeneity, ethnic's heterogeneity, and grouping criteria heterogeneity. It is known that the increased expression of $CD38^+$ B cells is closely associated with memory B cells hyperactivation and differentiation, while the increased expression of CD95 on the B cells may induce B cell apoptosis [24]. Further study is required to reveal the clinical relevance of the increased $CD38^+CD95^+$ B cells in the pathogenesis and progression of SLE.

Abnormal B cell activation is a prominent feature of the SLE [25]. Increased lipid raft expression on the plasma membrane of B cells may constitute a way to promote B cell activation [26]. In our study, we found that the lipid raft levels in B cells were dramatically increased in SLE patients, and the lipid raft levels in B cells were positively correlated with SLEDAI and dsDNA antibody titers. This suggests that the changes of lipid raft expression pattern on the B cells may contribute to B cell overactivation and disease activity in the SLE patients. Moreover, our study showed that the percentage

of $CD38^+GM1^+$ B cells in SLE was significantly increased. CD38 is associated with the synthetic metabolism of B cells and also participates in B cell activation and proliferation through regulating gene transcription and protein phosphorylation. CD38 expression can also lead to increase of intracellular Ca^{2+} level and enhanced BCR signaling [27]. We speculated that the increased CD38 expression on B cells in SLE patients might decrease the threshold of B cell activation by promoting B cell lipid raft overexpression. The distinct expansion of the $CD38^+GM1^+$ B cells in SLE patients may be one of the major features of B cell inherent abnormality and related to SLE pathogenesis. Further mechanistic studies are required to reveal the relationship between CD38 and lipid rafts in B cells.

The actin cytoskeleton regulates lipid raft dynamics and coalescence in B cells. Depolymerization of the actin cytoskeleton could alter lipid rafts clustering and influence the threshold for cellular activation [28]. Whether lipid rafts and F-actin expression could be changed in B cells in SLE has not been reported so far. We found that lipid rafts were irregularly distributed on the plasma membrane and showed aggregation in SLE B cells, accompanied by a decrease in F-actin expression and abnormal distribution of F-actin, suggesting the abnormal expression of LRs and F-actin may contribute to the hyperactivation of B cells in SLE patients.

LEF is a potent effective disease-modifying antirheumatic drug and has been recently demonstrated to be safe and effective for the treatment of SLE and even refractory lupus and lupus nephritis [29, 30]. But it was still not recommended by the latest ACR/Eular treatment guidelines for SLE [31]. Our data showed that LEF treatment can reduce the lipid rafts levels and reverse the abnormal distribution of lipid rafts and F-actin of B cells in the active SLE patients. This finding suggests that regulating the abnormal expression of lipid rafts and F-actin in B cells may be a new mechanism for LEF to effectively treat SLE and that modulation of abnormalities of lipid rafts and F-actin expression on B cells could be an important new target in the SLE therapy.

5. Conclusions

Although this study was somewhat limited in statistical power because of the small sample size, our data showed that there were significant abnormalities in the B cell subsets and their expression pattern of lipid rafts and F-actin from SLE patients. Modifying these abnormalities may represent a new mechanism for LEF in effective treatment of SLE. The altered lipid rafts and F-actin expression pattern or their signaling pathways may be used as new targets in SLE treatment.

Abbreviations

SLE: Systemic lupus erythematosus
pSS: Primary Sjögren's syndrome
LEF: Leflunomide
LRs: Lipid rafts
SLEDAI: SLE Disease Activity Index
CTB: Cholera toxin B subunit
dsDNA: Double-stranded deoxyribonucleic acid
A771726: The active metabolite of leflunomide.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Authors' Contribution

Guang Fu Dong designed and performed research, analyzed and interpreted data, and wrote the paper; De Ning He performed research, interpreted data, and wrote the paper; Xiao Zhang designed research and revised the paper; Ling Li and Guang Feng Zhang performed research.

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Review Article

Clinical Characteristics of Concomitant Systemic Lupus Erythematosus and Primary Biliary Cirrhosis: A Literature Review

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Although autoimmune diseases often coexist, concomitant cases of systemic lupus erythematosus (SLE) and primary biliary cirrhosis (PBC) are uncommon. In this review paper, 34 cases of SLE with concomitant PBC found in English and Japanese scientific literature and Japanese proceedings were reviewed and summarized, including cases with liver dysfunction complicated by SLE. Of the 34 reported concomitant cases of SLE and PBC, 97.1% (33/34) were females, and PBC was diagnosed initially in 69.0% (20/29), except for five cases in which both SLE and PBC were simultaneously diagnosed. Sjögren's syndrome was the most common autoimmune disease complicating concomitant SLE and PBC (23.5%, 8/34). Five deaths have been reported: two elderly patients died of liver failure because of the worsening of PBC, and another two patients died from pulmonary infection associated with SLE pharmacotherapy. It is uncertain whether concomitant cases occur by chance or share a common immunological or genetic basis.

1. Introduction

Autoimmune diseases exhibit an increased immune response to self-antigens and predominantly occur in females [1]. Autoimmune diseases share some similar pathological pathways or genetic etiologies, and it is common that more than one autoimmune condition may occur in a single patient [2].

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease that results from a combination of genetic, environmental, and hormonal factors [3–5]. It is characterized by the presence of pathogenic autoantibodies such as anti-double stranded DNA or anti-histones and immune complexes in the serum and target tissues, inducing serious inflammatory conditions by the activation of the complement system [3, 4]. The pathogenesis of SLE is mainly due to the deficiency of several immunological mechanisms, including inappropriate function of the innate immune system, altered self-tolerance mechanisms, and apoptotic cell clearance [4, 5]. Furthermore, SLE often coexists with other autoimmune diseases or collagen disorders, such as rheumatoid arthritis (RA) and Sjögren's syndrome (SjS) [6, 7].

Conversely, the liver is a target of autoimmune reactions, as observed in autoimmune diseases, such as autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC). The liver involvement in SLE patients includes several liver diseases or SLE itself, and it is necessary to discriminate between the causes of liver dysfunction or abnormal liver enzyme values. However, it is sometimes challenging to discriminate between the causes of the liver involvement in SLE patients, and the use of immunosuppressive therapy for SLE may distort the clinical and laboratory findings of underlying autoimmune liver disease, leading to diagnostic delay or occult progression of liver cirrhosis (LC) [8].

PBC is considered an autoimmune disease of unknown etiology with organ-specific disturbance characterized by chronic progressive cholestasis with the destruction of the intrahepatic small bile ducts, particularly the interlobular bile ducts [9–13]. The causes of PBC appear to involve environmental and genetic factors [14, 15]. Moreover, it is well recognized that PBC is an overlapping condition between the autoimmune hepatology and rheumatology [16].

Reported concomitant cases of SLE and PBC are relatively rare [8, 9, 17–32]. Some patients with SLE and PBC may have a common genetic susceptibility toward developing these diseases [33]; however, it is also thought that the coincidence of SLE and PBC is purely incidental.

Currently, it is uncertain whether concomitant SLE and PBC occurs by chance or whether this has a shared common immunological and/or genetic basis.

To date, there have been few systematic literature reviews of concomitant cases of SLE and PBC, and the clinical features and pathophysiology of concomitant cases of SLE and PBC remain unclear. However, it may be necessary to discriminate between the complications of PBC in SLE patients with liver involvement because some PBC patients may progress to occult end-stage liver failure, and prolonged use of glucocorticosteroids for SLE is potentially associated with significant adverse effects, including osteoporosis, which ranges from 20% to 44% in PBC patients, increasing with the progression of PBC [34].

In this report, a literature search and a review of cases of SLE with concomitant PBC, including autoimmune liver diseases, were conducted to clarify the clinical features of concomitant SLE and PBC cases.

2. Methods

A literature search on scientific articles in English and Japanese and Japanese proceedings was conducted using the PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and Japana Centra Revuo Medicina (Igaku Chou Zasshi) (http://edb.kulib.kyoto-u.ac.jp/dbe/JA.html) databases, respectively, retrieving cases of concomitant SLE and PBC published to date.

Suspected cases of SLE and those of drug-induced lupus-like syndromes were excluded. Moreover, the case report by Tunccan et al. [35], which is a case of leishmaniasis with clinical and laboratory features mimicking SLE and autoimmune liver disease, was also excluded from this analysis, because autoimmune reactions induced by leishmaniasis are common, and autoimmune reactions were indeed thought to be induced by leishmaniasis in this particular case [35]. Cases of SLE diagnosed according to the American Rheumatism Association criteria were included, with patients required to have at least 4/11 criteria for receiving a diagnosis of SLE [36].

2.1. Liver Dysfunction and Abnormal Liver Enzyme Values in Patients with SLE. Liver dysfunction is not considered as the main organ pathology in SLE [37, 38], and liver function abnormalities are not included in the classification and diagnostic criteria of SLE [36, 38]. The frequency of liver dysfunction or abnormal liver enzyme values during the course of SLE ranged from 19% to 60% [37–49]. Moreover, the prevalence of hepatomegaly in SLE patients ranged from 12% to 55% [48, 49]. Fluctuations in alanine transaminase values corresponding to SLE activity have been reported in some patients with SLE [45, 49]; however, no correlation between SLE activity and the incidence of liver disease was identified in any of the reviewed cases [37, 47]. This discrepancy may be attributed to the different causes of liver dysfunction or abnormal liver enzyme values in SLE patients.

Several authors have suggested a role for SLE in triggering an often subclinical hepatopathy, referred to as lupus hepatitis (SLE-related hepatitis), describing this disease as hypertransaminasemia, frequently associated with exacerbation of the lupus disease, which returns to normal values after glucocorticosteroid therapy [49]. Lupus hepatitis is a distinct manifestation in 3–9% of SLE patients [45, 49, 50]. In cases of lupus hepatitis, the fluctuations in alanine transaminase values occur parallel to the activity of SLE and are generally subclinical, with few cases progressing to end-stage liver disease [49, 50]. Conversely, no obvious correlation between SLE activity and the incidence of liver disease has been identified in liver involvement except lupus hepatitis [37, 47].

Hence, the identification of the etiology of liver dysfunction in SLE (other than lupus hepatitis) is often difficult [38, 47] because of the existence of several potential causes, including AIH, PBC, hepatic steatosis, nonalcoholic fatty liver disease, viral hepatitis, and drug-induced liver diseases such as those induced by glucocorticosteroids [42, 44, 47, 51]. The prevalence of drug-induced hepatitis is relatively high in SLE patients [49], and Takahashi et al. [44, 47] suggested that drug-induced hepatitis may be a common cause (approximately 30%) of liver dysfunction in SLE patients in Japan. Statistical analyses indicate that exposure to large doses of glucocorticosteroids is a significant factor in the etiology of severe fatty liver disease [52]. The causes of drug-induced hepatitis often include therapeutic drugs for SLE, such as nonsteroidal anti-inflammatory drugs, methotrexate, azathioprine, and other nonglucocorticosteroids [49].

In addition, other reports have revealed that liver dysfunction is not a major prognostic factor of SLE [42, 45, 47, 53]. Large multicenter studies focusing on the mortality in SLE have shown that liver disease does not influence morbidity or mortality in SLE patients [53]. One of the possible explanations for this may be that end-stage liver dysfunction with concomitant SLE is generally rare [2, 38, 39, 45]. A review by Matsumoto et al. [52] revealed that LC was evident in only 1.1% of liver biopsies from 1,468 Japanese patients with SLE.

2.2. Characteristics of PBC

2.2.1. Pathogenesis of PBC. Although the etiology and pathogenesis of PBC remain largely unknown, there is an increasing evidence of interplay of genetic associations, such as specific human leukocyte antigen alleles and environmental factors, in individual host susceptibility [54, 55]. PBC is characterized by the selective destruction of intrahepatic cholangiocytes [54], and PBC results from an immunologic response toward an immunodominant mitochondrial autoantigen, the E2 component of the pyruvate dehydrogenase complex (PDC-E2) [54]. The characteristics of the disease also include the presence of disease-specific antimitochondrial autoantibodies (AMAs) and autoreactive lymphocytes. Associations with the genetic background and environmental exposure and other suspected agents, such

as xenobiotics and microbes, appear to influence PBC by facilitating molecular mimicry, leading to loss of tolerance to PDC-E2, development of AMA, and subsequent development of autoimmunity and subsequent biliary injury in PBC [55, 56].

2.2.2. Clinical Features of PBC. PBC affects middle-aged (penta- and sexagenarians) women much more commonly than men (female : male ratio, 9-10 : 1) [11–13, 15, 34]. Its prevalence is 19–402 cases per million [13, 15, 34]. Comorbidity with other autoimmune disease, familial incidence, past or present smoking habits, a history of urinary tract infection, and repetitive use of cosmetic products such as hair dyes are significant risk factors for developing PBC [13, 14, 16].

The clinical features and natural history of PBC range significantly from asymptomatic to progressive conditions [13]. Jaundice, pruritus derived from cholestasis, and general fatigue are typical symptoms in patients with PBC. However, up to 60% of patients may have no clinical symptoms (asymptomatic PBC). PBC represents approximately 1% of all cases of LC [34], potentially reflecting the relatively high frequency of asymptomatic cases.

2.2.3. Histopathological Features of PBC. With regard to the histopathological features of PBC, florid bile duct lesions, such as chronic, nonsuppurative, and destructive cholangitis, and epithelioid granuloma formation are well-known and useful histological findings in the diagnosis of PBC [13]. Other histopathological findings include portal inflammation, chronic cholestasis, hepatic changes (interface hepatitis or lobular hepatitis), and bile duct loss. The classifications by Scheuer or Ludwig are globally used for disease staging and are based on the histopathological findings of PBC.

2.2.4. Diagnosis of PBC. The diagnosis of PBC is established if two of the three objective criteria are present: (1) elevated serum alkaline phosphatase; (2) presence of AMA, which is useful for the serological diagnosis of PBC (90%–95% of patients with PBC being AMA-positive [25, 57]); and (3) liver histology findings (presence of chronic, nonsuppurative, and destructive cholangitis) [13].

2.2.5. Prognosis of PBC. The prognosis of PBC is often dependent on the development of portal hypertension or cirrhosis, indicating liver failure. However, disease progression may in some cases be significantly inhibited by treatment with ursodeoxycholic acid (UDCA) [13, 58]. Meanwhile, patients with end-stage liver failure require organ transplantation [34]. In such cases, prognostic models, such as Mayo risk scores and bilirubin levels, are useful to determine the appropriate timing of liver transplantation [58]. Although the incidence of hepatocellular carcinoma (HCC) with concomitant PBC is relatively low, several studies have reported incidence rates of <1.6% [9, 59, 60]. Floreani et al. [61] reported that the prevalence of HCC in patients with PBC was 3.0% (11/361) after a mean follow-up period of 8 ± 6.9 years. According to Harada and Nakanuma [10], the incidence of HCC in patients with PBC has increased over the recent decades in Japan. The development of HCC may be associated with refractory to UDCA [58].

2.2.6. PBC/AIH Overlap. PBC/AIH overlap is a relatively rare condition, affecting <10% of patients with AIH or PBC [62, 63]. Recently, Efe et al. [64] reported that, among 1,065 patients diagnosed with PBC (n = 483) and AIH (n = 582), a progressive development of PBC-AIH after a mean of 6.5 years of follow-up was observed in 19 patients (1.8%). Moreover, the combination of UDCA and immunosuppression appears to be an appropriate therapy in cases of PBC-AIH overlap [64].

2.2.7. Extrahepatic Autoimmune Disease Complicated by PBC. SjS appears to be the most common autoimmune disorder concomitantly presenting with PBC [18, 22, 24, 65]. Similarly, RA, systemic sclerosis (SS), Raynaud's syndrome, and chronic thyroiditis (Hashimoto's thyroiditis or Hashimoto's disease) are also conditions that commonly coexist with PBC [2, 14, 16, 22, 24–26, 37, 46, 57, 61].

Floreani et al. [61] reported that, among 361 patients with PBC, 221 (61.2%) had at least one extrahepatic autoimmune disease (follow-up of 8 ± 6.9 years). The authors found a significantly positive association between the female gender and complications by extrahepatic autoimmune conditions in patients with PBC, whereas there were no significant correlations between positive AMA, histological stage, and mean age at diagnosis and PBC with and without extrahepatic autoimmune conditions [61]. Furthermore, they reported that extrahepatic complications of autoimmune diseases did not reduce patient survival, and there were no significant differences between HCC occurrence or extrahepatic malignancy and PBC in patients with and without concomitant extrahepatic autoimmune diseases [61]. Moreover, it has been reported that PBC patients with concomitant SS have a slower disease progression than matched patients with PBC alone [66]. These findings may be useful for the clinical management of SLE patients with concomitant PBC.

2.3. Concomitant Occurrence of PBC and SLE

2.3.1. Concomitant Occurrence of PBC in SLE Patients. Several reports indicate that the incidence of coexisting PBC in patients with SLE is ≤2%, with results ranging from 0% to 2.7% [2, 25, 37, 41, 42, 44, 45, 47, 57], although there were differences in the duration of follow-up time in these reports. Moreover, the frequency of PBC in patients with concomitant SLE who have abnormal liver enzyme values or liver dysfunction is reportedly 0%–7.5% [37, 42, 44, 45, 47]. Additionally, no obvious correlations between SLE activity and the incidence of PBC have been reported in patients with SLE [23, 25, 26]. In SLE patients with concomitant PBC, SLE flare-ups are unusual [25, 26].

2.3.2. Concomitant Occurrence of SLE in PBC Patients. Several reports indicated that the incidence of SLE during the follow-up of PBC patients is ≤2%, with the incidence ranging from 0% to 3.7% [2, 16, 17, 20, 25, 61, 67, 68], although there

were differences in the duration of follow-up time in these reports. It is unclear whether the incidence of SLE during the follow-up of PBC is significantly higher than that in the general population without autoimmune diseases. However, a large-scale study by Gershwin et al. [14] reported that among 1,032 patients with PBC, 27 (2.61%) also had SLE, and the incidence of SLE in patients with PBC (27/1032, 2.61%) was significantly higher than that in the controls (5/1,041, 0.48%).

2.3.3. Autoantibodies in PBC and SLE Patients. AMAs, particularly M2 antibodies, are useful for serological diagnosis of PBC. Although the percentage of AMA-positive cases in collagen diseases (other than PBC) is low [23, 25, 69], 90%–95% of patients with PBC are AMA-positive [25, 57]. However, patients with AMA-negative PBC exhibit a clinical course similar to their seropositive PBC counterparts [15]. Serological studies of large, presumably healthy cohorts indicated that the prevalence of AMA can be as high as 0.5% [13, 15]. Picceli et al. [70] reported that there was no significant difference in the frequency of AMA positivity between patients with SLE and healthy controls.

Despite this, AMA antibody titers reportedly decrease and undergo negative conversion over time in approximately 1/3 of the SLE patients with concomitant AMA-positive PBC [22, 23, 57, 62]. Matsumoto et al. [57] reported that 2/73 (2.7%) patients with SLE had concomitant PBC; however, both cases were AMA-negative. It may therefore be important to consider AMA-negative PBC in patients with SLE and liver dysfunction. Moreover, anti-double stranded DNA and anti-ribosomal-P antibodies, two serological markers of SLE, were detected in 22% and 5%, respectively, of patients with PBC without other autoimmune diseases [49].

2.3.4. Suspected Common Genetic Susceptibility in Concomitant Cases of SLE and PBC. Recent genome-wide studies have provided an insight into the genetic background of the pathogenesis of autoimmune diseases, including PBC and SLE, and have identified risk loci, such as IRF5-TNPO3, which may be associated with the genetic susceptibility to both SLE and PBC [71–73].

Moreover, osteopontin (OPN), a pleiotropic protein, is important in the immune system signaling, and OPN expression is influenced by the genetic polymorphisms of its promoter, hormones, and cytokines [74]. OPN was reported to be highly expressed in MRL/lpr mice, recognized as one of the spontaneous autoimmune models of SLE [26, 74]. A large number of publications suggested that OPN participates in the pathogenesis of several autoimmune diseases [74], including SLE, and a number of studies demonstrated that an increased plasma concentration, as a result of OPN gene polymorphism and increased protein expression, is associated with SLE susceptibility and/or clinical manifestations of the disease in humans [74, 75]. OPN may also be involved in the susceptibility to PBC [25, 26]. It has been reported that OPN is involved as a chemoattractant cytokine in the recruitment of macrophages and T lymphocytes in liver granulomas, also playing an important role in the production of autoantibodies in PBC [76]. However, although a single-nucleotide polymorphism (SNP) at nt position 9,250 (C–T) in exon 7 in OPN was highly associated with SLE, Kikuchi et al. [77] reported that symptoms and pathologic stages of PBC did not correlate with the variation of this SNP, suggesting no associations between this polymorphism and susceptibility to PBC in Japan.

To our knowledge, these genetic factors were not investigated or not discussed in previously reported cases of concomitant SLE and PBC.

Therefore, it is uncertain whether some patients with concomitant SLE and PBC may have a common genetic susceptibility and/or immunological background favoring the development of these diseases [33].

2.4. Cases of Concomitant SLE and PBC

2.4.1. Reported Cases of Concomitant SLE and PBC. Cases of concomitant SLE and PBC retrieved from the English and Japanese scientific literature and Japanese proceedings amount to a total of 34, which are summarized in Table 1 (20 citations in English [17–26] and Japanese [9, 27–32]) and Table 2 (14 Japanese proceedings).

2.4.2. Characteristics of Cases of Concomitant SLE and PBC. Thirty-three of 34 concomitant cases were seen in females. This proportion (97.1%, 33/34) appears high, although PBC and SLE are more common in females. These findings may be in accordance with those reported by Floreani et al. [61], who indicated that there was a significantly positive association between the female gender and the presence of extrahepatic autoimmune conditions in patients with PBC.

Because PBC is more common in middle-aged women and rare in teenagers and because SLE usually affects women of the childbearing age [2, 26], it is assumed that SLE is more likely to be first diagnosed in younger PBC patients with concomitant SLE. However, in the 34 patients with concomitant SLE and PBC, PBC was first diagnosed in 58.8% (20/34) and SLE in 26.5% (9/34); 14.7% (5/34) of the cases were simultaneously diagnosed with SLE and PBC. In the 20 patients in whom PBC first occurred, the interval from the diagnosis of PBC to that of SLE ranged from seven months to 10 years [9, 17, 18, 20–22, 24, 27, 29, 30, 32]. Meanwhile, in nine patients with preceding SLE, PBC was diagnosed 1–19 years after the diagnosis of SLE [19, 23, 25, 26, 31, 32].

Other disease complications in SLE patients with concomitant PBC are listed in Tables 1 and 2. The most common disease presenting concomitantly with SLE and PBC is SjS (23.5%, 8/34), a common complication of both SLE and PBC. Immune thrombocytopenia was diagnosed in three patients (8.8%), and RA, Hashimoto's thyroiditis, and pulmonary hypertension were diagnosed in two patients (5.9%). One patient represented a case of familial PBC [31]. Sato et al. [31] reported a case of a Japanese female who developed asymptomatic PBC at the age of 44, after presenting with SLE. In addition, her father was diagnosed with PBC, indicating a case of familial PBC.

As mentioned above, LC is uncommon in SLE patients with concomitant liver dysfunction. In fact, only one (5%)

Table 1: Characteristics of 20 systemic lupus erythematosus patients with concomitant primary biliary cirrhosis derived from the literature in English and Japanese.

Case	Sex	Age at diagnosis of SLE (years)	Age at diagnosis of PBC (years)	PBC prior to SLE	Remarks	References
1	F	39	33	+		[17]
2	F	58	53	+		[18]
3	M	53	50	+		[18]
4	F	39?	35	+	Sudden death (etiology?)	[18]
5	F	25	29	−	Lupus nephritis (renal failure)	[19]
6	F	60	53	+	Liver failure	[20]
7	F	65	64?	+		[21]
8	F	41?	37	+		[22]
9	F	54	72	−	Liver failure	[23]
10	F	57	47	+		[24]
11	F	21	29	−		[25]
12	F	69	70	−		[26]
13	F	63 or 64	62	+	RA and Sjögren's syndrome	[27]
14	F	41	41	Sim	Immune thrombocytopenia	[28]
15	F	34 or 35	31	+	Lupus nephritis	[29]
16	F	48	40	+	Sjögren's syndrome	[30]
17	F	27	44	−	Familial PBC case	[31]
18	F	46?	65	−	Sjögren's syndrome	[32]
19	F	55	52?	+	Sjögren's syndrome	[32]
20	F	81	80	+	Hepatocellular carcinoma	[9]

SLE: systemic lupus erythematosus; PBC: primary biliary cirrhosis; F: female; M: male; Sim: simultaneous; RA: rheumatoid arthritis.

Table 2: Characteristics of 14 systemic lupus erythematosus patients with concomitant primary biliary cirrhosis derived from Japanese proceedings.

Case (Year)	Sex	Age at diagnosis of SLE (years)	Age at diagnosis of PBC (years)	PBC prior to SLE	Remarks
1 (1984)	F	33?	28	+	Immune thrombocytopenia and pulmonary hypertension
2 (1987)	F	41	41	Sim	
3 (1991)	F	51	50	+	Immune thrombocytopenia
4 (1993)	F	?	46	−	Sjögren's syndrome
5 (1993)	F	51	51	Sim	
6 (1996)	F	39	46	−	Hashimoto's thyroiditis
7 (1999)	F	46	39	+	RA, Sjögren's syndrome, and lupus nephritis (death)
8 (2000)	F	51	48	+	Sjögren's syndrome
9 (2001)	F	45	51	−	Lupus nephritis
10 (2003)	F	48	47	+	HUS/TTP, DIC, and pneumonia (death)
11 (2005)	F	54	54	Sim	
12 (2008)	F	59<	?	+	Sjögren's syndrome and pulmonary hypertension
13 (2013)	F	64	64	Sim	
14 (2013)	F	65	64	+	Hashimoto's thyroiditis and lupus nephritis

SLE: systemic lupus erythematosus; PBC: primary biliary cirrhosis; F: female; M: male; Sim: simultaneous; RA: rheumatoid arthritis; HUS/TTP: hemolytic uremic syndrome/thrombotic thrombocytopenic purpura; DIC: disseminated intravascular coagulation.

of 20 SLE patients with concomitant PBC undergoing liver biopsy at the time of PBC diagnosis was found to present with stage IV according to the Scheuer classification, indicating cirrhosis. Similarly, few PBC patients with concomitant SLE develop LC at the time of PBC diagnosis. PBC patients with concomitant SLE who clinically presented with LC (PBC occurred first and SLE subsequently occurred) have also been reported [21].

Ishiguro et al. [9] reported a case of an 81-year-old Japanese female who developed SLE and HCC approximately one year after the diagnosis of PBC. HCC is relatively rare in patients with PBC [9, 59, 60] and, to the best of our knowledge, this is the only case report of PBC with concomitant SLE that concurrently occurs with the development of HCC.

Among the five deaths, two patients presented liver failure secondary to PBC (both were elderly females) [20, 23]. One of these patients died of liver failure two years later, although a liver biopsy at the time of PBC diagnosis indicated that the patient had stage I disease [23]. Liver biopsy of the other patient showed no obvious abnormalities at the time of diagnosis; however, the patient died of liver failure 15 years later [20]. One patient experienced sudden death (unknown etiology) [18]. The other two deaths were attributed to severe conditions: (1) complicated pneumonia and disseminated intravascular coagulation and (2) complicated pneumonia and hemolytic uremic syndrome/thrombotic thrombocytopenic purpura. One of these fatalities was presumably caused by the immunosuppressive condition of the host associated with immunosuppressive therapies.

2.5. Cases of Concomitant SLE and Other Autoimmune Liver Diseases

2.5.1. Concomitant of SLE and AIH. The diagnosis of AIH is based on elevated liver enzymes, hypergammaglobulinemia, presence of autoantibodies, and characteristic histological changes [78]. AIH can be divided into two subtypes (type 1 and type 2); type 1 AIH is characterized by the presence of anti-nuclear antibodies (ANA) and/or anti-smooth muscle antibodies, whereas type 2 AIH is characterized by anti-liver/kidney microsome type 1 antibody and/or anti-liver cytosol type 1 antibodies [78]. Coexisting SLE and AIH are rare; the frequency of AIH during the course of SLE appears to range from 2.1% to 3.7% [37, 38, 41, 44, 45, 47, 57]. Teufel et al. [79] reported that the prevalence of additional autoimmune disease in AIH patients was 39.9% (111/278) and the prevalence of SLE was 0.7% (2/278). Patients with AIH sometimes progress to fulminant hepatic failure and advanced LC; hence, differentiating between AIH and lupus hepatitis is critical [47]. However, it is sometimes difficult to discriminate between AIH and lupus hepatitis because immunosuppressive therapy hampers the differential diagnostic considerations, and anti-ribosomal P antibody is not a marker of lupus hepatitis only [38]. Patients with AIH/SLE overlapping or AIH alone also test positive [47]. Moreover, although anti-double stranded DNA antibodies were reported to be specific for SLE, these are also common in patients with ANA-positive type 1 AIH [80]. Further, the diagnostic criteria of SLE do not appear useful for discriminating AIH from lupus hepatitis [81]. Therefore, histological examination of the liver is instrumental in establishing the differential diagnosis between lupus hepatitis and AIH [51, 81, 82]. Periportal piecemeal necrosis associated with lobular activity, rosetting of liver cells, or dense lymphoid infiltrates are prominent in AIH, whereas in SLE inflammation is usually lobular and occasionally periportal with a paucity of lymphoid infiltrates [82].

It should be noted that a higher incidence of AIH is seen in patients affected by juvenile-onset SLE [41, 45, 48, 78].

2.5.2. Concomitance of SLE and PSC. PSC is also a cholestatic liver disease associated with autoimmune processes [58]. Although PBC and PSC are associated with chronic cholestatic liver disease, there are many clinical and epidemiological differences [58]. PSC is best known for its hepatobiliary manifestations accompanied by ulcerative colitis. Additionally, the prevalence of SLE patients with concomitant PSC appears to be extremely rare. To the best of our knowledge, only four reports are available on SLE patients with concomitant PSC to date [3, 83–85]. One of the reasons for the scarcity of reported cases is the low prevalence of PSC itself, which ranges from 4 to 16 per million [86]. Conversely, the reported prevalence of PBC is 19–402 per million [13, 15, 34]. Whether SLE with concomitant PSC occurs by chance or whether these entities have a common immunological basis remains unclear.

3. Conclusions

In this paper, 34 cases of SLE with concomitant PBC published in English and Japanese, including Japanese proceedings, were reviewed and summarized. Of the concomitant cases, 97.1% (33/34) were represented by females, and PBC was diagnosed first in 69.0% (20/29), except for five patients in whom both SLE and PBC were almost simultaneously diagnosed. The most common autoimmune disease present in SLE patients with concomitant PBC was SjS (23.5%, 8/34). Moreover, in SLE patients with concomitant PBC, no death cases resulting from the aggravation of the initially diagnosed disease that occurred soon after the onset of the subsequent disease have been reported.

However, it remains uncertain whether concomitant cases occur by chance or share a common immunological or genetic basis. Further studies are warranted to better understand these concomitant autoimmune diseases.

Conflict of Interests

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Clinical Study

Methotrexate, Cyclosporine A, and Biologics Protect against Atherosclerosis in Rheumatoid Arthritis

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Introduction. The risk of cardiovascular disease is increased in rheumatoid arthritis (RA). A meta-analysis showed increased intima media thickness (IMT) in RA. It has been shown that disease modifying antirheumatic drugs (DMARDs) may influence the progression of atherosclerosis. However, it was suggested that biologics may be more efficient than other DMARDs (including methotrexate—MTX) in protecting against atherosclerosis. *Objectives*. The aim of this study was to assess the influence of different RA characteristics and treatment regimens on IMT and atherosclerotic plaques. *Patients and Methods*. 317 RA patients and 111 controls were included in the study. IMT was measured in carotid (CIMT) and femoral (FIMT) arteries. Arteries were screened for the presence of plaques. *Results*. CIMT, FIMT, and prevalence of plaques were lower in patients treated with methotrexate (MTX) ≥ 20 mg/wk, cyclosporine (CsA), or biologics than in patients treated with lower doses of MTX and other disease modifying antirheumatic drugs. No differences in IMT between patients treated with MTX ≥ 20 mg/wk, biologics, or CsA were found. *Conclusions*. We found a beneficial effect of MTX ≥ 20 mg/wk, biologics, and CsA on atherosclerosis. We do not confirm a stronger influence of biologics on IMT compared with therapeutic doses of MTX.

1. Introduction

Cardiovascular disease (CVD) morbidity and mortality rates are increased in RA patients compared to general population [1]. It is estimated that CVD in RA leads to an excess 35–50% of the mortality rate in comparison to general population and reduces life expectancy by 5–10 years [2, 3]. The pathogenesis of accelerated atherosclerosis in RA is postulated to be multifactorial. It has been shown that traditional CV risk factors like hypertension, diabetes, and hyperlipidemia contribute to the development of atherosclerosis in RA. However, the excess CV risk in RA persists after adjustment for established CV risk factors; thus RA is considered as an independent CV risk factor [4–7].

Several noninvasive diagnostic tools such as assessment of endothelial function, measurement of carotid intima media thickness (CIMT), and assessment of coronary artery calcification score may be used to detect subclinical atherosclerosis. A meta-analysis showed that CIMT predicts future vascular events in healthy individuals [8]. Some studies suggest that carotid and femoral arteries respond differently to CV risk factors and that inclusion of femoral artery IMT measurements would add information to that provided by the common carotid artery [9].

Studies in RA patients showed a decrease in flow mediated dilatation and an increase in augmentation index and pulse wave velocity, which suggests endothelial dysfunction [10–13]. Several studies have also shown increased CIMT and

formation of plaques within the carotid artery in RA [10, 13, 14]. Increased CIMT in RA patients was also confirmed by a meta-analysis [15].

Several studies have focused on the influence of classical CV risk factors and disease-related factors on atherosclerosis in RA. Smoking is one of the most important CV risk factors but it is also known as a risk factor for the development of RA [16]. Thus smoking is frequently seen in RA patients and may provide a potential bias in studies on RA and CVD [17]. The relation between body mass index (BMI), RA, and CVD is also complex. On the one hand obesity is associated with CV morbidity and mortality [18, 19]. On the other hand, CV mortality is also increased in RA patients with a BMI below 20 kg/m^2 [20]. A possible explanation for this excess CV risk is that low BMI may indicate the presence of rheumatoid cachexia [17]. Hypertension, another classical CV risk factor, is common in RA and was shown to be associated with atherosclerosis [17, 21]. The relation between lipid profile and CVD in RA is complex. The active inflammatory state of RA may lower levels of circulating lipids (i.e., total, LDL and HDL cholesterol, and triglycerides) [22]. However, these changes in lipid profile are associated with increased CV risk. This phenomenon is called lipid paradox and is probably due not only to low levels of HDL cholesterol but also to structural and functional changes of HDL [23, 24]. Treatment with disease modifying antirheumatic drugs (DMARDs) was shown to increase lipid levels. However, it is believed that these changes may reflect normalization of the lipid profile. Thus, the interpretation of lipid levels for predicting CV risk in RA patients should be cautious [25].

Among RA-related factors influencing atherosclerosis inflammation seems to play a major role. Wållberg-Jonsson et al. found that high disease activity was associated with increased risk of CV event and decreased life span [26]. A study by Maradit-Kremers et al. showed that markers of systemic inflammation confer additional risk for CV death among patients with RA [2]. Innala et al. reported that erythrocyte sedimentation rate (ESR) and cumulative disease activity (defined as area under the curve DAS28) increased a hazard rate for a new CV event [27]. Several studies also found the association between markers of inflammation and subclinical atherosclerosis. ESR and C-reactive protein (CRP) were found to be associated with CIMT, atherosclerotic plaques, arterial stiffness, flow-mediated dilatation (FMD), and glyceryl trinitrate-mediated dilatation (GMD) [5, 28–31]. On the other hand some studies failed to find the association between cumulative inflammation and markers of atherosclerosis [32–34]. Other RA-related factors which may predict progression of atherosclerosis are rheumatoid factor (RF), anti-citrullinated peptide antibodies (ACPA), disease duration, and radiological damage index [5, 34, 35].

It is well established that DMARDs therapy decreases CV morbidity and mortality. However, most studies focused on methotrexate (MTX) and biologics, while little is known about other DMARDs. Moreover, only few reports compared influence of different DMARDs on subclinical atherosclerosis. A prospective study by Choi et al. showed that treatment with methotrexate reduces CV mortality in RA patients [36]. A systematic review confirmed that the use of MTX decreases CV morbidity and mortality [37]. Few small studies found a beneficial effect of combined DMARDs therapy (MTX, hydroxychloroquine, and sulfasalazine) on CIMT, FMD, and GMD [31, 38, 39]. Several studies showed a beneficial effect of anti-TNF-α therapy on subclinical atherosclerosis [40–42] and systematic review by Westlake et al. confirmed that anti-TNF-α therapy reduces the likelihood of CVD in RA [43]. Interestingly, some studies suggest that biologics may be more efficient than MTX in protecting against atherosclerosis in RA. A study by Giles et al. showed slower progression of CIMT in patients treated with anti-TNF-α compared to those not receiving treatment; such association was not observed with other DMARDs [44]. Similarly, an analysis of large RA registry (CORRONA) showed reduction of CV events risk in patients treated with anti-TNF-α compared with patients treated with MTX and other nonbiological DMARDs [45].

The aim of this study was to assess the influence of different RA characteristics and treatment regimens on CIMT, FIMT, and atherosclerotic plaques.

2. Materials and Methods

The study was approved by the local ethical committee. All participants signed an informed consent form.

2.1. Patients. 317 RA patients fulfilling the 1987 ACR criteria were recruited. Exclusion criteria comprised diabetes mellitus, coronary artery disease, and history of stroke. A complete history, physical examination, and laboratory evaluation were performed and recorded in a standard protocol (Table 1). All DMARDs ever used were recorded unless treatment duration was <3 months. Patients were divided into 2 groups: continuously (cDMARDs) and discontinuously (dDMARDs) treated with DMARDs (treatment with DMARDS ≥ 90 and <90% of RA duration, resp.). Hands and feet X-rays were performed in most patients. RA activity was assessed with DAS28. Framingham 10-year risk score (FSS) was used to estimate general CV risk related to classical risk factors [46].

2.2. Controls. 111 age- and sex-matched healthy individuals were included in the control group. Clinical and laboratory data are summarized in Table 1.

2.3. Ultrasonography. IMT was measured on the far wall of the common carotid and superficial femoral arteries. Atherosclerotic plaque was defined as a focal structure that encroaches into the arterial lumen of at least 0.5 mm or 50% of the surrounding IMT or demonstrates a thickness of ≥1.5 mm as measured from the media-adventitia interface to the intima-arterial lumen interface. CIMT and FIMT were defined as a mean value of 6 measurements (CIMT: 1, 2, and 3 cm proximal to the bifurcation bilaterally; FIMT: 1, 2, and 3 cm distal to the bifurcation bilaterally). Common carotid and superficial femoral arteries were investigated for the presence of plaques.

Table 1: Study and control group characteristics.

	RA (n = 317)	Controls (n = 111)	P value
Age, years	57.61 (12.62)	55.50 (9.37)	0.1
Males	58 (18.30%)	22 (19.81%)	0.7
Ever-smokers	140 (44.16%)	62 (55.86%)	0.04
Pack-years	9.66 (16.04)	13.13 (18.20)	0.06
BMI, kg/m^2	25.54 (4.37)	27.46 (4.69)	0.0001
Hypertension	137 (43.22%)	38 (34.23%)	0.1
Creatinine, mg/dL	0.73 (0.27)‡	0.65 (0.07)$^{\blacktriangledown}$	0.3
ESR, mm/h	31.77 (23.64)	10.63 (9.19)	$<1 \times 10^{-6}$
CRP, mg/dL	2.15 (3.2)	0.43 (0.641)	$<1 \times 10^{-6}$
Total cholesterol, mg/dL	203.8 (41.3)	215.8 (44.3)	0.01
LDL cholesterol, mg/dL	115.7 (34.3)†	127.4 (40.5)	0.004
HDL cholesterol, mg/dL	63.2 (19.7)†	66.5 (19)	0.1
Triglycerides, mg/dL	128.6 (60.5)†	116.2 (56.8)	0.06
Framingham 10-year risk score	7.17 (5.4)†	7.86 (6.32)	0.3
Presence of atherosclerotic plaques in carotid and/or femoral arteries	74 (23.34%)	14 (12.61%)	0.015
CIMT, mm	0.718 (0.181)	0.682 (0.167)	0.07
FIMT, mm	0.516 (0.168)	0.457 (0.099)	0.0005
Disease duration, years	10.74 (8.98)		
Methotrexate ever	303 (95.58%)		
Sulphasalazine ever	148 (46.69%)		
Hydroxychloroquine or chloroquine ever	98 (30.91%)		
Gold salts ever	46 (14.51%)		
Azathioprine ever	18 (5.68%)		
Cyclophosphamide ever	7 (2.21%)		
Cyclosporine A ever	77 (24.29%)		
Leflunomide ever	123 (38.8%)		
Biologic agents ever	61 (19.24)		
Infliximab ever	24 (7.57%)		
Adalimumab ever	12 (3.78%)		
Etanercept ever	39 (12.3%)		
Rituximab ever	13 (4.1%)		
Continuous treatment with DMARDs	141 (45.19%)¶		
RF positivity	217 (70.68%)$^{\#}$		
ACPA positivity	211 (77.29%)§		
DAS28	4.7 (1.55)		
Presence of erosions in hand and/or feet X-ray	176 (70.97%)$^{\blacktriangle}$		

Data is presented as mean (standard deviation) for continuous variables and number (percentage) for categorical variables. ¶Data available for 312 patients. $^{\#}$Data available for 307 patients. §Data available for 273 patients. ‡Data available for 286 patients. †Data available for 302 patients. $^{\blacktriangle}$Data available for 248 patients. $^{\blacktriangledown}$Data available for 102 patients.

2.4. Statistical Analysis. All statistical tests were performed with STATISTICA 10.0 (StatSoft). Results are reported as mean (SD) for continuous variables and n (%) for categorical variables. According to data distribution, a parametric (t-test) or nonparametric (U Mann-Whitney) test was used. Categorical variables were compared with chi square exact test. A P value < 0.05 was considered significant.

3. Results and Discussion

3.1. Results. Patients and controls were age- and sex-matched. The percentage of ever-smokers, LDL, and total cholesterol concentrations and BMI were higher in controls than in RA (Table 1). However, total CV risk calculated with FSS was similar in both groups. CIMT and FIMT were higher in RA but only the difference in FIMT was significant. Atherosclerotic plaques were more prevalent in RA.

The presence of plaques in RA was positively correlated with ESR, creatinine concentration, FSS, and presence of rheumatoid factor (RF) (Table 2). Analysis for associations between plaques and treatment with DMARDs showed a significant negative correlation between presence of plaques and treatment with methotrexate (MTX), cyclosporine A (CsA), and biologics. Plaques were insignificantly

Table 2: Associations between presence of atherosclerotic plaques and clinical, laboratory, and radiological characteristics and use of different DMARDs.

	Presence of atherosclerotic plaques in carotid and/or femoral arteries ($n = 74$)	Lack of atherosclerotic plaques in carotid and femoral arteries ($n = 242$)	P
RA duration, years	10.54 (9.41)	10.76 (8.86)	0.8
ESR, mm/h	31.17 (24.39)	22.49 (21.4)	0.0008
CRP, mg/dL	1.99 (2.9)	1.42 (2.66)	0.07
Creatinine, mg/dL	0.81 (0.3)	0.7 (0.21)	0.0007
DAS28	4.63 (1.51)	4.73 (1.57)	0.6
Framingham 10-year risk score	10.1 (6.22)	6.01 (5.16)	$<1 \times 10^{-6}$
Methotrexate ever	65 (87.84%)	237 (97.93%)	0.0002
Sulphasalazine ever	30 (40.54%)	117 (48.35%)	0.2
Hydroxychloroquine or chloroquine ever	22 (29.72%)	76 (31.4%)	0.8
Gold salts ever	8 (10.81%)	38 (15.7%)	0.3
Azathioprine ever	5 (6.76%)	13 (5.37%)	0.7
Cyclophosphamide ever	2 (2.7%)	5 (2.07%)	0.7
Cyclosporine A ever	10 (13.51%)	67 (27.69%)	0.01
Leflunomide ever	28 (37.84%)	95 (39.26%)	0.8
Biologic agents ever	5 (6.76)	56 (23.14%)	0.002
Infliximab ever	1 (1.35%)	23 (9.5%)	0.02
Adalimumab ever	0 (0%)	12 (4.96%)	0.0503
Etanercept ever	2 (2.7%)	37 (15.29%)	0.004
Rituximab ever	0 (0%)	13 (5.37%)	0.04
Continuous treatment with DMARDs	27 (36.49%)	114 (47.11%)	0.1
RF positivity	59 (79.73%)	158 (67.81)▲	0.0497
ACPA positivity	49 (76.56%)#	162 (77.51%)†	0.9
Presence of erosions in hand and/or feet X-ray	42 (68.58%)‡	134 (71.66%)§	0.8

Data is presented as mean (standard deviation) for continuous variables and number (percentage) for categorical variables. #Data available for 64 patients. ‡Data available for 61 patients. ▲Data available for 233 patients. †Data available for 209 patients. §Data available for 187 patients.

more prevalent in dDMARDs group than in cDMARDs group.

We found a positive correlation between CIMT, FIMT, and FSS ($r = 0.488$, $P < 0.001$, and $r = 0.434$, $P < 0.001$, resp.), ESR ($r = 0.132$, $P = 0.018$, and $r = 0.199$, $P < 0.001$), and creatinine concentration ($r = 0.2$, $P < 0.001$, and $r = 0.212$, $P < 0.001$). However, after adjustment for age, associations with creatinine became insignificant. No significant associations were found between CIMT, FIMT, and RA duration, CRP concentration, and DAS28 (data not shown).

CIMT and FIMT were significantly lower in cDMARDs group compared with dDMARDs group (Table 3). The association remained significant after adjustment for classical CV risk factors. The use of MTX was associated with lower FIMT. Comparison of different doses of MTX revealed significantly lower CIMT and FIMT in patients treated with doses ≥ 20 mg/wk; correlation remained significant after adjustment for classical CV risk factors. CIMT was also significantly lower in patients treated with CsA and biologics. A similar correlation was observed between CsA, biologics, and FIMT but it became insignificant after correction for CV risk factors. We did not find significant differences in CIMT and FIMT in pairwise comparisons between patients treated with MTX ≥ 20 mg/wk, biologics, or CsA (further named MTX20(+)/CsA(+)/biologics(+) group); a comparison of this group with patients treated with different DMARDs/lower doses of MTX (further named MTX20(−)/CsA(−)/biologics(−) group) revealed a robust difference in CIMT (0.104 mm, $P = 1 \times 10^{-6}$) and FIMT (0.081 mm, $P = 5 \times 10^{-5}$). Interestingly, RA activity (measured by DAS28) was similar in both groups: 4.64 (1.54) versus 4.83 (1.57), $P = 0.3$. No significant differences in classical CV risk factors were found between patients treated with MTX ≥ 20 mg/wk, biologics, or CsA. CIMT in MTX20(+)/CsA(+)/biologics(+) was comparable to controls. FIMT was slightly higher in MTX20(+)/CsA(+)/biologics(+) group than in controls. No correlations were found between CIMT and FIMT and presence of RF, ACPA, and bone erosions.

3.2. Discussion. RA patients are at higher risk of CVD than an age-matched general population. It is estimated that CV risk in RA is increased to a similar magnitude to that seen in type 2 diabetes [47]. Studies assessing IMT in RA showed conflicting results but two meta-analyses

Table 3: Associations between IMT and use of different DMARDs, treatment regimen, presence of RF, ACPA, and erosions.

	Significant differences in classical CV risk factors (i.e., age, BMI, smoking, hypertension, lipid profile, and FSS) between groups	CIMT			FIMT		
		CIMT, mm	P	P_{adj}	FIMT, mm	P	P_{adj}
MTX(+) versus MTX(−)	—	0.716 (0.178) versus 0.784 (0.235)	0.2		0.511 (0.159) versus 0.628 (0.293)	0.01	0.03
MTX ≥ 20 mg/wk versus MTX < 20 mg/wk	MTX ≥ 20 mg/wk group was younger (55.77 yrs versus 59.85 yrs) and had lower FSS (6.57 versus 7.92)	0.687 (0.171) versus 0.758 (0.186)	0.0005	0.009	0.492 (0.126) versus 0.546 (0.206)	0.004	0.04
Sulphasalazine(+) versus sulphasalazine(−)	—	0.707 (0.168) versus 0.731 (0.192)	0.2		0.505 (0.138) versus 0.526 (0.192)	0.3	
Hydroxychloroquine/chloroquine(+) versus hydroxychloroquine/chloroquine(−)	—	0.703 (0.181) versus 0.727 (0.181)	0.3		0.517 (0.163) versus 0.515 (0.181)	0.9	
Gold salts(+) versus gold salts(−)	—	0.682 (0.139) versus 0.726 (0.187)	0.1		0.494 (0.166) versus 0.520 (0.169)	0.3	
Azathioprine(+) versus azathioprine(−)	—	0.731 (0.216) versus 0.719 (0.179)	0.8		0.518 (0.110) versus 0.516 (0.172)	0.96	
Cyclophosphamide(+) versus cyclophosphamide(−)	—	0.745 (0.205) versus 0.719 (0.181)	0.7		0.669 (0.427) versus 0.512 (0.158)	0.01	0.2
Cyclosporine(+) versus cyclosporine(−)	CsA(+) group was younger (53.03 yrs versus 58.46 yrs) and had lower FSS (5.91 versus 7.59), and hypertension was less prevalent in this group (32.47% versus 46.44%)	0.665 (0.165) versus 0.737 (0.183)	0.002	0.03	0.471 (0.095) versus 0.530 (0.184)	0.007	0.06
Leflunomide(+) versus leflunomide(−)	—	0.719 (0.174) versus 0.720 (0.186)	0.96		0.514 (0.161) versus 0.517 (0.174)	0.9	
Biologic agents(+) versus biologic agents(−)	Biologic agents(+) group was younger (53.24 yrs versus 58.31 yrs)	0.663 (0.165) versus 0.733 (0.182)	0.006	0.04	0.477 (0.124) versus 0.525 (0.177)	0.04	0.2
MTX20(+)/CsA(+)/biologics(+) versus MTX20(−)/CsA(−)/biologics(−)	MTX20(+)/CsA(+)/biologics(+) group was younger (55.47 yrs versus 61.59 yrs) and had lower FSS (6.38 versus 8.82)	0.684 (0.169) versus 0.788 (0.186)	1×10^{-6}	2×10^{-5}	0.489 (0.123) versus 0.570 (0.227)	5×10^{-5}	6×10^{-4}
MTX20(+)/CsA(+)/biologics(+) versus controls	—	0.684 (0.169) versus 0.682 (0.167)	0.94		0.489 (0.123) versus 0.457 (0.099)	0.02	0.02
MTX20(+)/biologics(−) versus biologics(+)MTX20(−)	—	0.703 (0.173) versus 0.708 (0.172)	0.9		0.493 (0.128) versus 0.458 (0.123)	0.2	
Biologics(+)/CsA(−) versus CsA(+)/biologics(−)	—	0.655 (0.181) versus 0.662 (0.176)	0.9		0.476 (0.155) versus 0.467 (0.102)	0.3	
CsA(+)/MTX20(−) versus MTX20(+)/CsA(−)	—	0.661 (0.144) versus 0.696 (0.168)	0.3		0.473 (0.076) versus 0.500 (0.134)	0.3	
MTX ≥ 20/biologics(−)/CsA(−) versus MTX < 20/biologics(−)/CsA(−)	MTX ≥ 20/biologics(−)/CsA(−) group was younger (56.41 yrs versus 60.39 yrs) and had lower FSS (6.71 versus 8.01)	0.708 (0.164) versus 0.791 (0.188)	0.0009	0.008	0.502 (0.130) versus 0.577 (0.227)	0.004	0.02
cDMARDs versus dDMARDs	cDMARDs group was younger (54.53 yrs versus 59.62 yrs)	0.683 (0.177) versus 0.746 (0.182)	0.002	0.02	0.485 (0.114) versus 0.544 (0.200)	0.002	0.01
RF(+) versus RF(−)	—	0.733 (0.189) versus 0.690 (0.160)	0.06		0.524 (0.164) versus 0.506 (0.182)	0.4	
ACPA(+) versus ACPA(−)	—	0.720 (0.180) versus 0.702 (0.202)	0.5		0.525 (0.171) versus 0.480 (0.148)	0.06	
Bone erosions(+) versus bone erosions(−)	—	0.730 (0.190) versus 0.721 (0.174)	0.8		0.530 (0.184) versus 0.501 (0.121)	0.2	

Data is presented as mean (standard deviation). Biologics(+)/MTX20(−): patients treated with biologics but never treated with MTX ≥ 20 mg/wk. Biologics(+)/CsA(−): patients treated with biologics but never treated with CsA. CsA(+)/MTX20(−): patients treated with CsA but never treated with MTX ≥ 20 mg/wk. CsA(+)/biologics(−): patients treated with CsA but never treated with biologics. MTX20(+)/biologics(−): patients treated with MTX ≥ 20 mg/wk but never treated with biologics. MTX20(+)/CsA(−): patients treated with MTX ≥ 20 mg/wk but never treated with CsA. MTX20(+)/CsA(+)/biologics(+): patients treated with MTX ≥ 20 mg/wk and/or CsA and/or biologic agents. MTX20(−)/CsA(−)/biologics(−): patients never treated with CsA, biologic agents, and MTX ≥ 20 mg/wk. MTX ≥ 20/biologics(−)/CsA(−): patients treated with MTX ≥ 20 mg/wk but never treated with biologics and CsA. MTX < 20/biologics(−)/CsA(−): patients treated with MTX < 20 mg/wk but never treated with biologics and CsA. P_{adj}: P value for analysis adjusted for classical CVD risk factors.

confirmed increased IMT in RA [15, 48]. We also observed increased IMT in carotid and femoral arteries in RA patients, but only the difference in femoral arteries was significant. Atherosclerotic plaques were more frequently found in RA than in controls.

We found a strong correlation between FSS and IMT and presence of plaques. This finding underlines the role of classical CV risk factors in pathogenesis of atherosclerosis in RA.

CIMT, FIMT, and presence of plaques were associated with ESR. However, no correlation was found between atherosclerosis markers and DAS28. It may be explained by the fact that DAS28 comprises two parameters which are not completely objective: VAS and tender joints count. Moreover, swollen joints count is related to the local inflammation (synovitis), while progression of atherosclerosis in RA is thought to be due to systemic inflammation. Thus ESR, as a more objective parameter and a marker of systemic inflammation, may be a better predictor of increased risk of atherosclerosis in RA. In this context lack of association with CRP is intriguing. CRP was found to be a powerful predictor of cardiovascular disease in general population [49]. The absence of association in our study may be explained by the use of conventional CRP assay, since the involvement of CRP in atherosclerosis has been demonstrated by high sensitivity CRP assay. It must be also emphasized that RA is a disease characterized by periods of exacerbations and remissions. Thus, a single measure of disease activity may not reflect intensity of disease in a longer period of time. Indeed, several studies suggest that assessment of the cumulative inflammation for the whole duration of RA may be a better predictor of atherosclerosis [34].

Creatinine concentration was significantly higher in patients with plaques and correlated positively with CIMT and FIMT. However, after adjustment for age these associations became insignificant (data not presented). It is not surprising as a concentration of creatinine increases with age and age is a strong risk factor for atherosclerosis.

CIMT, FIMT, and prevalence of plaques were lower in patients treated with MTX $\geq$ 20 mg/wk, CsA, and biologics. This effect seems to be independent of disease activity as DAS28 was similar in MTX20(+)/CsA(+)/biologics(+) and MTX20(−)/CsA(−)/biologics(−) groups. Analysis of different combinations of DMARDs did not reveal any significant correlations (data not shown). There is a lot of evidence supporting a beneficial effect of biologics on atherosclerosis, while data concerning MTX are conflicting. Several studies showed a beneficial influence of anti-TNF-α therapy on subclinical atherosclerosis [40–42]. A protective effect of anti-TNF-α therapy was also confirmed in meta-analyses [43, 50]. A study based on data from British Society for Rheumatology Biologics Register showed no overall difference in the risk of myocardial infarction between patients treated with anti-TNF-α and nonbiologic DMARDs; however, the authors reported a reduced risk of myocardial infarction in TNF-α-responders [51]. Giles et al. observed slower CIMT progression in patients treated with anti-TNF-α, but not in users of other RA treatments [44]. Analysis of CORRONA registry showed reduction of CV events risk in patients treated with anti-TNF-α compared with patients treated with MTX and other nonbiological DMARDs [45]. On the other hand a systematic review by Westlake et al. found that use of MTX is associated with reduced risk of CV events [37]. Our results suggest that the observed discrepancy in the literature may be due to different doses of MTX. For last three decades doses of MTX used in RA have increased from 5–7.5 mg/wk to 30 mg/wk. A study by Giles et al. enrolled patients between 2004 and 2006 and enrolment to CORRONA registry took place between 2001 and 2006; data concerning average dose of MTX in these studies is missing in the publications; however, we may speculate that it was below 20 mg/wk. We observed a significant difference in CIMT and FIMT between patients treated with MTX $\geq$ 20 mg/wk and $<$ 20 mg/wk; it should be emphasized that the difference remained significant after exclusion of patients treated with other drugs influencing IMT (i.e., CsA and biologics). We did not observe significant differences in IMT between patients treated with MTX $\geq$ 20 mg/wk (but never using biologics) and patients treated with biologics (but never using MTX $\geq$ 20 mg/wk). It suggests that the impact of MTX $\geq$20 mg/wk on IMT was comparable to that of biologics.

A beneficial influence of CsA on atherosclerosis in RA is a novel finding. Few studies reported a protective effect of CsA on IMT in lupus patients [52]. Surprisingly, other synthetic DMARDs recommended in RA (leflunomide, sulphasalazine) showed no effect on IMT and presence of plaques.

The differences in CIMT and FIMT between MTX20(+)/CsA(+)/biologics(+) and MTX20(−)/CsA(−)/biologics(−) groups were robust (0.104 mm, $P = 1 \times 10^{-6}$, and 0.081 mm, $P = 5 \times 10^{-5}$). A large study in general population found that an absolute carotid IMT difference of 0.1 mm is associated with a 10–15% higher risk of myocardial infarction and 13–18% higher risk of stroke [53]. Thus, the observed effect of MTX, biologics, and CsA seems to be important.

Another factor influencing the atherosclerosis status is regularity of treatment. Patients treated continuously with DMARDS had a lower CIMT and FIMT. This finding is not surprising as good RA control is a widely accepted predictor of slower atherosclerosis progression.

Lekakis et al. suggested that combined assessment of carotid and femoral IMT might provide additional information compared with analysis of carotid IMT only [9]. Our results suggest that this hypothesis may not be applicable to RA population.

4. Conclusions

In conclusion, we found a beneficial effect of MTX $\geq$ 20 mg/wk, biologics, and CsA on atherosclerosis. We do not confirm a stronger influence of biologics on IMT compared with MTX (in doses $\geq$20 mg/wk).

Conflict of Interests

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

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Review Article

Interleukin-23R rs7517847 T/G Polymorphism Contributes to the Risk of Crohn's Disease in Caucasians: A Meta-Analysis

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The association between *Interleukin-23R* gene polymorphism and Crohn's disease (CD) in Caucasians is still controversial. Thus, a meta-analysis was performed to evaluate the correlation between this gene variant and CD risk. We retrieved the available data from EMBASE and PUBMED until May 1, 2014, and evaluated the effect of rs7517847 in Caucasians. The significant associations were confirmed between rs7517847 and CD risk in dominant models (TT/TG versus GG: OR = 1.652, 95% CI 1.277, 2.137), allelic model (T allele versus G allele: OR = 1.327, 95% CI 1.198, 1.469), homozygote comparison (TT versus GG: OR = 1.890, 95% CI 1.465, 2.437), heterozygote comparison (TG versus GG: OR = 1.509, 95% CI 1.161, 1.960), and recessive model (TT versus TG/GG: OR = 1.409, 95% CI 1.279, 1.552). In conclusion, this meta-analysis demonstrates that rs7517847 is associated with the risk of CD in Caucasians. These findings show that IL-23R genes confer susceptibility to CD in the Caucasians.

1. Introduction

Crohn's disease (CD) is a form of inflammatory bowel disease (IBD) that primarily affects the Caucasian population [1, 2]. It is a heritable disease which is influenced by many genetic risk factors [3]. Therefore, identification of gene risk factors of CD is beneficial for the clinical treatment of patients.

Interleukin 23 (IL-23) plays an important role in the inflammatory response against infection as a regulator of immune cells [4]. IL-23R which interacts with IL-23 is a protein consisting of an IL-12β1 and an IL-23R chain [5]. Recently, the mechanisms of IL-23R variants have been investigated in different autoimmune diseases [6–9]. Studies also have shown that rs7517847, the single nucleotide polymorphisms (SNPs) of the *IL23R* gene, are associated with CD occurring rate [10, 11]. However, the association between IL-23R polymorphisms and CD susceptibility are inconclusive and controversial due to small sample size in each of the published studies.

To better understand the association of IL-23R polymorphisms and CD susceptibility in Caucasians, we conducted a meta-analysis of all eligible studies and hope to yield more accurate and robust estimates.

2. Materials and Methods

2.1. Search Strategy. We searched for relevant studies in the following databases: EMBASE and PUBMED. Available studies for IL-23R polymorphism and CD were collected by different combinations of various key words: Interleukin-23 receptor, IL-23R; polymorphism, variant, or mutation; Crohn's disease, CD. Languages restriction was not imposed in this research and only published studies with full text were included in this meta-analysis.

2.2. Inclusion and Exclusion Criteria of Trials. In the meta-analysis, the following inclusive selection criteria were set: (a) study design evaluating the association between IL-23R polymorphism and CD risk; (b) case control design; (c) Caucasians design. The following exclusive selection criteria were set: (a) no control cases; (b) duplication of the previous

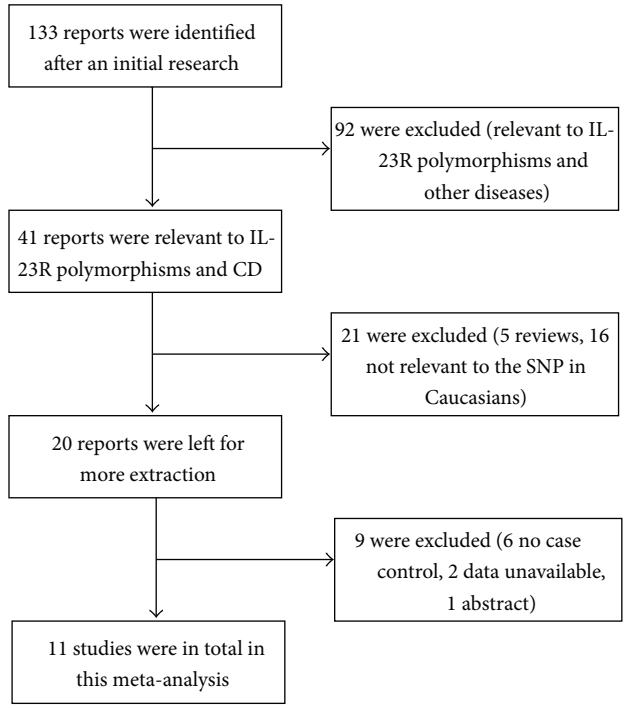

Figure 1: Flow diagram of the study selection process and specific reasons for exclusion.

publication; (c) no available genotype frequency; for studies with overlapped or repeated data (d) no Caucasians.

2.3. Data Extraction. Eligible studies were extracted by 2 reviewers (Li Zhang and Yunjie Lu) independently according to the predesigned data collection form. The following information was extracted: first author's name, publication year, country, ethnicity, immune suppressive protocol, number of cases and controls, and genotype distribution in both groups. Disagreement was resolved by discussion with a third reviewer (Guozhong Yao).

2.4. Statistical Analysis. For each trial, odds ratio (OR) with the 95% confidence interval (95% CI) of the survival rate was derived and calculated. Increased or decreased risk of CD was indicated by 95% CI without 1 for OR. The pooled ORs were estimated for allelic model (T allele versus G allele), homozygote comparison (TT versus GG) and heterozygote comparison (TG versus GG), dominant models (TT/TG versus GG), and recessive model (TT versus TG+GG). Z test was performed to assess the significance of the pooled OR. Between-study heterogeneity was assessed by the Cochran's Q statistic and I^2 tests [12]. The random effects model was conducted if the Q test exhibited a $P < 0.05$ or the I^2 test showed >50%. Otherwise, the fixed effects model would be conducted. For publication bias, the Begg's funnel plot and Egger's linear regression test were conducted, and $P < 0.05$ was considered significant.

A fixed-effect model (based on Mantel-Haenszel method) was utilized to pool the data from different studies if the between-study heterogeneity was absent, or a random-effect model (based on DerSimonian and Laird method) was applied.

The statistical analysis was performed by STATA 10.0 (Stata Corp LP, College Station, TX, USA). All P values are two-side.

3. Results

3.1. Selection of the Included Studies and Characteristics. The flow diagram of studies selection and exclusion reasons were represented in Figure 1. A total of 133 studies were identified by our first research; a number of 41 were preliminarily yielded out after excluding inappropriate studies and screening abstract-screening, full-text assessment. In these 41 studies, 30 were excluded, 11 articles containing rs7517847 in Caucasians were recruited for detailed analysis (Table 1), and these data built Table 1 [13–23]. Each of them was independent. Thus, a total of 3279 CD cases and 4136 healthy controls were included in our meta-analysis. All of them were Caucasian and the diagnosis of CD was based on clinical manifestations and laboratory examinations and further biopsy.

3.2. Evaluation of the Association. The OR from all models indicated a significant association between rs7517847 and CD. After pooling all the eligible studies in Table 2, we found that the risk of CD was significantly associated with rs7517847 in dominant models (TT/TG versus GG: OR = 1.652, 95% CI 1.277, 2.137), allelic model (T allele versus G allele: OR = 1.327, 95% CI 1.198, 1.469), homozygote comparison (TT versus GG: OR = 1.890, 95% CI 1.465, 2.437, Figure 2),

Table 1: Characteristics of eligible studies included in the meta-analysis.

Author	Year	Country	Ethnicity	Genotyping methods	Case			Control			HWE
					TT	TG	GG	TT	TG	GG	
Safrany et al. [13]	2013	Hungary	Caucasian	PCR–RFLP	72	110	17	74	138	41	0.081
Szabo et al. [14]	2013	Hungary	Caucasian	PCR–RFLP	150	182	64	57	99	26	0.104
Ferguson et al. [15]	2010	New Zealand	Caucasian	MassARRAY	108	172	29	113	183	72	0.892
Lauriola et al. [16]	2011	Italy	Caucasian	PCR	9	4	6	13	6	1	0.78
Lacher et al. [17]	2010	Germany	Caucasian	RT-PCR	81	101	39	78	125	50	0.995
Latiano et al. [18]	2008	Italy	Caucasian	PCR-RFLP	366	305	52	280	328	108	0.459
Márquez et al. [19]	2008	Spain	Caucasian	RT-PCR	145	161	36	192	260	94	0.71
Baptista et al. [20]	2008	Brazil	Caucasian	RT-PCR	59	95	28	79	120	43	0.825
Newman et al. [21]	2009	England	Caucasian	MassARRAY	195	204	40	300	436	164	0.799
Okazaki et al. [22]	2008	Canada	Caucasian	RT-PCR	84	91	36	91	157	66	0.91
Oliver et al. [23]	2007	Spain	Caucasian	Taqman PCR	101	119	18	121	153	68	0.124

Table 2: Stratified analysis of rs7517847 polymorphism and CD risk in eligible studies.

Author	Year	T versus G allele	TT versus GG OR (95% CI)	TG versus GG OR (95% CI)	TT + TG versus GG (95% CI)	TT versus TG + GG (95% CI)
Total		**1.327 (1.198, 1.469)**	**1.890 (1.465, 2.437)**	**1.509 (1.161, 1.960)**	**1.652 (1.277, 2.137)**	**1.409 (1.279, 1.552)**
Safrany et al. [13]	2013	1.357 (1.036, 1.777)	2.347 (1.223, 4.503)	1.922 (1.036, 3.568)	2.070 (1.137, 3.769)	1.371 (0.923, 2.038)
Szabo et al. [14]	2013	1.102 (0.856, 1.419)	1.069 (0.618, 1.850)	0.747 (0.445, 1.253)	0.865 (0.528, 1.417)	1.337 (0.921, 1.942)
Ferguson et al. [15]	2010	1.349 (1.084, 1.678)	2.373 (1.432, 3.933)	2.334 (1.446, 3.766)	2.349 (1.481, 3.724)	1.213 (0.879, 1.673)
Lauriola et al. [16]	2011	0.344 (0.126, 0.941)	0.115 (0.012, 1.129)	0.111 (0.009, 1.309)	0.114 (0.012, 1.062)	0.485 (0.134, 1.754)
Lacher et al. [17]	2010	1.176 (0.908, 1.523)	1.331 (0.790, 2.243)	1.036 (0.632, 1.698)	1.149 (0.723, 1.828)	1.298 (0.886, 1.902)
Latiano et al. [18]	2008	1.553 (1.328, 1.816)	2.715 (1.884, 3.913)	1.931 (1.340, 2.784)	2.292 (1.618, 3.248)	1.596 (1.295, 1.968)
Márquez et al. [19]	2008	1.347 (1.104, 1.643)	1.972 (1.269, 3.063)	1.617 (1.050, 2.489)	1.768 (1.172, 2.665)	1.357 (1.029, 1.791)
Baptista et al. [20]	2008	1.045 (0.793, 1.377)	1.147 (0.640, 2.055)	1.216 (0.704, 2.100)	1.188 (0.706, 2.000)	0.990 (0.656, 1.492)
Newman et al. [21]	2009	1.542 (1.302, 1.827)	2.665 (1.805, 3.935)	1.918 (1.307, 2.815)	2.223 (1.541, 3.207)	1.598 (1.265, 2.019)
Okazaki et al. [22]	2008	1.355 (1.054, 1.741)	1.692 (1.024, 2.798)	1.918 (1.307, 2.815)	1.294 (0.825, 2.029)	1.621 (1.122, 2.342)
Oliver et al. [23]	2007	1.515 (1.187, 1.935)	3.153 (1.761, 5.648)	2.938 (1.658, 5.207)	3.033 (1.752, 5.252)	1.347 (0.959, 1.891)

heterozygote comparison (TG versus GG: OR = 1.509, 95% CI 1.161, 1.960), and recessive model (TT versus TG/GG: OR = 1.409, 95% CI 1.279, 1.552). These data demonstrate that rs7517847 increases the risk of CD among Caucasians with hospital-based studies.

3.3. Publication Bias. Begg's funnel plot and Egger's test were both performed to assess the publication bias of this meta-analysis. The shape of the funnel plots for homozygote comparison models seemed symmetrical (Figure 3). Then, the Egger's test was used to provide statistical evidence of funnel plot symmetry. The results still did not suggest any evidence of publication bias. Thus, publication bias was not evident in present meta-analyses.

4. Discussion

CD is associated with JAK2 signaling pathway which is activated by IL-23 and IL-23R receptor [24]. Previous studies suggested that the interruption of IL-23R SNPs might lead to the dysregulation of intestinal inflammation [25]. IL-23R gene variants also play an essential role in the development of many autoimmune diseases such as ankylosing spondylitis (AS), inflammatory bowel disease (IBD), and systemic lupus erythematosus (SLE) [7, 26, 27]. Therefore, researchers are focusing on observing the relationship between IL-23R gene polymorphisms and the risk of CD. However, the results are conflicting and controversial due to the different races and insufficient sample size. After pooling data for 11 studies in this meta-analysis, our results firstly demonstrate that T allele of rs7517847 was highly susceptible to CD in Caucasians.

One previous study showed that rs7517847 is a protective factor in rheumatoid arthritis (RA) in European population. Interestingly, RA is a systemic autoinflammatory disease which is associated with PTPN22/C1858T, while the organ-specific autoimmune disease CD is not [28, 29]. Thus, the mechanism of this genetic variant may not play a common role in different autoimmune diseases. More researches are required to observe the exact mechanisms of IL-23R gene polymorphism.

We should also mention the limitations of this meta-analysis. Primarily, all the studies were limited to the Caucasian. The allelic frequencies may be different in other ethnic groups. Secondly, publication bias might occur even if there is no significance in statistical test due to extracting published studies. Ultimately, owing to methodological limitations,

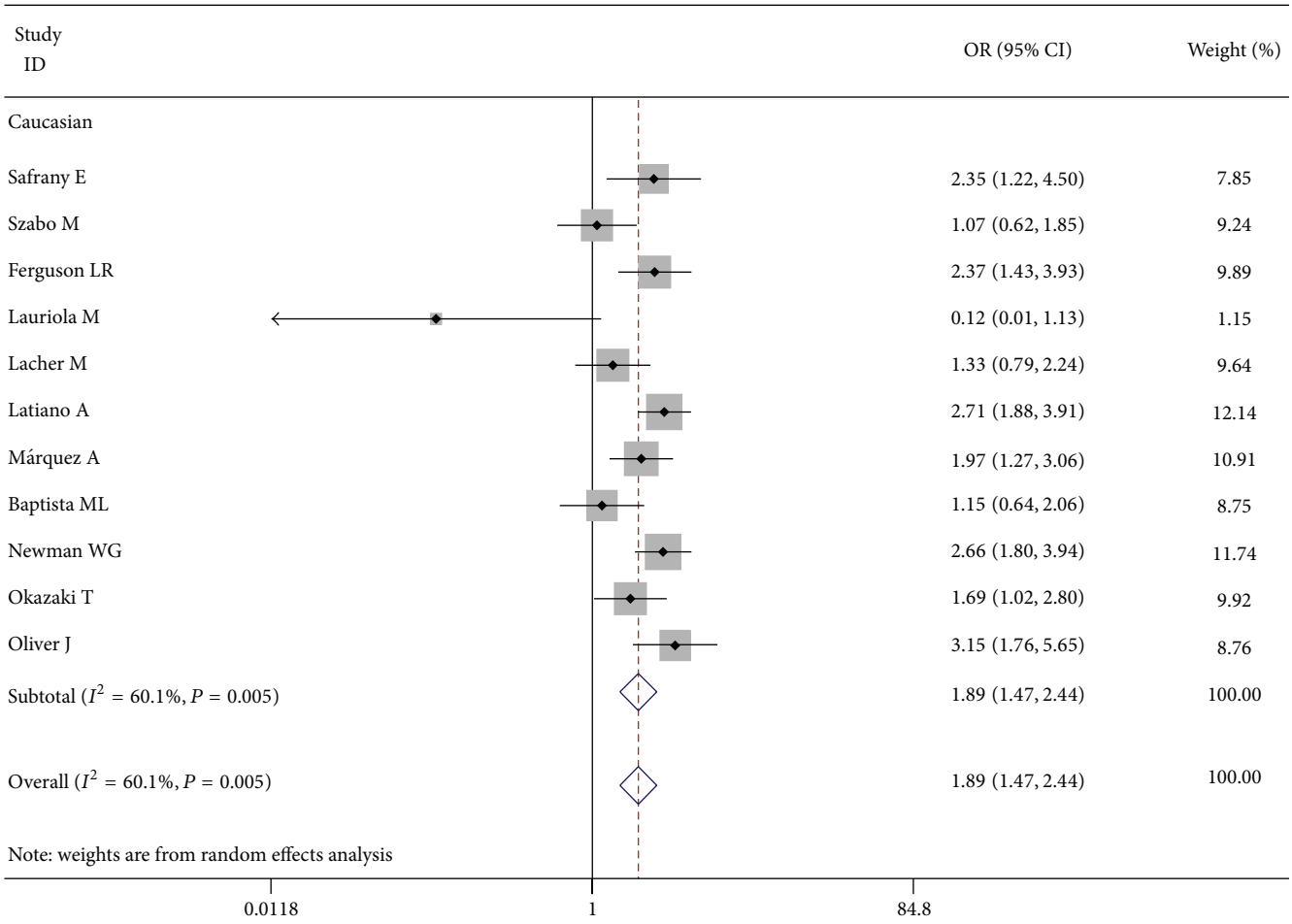

Figure 2: Forest plot for risk of CD associated with rs7517847 in Caucasian (TT versus GG. For each study, the estimate of OR and its 95% CI is plotted with a box and a horizontal line. Filled diamond pooled OR and its 95% CI).

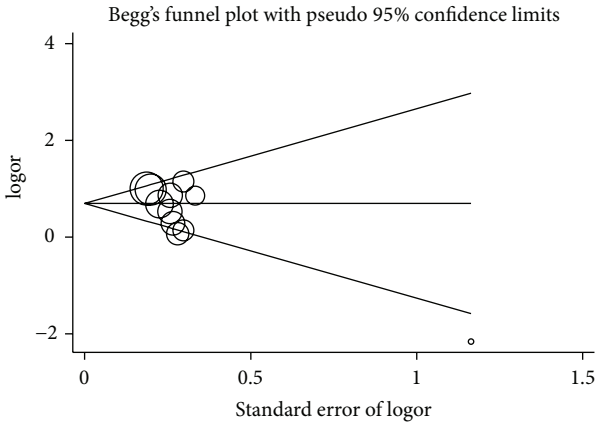

Figure 3: Begg's funnel plot for publication bias test (TT versus GG for rs7517847).

this meta-analysis is retrospective. Two independent authors performed the process of study selection and data extraction and a third author resolved the discrepancy to minimize the bias.

In conclusion, our meta-analysis suggests that IL-23R rs7517847 confers susceptibility to CD in the Caucasians. Furthermore, more studies with larger scale are required to confirm these associations.

Conflict of Interests

All the authors declare that they do not have any commercial or associative interest that represents a conflict of interests in connection with the work submitted.

Authors' Contribution

Li Zhang and Yunjie Lu contributed equally to this work and both are co-first authors.

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Review Article

Pregnancy Associated with Systemic Lupus Erythematosus: Immune Tolerance in Pregnancy and Its Deficiency in Systemic Lupus Erythematosus—An Immunological Dilemma

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Pregnancy is a physiological condition that requires immune tolerance to the product of conception. Systemic lupus erythematosus (SLE) is a disease with well-represented immune mechanisms that disturb immune tolerance. The association of pregnancy with systemic lupus erythematosus creates a particular immune environment in which the immune tolerance specific of pregnancy is required to coexist with alterations of the immune system caused by SLE. The main role is played by T regulatory (Treg) cells, which attempt to regulate and adapt the immune system of the mother to the new conditions of pregnancy. Other components of the immune system also participate to maintain maternal-fetal immune tolerance. If the immune system of pregnant women with SLE is not able to maintain maternal immune tolerance to the fetus, pregnancy complications (miscarriage, fetal hypotrophy, and preterm birth) or maternal complications (preeclampsia or activation of SLE, especially in conditions of lupus nephritis) may occur. In certain situations this can be responsible for neonatal lupus. At the same time, it must be noted that during pregnancy, the immune system is able to achieve immune tolerance while maintaining the anti-infectious immune capacity of the mother. Immunological monitoring of pregnancy during SLE, as well as of the mother's disease, is required. It is important to understand immune tolerance to grafts in transplant pathology.

1. Introduction

The association of systemic lupus erythematosus (SLE) with pregnancy represents a particular situation in immunopathology.

This is closely related to specific immune changes of the maternal body during pregnancy that ensure immune tolerance to the product of conception which presents paternal antigens and therefore represents a semiallogeneic graft for the host. In fact, pregnancy is considered "a major challenge to the maternal immune system" [1].

Important immune alterations occur in patients with SLE, including deficiencies of the immune system as well as immune tolerance.

The association of pregnancy with a modified immune system adapted to immune tolerance to fetal antigens with a disease with a strongly impaired immune system, with deficiencies concerning immune tolerance mechanisms, represents an entirely special aspect in immunopathology.

The cornerstone of the relationship between the immune system in pregnancy and the immune system in SLE is represented by T regulatory (Treg) cells. Pregnancy-related

hormonal changes such as hyperestrogenism are added to this relationship, and the immune cells are sensitive to these changes. One can also observe a relationship between the immune system and hormonal factors, mainly estrogens, among patients with SLE.

In cases of pregnancy associated with lupus erythematosus, important interrelations occur between the immune system of the mother and the immune system of the fetus. Alterations in immune mechanisms can have severe consequences both for the fetus, including a risk of miscarriage or disease transmission (neonatal lupus), and for the mother, including activation of SLE.

The aim of this paper is to present the interrelationship between the immune mechanisms in pregnancy and the immune mechanisms in SLE in cases of pregnancy associated with lupus erythematosus. Although many elements remain unknown, we consider an updated presentation useful.

2. Specifics of Immunology of Pregnancy

From an immunological point of view, pregnancy is an allograft with the following particularities.

(i) The fetus has 50% paternal antigens.

(ii) The fetus is separated from the mother by a maternal-fetal interface. Among the components of this interface, we distinguish the trophoblast, which represents a cellular layer that does not allow contact between fetal antigens and maternal antigens.

(iii) The specific hormonal environment is represented by high levels of estrogens and progesterone.

In this situation, the maternal immune system has to achieve conditions of immune tolerance, while also maintaining its anti-infectious capacity.

This characteristic of the immune system, which on the one hand ensures immune tolerance and on the other hand maintains reactivity against pathogens, demonstrates its particular adaptability.

The maternal immune system ensures antibacterial activity mainly by means of antibodies. Bacterial antigens are taken up by antigen-presenting cells. Stimulation of B cells occurs with production of antibodies. T helper cells participate as costimulatory cells.

A shift at the Th 1 and Th 2 helper cell level occurs. Th 2 cells dominate in pregnancy and also suppress the response of cytotoxic T cells. The Th 1-Th 2 shift leads to suppression of antifetal antigen-mediated immune responses.

The hormonal system participates in the suppression of cell-mediated immunity, and thus immune tolerance.

A tight cooperation for preventing a response to fetal antigens occurs between the trophoblast and the maternal immune system.

According to Mor and Cardenas, immune mechanisms in pregnancy combine a "signal of response of the maternal immune system and fetal-placental immune system." They suggest that the fetal-placental immune system could play an important mediating role for the maternal immune system [2].

3. SLE

SLE is an autoimmune disease that predominantly affects women during their reproductive years.

The intervention of hormonal factors is involved in SLE. Alterations in estrogen metabolism occur, with high levels of 16-alpha-hydroxyestrone. The estrogen receptor and its relationship with cytokines are very important [3]. Additionally, the level of androgens diminishes.

Increased estrogens influence the immune system. The proliferation of B-cells and the production of antibodies increase, influencing the proliferation of T cells. Prolactin, an immunostimulatory hormone, is also affected, as is the gonadotropin-releasing hormone, the hypothalamus-pituitary axis being defective in SLE.

Humoral immune mechanisms are impaired, with formation and deposition of immune complexes and components of the complement system in tissues.

Apoptosis leads to elimination of molecules from the nucleus, the cytoplasm, and the cell surface. The clearance of these products is deficient [4]. The products are taken up by macrophages and presented to T cells, subsequently activating them. Dendritic cells are also involved as antigen-presenting cells.

Autoantibodies are produced as a consequence of B-cell activation. The most representative are antinuclear antibodies. Anti-double-stranded DNA (ds-DNA) and anti-Sm antibodies are specific for SLE.

4. Dysregulation of the Immune Response

Immune system abnormalities involve B cells, T cells, and monocytes, with polyclonal B activation and increased autoantibody production.

In SLE, alterations of the immune system affect T regulatory cells, which seem to play an important role in the dysfunction of the immune system.

5. Pregnancy and Systemic Lupus Erythematosus

The function of the immune system as a whole is altered in SLE, which results in alteration of immune tolerance not only to self-antigens. Pregnancy is founded on tolerance of the maternal immune system to paternal antigens present in the fetus.

In cases of pregnancy associated with SLE, the main immune alteration involves the function of Treg cells [5].

In SLE, the number of Treg cells and their functions are limited. However, Treg cells have an important role in regulating the immune system and in maintaining self-tolerance. In pregnancy, immune tolerance to the fetus is mainly ensured by Treg cells. In cases of pregnancy associated with SLE, the immune system is confronted with the dilemma of how to adapt to ensure favorable development of the product of conception. How does tolerance to fetal antigens occur in the presence of a disease with altered tolerance to self-antigens?

The relationship between pregnancy and SLE is even more complex because estrogens are involved in the immunopathology of SLE. Pregnancy also presents an immune system under the influence of increased estrogen levels. Other hormonal factors, including progesterone and prolactin, are also involved.

It should be noted that numerous factors of the immune system that will be presented here play an important role in shaping the immune tolerance in cases of pregnancy associated with SLE. We specifically mention that the presence of maternal antiphospholipid antibodies can adversely affect pregnancy. Additionally, maternal anti-Ro and anti-La antibodies can cross the placental barrier and can cause neonatal lupus.

It is worth mentioning that in cases of SLE, the loss of immune tolerance to the fetus can have both fetal (miscarriage, intrauterine growth restriction (IUGR), preterm birth, and neonatal lupus) and maternal (preeclampsia and activation of SLE) consequences [6].

Our paper aims to analyze the main immune factors that participate in cases of SLE associated with pregnancy.

6. T Regulatory Cells (Treg Cells)

One of the functions of T helper cells is to modulate the immune response. Their function is regulated by T regulatory cells (Treg cells). According to Saito et al., there is a new paradigm of T cells, namely, Th 1/Th 2/Th 17, which is related to Treg cells. Treg cells, by means of immunomodulatory cytokines such as TGF-beta, can suppress the capacity of these cells to produce cytokines [7].

Two types of T regulatory cells are described; some originate at the level of the thymus (natural Treg cells), while others are induced in the periphery (induced Treg cells).

Their role is to control effector functions of immune cells such as macrophages, cytotoxic CD8 cells, and NK cells. They also have a role in regulating immune responses, and they are involved in cytokine secretion (IL 10, TGF-beta, etc.).

Treg cells bear the CD25 antigen, which is a marker of their activation [5].

Adaptive Treg cells have the following subtypes: IL 10-producing Trl cells, Th 3 cells (TGF-producing Treg cells), and $CD4^+$ $CD25^+$ Foxp3 Treg cells [8].

$CD4^+$ $CD25^+$ Treg cells suppress the potential action of autoreactive cells [9].

$CD4^+$ $CD25^+$ Treg cells express a protein, Foxp3, which is a marker of their activation [9]. Another marker of Treg cells is CTLA-4, which is associated with suppressive functions.

After stimulation and activation, Treg cells are susceptible to action. T cells originating in the thymus can intervene to protect against Th 1-mediated responses to autoantigens [10].

Treg cells accumulate in the decidua. At the same time, they are numerous in maternal blood during the first trimester.

Treg cells act at the maternal-fetal interface. At this level, they regulate immune cell responses by interacting with other cells and regulating the expression of immune regulatory molecules [11].

According to Aluvihare et al., maternal Treg cells suppress an aggressive allogeneic response directed against the fetus. Their absence could impair the continuation of pregnancy by resulting in immune rejection of the fetus [1].

Abnormalities of $CD4^+$ $CD25^+$ Foxp3 Treg cells could contribute to T- and B-cell hyperactivity in SLE [12].

The number of Treg cells increases in normal pregnancy and decreases in cases of pregnancy loss and preeclampsia [11].

The decrease in the number and functionality of Treg cells can predispose women with systemic lupus erythematosus to pregnancy complications [13].

Pregnancy associated with SLE represents a special situation for Treg cells. On the one hand, pregnancy benefits from the contribution of Treg cells, which ensure maternal-fetal tolerance. On the other hand, Treg cells are defective in SLE.

In cases of pregnancy associated with inactive SLE, Treg cells might ensure maternal-fetal tolerance because functional Treg cells predominate.

It is also possible that in cases of pregnancy associated with SLE, inactive Treg cells impair maintenance of fetal immune tolerance and result in complications such as miscarriage, preterm birth, or preeclampsia [1].

Tower suggests that women with SLE have dysfunctional tolerance capabilities that compromise their adaptability to pregnancy [11].

This is why women with SLE are dissuaded from becoming pregnant prior to remission of SLE.

7. Th 17 Cells

T helper cells are regulated by Treg cells. The Th 17 cell subset mainly produces IL 17. Other interleukins that are produced by these cells include IL 21, IL 22, and IL 17F.

Th 17 cells play a protective role against pathogenic germs and are central to the relationship between the self-antigens of inflammation and those of autoimmunity [14]. Th 17 cells have a role in inflammatory processes in autoimmune diseases [15].

Th 17 cells intervene in recurrent pregnancy loss and preeclampsia [16]. Changes in the ratio of Th 17 cells to Treg cells may be related to spontaneous abortion and premature birth [7].

Th 17 cells possess great plasticity. Cells which mainly produce IL 17A and IL 17F can turn into cells that produce interferon gamma [17].

Torricelli et al. found that pregnant women with SLE demonstrated increased levels of IL 17 together with other cytokines, including IL 6, IL 10, and TNF. This may indicate a hyperactive immune system among pregnant women with SLE, and this may be related to the placenta [18].

Estrogens play a role in Th cell secretion of IL 17, as demonstrated in mice [19]. They inhibit the secretion of Th 17 [20]. Estrogen levels are high during pregnancy, and patients with unexplained recurrent abortion have high levels of Th 17 cells in their blood and decidua [21].

Nakashima et al. found a high number of IL 17-positive cells in the decidua of abortion cases and suggested that Th

17 cells could be involved in the induction of inflammation in the late stage of abortion [22].

By promoting Th 2 responses, estrogens in pregnancy tend to worsen Th 2-mediated diseases such as SLE [23]. Torricelli showed high levels of serum IL 17 in pregnant women with SLE [18].

Th 17 and estrogens are involved in processes of osteogenesis. Th 17 cells are considered to be "a new candidate in the pathogenesis of osteoporosis." Estrogen deficiency was found to play a role in inducing the differentiation of IL 17-secreting Th 17 cells [20].

Progesterone is involved in autoimmune processes. Its action consists in the suppression of Th 17- and Th 1-related responses [24].

In pregnancy, progesterone contributes to a favorable environment for pregnancy through its favorable effects on Th 2 and Treg cells and its suppression of Th 1 and Th 17 cells [21].

The immune response to antigens such as candida albicans via Th 17 cytokines is diminished in the presence of high levels of estradiol, while progesterone associated with lower estradiol levels restore the pathogen-host equilibrium, a fact demonstrated by experimental studies in mice [25].

8. B Cells

B cells have an important role in producing antibodies. In SLE, they produce autoantibodies, which can play a pathogenic role.

According to Fettke et al., B cells participate in maternal immune tolerance to the fetus, with IL 10, a modulating cytokine produced by B cells, playing an important role [26]. These cells (B10) are a subset of B2 cells called regulatory B cells. Thus, B cells play an adaptive role. It was found that maternal lymphocytes specific for the paternal histocompatibility antigens undergo a process of partial deletion. The important role played by B cells could be reflected in the success of the fetal allograft [27].

According to Fettke et al., aberrant B cell behavior is related to obstetric pathology [26].

In SLE, B cells participate in the production of antibodies, which play an important role in the initiation, evolution, and exacerbation of the disease. This fact led to the use of rituximab in SLE therapy, the depletion of B cells with rituximab being accompanied by a decrease in autoantibody production [28].

The control of B cell proliferation depends strictly on Treg cells. In SLE, the control of autoantibody production is lost, but regulatory T cells play an important role in SLE and pregnancy [11]. Muzzio et al. consider "the role of B cells in pregnancy: the good and the bad." A balance between activation and tolerance is present. B cells participate in the normal evolution of pregnancy and in pregnancy-associated pathology [29].

Although therapeutic modulation with rituximab has proven to be effective in SLE, its use during pregnancy is not allowed.

It is possible that future risk-free immunomodulatory medications may be developed for SLE that target B cells and allow for favorable pregnancy development [30].

9. Autoantibodies

In SLE, impaired removal of apoptotic cellular material occurs due to deficient clearance. This leads to the production by B cells of antibodies against these structures, which are referred to as autoantibodies. These autoantibodies are common in SLE and vary according to the structures affected by SLE.

The most important autoantibodies in SLE are those against nuclear structures, referred to as antinuclear antibodies. They are used in the diagnosis of SLE.

Anti-double-stranded DNA antibodies are very specific for SLE. Anti-single-stranded DNA antibodies and anti-RNA antibodies can also be present in SLE.

Anti-Sm antibodies are increasingly found during renal involvement in SLE [31]. Anti-Ro and anti-La antinuclear antibodies are very important in pregnancy. They can cross the placenta and can induce fetal injury, causing neonatal lupus [32].

Some autoantibodies have organ specificity. For example, anti-N-methyl-D-aspartame (NMDA) antibodies are present in central nervous system lupus.

Autoantibodies against red blood cells (producing hemolytic anemia), as well as antiplatelet antibodies, have been described.

The diversity of antibodies in SLE can be explained by the complexity of immune mechanisms.

Pregnancy imposes immune tolerance to paternal antigens. However, SLE triggers immune reactions with cytotoxic characteristics. In this situation, the protective maternal immune mechanisms developed during pregnancy compete with the cytotoxic characteristics. The variety of autoantibodies present in SLE can also influence pregnancy outcome.

Thus, antiphospholipid antibodies can play a detrimental role in the development of pregnancy, as can the above-mentioned anti-Ro and anti-La antibodies, which can induce neonatal lupus.

However, it is recommended that women with SLE do not become pregnant during the 6-month period following an SLE flare-up, as autoantibody titers are low when the disease is inactive.

10. Antiphospholipid Antibodies

Antiphospholipid antibodies (aPLs) are associated with SLE and other autoimmune diseases. They can have fetal and maternal consequences during pregnancy.

Antiphospholipid antibodies are associated with miscarriage, fetal death, intrauterine growth restriction (IUGR), preterm birth, and preeclampsia [33], and an association with recurrent miscarriage has also been reported [34].

Antiphospholipid syndrome is frequently associated with the HELLP syndrome, an association mentioned by Le Thi Thuong et al. [35]. Placental insufficiency can occur in the presence of aPLs [36].

The importance of aPLs was shown in a study of McNeil et al., who reported fetal demise in 38–59% of pregnancies associated with SLE, compared to 16–20% of pregnancies without these antibodies [37].

Maternal and fetal complications are considered to be related at least partly with the presence of aPLs [38].

Immunoglobulin, heparin, and small doses of aspirin are used to treat the anticardiolipin syndrome [39].

11. Innate Lymphoid Cells (ILC)

A new type of cells involved in innate immunity, the ILC cells, has recently been defined. They are not homogenous, and they consist of 3 main groups. Group 1 contains NK cells, group 2 contains ILC 2 cells, and group 3 contains ILC 3 cells and a subgroup defined as lymphoid tissue inducer cells (LTi).

ILC cells play a distinct part in innate immune responses associated with the production of Th 1, Th 2, and Th 17 cytokines [40].

Group 1 is related to the intervention of NK cells in pregnancy and in SLE.

NK cells are considered to be a "founding member of the innate lymphoid cell family" [41]. Thus, NK cells intervene in maintaining homeostasis, secreting both protective and proinflammatory cytokines [42].

NK cells participate in immune regulation processes by playing a role in regulating production of antibodies that are dependent on T cells involved in autoimmune disease [43]. NK cells also participate in host defense and play an important role in infections.

NK cells have an important role both in fertility and in development of pregnancy, and they also play an important role in implantation [43].

According to Baxter and Smyth, NK cell populations are lower among patients with SLE than in controls [44].

Regarding the serum values of NK cells, Su et al. found no difference between the number of NK cells in pregnant women with and without SLE [45].

In SLE, there is a reduction in NK activity, which decreases markedly in severe cases and in cases of lupus nephritis [44].

There are higher concentrations of decidual NK cells in complicated pregnancies and in cases of recurrent miscarriage; treatment with glucocorticoids is currently being discussed [46].

Decidual cells, which are located at the fetal-maternal interface, have the capacity to produce cytokines, including IL 1B and TGF. They can participate in autoimmune processes at this level via these cytokines [47]. During pregnancy, NK cells are in close contact with the fetal interface. NK cells in the decidua have limited activity compared to NK cells in the trophoblast and in the blood [48].

NK cells are found at decidual level and around spiral arteries. They play a role in modulating trophoblast invasion and vascular remodeling, and they are interrelated with cells at the level of the decidua. This relationship is impaired in preeclampsia. In fact, it is generally known that uterine NK cells intervene in the development of normal pregnancy [49].

Wallace et al. noticed that NK cell receptor expression is altered in pregnancies at higher risk of preeclampsia [50].

According to Pereira et al., while the innate immune system is active in pregnancy, there is suppression of adaptive immunity [43].

NK cells can also regulate the target cells by apoptosis.

Total apoptosis in SLE is low. NK cells have a prolonged life cycle, which could represent a higher immune stimulus for activating apoptotic processes [43].

Group 2, consists of Th 2-type innate lymphocytes that produce the following type 2 cytokines: IL 5 and IL 13 [51].

Group 2 cells are found in the human respiratory and gastrointestinal tract, in the skin and in the spleen. ILC 2 cells intervene in antiparasite immunity, allergy, asthma, and atopic dermatitis.

Classic NK cells and Th 2 type ILC cells intervene in the production of Th 1, Th 2, and Th 17 cytokines [40].

There are no studies on the intervention of ILC 2 in pregnancy and SLE.

Group 3 (innate lymphoid cells 3/ILC3) are present at the level of the mucosa, at the level of Peyer's patches and gut-associated lymphoid tissue (GALT). Small quantities are found in the spleen and in the lungs.

A subset of ILC 3 cells is represented by lymphoid tissue inducer (LTi cells), which produce IL 17 and IL 22. The profile of LTi cells is similar to that of Th17 cells.

These cells are involved in the development of lymphoid nodes and Peyer's patches, which are programmed during the fetal period [52].

They ensure the relationship between ILC and NK cells, facilitating via a lymphotoxin NK cell development.

Until recently, only NK cells have been identified and functionally characterized at the level of the human decidua. However, in 2014, Vacca et al. identified two subsets of the ILC 3 decidual group. One of these subsets produces IL 17 and TNF, and the other produces IL 2a and IL 8 [53].

They could contribute to innate defense and tissue building, thus playing a part in the continuation of pregnancy.

ILC cells require further study to elucidate their relationship with the immune system in pregnancy.

12. Gamma/Delta Cells

Numerous gamma/delta cells are located at the level of the maternal-fetal interface [54]. According to Szekeres-Bartho et al., HLA G presents antigens to gamma/delta cells. At the same time, gamma/delta cells can recognize unprocessed foreign antigens without the MHC [55].

The response of lymphocytes that bear gamma/delta receptors is related to progesterone, being considered as progesterone-dependent immunomodulation [56].

According to Su et al., gamma/delta cells play a role in immune responses being involved in autoimmune diseases [57].

Fujii et al. identified in lupus-prone mice a gamma/delta cells line that suppresses autoantibody synthesis [58].

13. Monocytes/Macrophages

During pregnancy, peripheral monocytes undergo adaptive phenomena. They consist of decreased expression of chemokine receptors, as well as alteration of the response to microbial stimuli. The trophoblast cells secrete chemokines and recruit monocytes [59].

Björkander et al. suggest that the expression of monocyte chemokine receptors creates a unique chemokine milieu during pregnancy. In SLE, there is increased expression of CCR5 on $CD16^{+}$ monocytes [59].

Decidual macrophages express inhibitory receptors that can bind to HLA-G, being expressed on invading extravillous trophoblasts.

Afterwards, transmission of a negative signal can block their action resulting in immune tolerance and induction of anti-inflammatory cytokines [60].

14. Dendritic Cells

Palucka et al. reported alterations in immune cell homeostasis that involve dendritic cells. SLE is considered to present continuous activation of myeloid dendritic cells produced by plasmacytoid dendritic cells via alpha interferon [61].

Dendritic cells are involved in controlling immunity and immune tolerance, and dendritic cells play an important role at the level of the maternal-fetal interface [62].

In SLE, maturation of myeloid dendritic cells secondary to deficient apoptosis will cause induction of Th 17 cells with production of IL 17 [63].

During pregnancy, myeloid dendritic cells induce differentiation of Th 1 cells, and plasmacytoid cells induce differentiation of Th 2 cells. T regulatory cells have suppressive activity.

In SLE, the ratio of Treg cells to dendritic cells maintains maternal tolerance. This alteration could be the basis of pregnancy complications associated with SLE [64].

15. Estrogens and Progesterone in the Immune Responses of Pregnancy and SLE

Estrogens are related to the immune system. Immune cells possess estrogen receptors. According to Tanriverdi et al., they are expressed in primary lymphoid organs and peripheral immune cells, and they can also play an active role in immune diseases [65]. Estrogens are considered to play an immunomodulatory role [66].

SLE is under the influence of estrogens. SLE predominantly affects women during their reproductive years, and there can be an increase in some active estrogen metabolites in this disease. During pregnancy, high levels of estrogens can be related to the Th 1/Th 2 ratio, which alters in favor of Th2. In cases of associations with SLE, there are consequences for the evolution of pregnancy. In SLE, estradiol levels decrease during the third trimester of pregnancy secondary to placental impairment. As a consequence of this process, the immune response, as well as SLE activity, decreases [67].

Iaccarino et al. noted that polarization towards Th 2 of the immune response during pregnancy and SLE is markedly less than in normal pregnancies [68].

It should also be noted that 17-beta-estradiol influences the production of IgG anti-double-stranded-DNA antibodies [69].

There is also hormonal regulation of B cell function in SLE [70].

16. Progesterone

In the second part of gestation, progesterone inhibits Th1-type cytokine production and induces production of Th2 cytokines and IL 10, which stimulate the humoral immune response.

According to Zen et al., high progesterone levels at the fetal-maternal interface level could play a role in the favorable development of pregnancy [71].

Hughes and Choubey suggest a relationship between estrogen and progesterone in SLE, in which the balance between the two determines disease expression [72].

Other hormones, such as prolactin and gonadotropin, also play a role in modulating immune responses.

17. The Maternal-Fetal Interface

During pregnancy, there is a special relationship between the mother and the fetus, as the fetus contains 50% paternal antigens.

This requires a barrier between the mother and the fetus to separate the fetal cells, which contain paternal antigens that are alien to the mother, from her immune system.

The relationship between the mother and the fetus is ensured via the placenta, which offers the nutritive substances needed by the fetus. One of the placental layers consists of the trophoblast, which separates the immune system of the mother from the fetal antigens and thus allows the development of the fetus. The maternal-fetal interface involves both the anatomic barrier formed by the trophoblast and the local immune changes that play a role in maintaining the fetal allograft.

According to Du et al., there is permanent cross-talk between the mother and the fetus at this interface [73]. Immune cells are present at the maternal-fetal interface, and there are several types of chemokines with the following functions:

(i) selective leukocyte traffic at placental level,

(ii) trophoblast invasion,

(iii) placental angiogenesis,

(iv) recruitment and instruction of immune cells that ensure preservation of an environment that is favorable to pregnancy [73].

Numerous immune cells are found at the level of the maternal-fetal interface, with Treg cells playing a leading role [5].

Chorionic gonadotropin produced by the trophoblast also plays a role.

As far as histocompatibility antigens are concerned, as opposed to the HLA Class I molecules which are present on

most somatic cells and present peptide antigens to T cells, nonclassical HLA G and HLA E are expressed. The main HLA molecule with strong expression in pregnancy is HLA G. It is expressed only at placental level. HLA G could be essential in maintaining immune tolerance to fetal antigens [74].

Immune tolerance is achieved by the activity of HLA molecules that act as ligands for receptors of NK cells and of macrophages [74].

Placental changes in SLE can adversely affect fetal development. Antiphospholipid antibodies can alter the placental phospholipid membrane. This happens mainly when antiphospholipid antibodies or other procoagulant conditions are present [75].

Vascular impairment of the placenta is evidenced by fibrin deposits caused by impairment of coagulation processes in which procoagulant factors also participate. One can detect coagulant changes related to the antiphospholipid syndrome, consisting of vascular changes or coagulation abnormalities. These can lead to villous thrombosis. Infarctions and edema/swelling can also be observed at placental level [76].

The participation of immune processes in the production of placental injury during pregnancy and SLE is documented by the presence immunoglobulins IgG, IgA, IgM, and C_3 complement deposits. DNA-anti-DNA deposits can also be detected in pregnancy and SLE. The volume of the placenta decreases along with the number and size of the villi. Placental insufficiency can also be observed [77].

18. Complications of SLE During Pregnancy

Complications can involve the fetus, the mother, or sometimes both. Both maternal and fetal complications associated with SLE are related to the participation of immune mechanisms.

Pregnancy is considered an allograft. At the moment in which immune mechanisms no longer ensure the mother's tolerance to the product of conception, miscarriage or premature delivery occurs.

Among women with SLE, fetal loss occurs in up to 50% of pregnancies, though the incidence is lower at present.

Normal pregnancies are associated with functional placentas. In SLE, placental insufficiency is common. As a consequence, insufficient development of pregnancy occurs, which may result in IUGR, fetal death, or stillbirth [78].

Antiphospholipid antibodies are among the factors involved in fetal complications, and they are frequently associated with fetal loss. They could be the main cause of spontaneous miscarriage and recurrent miscarriage [79].

Anti-DNA antibodies cross react with laminin, which participates in placental implantation. Anti-DNA antibodies inhibit trophoblast attachment and migration, with implications in recurrent pregnancy loss in SLE [80].

Thrombocytopenia could also be involved in pregnancy loss [81].

The risk of fetal loss is high in pregnancies associated with active SLE and with lupus nephritis.

The role of the T helper cells should be mentioned. Th 1-mediated response via the intervention of proinflammatory cytokines could participate in producing miscarriage. They could counteract the anti-inflammatory cytokines produced by Th 2, Th 17, and Treg cells [82].

19. Neonatal Lupus

Children born to mothers with antibodies against Ro/SSA and La/SSB can present skin, cardiac, and systemic abnormalities, which are classified as neonatal lupus erythematosus [83].

These autoantibodies can cross the placenta to cause lesions in the fetus [84].

The main fetal injuries produced by these autoantibodies involve the heart, producing congenital heart block.

These lesions could originate in the activity of anti-Ro/SSA and anti-La/SSB autoantibodies, which act on calcium channels related to their regulatory proteins. The conduction abnormalities and the inhibition of L-type calcium channels have been highlighted by experimental studies in the rat heart model [85].

According to Wisuthsarewong et al., injuries produced by maternal autoantibodies can impair atrial fetal cells where inflammatory lesions appear with subsequent fibrosis and scarring of the atrioventricular node, the sinus node, and the His fascicle [86].

Anti-U_1 RNP autoantibodies can be involved in the production of skin lesions. In these situations, skin lesions are not associated with cardiac ones.

Anti-U_1 RNP autoantibodies, according to Okawa-Takatsuji et al., can be the basis for endothelial cell-binding activity in patients with connective tissue disease [87].

Shahian et al. found anti-Ro/SSA and anti-La/SSB autoantibodies in 98% of mothers who had given birth to children with neonatal SLE; however, only 1%-2% of mothers with these antibodies have neonates with neonatal lupus [88].

Meyer also reports that congenital heart block is rare and is present in only 1% of neonates of pregnant women with anti-Ro/SSA or anti-La/SSB autoantibodies [89].

In general, skin, liver, and blood (thrombocytopenia) complications tend to regress spontaneously after 4–6 months [90].

20. Maternal Complications

20.1. Preeclampsia. It is one of the complications occurring in SLE that can worsen the disease. Preeclampsia occurs most frequently in cases of lupus nephritis in the presence of anticardiolipin antibodies, thrombocytopenia, diabetes mellitus, and so forth. Preeclampsia is also observed in pregnant women with a history of preeclampsia in previous pregnancies [91].

In preeclampsia, there is a generalized inflammatory reaction related to the secretion of inflammatory factors by the placenta, which results in activation of neutrophils, monocytes, and endothelial cells [60].

SLE is also a complex inflammatory condition. Immune changes are found in preeclampsia, the most important being the Th 1–Th 17/Th 2-Treg imbalance (Perez-Sepulveda et al). In SLE, there is an important alteration of the relationship between Th 1 and Th 2 cells [92].

Lupus nephritis presents a special condition in pathology when associated with pregnancy. In lupus nephritis, immune mechanisms mediated by immune complexes, mostly composed of anti-DNA antibodies, and elements of the complement system participate. Cellular immunity with alteration of Treg cell functions also plays an important role.

In cases of active disease, the newly formed complexes can lead to activation of lupus nephritis. This is why pregnancy is recommended only after lupus nephritis has been inactive for 6 months.

Active lupus nephritis is frequently associated with fetal complications of IUGR, preterm birth, and fetal death, as well as with maternal complications such as preeclampsia.

According to Fatemi et al., the absence of lupus nephritis is important to prevent SLE activation [93].

20.2. SLE Flares. SLE may develop before or during pregnancy. Pregnancy that is superimposed on active SLE can worsen the disease. According to Ruiz-Irastorza et al., flares are more frequent during the second and third trimester and during the postpartum period [94].

Women with SLE are counselled to avoid pregnancy for 6 months from the last flare-up. Pregnancy can activate SLE, and it may progress to severe disease either during pregnancy or in the postpartum period.

Some clinical symptoms of pregnancy can be mistaken for activation of SLE, but the presence of anti-DNA, anti-Sm, anti-Ro, and anti-La antibodies indicates activation of disease, as is a low C3 level. SLE activation during pregnancy can be difficult to differentiate from preeclampsia, as both present common symptoms such as hypertension, proteinuria, and edema.

SLE activation during pregnancy can be accompanied by fetal or maternal complications that are related to the loss of immune tolerance to the product of conception. Changes in cellular immunity, mainly in Treg cells, and activation of other immune mechanisms play an important role.

21. Conclusions

In pregnancy associated with SLE, immune tolerance to the semiallogeneic graft (the fetus) has to be operational in conditions of a disease with disturbed tolerance to self-antigens (SLE).

Important innate and adaptive immune changes occur. The cornerstone of these changes is represented by Treg cells.

The association of pregnancy with SLE represents a real dilemma in which the human body can either favor pregnancy or result in fetal complications (miscarriage, IUGR, preterm birth, and neonatal lupus) or maternal complications (preeclampsia; activation of SLE, mainly of lupus nephritis).

The encounter in clinical practice of pregnancy which requires immune tolerance to paternal antigens and SLE, a disease with altered tolerance to self-antigens, represents a special situation that has to be known, as it can prove useful for understanding immune tolerance mechanisms important in transplant pathology.

Conflict of Interests

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

Authors' Contribution

Cristina Gluhovschi and Gheorghe Gluhovschi contributed equally to this paper.

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Research Article

Distribution of Peripheral Lymphocyte Populations in Primary Sjögren's Syndrome Patients

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Purpose of this study was to evaluate the lymphocyte populations' distribution changes in peripheral blood of patients with primary Sjögren's syndrome (pSS). Lymphocyte populations' distribution changes in peripheral blood of pSS patients were investigated in 52 patients with pSS and in 28 healthy controls by flow cytometry. We found decreased absolute count of $CD3^+$ T cell population in pSS patients. Analysis of $CD4^+$ T cell population showed significant proportion and absolute count differences in pSS patient's blood with SSA/SSB antibodies (Abs) in comparison to controls. No significant differences were observed analyzing $CD4^+$ and $CD8^+$ Treg subpopulation. Proportion and absolute counts of Th17 cells were significantly lower in pSS patient's blood. Absolute counts of $CD8^+$ T cells were significantly lower in pSS patients in comparison to controls and also impaired proportion and absolute counts of $CD8^+$ subpopulations according to $CD27^+$ and $CD57^+$ were observed. Absolute counts of NKT and NK cells were decreased in pSS with Abs. B cells proportion was increased only in blood of pSS with Abs. Lymphocyte distribution impairment can be due to genetically determined lymphopenia or lymphocyte migration from periphery to inflammatory sites or/and increased susceptibility to apoptosis.

1. Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disorder that affects secretory organs and is characterized by ocular and mouth dryness, fatigue, and pain, as well as extra-glandular manifestations that reveal the severity of this disorder [1, 2]. Patients with pSS also present broad spectrum analytical features (cytopenias, hypergammaglobulinemia, and cryoglobulins). Biological signatures of the disease are B-lymphocyte activation, which could be triggered by the dysregulation of B-cell activating factor (BAFF) [1]. It is suggested that BAFF is influential in driving antibody production in autoimmune diseases [2]. One of the objective classification criteria for pSS is serum SSA/SSB antibodies (Abs) [2]. Recent research studies suggest that these antibodies may also be the biomarkers of disease activity [3]. Some studies indicate that anti-SSA/SSB seropositive patients have the increased amount of B-cell activation markers, such as BAFF, free immunoglobulin light chain, beta-2 microglobulin, and IgG [3–7]. Thus, the spectrum of the disease ranges widely from minimal local symptoms of the eyes and oral mucosa to systemic involvement and development of malignant lymphoma; the latter are being the most worrisome complication of pSS [2]. Pathophysiology of Sjögren's syndrome is not yet fully understood. Recently, much attention has been focused on the relationship between innate responses and subsequent activation of specific adaptive-immunity in an attempt to understand subsequent immune dysregulation

Table 1: Clinical and serological characteristics of pSS patients.

Features	[1]pSS Abs$^-$ ($n = 29$)	[2]pSS Abs$^+$ ($n = 23$)	P^* I-II
Age, mean ± SD years	56 ± 12	56 ± 13	0.873
Schirmer's *I* test (mm/5 min), mean ± SD	3.17 ± 1.20	1.25 ± 1.12	<0.0001
Unstimulated salivary flow (mL/15 min), mean ± SD	1.22 ± 0.20	0.83 ± 0.39	0.002
Biopsy focus score (number of lymphocytic foci/4 mm^2), mean (min/max)	1.17 (1/2)	2.40 (1/3)	<0.0001
Anti-SSA$^+$, *n* (%)	0 (0)	6 (26)	
Anti-SSB$^+$, *n* (%)	0 (0)	2 (9)	
BAFF, mean ± SD, ng/mL	4230 (1748)	5014 (2518)	0.187
Anti-SSA/SSB$^+$, *n* (%)	0 (0)	15 (65)	
ESSPRI, mean (min/max)	8.39 (6/10)	9.1 (7/10)	0.075
ESSDAI, mean (min/max)	24.83 (21/30)	33.50 (27/39)	<0.0001

*Mann-Whitney test. [1]pSS Abs$^-$: pSS patients without anti-SSA/SSB; [2]pSS Abs$^+$: pSS patients with anti-SSA and/or anti-SSB. ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's syndrome patient reported index.

[8–10]. Specific cytotoxic lymphocyte populations can lead to the formation of autoimmune diseases, whereas suppressive/regulatory cell populations may lead to suppression of autoimmunity and disease remission [11, 12]. However, the pathological role of T cells in pSS remains to be elucidated.

The aim of the study was to perform a detailed quantitative analysis of peripheral blood CD4$^+$ and CD8$^+$ T lymphocyte subpopulations in patients with Sjögren's syndrome with special emphasis on Treg, Th17, NKT lymphocytes, NK cells, and B cells and expression of CD57 and CD27 markers on CD8^{high} lymphocytes.

2. Patients and Methods

2.1. Patients. In total, 52 patients with pSS and 28 healthy controls were recruited at the State Research Institute Center for Innovative Medicine for this study. Patients with pSS were grouped in two groups: pSS Abs$^-$ group, 29 without anti-SSA and/or anti-SSB Abs, and pSS Abs$^+$ group, 23 patients with anti-SSA and/or anti-SSB Abs. The average age of the patients groups and healthy controls was accordingly: 57 ± 13 years, 56 ± 13 years, and 53 ± 11 years. The majority of enrolled patients in our study were Lithuanian women. Only 1 Lithuanian man (1 in pSS Abs$^+$ group) was enrolled in pSS group. Nobody of the control group had connective tissue diseases, anti-SSA or anti-SSB Abs. Primary SS was diagnosed according to American-European Consensus Group Classification criteria for Sjögren's syndrome [2]. All patients underwent serologic evaluations, which included test for the presence of antibodies against SSA and SSB, Schirmer's *I* test, unstimulated whole salivary flow test, and histology of minor salivary glands. Disease activity was assessed using EULAR Sjögren's syndrome disease activity index (ESSDAI) [13] and EULAR Sjögren's syndrome patient reported index (ESSPRI) [14]. The characteristics of the pSS patients included in the study are summarized in Table 1. Informed and written consent was obtained from all participants of this study. The study has been approved by the Lithuanian Bioethics Committee (no. 158200-03-299-73).

2.2. Lymphocyte Populations' Proportion and Absolute Counts Determination in Peripheral Blood. Blood samples were collected from heparinized venous blood. Absolute counts of white blood cells (WBC) were determined with a haemocytometer and used for calculation of absolute numbers of lymphocyte populations (numbers of cells/μL peripheral blood). For cell surface staining, the following mAbs were used: anti-CD3 FITC (Exbio, Czech); anti-CD4 PerCP (BD, USA); anti-CD8-PerCP (BD); anti-CD-16+56-PE (Exbio, Czech); anti-CD-19 PerCP (Exbio, Czech); anti-CD57 FITC (BD, USA); and anti-CD27-APC (Exbio, Czech). For isotype controls staining, mouse anti-IgG1-FITC (BD, USA), IgG1-PerCP (BD, USA), and IgG2a-PE (BD, USA) were used. Staining was performed at room temperature for 30 min. Cell staining was followed by red blood cell lysis using Pharm Lyse (BD, USA) lysing solution for 15 min at room temperature in the dark. Leukocytes were then centrifuged (500 g for 10 min) and washed two times with CellWash (BD, USA) and resuspended in FBS (BD, USA). Samples were examined immediately after staining without fixation.

For the evaluation of intracellular cytokine IL-17A of CD4$^+$ T cells, 1 mL of whole heparinized blood was diluted 1 : 2 in RPMI-1640 supplemented with 80 mg/L gentamycin and 2 nM glutamine. Cells were stimulated using 50 ng/mL phorbol-myristate-acetate (Sigma Aldrich, St. Louis, MO, USA) and 1 ng/mL ionomycin (Sigma Aldrich) in the presence of 0.7 μL/mL monensin (GolgiStop (BD, USA)) for 4.5 h at 37°C in an atmosphere containing 5% CO_2. Unstimulated cells served as controls. Following stimulation, cells were stained for CD4 for 30 min at room temperature. Cell staining was followed by red blood cell lysis using Pharm Lyse (BD, USA) lysing solution for 15 min at room temperature in the dark. Leucocytes were then centrifuged (at 500 g for 10 min) and washed two times with CellWash (BD). Then, cells were fixed and permeabilized with Cytofix/Cytoperm (BD, USA) solution for 20 min, washed two times with Perm/Wash (BD, USA) solution, and incubated further for 30 min in the dark with the specific mAbs anti-IL17A APC (BD, USA) and isotypic control anti-IgG1 APC (BD, USA). Following the

incubation with mAbs, the cells were washed two times with Perm/Wash solution and resuspended in FBS (BD, USA).

For the evaluation of intracellular FoxP3 marker of $CD4^+$ and $CD8^+$ T cells, cells were stained with anti-CD4 PerCP (BD, USA), anti-CD25 FITC (Exbio, Czech), and anti-CD8-PerCP (BD, USA) for 30 min at room temperature. Cell staining was followed by red blood cell lysis using Pharm Lyse (BD, USA) solution for 15 min at room temperature in the dark. Leucocytes were then centrifuged (at 500 g for 10 min) and washed two times with CellWash (BD, USA). Then, cells were fixed and permeabilized with Cytofix/Cytoperm (BD, USA) solution for 20 min, washed two times with Perm/Wash (BD, USA) solution, and incubated further for 30 min in the dark with the specific mAbs anti-FoxP3-PE (BD, USA) and isotypic controls anti-IgG1-FITC (BD, USA), IgG1-PerCP (BD, USA), and IgG2a-PE (BD, USA). Following the incubation with mAbs, the cells were washed two times with Perm/Wash (BD, USA) solution and resuspended in FBS (BD, USA). Lymphocytes were gated and separated based on their morphological properties.

Flow cytometry was performed on FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) calibrated with CaliBRITE beads (BD Biosciences, San Jose, CA, USA) using CELL-Quest software (BD Biosciences, San Jose, CA, USA). Data for each sample were acquired until 100,000 leukocytes were analyzed.

2.3. Determination of BAFF Level in Serum. Serum samples were analyzed using commercial BAFF, Soluble (human) ELISA Kit (hypersensitive) (AdipoGen, Switzerland). From collected blood samples, serum was separated and stored at −80°C until analysis. Serum dilutions and enzyme-linked immunoassay was carried out in the strict accordance with the manufacturer's instructions and sets recommendations. The results were evaluated by spectrophotometer (BioTek Instruments, USA). The concentrations of analytes in ELISA assays were quantified using standard curves. A regression analysis was performed to derive an equation that was then used to predict the concentration of the unknown samples with Gen5 Microplate Data Collection & Analysis Software (BioTek Instruments, USA). The results are shown in Table 1.

2.4. Statistics. Statistical differences were analyzed using the Mann-Whitney U test. Correlations were assessed by the Spearman's rank test using standard program GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). P values less than 0.05 were considered significant.

3. Results

3.1. Main Lymphocyte Populations. We found that absolute count of $CD3^+$ T cell population was significantly decreased in pSS patients in comparison to healthy controls. No differences were observed between pSS groups. Significant decrease of $CD3^+$ cells was found in pSS Abs^- ($P = 0.027$) and pSS Abs^+ ($P = 0.0002$) groups when compared to controls; however, no differences in the $CD3^+$ cells proportion of WBC were found between pSS groups. Analysis of $CD4^+$ T cell population showed significant proportion differences in pSS Abs^+ patient's blood when compared to pSS Abs^- ($P = 0.036$) and control group ($P = 0.0036$). But absolute counts of $CD4^+$ cells were significantly lower in both pSS groups than in control group, pSS Abs^- ($P = 0.015$) and pSS Abs^+ ($P < 0.0001$), and also absolute counts of $CD4^+$ cells were significantly lower in pSS Abs^+ than in pSS Abs^- ($P = 0.01$) patients' blood. No significant differences in the proportion of $CD8^+$ T cell population were found between pSS patients and controls. But analysis of absolute counts showed significantly lower counts of $CD8^+$ T cells in pSS Abs^- ($P = 0.014$) and pSS Abs^+ ($P = 0.006$) in comparison to controls; no differences were found between pSS groups (Table 2, Figure 1). Analysis of NKT ($CD3^+CD16/56^+$) and NK ($CD3^-CD16/56^+$) cells showed only decreased absolute counts in pSS Abs^+ group (resp., $P = 0.009$ and $P = 0.036$) in comparison to controls. Increased proportion of $CD3^-CD19^+$ (B cells) in pSS Abs^+ ($P = 0.045$) was found when compared to controls, but no differences were found analyzing absolute counts among pSS groups and controls (Table 2).

3.2. $CD4^+$ Lymphocyte Subpopulations. Analysis of $CD4^+$ lymphocyte subpopulations according to their expression of CD25 and FoxP3 markers showed significant reduced absolute counts of $CD4^+CD25^+$ ($P = 0.036$) and $CD4^+CD25^{low}$FoxP3 ($P = 0.017$) cells when comparing pSS Abs^+ with controls. No significant differences were observed analyzing other $CD4^+$ subpopulations according to their expression of CD25 and FoxP3 markers (Table 3). Analysis of $CD4^+$IL-$17A^+$ (Th17) cells showed significant lower proportion and absolute counts of these cells in pSS patients in comparison to control group's results (Figure 2). Proportion was significantly altered in pSS Abs^- ($P = 0.0003$) and pSS Abs^+ ($P = 0.004$), and also absolute counts of Th17 cell were significantly lower in pSS Abs^- ($P < 0.0001$) and pSS Abs^+ ($P < 0.0001$) patients' blood in comparison to controls. No significant differences were found analyzing proportion and absolute counts of this subpopulation between pSS groups (Table 3, Figure 1).

3.3. $CD8^+$ Lymphocyte Subpopulations. $CD8^{high}$ lymphocyte population was differentiated to subpopulations according to the markers CD57 and CD27 that defines replicative senescence (Figure 1). Analysis of $CD8^{high}CD57^+CD27^-$ subpopulation showed no differences in proportion and absolute counts among pSS and control groups. In pSS patients with Abs, we observed significantly increased proportion ($P = 0.032$) and absolute counts ($P = 0.011$) of $CD8^{high}CD57^+CD27^+$ subpopulation in comparison to pSS without Abs. No significant differences were found when comparing results of pSS Abs^+ and pSS Abs^- to controls. $CD8^{high}CD57^-CD27^+$ population's proportion was significantly reduced only in pSS without Abs patients' blood ($P = 0.026$) when compared to controls and no significant differences between pSS groups were observed. Absolute counts of this subpopulation were significantly reduced both pSS Abs^- ($P = 0.0003$) and pSS Abs^+ ($P = 0.005$) in comparison to controls. Proportion of $CD8^{high}$ subpopulation lacking CD57

Table 2: Distribution of lymphocyte populations in peripheral blood of pSS patients and controls.

Patients	[1]I-controls	[2]II-pSS Abs$^-$	[3]III-pSS Abs$^+$	P^* I-II	P^* I-III	P^* II-III
Main lymphocyte populations						
CD3$^+$ T cells						
%[4]	75.36 ± 6.93	72.16 ± 9.81	73.03 ± 8.47	NS	NS	NS
A. count[#]	2530 ± 811.0	2154 ± 1133	1868 ± 1052	**0.027**	**0.0002**	NS
CD4$^+$ T cells						
%[4]	49.33 ± 9.12	46.20 ± 8.49	39.37 ± 12.39	NS	**0.0036**	**0.036**
A. count[#]	1688 ± 654.9	1391 ± 804.3	1089 ± 975.4	**0.015**	**<0.0001**	**0.01**
CD8$^+$ T cells						
%[4]	26.73 ± 7.29	26.25 ± 8.82	29.87 ± 11.64	NS	NS	NS
A. count[#]	939 ± 326.4	760.5 ± 433.5	690.2 ± 259.6	**0.014**	**0.006**	NS
B cells						
%[4]	11.86 ± 4.43	13.82 ± 9.13	14.52 ± 5.15	NS	**0.045**	NS
A. count[#]	401.3 ± 216.5	351.2 ± 197.8	393.5 ± 294.1	NS	NS	NS
NK						
%[4]	12.19 ± 5.67	12.99 ± 6.84	12.10 ± 6.05	NS	NS	NS
A. count[#]	415.4 ± 186.7	365.7 ± 210.7	306.1 ± 201.7	NS	**0.036**	NS
NKT						
%[4]	6.79 ± 4.26	8.44 ± 8.51	6.36 ± 5.86	NS	NS	NS
A. count[#]	223.4 ± 132.0	206.7 ± 168.6	137.7 ± 127.1	NS	**0.009**	NS

[1]I group: healthy controls; [2]II group: pSS patients without anti-SSA/SSB; [3]III group: pSS patients with anti-SSA and/or anti-SSB; %[4]: proportion of all CD4$^+$ lymphocytes; A. count[#]: absolute count of cells in 1 μL of blood. *Mann-Whitney test. NS: not significant.

and CD27 markers (CD8^{high}CD57$^-$CD27$^-$) was significantly increased only in pSS Abs$^-$ patients' blood when compared to controls. Differences in proportion or absolute counts of subpopulation expressing FoxP3 marker were not observed (Table 4).

3.4. Correlation between Cell Populations' Changes and Clinical Parameters in pSS Abs$^-$ Patients. Focus score correlated with absolute counts of CD3$^+$ ($P = 0.047$, $r = 0.474$), CD4$^+$ ($P = 0.033$, $r = 0.503$), B cell ($P = 0.023$, $r = 0.532$), and CD8$^+$CD57$^-$CD27$^+$ ($P = 0.016$, $r = 0.560$) cell populations. ESSPRI correlated with NK ($P = 0.034$, $r = 0.501$). ESSDAI correlated with NK ($P = 0.043$, $r = 0.482$) and CD4$^+$CD25^{high}FoxP3 ($P = 0.047$, $r = 0.474$) cell absolute counts. In pSS patients without Abs, we found that Schirmer's *I* test correlated with proportion ($P = 0.033$, $r = 0.504$) and absolute counts ($P = 0.015$, $r = 0.562$) of Th17 cells. Serum BAFF concentration correlated with proportion of CD8$^+$ ($P = 0.032$, $r = 0.415$), NK ($P = 0.009$, $r = 0.491$), and CD8^{high}CD57$^+$CD27$^-$ ($P = 0.014$, $r = 0.469$) cell populations and negatively correlated with B cells ($P = 0.026$, $r = -0.429$), CD8^{high}CD57$^+$CD27$^+$ ($P = 0.036$, $r = -0.404$), and CD8^{high}CD57$^-$CD27$^+$ ($P = 0.009$, $r = -0.493$). Analyzing absolute counts of cells populations observed negative correlation between BAFF and CD4$^+$ ($P = 0.025$, $r = -0.430$), B cell ($P = 0.007$, $r = -0.509$), CD8^{high}CD57$^+$CD27$^+$ ($P = 0.028$, $r = -0.424$), and CD8^{high}CD57$^-$CD27$^+$ ($P = 0.017$, $r = -0.454$) cells.

3.5. Correlation between Cell Populations' Changes and Clinical Parameters in pSS Abs$^+$ Patients. Schirmer's *I* test correlated with proportion of B cells ($P = 0.038$, $r = 0.467$) and CD8^{high}CD57$^-$CD27$^+$ ($P = 0.006$, $r = 0.589$) and negatively correlated with CD3$^+$ ($P = 0.006$, $r = -0.590$) and CD8^{high}CD57$^-$CD27$^-$ ($P = 0.033$, $r = -0.479$) cells. Also correlation between Schirmer's *I* test and absolute count of CD8^{high}CD57$^-$CD27$^+$ ($P = 0.021$, $r = 0.512$) and negative correlation with CD8^{high}CD57$^-$CD27$^-$ ($P = 0.017$, $r = -0.525$) cell counts were observed. Unstimulated salivary flow rate correlated with proportion of CD8$^+$ ($P = 0.017$, $r = 0.525$) and Th17 ($P = 0.015$, $r = 0.535$) and also negatively correlated with absolute counts of CD3$^+$ ($P = 0.011$, $r = -0.555$) and CD4$^+$ ($P = 0.017$, $r = -0.526$) cells. Focus score negatively correlated with proportion and absolute counts of CD8^{high}CD57$^+$CD27$^-$ cells, respectively, ($P = 0.020$, $r = -0.517$) and ($P = 0.040$, $r = -0.462$). ESSPRI correlated with proportion of CD3$^+$ ($P = 0.015$, $r = 0.535$), CD4$^+$FoxP3 ($P = 0.044$, $r = 0.454$), and CD4$^+$CD25$^+$FoxP3 ($P = 0.041$, $r = 0.461$) cells and negatively correlated with proportion of CD4$^+$CD25$^+$ ($P = 0.026$, $r = -0.496$) and CD4$^+$CD25^{high} ($P = 0.043$, $r = -0.456$) cells. ESSDAI negatively correlated with proportion of Th17 ($P = 0.001$, $r = -0.675$) cells and also with absolute counts of CD8$^+$ ($P = 0.042$, $r = -0.459$), Th17 ($P = 0.001$, $r = -0.673$), and CD8^{high}CD57$^+$CD27$^+$ ($P = 0.028$, $r = -0.492$) cells. No correlation was observed between serum BAFF concentration and cell populations changes in pSS patients with Abs.

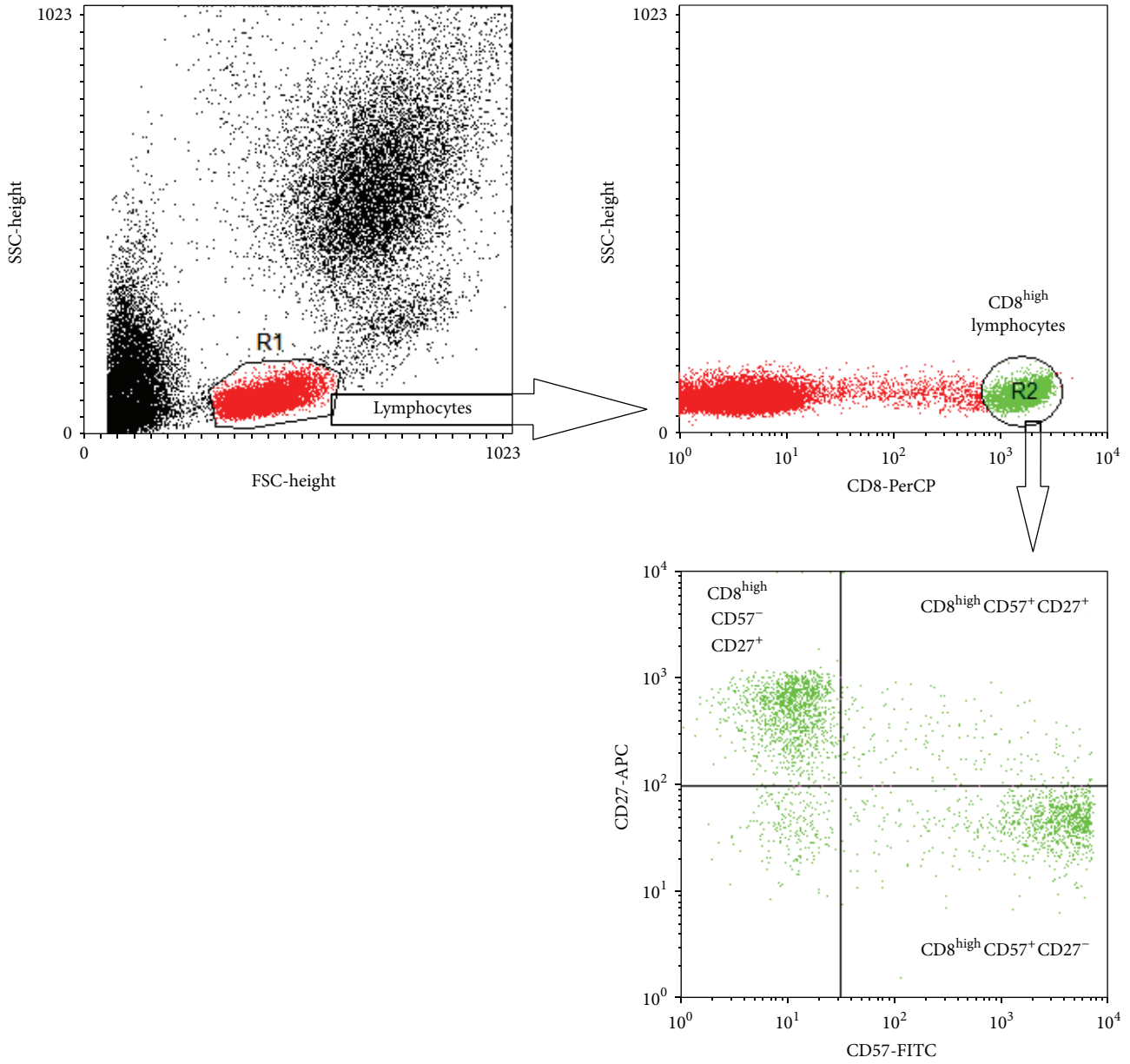

Figure 1: Representative dot plots. $CD8^{high}$ lymphocytes in the $CD8^+$ subset were determined in the flow cytometric SSC/CD8-PerCP dot plot and percentages of $CD8^{high}CD57^-CD27^+$ lymphocytes were determined in gated $CD8^{high}$ subset in the flow cytometric CD57-FITC/CD27-APC dot plot.

4. Discussion

Despite systemic B-cell hyperactivity, T and B cells constitute the vast majority of infiltrating mononuclear cells at the minor salivary glands inflammatory lesions of pSS, with their prevalence varying according to the severity of the infiltrates. The majority of these T cells are $CD4^+$ and show an activated phenotype. $CD8^+$ T cells with cytotoxic activity, as manifested by their expression of granzymes, constitute around 15% of infiltrating cells. T cells predominate in mild lesions, whereas in severe lesions B cells constitute the main population. The prevalence of $CD4^+$ T cells decreases with lesion severity, whereas the prevalence of $CD8^+$ T cells remains unchanged. The prevalence of regulatory T cells associates with lesion severity, with the higher values to be observed at intermediate lesions. NK cells comprise a small but considerable portion of the infiltrating mononuclear cells, and their percentage correlates with the grade of the lesions [15, 16].

In our study, T lymphocyte identification by CD4 and CD8 markers showed a statistically significant decrease in the absolute counts of $CD4^+$ and $CD8^+$ T lymphocytes in the peripheral blood of pSS patients in comparison to the control group. This shows that the decline of $CD3^+$ T lymphocyte population in the peripheral blood of pSS patients is influenced by a decrease of both $CD4^+$ and $CD8^+$ T lymphocyte absolute counts. The decrease of the total

Table 3: Distribution of $CD4^+$ lymphocyte subpopulations in peripheral blood of pSS patients and controls.

Patients	[1]I-controls	[2]II-pSS Abs^-	[3]III-pSS Abs^+	P^* I-II	P^* I-III	P^* II-III
$CD4^+$ lymphocyte populations						
$CD4^+CD25^+$						
%[4]	10.00 ± 10.94	9.72 ± 6.1	11.64 ± 9.38	NS	NS	NS
A. count[#]	183.6 ± 222.3	132.7 ± 106.0	106.1 ± 75.89	NS	**0.036**	NS
$CD4^+CD25^{high}$						
%[4]	1.97 ± 1.342	2.45 ± 1.71	2.71 ± 1.83	NS	NS	NS
A. count[#]	32.13 ± 25.88	31.81 ± 25.92	24.39 ± 13.50	NS	NS	NS
$CD4^+$FoxP3						
%[4]	4.56 ± 4.73	5.19 ± 6.69	5.012 ± 4.68	NS	NS	NS
A. count[#]	75.25 ± 82.62	59.45 ± 72.83	62.62 ± 105.0	NS	NS	NS
$CD4^+CD25^+$FoxP3						
%[4]	1.12 ± 2.09	1.10 ± 0.90	0.90 ± 0.63	NS	NS	NS
A. count[#]	19.12 ± 42.33	13.19 ± 10.79	10.21 ± 13.65	NS	NS	NS
$CD4^+CD25^{high}$FoxP3						
%[4]	0.27 ± 0.34	0.36 ± 0.31	0.35 ± 0.27	NS	NS	NS
A. count[#]	4.45 ± 6.9	4.039 ± 2.91	3.26 ± 2.81	NS	NS	NS
$CD4^+CD25^{low}$FoxP3						
%[4]	0.84 ± 1.75	0.78 ± 0.73	0.57 ± 0.51	NS	NS	NS
A. count[#]	14.57 ± 35.46	9.64 ± 9.612	6.71 ± 12.78	NS	**0.017**	NS
Th17						
%[4]	2.08 ± 1.31	0.85 ± 1.08	1.51 ± 2.89	**0.0003**	**0.004**	NS
A. count[#]	34.44 ± 21.02	9.12 ± 10.89	10.89 ± 22.71	<**0.0001**	<**0.0001**	NS

[1]I group: healthy controls; [2]II group: pSS patients without anti-SSA/SSB; [3]III group: pSS patients with anti-SSA and/or anti-SSB; %[4]: proportion of all $CD4^+$ lymphocytes; A. count[#]: absolute count of cells in 1 μL of blood. [*]Mann-Whitney test. NS: not significant.

amount of $CD4^+$ T lymphocytes in the peripheral blood of pSS patients is also confirmed by other authors [17]. In some pSS patients, low counts of $CD4^+$ lymphocytes or their dysfunction in peripheral blood maybe due to anti-CD4 antibodies. These autoantibodies in some pSS patients' serum were identified by Henriksson and colleagues [18]. The fact is that the proportion of $CD4^+$ was lower only in our pSS patients with Abs, and no differences in proportion of $CD3^+$ and $CD8^+$ in all pSS patients were observed; let us think that this lymphopenia can also be genetically determined. Apoptosis may also play a role in the pathogenesis of some extraglandular manifestations of pSS and peripheral $CD4^+$ lymphocytopenia [19, 20].

Th17 cells also appear to play a role in the development of pSS. Studies in patients with pSS and animal models of pSS have identified the presence of IL-17 in the lymphocytic infiltrates of the exocrine glands, as well as higher levels of circulating IL-17 in both serum and saliva [8, 21, 22]. On the one hand, our finding that Th17 lymphocyte counts decreased in the peripheral blood of patients with Sjögren's syndrome is quite unexpected. On the other hand, this result may be explained by the redistribution of Th17 lymphocytes, that is, increasing their concentration in tissues (salivary glands) and decreasing concentration in peripheral blood [8, 10]. Also, we cannot dismiss presumable apoptosis of peripheral Th17 cells.

Treg lymphocytes are characterized by autoimmune reaction-inhibiting properties [15, 23]. So they should be reduced in patients with SS [24]. However, in this study, we have not find more distinct Treg cells changes in the peripheral blood of pSS patients. No statistical significant differences were found analyzing $CD4^+CD25^{high}$FoxP3 cells, $CD4^+CD25^{high}$, or $CD4^+CD25^+$FoxP3 cells. Alike results were published by Sarigul and colleagues [25]. Sometimes it is hard to define what is what, when conflicting results have been reported. One of the problems is that different authors as Treg population define different pools of $CD4^+$ cells. Some researchers uses two markers $CD4^+$ and $CD25^{high}$ to identify Treg cells [26], while others also uses FoxP3 marker [15]. This is why we have checked more pools of $CD4^+$ that some authors define as Treg cells.

The role of cytotoxic T cells in pSS pathogenesis has not been studied in detail. Autoreactive cytotoxic T cells are seen in pSS targeting autoantigens. $CD8^+$ T-cell deficiency is a feature of many chronic autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, Sjögren's syndrome, systemic sclerosis, ulcerative colitis, Crohn's disease, psoriasis, etc. It also occurs in blood of healthy relatives of patients with autoimmune diseases, suggesting that it is genetically determined. These cells play critical roles in purging acute infections, limiting persistent infections, and conferring life-long protective immunity. $CD8^+$ T cell deficiency can prompt the development of chronic autoimmune diseases by impairing $CD8^+$ T cell control of virus infection [27]. It is known that viral infections

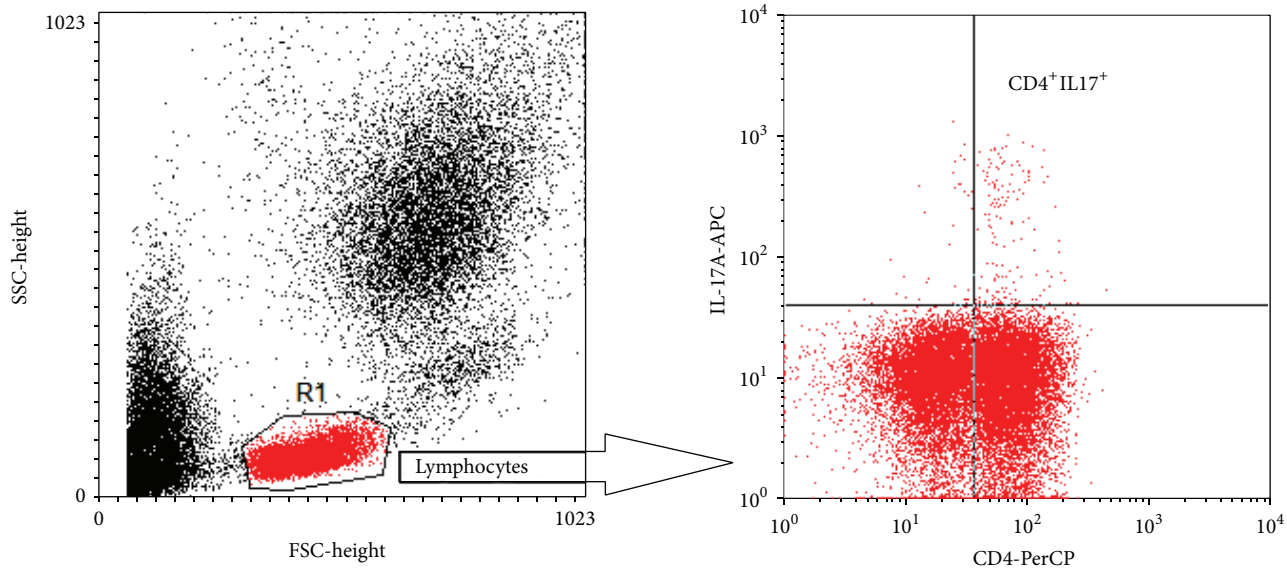

FIGURE 2: Representative dot plots. $CD4^+IL\text{-}17A^+$ positive cells were determined on CD4-PerCP versus IL-17A-APC dot plot by gating on lymphocyte on the forward-scatter versus side-scatter dot plot.

TABLE 4: Distribution of $CD8^+$ lymphocyte subpopulations in peripheral blood of pSS patients and controls.

Patients	[1]I-controls	[2]II-pSS Abs^-	[3]III-pSS Abs^+	P^* I-II	P^* I-III	P^* II-III
$CD8^+$ lymphocyte population						
$CD8^{high}CD57^+CD27^+$						
%[4]	5.306 ± 3.899	5.329 ± 5.431	8.239 ± 8.239	NS	NS	**0.032**
A. count[#]	34.26 ± 29.11	27.05 ± 33.57	42.59 ± 33.12	NS	NS	**0.011**
$CD8^{high}CD57^+CD27^-$						
%[4]	22.67 ± 13.33	25.52 ± 17.42	27.40 ± 19.55	NS	NS	NS
A. count[#]	150.6 ± 119.7	143.4 ± 157.7	155.2 ± 136.8	NS	NS	NS
$CD8^{high}CD57^-CD27^+$						
%[4]	59.13 ± 17.54	46.51 ± 22.00	47.16 ± 22.29	**0.026**	NS	NS
A. count[#]	387.3 ± 211.4	227.9 ± 186.6	224.8 ± 129.3	**0.0003**	**0.005**	NS
$CD8^{high}CD57^-CD27^-$						
%[4]	13.10 ± 8.112	22.60 ± 15.37	17.18 ± 11.20	**0.024**	NS	NS
A. count[#]	85.79 ± 73.99	119.1 ± 126.7	99.46 ± 85.96	NS	NS	NS
$CD8^+FoxP3$						
%[5]	10.59 ± 15.16	5.881 ± 6.673	6.498 ± 7.939	NS	NS	NS
A. count[#]	91.42 ± 126.1	51.19 ± 86.72	50.49 ± 60.37	NS	NS	NS

[1]I group: healthy controls; [2]II group: pSS patients without anti-SSA/SSB; [3]III group: pSS patients with anti-SSA and/or anti-SSB; %[4]: proportion of all $CD8^{high}$ lymphocytes; %[5]: proportion of all $CD8^+$ lymphocytes. A. count[#]: absolute count of cells in 1 μL of blood. *Mann-Whitney test. NS: not significant.

are the best candidates for the role of environmental triggers to autoimmune reactions [28]. It is proposed that, after activation in peripheral lymphoid organs by cross-reacting foreign antigens, autoreactive T cells enter the target organ where they are reactivated by B cells which provide costimulatory survival signals, thereby inhibiting the activation-induced T-cell apoptosis which normally occurs when autoreactive T cells enter the target organ [27]. Understanding $CD8^+$ T memory effector cells differentiation is essential for studying how virus-specific $CD8^+$ T cells control viral infection. Distinct stages of virus-specific $CD8^+$ T memory effector cells differentiation have been extensively characterized by phenotypic and functional analyses. Primed virus-specific $CD8^+$ T cells typically differentiate from the least mature memory stage ($CD27^+$) to the most mature effector stage when they start to lose CD27 and obtain CD57 marker and eventually become terminally differentiated effector cells which can be further defined by CD57 expression [29]. According to some studies, last stage of $CD8^+$ cells differentiation seems to be $CD8^+CD27^-CD57^-$ T cells subset with

high perforin and killing activity. Is this the true end-stage or terminally-differentiated state of cytotoxic T cells? This fact is still unclear [30]. We observed increased proportion of this population in pSS patients, but significant differences were observed only in pSS without Abs group. In pSS with Abs group, negative correlation between $CD8^{high}CD27^{-}CD57^{-}$ T cells subset absolute count and Schirmer's *I* test results was observed. This connection proposes that this subset can be involved in a pathogenic process which appears in the glandular tissue. Impaired proportion and absolute counts of $CD8^{high}CD27^{+}CD57^{-}$ T cells in blood of pSS patients can be the reason of lower counts of $CD8^{+}$ in blood of pSS patients. It is not known whether the proportion and absolute counts of this population are downregulated in blood by migration to inflammatory sites, or this can be due to increased apoptosis of these cells. There is hypothesis that more mature effector stage ($CD8^{high}CD27^{+}CD57^{+}$ and $CD8^{high}CD27^{-}CD57^{+}$) cells with lower capacity of proliferation are more resistant to apoptosis than least mature memory stage $CD8^{high}CD27^{+}CD57^{-}$ T cells [29–31]. All these observed changes in $CD8^{+}$ T cell subpopulations rearrangement prove that these subpopulations actively participate in pathological processes of pSS.

NKT lymphocytes and NK cells might function as regulatory T cells and are one of the autoimmune process preventing chains [32, 33]. According to literature, in patients with autoimmune rheumatic diseases, the decreased NKT and NK cell counts and functional characteristics are associated with the progression of autoimmune process and autoantibody production [34–37]. Nevertheless Szodoray et al. published results where they identified higher proportion of these cells in blood of pSS patients than in control group [38]. Our investigation of these populations showed a significant decrease of NKT and NK-cell absolute counts in the peripheral blood of pSS patients with Abs; however, the fact that a proportion of these cells were similar with the controls can indicate that lower absolute counts can be due to genetically determined lymphopenia. There is also possibility that low counts of these cell populations in periphery can be by reason of overall lymphocyte population migration to inflammatory sites or/and apoptosis.

In conjunction with the classical $CD4^{+}$ Tregs, we were also investigating $CD8^{+}$ suppressor cells that express FoxP3 marker, as FoxP3 confers suppressive properties and is confined to regulatory T cells. $CD8^{+}$FoxP3 cells represent a new regulatory population and ability of these $CD8^{+}$FoxP3 Treg to suppress $CD8^{+}$ responses far more effectively than $CD4^{+}$FoxP3 Treg [39]. This was shown in mice after experimental allogeneic bone marrow transplantation. Our study results did not show any significant differences in proportion or absolute count changes on these cells in pSS patients' peripheral blood in comparison to healthy controls.

We found negative correlation between BAFF and T and B cells in pSS patients without Abs. Increase of BAFF in serum can be due to negative regulation of BAFF secretion by monocytes [40]. This fact can indicate tight control of BAFF secretion. Whereas we do not found correlation between BAFF and lymphocyte populations changes in pSS patients with Abs group, what can be the indication of uncontrolled BAFF secretion and its homeostasis disturbance?

Despite recent knowledge, in many respects, the role of T cells and their subsets in pSS remains unexplained. Are cells in the infiltrate specific, or maybe many of them are just bystanders (with nonactivated phenotype) recruited from the periphery to the inflammatory sites? T cells undergo expansion within the gland, or does this occur elsewhere with subsequent migration? Is there migration in and out of the gland, or do T cells remain in the infiltrates once they arrive? Pointers to these questions could help us understand which processes are going on periphery. All this together could help us to understand pathogenesis of the primary SS.

One of future projects should be the immunohistochemistry for assessing cell populations' changes in salivary glands in parallel with blood analysis and apoptosis markers. Such analysis could help better to define changes of cell populations in periphery, is this due migration to the inflammatory sites or increased apoptosis, or maybe both.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Autoantibody to MDM2: A Potential Serological Marker of Systemic Lupus Erythematosus

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Introduction. Systemic lupus erythematosus (SLE) is one of the systemic autoimmune diseases characterized by the polyclonal autoantibody production. The human homologue of the mouse double minute 2 (MDM2) is well known as the negative regulator of p53. MDM2 has been reported to be overexpressed in SLE animal model and to promote SLE. Since abnormally expressed proteins can induce autoimmune response, anti-MDM2 autoantibody was examined in SLE patients. *Methods.* Anti-MDM2 antibody in sera from 43 SLE patients and 69 healthy persons was investigated by ELISA. Positive samples were further confirmed by western blotting. The immunological feathers of anti-MDM2 positive sera were analyzed by indirect immunofluorescence assay. Anti-p53 was also investigated in SLE patients by ELISA, and the correlation of anti-MDM2 and anti-p53 was analyzed. *Results.* The presence of anti-MDM2 in SLE patients was 23.30%, much higher than normal healthy persons (4.30%). These anti-MDM2 positive sera present a nuclear staining pattern. The presence of anti-p53 in SLE patients was 39.50%, and the titer of anti-MDM2 was positively correlated with anti-p53 in SLE patients. *Conclusions.* Anti-MDM2 autoantibody was detected at high prevalence in SLE patients. The detection of anti-MDM2 in SLE patients should be clinically useful.

1. Introduction

Systemic lupus erythematosus (SLE) is one of the systemic autoimmune diseases characterized by the production of autoantibodies to cellular constituents [1]. Autoantibodies are widely used as biomarkers in many types of autoimmune diseases and other diseases such as cancer. One of the most important research areas in which autoantibodies are used is diseases diagnosis.

Besides its use in diagnosis, the detection of autoantibodies can also provide information about clinical manifestations or prognosis of some autoimmune diseases. The study on biological functions of autoantibody or its antigens can provide us with a better understanding of the mechanism of pathogenesis of autoimmune diseases and thus may give us new insights into the new strategies in autoimmune diseases treatment.

Several autoantibodies have been well characterized in SLE. Some autoantibodies are considered to be highly specific to SLE, such as anti-Sm and antiribosomal P. However, these autoantibodies are present in only about 15% and 10% of SLE patients, respectively [2, 3]. Though anti-dsDNA antibodies are found to be highly presented in SLE patients with prevalence of about 70%, its level fluctuates significantly according to disease activity and treatment [4]. Patients with SLE are still heterogeneous in clinical manifestations and serological characteristics. More new autoantibodies in SLE still need to be identified in order to further classify this disease or to better understand its pathogenesis.

The human homologue of the mouse double minute 2 (MDM2), also known as E3 ubiquitin-protein ligase, is known to degrade several central cell cycle regulators including p53 and retinoblastoma (Rb) protein which are involved in important processes such as cell apoptosis [5]. It was

interesting that DNA viruses can specifically induce MDM2 expression and then cause B cell lymphoma [6]. This is a mechanism that might contribute in a similar manner to lymphoproliferation in SLE induced by self-DNA. It was further demonstrated that cytosolic DNA can trigger the expression and activation of MDM2. In MRL-Fas^{lpr} mice, an animal model of SLE, the expression level of MDM2 was found to be increased and to correlate with disease progression [7], which provides us with a new molecular target in SLE. Since abnormally expressed proteins can induce autoimmune response, overexpression of MDM2 in lupus may trigger the production of autoantibody which may serve as a new serologic marker in SLE.

In this study, we investigated the presence of autoantibody to MDM2 in sera of SLE patients and normal human sera (NHS). We found that autoantibody to MDM2 was highly presented in SLE patients, which may be used as a new serological marker or therapeutic target in SLE.

2. Materials and Methods

2.1. Sera and Patients. In the current study, 69 normal human sera (NHS) and 43 SLE patient sera were examined. These sera were obtained from the serum bank of Cancer Autoimmunity and Epidemiology Research Laboratory at University of Texas at El Paso (UTEP), which were originally provided by our clinical collaborators. The diagnosis of SLE was established according to the American College of Rheumatology criteria [8, 9]. The Institutional Review Board of UTEP and Collaborating Institutions has approved this study.

2.2. Expression and Purification of Recombinant MDM2 and p53. Recombinant protein of MDM2 and p53 was derived from our previous studies [10]. MDM2 and p53 cDNAs were subcloned into pET28a vector producing fusion proteins with NH-terminal 6x histidine and T7 epitope tags. Recombinant protein was further expressed in *E. coli* BL21 (DE3) and then purified using nickel column chromatography (Qiagen, Valencia, USA). Reactivities of the purified recombinant protein have been analyzed by electrophoresis on SDS-PAGE and determined with polyclonal anti-MDM2 antibody (GeneTex, Irvine, USA).

2.3. Enzyme-Linked Immunosorbent Assay (ELISA). Standard protocol for ELISA was conducted as described in our previous study [11]. In brief, a 96-well microtiter plate was coated with recombinant MDM2 or p53 protein overnight at 4°C with a final concentration of 0.5 μg/mL in phosphate-buffered saline (PBS). The antigen-coated wells were blocked with gelatin postcoating solution at room temperature for 2 h. Human sera were diluted at 1 : 100 and then incubated for 2 h at room temperature in the antigen-coated wells, followed by HRP-conjugated goat anti-human IgG. The substrate 2,2′-azino-bis-3-ethylbenzo-thiazoline-6-sulfonic acid (ABTS, Sigma-Aldrich, St. Louis, USA) was used as detecting reagent. The average optical density (OD) value at a wavelength of 405 nm was applied as data analysis. The cutoff value used to designate a positive sample was the mean OD value of 69 NHS + 2SD.

2.4. Western Blotting. Denatured recombinant MDM2 protein was electrophoresed on 10% SDS-PAGE and transferred to nitrocellulose membranes. After blocking in PBS with 5% nonfat milk and 0.05% Tween-20 for 1 h at room temperature, the nitrocellulose membrane was incubated overnight with 1 : 200 dilution of human sera at 4°C. HRP-conjugated goat anti-human IgG (Santa Cruz, USA) was then applied as secondary antibody at a 1 : 10,000 dilution. The immunoreactive bands were detected by ECL kit according to the manufacturer's instructions (Thermo Scientific, Waltham, USA).

2.5. Absorption of Antibodies with Recombinant Protein. The diluted SLE sera (1 : 80) were incubated with recombinant protein MDM2 (final concentration of recombinant proteins in the diluted human sera was 0.01 μg/μL) overnight at 4°C and then centrifuged at 10,000 ×g for 15 min. The supernatant was used for immunofluorescence assay.

2.6. Indirect Immunofluorescence Assay (IIFA). Hep-2 antigen substrate for IIFA test system was incubated with diluted sera (1 : 80) and preabsorbed sera overnight at 4°C. FITC-conjugated goat anti-human IgG was then used as secondary antibody at a 1 : 100 dilution. Fluorescence microscope (Leica DM1000, Germany) was used for examination.

2.7. Statistical Analysis. All data were represented as mean ± standard deviation (SD). The frequency of autoantibody to MDM2 in the sera was compared using the χ^2 test with Fisher's exact test. Correlation coefficients were calculated using the Spearman rank correlation analysis. Statistical analysis was performed in SPSS13.0 software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. The Prevalence of Autoantibody to MDM2 in SLE. Serum level of autoantibody to MDM2 in SLE patients and normal human sera was determined by ELISA. The mean titer of autoantibody to MDM2 was significantly higher than that in NHS (Figure 1).

We then used the mean OD value plus 2SD of NHS as the cutoff value to determine the frequency of anti-MDM2 autoantibody positive sera in these three groups. The frequency of anti-MDM2 positive sera was significantly higher in SLE patients group (23.30%) than NHS group (4.30%) (Table 1).

In order to confirm the presence of anti-MDM2 in SLE patients, anti-MDM2 autoantibody positive sera were further confirmed by western blotting. These sera also had strong reactivity with MDM2 recombinant protein in western blotting analysis (Figure 2).

3.2. Immunofluorescence Staining Pattern of MDM2 in Hep-2 Cells. To further confirm the reactivity of autoantibodies

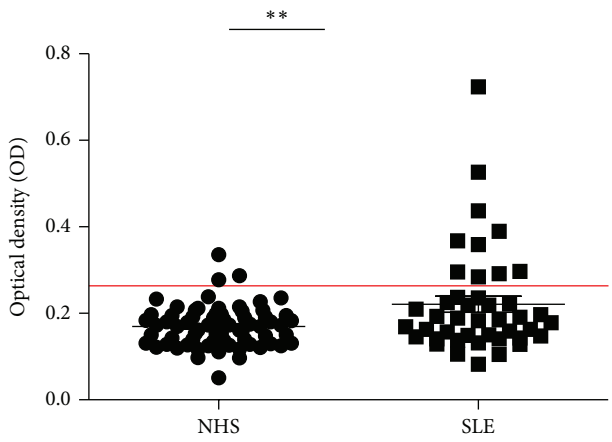

Figure 1: Titer of autoantibody against MDM2 in human sera by ELISA. The range of antibody titers to MDM2 was expressed as optical density (OD) obtained from ELISA. The mean + 2SD of NHS was shown in relationship to all serum samples. Titer of anti-MDM2 in SLE serum was much higher than that in NHS ($P < 0.01$). The cutoff value line for positive samples is indicated in the figure.

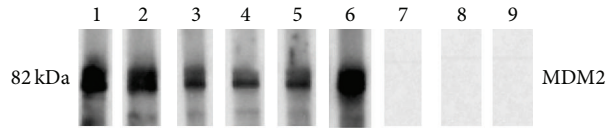

Figure 2: Western blotting analysis with representative sera in ELISA. Lanes 1–6: six representative SLE sera which were positive in ELISA and also had strong reactivity with MDM2 recombinant protein in western blotting analysis. Lanes 7–9: three randomly selected NHS had negative reactivity with MDM2 recombinant protein.

Table 1: Frequency of autoantibody against MDM2 in human sera by ELISA.

	Number	Anti-MDM2 (+)	Frequency
NHS	69	3	4.30%
SLE	43	10*	23.30%*

NHS: normal human sera; SLE: systemic lupus erythematosus.
*$P < 0.05$.

against MDM2 in SLE sera and the intracellular localization of MDM2, commercially available Hep-2 cell slides were used in indirect immunofluorescence assay to examine anti-MDM2 autoantibody positive SLE sera. As shown in Figure 3, the anti-MDM2 positive sera had the nuclear staining patterns, while the normal human serum had very weak staining. The fluorescent staining was significantly reduced when the same serum was preabsorbed with recombinant anti-MDM2 protein.

3.3. Association of Anti-MDM2 and Anti-p53 in SLE Patients. Since MDM2 has been demonstrated as an important negative regulator of p53 and anti-p53 was also been reported to be found in SLE patients [12], whether there was an association between anti-MDM2 and anti-p53 is still unknown. We further investigated the presence of anti-p53 in these SLE patients by ELISA.

We used the mean OD value + 2SD of NHS as the cutoff value to determine the frequency of anti-p53 autoantibody positive sera. Consistent with results reported in other studies, the frequency of anti-p53 positive sera was significantly higher in SLE patients group (39.50%) than NHS group (5.90%) (Figure 4), and the titer of anti-MDM2 was positively correlated with anti-p53 (Figure 5).

4. Discussion

The present study showed that anti-MDM2 autoantibody was presented in 23.30% SLE patients, significantly higher than normal heathy humans. The titer of anti-MDM2 was positively associated with anti-p53. This suggests that the anti-MDM2 autoantibody might be used as a new serologic marker for SLE.

The MDM2 protein (also known in humans as Hdm2) was first identified as the product of a gene amplified over 50-fold on acentromeric extrachromosomal bodies (called "double minutes") found in a 3T3DM spontaneously transformed mouse cell line [13, 14]. MDM2 was known as an important negative regulator of p53. Besides its regulation on p53, it was found that MDM2 also interacted with many proteins in addition to p53 such as NF-κB [15, 16].

The role of MDM2 in cancer has been well studied. It was described as one of the tumor associated antigens (TAA) as MDM2 was overexpressed in several kinds of tumors [17]. It was demonstrated that MDM2 could elicit a functional autologous immune response in human [18]. It has also been reported that autoantibody to MDM2 can be found in patients with esophageal squamous cell carcinoma [19].

The role of MDM2 in immune regulation can be speculated by its regulation on p53. In recent years, p53 has been found to be important in both innate and acquired immune regulation [20, 21], and it was necessary in the inhibition of autoimmune inflammation [22]. Several studies have found that the presence of anti-p53 in SLE patient, with a prevalence of about 26%–59%, showed that anti-p53 was related to anti-DNA antibodies and can be used as a marker for detecting the disease activity of SLE [12, 23, 24]. Our study showed that anti-p53 was presented in 39.50% of SLE patients, and the titer of anti-p53 was positively correlated with anti-MDM2.

The direct role of MDM2 in immune regulation has been showed by several studies. Gasparini et al. showed that MDM2 can modulate dendritic cell-induced T cell proliferation [25] and Mulay et al. proved that MDM2 was required to induce mRNA expression and secretion of NF-κB-dependent cytokines upon Toll-like receptor stimulation [26], which were important processes in SLE pathogenesis. Another study showed that MDM2 can promote SLE and inhibition of MDM2 can suppress the abnormal expansion of all T cell subsets, without causing myelosuppression effect on splenic regulatory T cells, neutrophils, dendritic cells, or monocytes [7]. These data suggest a new promising therapeutic target in SLE treatment. Our study further confirmed the importance of MDM2 in the pathogenesis of SLE and provided a new serological marker for SLE.

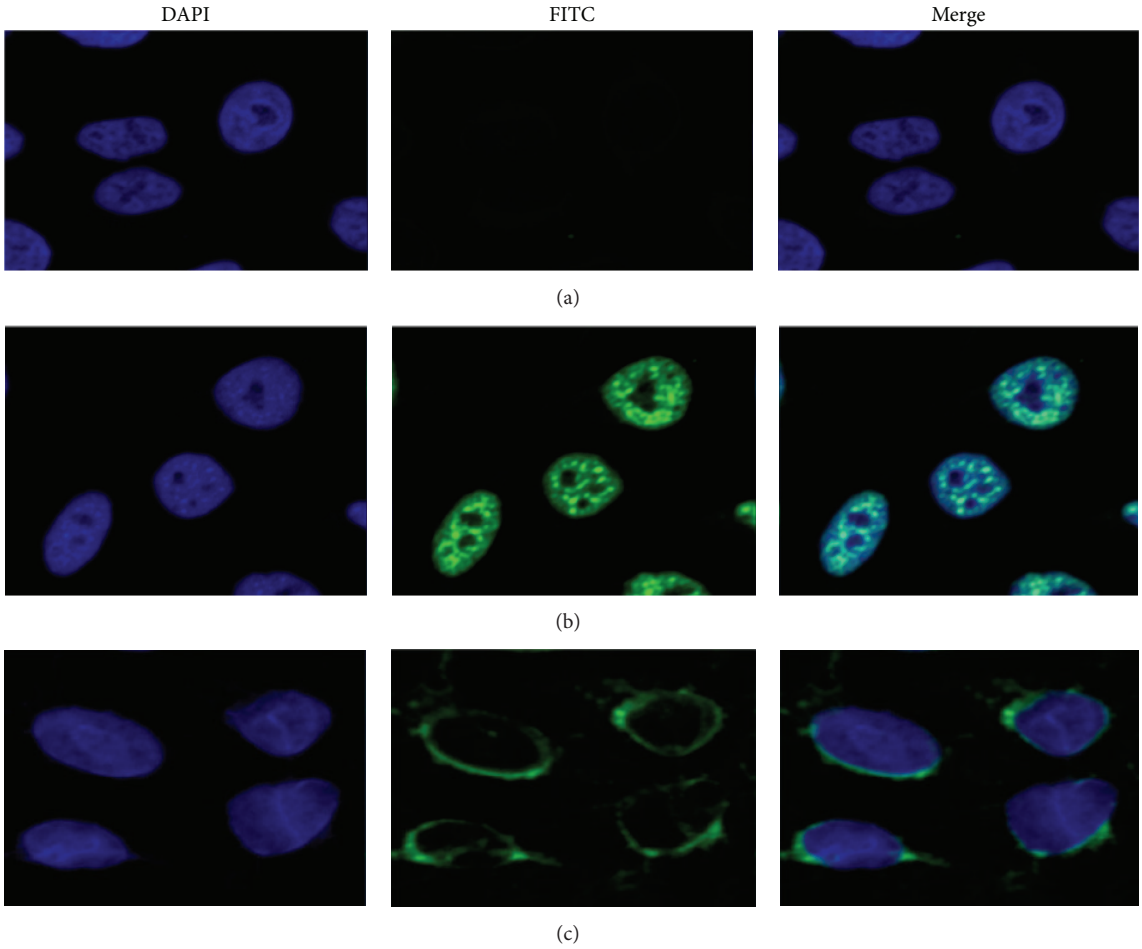

Figure 3: Representative immunofluorescence staining pattern from a SLE serum with anti-MDM2 autoantibody positive (performed on Hep-2 antinuclear antigen tissue slides). (a) NHS was used as negative control; (b) a representative SLE serum with anti-MDM2 autoantibody positive demonstrated an intense nuclear staining pattern; (c) the same SLE serum used in panel (b) was preabsorbed with recombinant MDM2, and the nuclear fluorescent staining was significantly reduced.

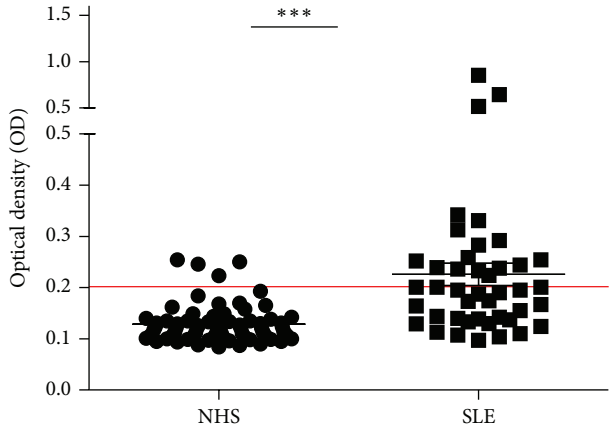

Figure 4: Titer of autoantibody against p53 in human sera by ELISA. The range of antibody titers to p53 was expressed as optical density (OD) obtained from ELISA. The mean + 2SD of NHS was shown in relationship to all serum samples. Titer of anti-p53 in SLE serum was much higher than that in NHS ($P < 0.01$). The cutoff value line for positive samples is indicated in the figure.

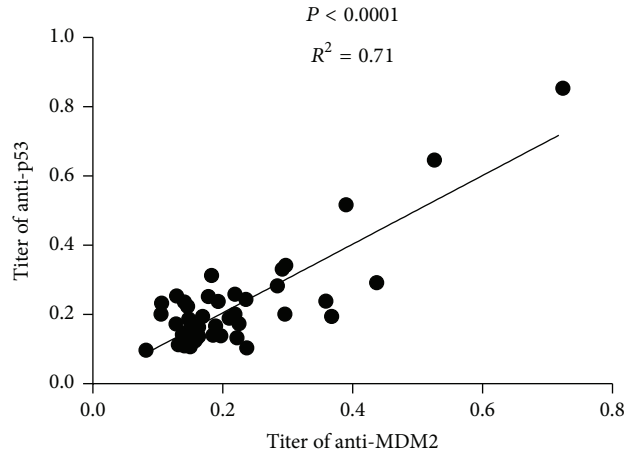

Figure 5: Correlation between anti-MDM2 and anti-p53 antibodies in SLE patients. A positive correlation between the titers of anti-MDM2 and anti-p53 antibodies was found in 43 SLE patient sera.

An increased risk of cancer in SLE patients has been observed [27, 28]. However, the mechanism underlying the association between SLE and cancer remained largely unknown. Whether the high prevalence of autoantibodies to MDM2 and p53 was related to the high risk of cancer in SLE patients still needs to be clarified.

Our study is the first one to demonstrate the presence of anti-MDM2 antibody in SLE patients. However, an obvious limitation of the present study is the limited clinical information of SLE patients included in this study. Analysis regarding the association of anti-MDM2 autoantibody and the clinical manifestations was hard to conduct due to the limited clinical information. Studies on anti-MDM2 in more SLE patients with detailed clinical information are needed to further ensure the role of anti-MDM2 in SLE diagnosis, disease activity evaluation, or prognosis prediction. The presence of anti-MDM2 still needs to be investigated in other autoimmune diseases such as rheumatoid arthritis, systemic sclerosis, Sjögren's syndrome, and dermatomyositis.

5. Conclusion

In conclusion, the present study reported a high prevalence of anti-MDM2 in SLE patients, which suggests the role of MDM2 in the pathogenesis of SLE. The detection of anti-MDM2 autoantibody may provide a new serological marker in SLE diagnosis.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Yuan Liu and Liping Dai contributed equally to this study.

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Research Article

Body Image Disturbances Have Impact on the Sexual Problems in Chinese Systemic Lupus Erythematosus Patients

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SLE might affect all aspects of life including sexual functioning; previous study found that body image disturbance (BID) was the most powerful predictors of impaired partner relationships and sexual function. The current study investigated the relationship among disease parameters, quality of life, the psychological status, BID, and sexual problems in Chinese patients with SLE. A self-report survey design was administered to 168 SLE patients and 210 healthy individuals. Our results showed that 86 (55.1%) SLE patients reported impaired relationships with a sexual partner or partners, and 100 (64.1%) patients reported impaired sexual function which were significantly higher than the control group (31.6%, 35.7%, rep.). Age, marital status, depression, and BIDQ were the most powerful predictors of impaired partner relationships, while BIDQ3 and education, disease activity, and depression were the most significant causes of impaired sexual function. The study for first time reported Chinese SLE patients had sexual problems and BID was associated with sexual problems. So, early detection and interventions might not only rehabilitate the patients and their loved ones, but also improve overall health outcomes and reduce the direct and indirect costs of their medical care.

1. Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that disproportionately affects young women; women are affected nine times more frequently than men. About two-thirds of patients develop cutaneous manifestations that may be visible, transient, or permanent rashes, scars, depigmentation, skin dimpling, photosensitivity, and hair loss. Arthritis, serositis, nephritis, and hematological and neuropsychiatric problems are common [1]. SLE can affect different aspects of patients' life, leading to an impairment of health-related quality of life (QoL). We have reported that Chinese SLE patients have impaired QoL and psychological health problems [2]. The disease causes characteristic physical problems (i.e., chronic pain and fatigue) and emotional problems (i.e., low self-esteem and depression), and these may decrease sexual interest and reduce intercourse frequency. Partnership difficulties arising from disease-related stress can also contribute to a less active sexual life [3]. For these reasons, SLE may affect all aspects of life including sexual functioning. However, the impact of SLE on sexual problems has been less studied. Tseng et al. have reported that 52.5% have impaired sexual function in Taiwan SLE patients [4]. However, to our knowledge, few studies have focused on the sexual problems in SLE patients, especially in Chinese mainland.

Body image disturbance (BID) is commonly defined as the distortion of perceptions or cognitions related to the weight or shape of the body [5]. BID plays an important role in anxiety/depression and reduces the quality of life (QoL) [6]. It has been reported that lymphedema patients with pain have higher levels of BID and decreased sexual drive [7]. Severe BID is well documented in chronic diseases, particularly those accompanied by deformities or disabilities, such as ankylosing spondylitis (AS). Jolly et al. have reported that BID is correlated with health-related quality of life in SLE patients [8].

Recently studies have reported that vaginal pain symptoms, poorer body image, and fatigue are independent predictors of sexual dysfunction in young breast cancer

survivors [9]. And sexual interest is associated with vaginal pain symptoms, body image, and weight problems. We have reported that Chinese AS patients exhibit BID, and significant relationships are found among BID, disease and psychological variables, and QoL [10]. Interestingly, we have found that disease activity and BID are the most powerful predictors of impaired partner relationships, while disease severity, BID, and physical function are the most important causes of impaired sexual function in Chinese AS patients [11]. The psychological problems, such as anxiety and depression, as well as BID occurrence, may impair sexual function and relationships. But no papers about BID and sexual problems in SLE patients have been published.

The current study examines the independent association of BID occurrence with sexual function in a Chinese population in order to provide a preliminary analysis of the clinical parameters, disease activity, physical functions, and psychological parameters associated with sexual problems in SLE patients. As a result, the factors most closely linked to a patient's sexual status can be documented and considered in the design of appropriate clinical treatment strategies.

2. Methods

2.1. Participants. SLE patients were recruited from The Second Affiliated Hospital of Nantong University from January 2010 to 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 conditions. (1) They did not complete the questionnaire; (2) they had comorbidities (e.g., serious infections or cardiac, respiratory, gastrointestinal, neurological, or endocrine diseases) that could influence SLE activity. This study was approved by the Ethics Committee of The Second Affiliated Hospital of Nantong University, and written informed consent was obtained from all participants.

2.2. Measures of Clinical Variables

2.2.1. The Revised Self-Rating Anxiety Scale (SAS) [12]. SAS was used to evaluate the level of anxiety-related symptoms during the week prior to the survey. This self-administered test had 20 questions, with 15 items reflecting increasing anxiety levels and 5 questions reflecting decreasing anxiety levels. Each question was scored on a scale of 1 to 4 (rarely, sometimes, frequently, and always, resp.). The scores ranged from 20 to 80. Scores greater than 70 suggested severe anxious symptoms, scores between 60 and 69 indicated moderate to marked anxiety, scores between 50 and 59 suggested minimal to mild anxiety, and scores less than 50 indicated no anxious symptoms.

2.2.2. The Revised Self-Rating Depression Scale (SDS) [13]. SDS was a 20-item questionnaire designed to assess mood symptoms over the past week (e.g., "I feel downhearted, blue and sad"). Each item was scored on a Likert scale ranging from 1 to 4; scores greater than 70 suggested severe depressive symptoms, scores between 60 and 69 indicated moderate to marked depression, scores between 53 and 59 suggested minimal to mild depression, and scores less than 53 indicated no depressive symptoms.

2.2.3. Measure of the Quality of Life [14]. The patient's general health status was measured using the Short Form- (SF-) 36 questionnaires, which measured eight multi-item dimensions: physical functioning (PF, 10 items); role limitations due to physical problems (RP, 4 items); role limitations due to emotional problems (RE, 3 items); social functioning (SF, 2 items); mental health (MH, 5 items); energy/vitality (VT, 4 items); body pain (BP, 2 items); and general health perception (GH, 5 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).

2.3. Clinical Measurement of Disease Activity. The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) was used to measure disease activity [15].

2.4. Questionnaire Design

2.4.1. Body Image Assessment. Body image was assessed using the Body Image Disturbance Questionnaire (BIDQ) containing seven scaled items scored from 0 (not affected) to 8 (extremely affected) pertaining to appearance-related concerns (BIDQ1); mental preoccupation (BIDQ2); emotional distress (BIDQ3); social, occupational, or functional impairment (BIDQ4); social life interference (BIDQ5) and educational, occupational, or other functional interferences (BIDQ6); and behavioural avoidance (BIDQ7), as previously described. Average Cronbach's alpha value is 0.82 [16, 17].

The questionnaire design was based on modified questions of numbers 13 and 14 of the Body Image Questionnaire provided in the digital form by the King's College of London, UK (http://psychology.iop.kcl.ac.uk/cadat/questionnaires/BIQ.pdf). Briefly, the questionnaire first required participants to report the magnitude of the effect of their current emotional status on sexual relationship(s) with current partners (Cronbach's $\alpha = 0.73$). Secondly, participants were required to indicate the impact of their current status on sexual function, including sexual enjoyment and frequency (Cronbach's $\alpha = 0.84$). For each question, participants provided ratings based on a 9-point scale ranging from 0 (not at all) to 8 (extremely).

2.5. Questionnaire and Measurement Administration. Questionnaires and other assessments were administered to participants from January 2010 to July 2011. Written questionnaires were provided on papers, and all participants completed the questionnaire under physician's supervision in a clinical setting. SLEDAI was evaluated by the same clinician for all patients. Nurses counted the results. The results were

Table 1: Demographic and psychological and disease characteristics in SLE patients and controls.

Variables	SLE patients ($N = 156$)	Control subjects ($N = 196$)	P
Female gender[a]	142 (91.2)	176 (89.8)	0.75
Age, years[b]	32.9 ± 10.2	35.0 ± 11.4	0.19
SAS (≥50)[a]	32 (20.51)	14 (7.1)	<0.01
SDS (≥53)[a]	52 (33.33)	28 (14.3)	0.003
SLEDAI	11.8 ± 9.5		
Marital status[b]			
Single	30 (19.23)	56 (18.6)	0.20
Married	126 (80.76)	140 (71.4)	
Education[b]			
<9 years	86 (55.12)	76 (49.0)	0.46
≥9 years	70 (44.87)	100 (51.0)	
Work status[b]			
Working	30 (19.23)	44 (22.5)	0.58
Unemployed	126 (80.77)	152 (77.5)	
Income/person[b]			
≤2000 yuan	100 (64.10)	118 (60.2)	0.68
>2000 yuan	56 (35.90)	78 (39.8)	
Menstrual history[b]			
Normal	95 (66.90)	102 (58.0)	0.25
Abnormal	47 (33.10)	74 (42.0)	

[a]Mean ± SD; [b]number (percentage). SLE: systemic lupus erythematosus; SAS: revised Self-Rating Anxiety Scale; SDS: revised Self-Rating Depression Scale; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.

added to a computer database by 2 research assistants and double-checked against the original data prior to analysis.

2.6. Statistical Analysis. All data were expressed as means ± SD for continuous variables and as frequencies (%) for categorical variables. The statistical package was included in the STATA v.10.0 (StatCorp, USA) software for all data management and analysis. Descriptive analyses were performed to investigate participant characteristics. Student's t-tests were applied to assess parametric variables of independent groups, while Spearman's correlation analysis was used to assess the correlation of parametric variables. Stepwise regression analyses were conducted for sexual problems and SF-36 scores separately in order to identify significant predictors of dysmorphic concern. A P value less than 0.01 or less than 0.001 ($P < 0.01$ or $P < 0.001$) was considered highly statistically significant, while P value less than 0.05 was considered statistically significant ($P < 0.05$).

3. Results

3.1. Characteristics of SLE Patients. A total of 170 patients met the eligibility criteria. 8.236% ($n = 14$) did not complete the full questionnaire due to the lack of interest, resulting in the enrollment of 156 eligible SLE patients. Table 1 presented the baseline participant characteristics included in our analysis.

Table 2: The sexual status in SLE patients in China.

SLE patients ($N = 156$)	Control subjects ($N = 196$)	
86 (55.1)	62 (31.6)	<0.001***
1.8 ± 2.0	1.2 ± 1.3	0.0008***
100 (64.1)	70 (35.7)	<0.001***
2.6 ± 2.7	1.7 ± 2.0	0.0004***

***$P < 0.001$.

Ages ranged from 18 to 60 and the average was approximately 32.9 (SD = 10.2). The majority of our study participants were female (91.2%), married (80.76%), and low-income (64.10%) and had received less than high school education (55.12%). The SLEDAI score of participants ranged from 2 to 55 (mean = 11.8, SD = 9.5). 32 (20.51%) SLE patients were at high risk for anxiety, and 52 (33.33%) exhibited signs of depression. Comparatively, only 14 (7.1%) healthy individuals had high risk for anxiety, and 28 (14.3%) exhibited signs of depression.

3.2. Sexual Status in SLE Patients. There were significant differences in sexual relationship impairment as observed between SLE patients and healthy individuals. 86 (55.1%) SLE patients reported impaired relationships with a sexual partner or partners, with an overall mean score of 1.8 ± 2.0 in SLE patients on the 0–8 scale. Comparatively, 62 (31.6%) healthy individuals reported impaired relationships with a partner with an overall mean score of 1.2±1.3 in SLE patients. 100 (64.10%) patients reported impaired sexual function. The frequency was significantly higher than healthy individuals (70, 35.7%) ($P < 0.001$). The overall mean scores for SLE patients regarding sexual function on the 0–8 scale were also significantly higher compared to those of healthy individuals (2.6 ± 2.7 versus 1.7 ± 2.0, resp.) (Table 2).

3.3. Associations between Sexual Problems and Overall Variables. The effect of SLE on sexual partner relationships showed significant correlation with scores for age ($P = 0.01$), marital status ($P = 0.03$), menstrual history ($P = 0.04$), appearance-related concerns ($P = 0.02$), distress ($P = 0.01$), impairment in social functioning ($P = 0.003$), and impairment in social life ($P = 0.009$). The effects of SLE on sexual function were significantly associated with scores of education ($P = 0.01$), appearance-related concerns ($P = 0.03$), BIDQ3 ($P = 0.006$), BIDQ4 ($P = 0.003$), BIDQ5 ($P < 0.001$), and BIDQ6 ($P = 0.00019$). These findings were detailed in Table 3.

3.4. Stepwise Regression Analysis for Sexual Problems. Stepwise regression analyses were used to confirm the variables most significantly correlated with psychological problems. The results showed that age, marital status, depression, and BIDQ 3,5,2,1 were the most powerful predictors of impaired partner relationships ($P < 0.05$) (Table 4). In contrast, BIDQ3 and education, disease activity, and depression were

Table 3: Relationships between psychological scores, disease parameters, and sexual problems in SLE patients.

	Partner relationships		Sexual functions	
	r	P	r	P
Age	0.29	0.01*	0.06	0.59
Sex	−0.19	0.09	−0.03	0.80
BMI	0.04	0.70	−0.07	0.55
Marital status	−0.25	0.03*	0.1	0.39
Education	−0.22	0.06	−0.28	0.01*
Work status	−0.06	0.59	0.07	0.53
Income/person	−0.12	0.29	−0.07	0.57
Menstrual history	−0.24	0.04*	−0.05	0.74
SLEDAI	0.24	0.04*	0.29	0.02*
SAS	0.09	0.42	0.1	0.36
SDS	0.21	0.045*	0.21	0.048*
BIDQ1	−0.27	0.02*	−0.25	0.03*
BIDQ2	0.33	0.003**	0.18	0.12
BIDQ3	0.29	0.01*	0.3	0.006**
BIDQ4	0.33	0.003**	0.33	0.003**
BIDQ5	0.29	0.009**	0.37	<0.001***
BIDQ6	0.19	0.09	0.38	<0.001***
BIDQ7	−0.14	0.20	−0.08	0.51

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
SLE: systemic lupus erythematosus; BMI: Body Mass Index; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; SAS: revised Self-Rating Anxiety Scale; SDS: revised Self-Rating Depression Scale.

Table 4: Stepwise regression analyses of medical and psychological variables and their relationship to partner relationships in SLE patients.

Partner relationships	Coef.	SE	t	P	95% CI
Age	0.07	0.03	2.73	0.009	0.02, 0.12
Marital status	−1.44	0.63	−2.28	0.027	−2.7, 0.2
SDS	0.77	0.46	2.86	<0.001	0.45, 0.89
BIDQ3	0.22	0.09	2.33	0.024	0.03, 0.40
BIDQ5	0.27	0.12	2.30	0.027	0.03, 0.52
BIDQ2	0.28	0.11	2.55	0.014	0.06, 0.50
BIDQ1	−0.37	0.10	−3.78	<0.001	−0.57, −0.17
_cons	1.19	0.17	2.41	0.034	2.1, 2.5

the most significant causes of impaired sexual function ($P < 0.05$) in SLE patients (Table 5).

4. Discussion

The associations among demographics, disease-related variables, psychological problems, BID, and sexual problem in Chinese SLE patients are examined, revealing that these SLE patients are much more likely to have impaired sexual health and partner relationships than their healthy counterparts. The current study is novel in that it assesses these parameters in a group representing the Chinese SLE population. These findings should be considered in clinical settings, where

Table 5: Stepwise regression analyses of medical and psychological variables and their relationship with sexual functions in SLE patients.

Sexual functions	Coef.	SE	t	P	95% CI
Q3	0.51	0.19	2.63	0.011	0.12, 0.90
Education	−1.40	0.69	−2.04	0.047	−2.79, −0.02
SLEDAI	0.55	0.37	2.91	0.003	0.37, 0.68
SDS	0.25	0.19	2.53	0.034	0.14, 0.51
_cons	3.43	1.21	2.83	0.007	1.00, 5.9

SLE: systemic lupus erythematosus.
SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; SDS: revised Self-Rating Depression Scale.

sexual health is often overlooked during the treatment of physical symptoms.

The underlying cause of disturbed sexual function in SLE patients is multifaceted, though the physical aspects are most easily assessed [18]. The physical symptoms of impaired function, pain, fatigue, and stiffness as well as psychological responses to chronic disease, such as depression and insecurity, can all contribute to sexual dysfunction [3]. Ryan et al. have reported that pain, fatigue, and stiffness may interfere with normal sexual functions [19]. SLE has been associated with substantial medical morbidity resulting in physical and occupational disability [3]. In a study by Boomsma et al., 20% of SLE patients believe that their illness would drive their family apart or worsen their relationship with their partners [20]. Partnership difficulties arising from disease-related stress can also contribute to a less active sexual life [21]. Curry et al. have found that when compared with the controls, patients with SLE have a significantly higher rate of abstention, a lower frequency of sexual activity among the sexually active, diminished vaginal lubrication, poor general sexual adjustment, and more depression [18]. Greater vaginal discomfort or pain during intercourse and difficulty in penetration due to vaginal tightness are also found. Liang et al. have reported 16% of 74 patients have sexual difficulties [22]. Stein et al. have demonstrated 10.5% of 120 patients have mild and 4% have major sexual problems [23]. Depression is a major cause of reduced quality of life in SLE patients of both genders [24]. It has been reported that depression may be the principal factor contributing to sexual dysfunction in SLE patients [25]. Questionnaires, as applied in the current study, have been shown to be the effective methods of psychological and sexual health [26]. The current study has founded that marital and education status may contribute to the sexual function of lupus. These findings are consistent with current results.

BID may adversely affect quality of life and result in psychosocial consequences, such as depression, social anxiety, impaired sexual functioning, and poor self-esteem. The effect has been previously demonstrated in body dysmorphic conditions and eating disorders, and it may also affect SLE patients with significant and chronic physical impairments [27]. Alba and Kes have reported the relation between body image and women's sexuality and the sexuality in women

with eating disorders [28]. Body image is significantly altered in Tunisian nonmetastatic breast cancer patients with sexual dysfunction [29]. This study indicates that BID has impact on the sexual problems. Previous studies have found that SLE patients report poor body image [30]. Similarly, we have found that Chinese SLE patients also have poor body image which is inversely correlated with depression (data not shown). Despite these initial indications, few published studies address the correlation between BID and sexual problems in SLE patients. The current results show that, except for "consequential behavioral avoidance," all other aspects of BID are significantly associated with impaired relationships with sexual partners. Sexual problems are shown to be common in Chinese SLE patients. The results of stepwise regression analysis in the current study demonstrates that age, marital status, and BID are most closely linked to impairments in the relationships of SLE patients and their sexual partners while BID and education are most closely linked with impaired sexual functioning in SLE patients. Interestingly, the current study reports that distress along with impairments in social functioning and social life correlates significantly with impaired sexual function. This suggests that the link between physical function and psychological factors may play a role in the sexual health and perhaps even the overall quality of life of SLE patients. Further studies will be required to assess whether psychological factors progress over time in Chinese SLE patient or if these symptoms have direct relationships with physical variables in such patients.

This study has several limitations. First, the single-center study design may mean results are not necessarily generalizable to a broader population. Second, BID and sexual problem are not separately analysed in men and women. This is a necessary next step as men and women have a dichotomy of physical build and self-perception of fitness. Third, current treatments of SLE, such as prednisone and toxic immunosuppressive drugs, may adversely affect body image. The study did not focus on the relationship between drug side effects and BID. Finally, psychological factors and sexual problem were analyzed with self-report questionnaires. Thus, further exploration of SLE patients' BID based on age, gender, and disease severity will be required to comprehensively supervise clinical prognostic guidelines, and further analyses should be conducted with assessment instruments weighted for use in SLE patients.

In summary, the study for first time has reported Chinese SLE patients have sexual problem and BID is associated with sexual problems. So, early detection and interventions may not only rehabilitate the patient and their loved ones, but also improve overall health outcomes and reduce the direct and indirect costs of their medical care. Discussions on changes in body image and the possible effects on patients with SLE need to be encouraged from both sides, by the patients and the physicians. This requires a major shift in the way we assess and provide medical care for these patients. Patients' beliefs that "doctors care for your body but do not care how you feel about your body" should challenge our approach towards the care of these patients. This requires interdisciplinary health care research, clinical collaboration, and, above all, shifting from a biomedical model to a biopsychosocial model. Increased awareness of the physical and psychological factors will aid rheumatologists and nursing specialists in initiating proper management of this subgroup of SLE patients. Sexual health may be improved by treating BID along with the physical symptoms of SLE.

Abbreviations

SLE: Systemic lupus erythematosus
BID: Body image disturbance
BIDQ: Body Image Disturbance Questionnaire
SLEDAI: Systemic Lupus Erythematosus Disease Activity Index
SAS: Self-Rating Anxiety Scale
SDS: Self-Rating Depression Scale.

Conflict of Interests

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

Authors' Contribution

Biyu Shen, Yan He, and Haoyang Chen contributed equally to this work.

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Review Article

The Emerging Functions of Long Noncoding RNA in Immune Cells: Autoimmune Diseases

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The long noncoding RNAs (lncRNAs) are RNA transcripts more than 200 nucleotides in length, which do not encode proteins. The lncRNAs are emerging as an important regulator of biological process, such as chromatin remodeling, gene transcription, protein transport, and trafficking through diverse mechanisms. The lncRNAs play crucial role in various multigenetics human diseases including cancers and neurological diseases and currently its role in autoimmune diseases is attracting many researchers. Recent studies have reported that differentiation and activation of immune cells, T cells, B cells, macrophages, and NK cells have correlation with lncRNAs, which have also an essential role in autoimmune diseases such as rheumatoid arthritis and SLE. Therefore, elucidation of the roles of lncRNAs in autoimmunity could be beneficial to understand the pathogenesis of autoimmune diseases. In this review article we attempt to highlight the recent progress regarding lncRNAs studies and summarize its role in autoimmune diseases.

1. Introduction

Among the 20,000 protein coding genes, only less than 2% of total human genome sequence has been reported [1]. Not surprisingly, at least 90% of the genome is actively transcribed into noncoding RNAs (ncRNAs), which have no protein coding potentiality [2]. A heterogeneous, novel class of long noncoding RNAs (lncRNAs) with length longer than 200 nucleotides is generally characterized as nonprotein transcript [3]. Over the past decade, with the speedy progress in high-throughput genetic sequencing technology more than 18,000 transcripts are annotated as lncRNAs that have been recognized in mammalian transcriptomes [4–6]. Based on it, various studies have revealed that lncRNAs are believed to form a major proportion of novel transcripts and known to be involved in number of functionally distinct biological and physiological processes including chromatin remodeling, gene transcription, RNA splicing, and protein transport diverse mechanisms [7, 8] and directly linked to human diseases including various cancers [9], Alzheimer's disease [10], and coronary artery disease [11]. Recently some studies have claimed that lncRNAs have certain roles in the different kinds of protein-coding and noncoding immune genes and their role in autoimmune diseases [12]. Furthermore, the lncRNAs act as a key regulator of inflammatory gene expression by a collaboration involving signal-dependent activation of transcription factors, transcriptional coregulators, and chromatin-modifying factors [13]. Nonetheless, to the date the exact mechanisms of lncRNA functions in autoimmunity are not well constituted. Here, we are going to review the lncRNAs functions associated with T cells, B cells, macrophages, and NK cells (Table 1), to the autoimmune diseases mainly in SLE, RA, and psoriasis (Table 2).

2. Classification and Characterization of LncRNAs

Long noncoding RNA is a new class of transcripts which has been found to be pervasively transcribed in the genome, mutations, and dysregulations of lncRNAs that lead to diverse

Table 1: LncRNAs associated transcription factors, genes, and cells in autoimmunity.

	LncRNAs	Cells	References
T-cells	TEMVPG1 (LincR-Ifng-3′AS)	CD8+	[14]
	NeST (WDR5)	CD8+/CD4+	[15]
	Lef1as (Wnt)	CD8+	[16]
	Ptpre (Jak-Stat)	CD8+	[16]
	Il2ra	CD8+	[16]
	LincR-Ccr2-5′AS, (GATA-3)	CD4+/Th2	[17]
	LincR-Gng2-5 (STAT4)	CD8+/Th1	[17]
	LincREpas1-3′s (STAT6)	CD4+/Th2	[17]
	LincR-Ifng-3′AS (T-bet)	CD8+/Th1	[17]
	Lnc-DC (STAT3)	CD4+	[18]
	TR-ZAFT	CD4+, CD8+	[19]
	SeCATs	CD4+	[20]
B cells	Linc-DC	CD20+B cells,	[18]
	SAS-ZAFT	CD19+B cells	[19]
	Igh locus (DJH)	pro-B cells	[21]
Macrophages	lincRNA-Cox2 (hnRNP-A/B, A2/B1)	Macrophages	[13]
	linc-DC	CD14 monocyte	[18]
	ptprj	CD148	[22]
	lincRNA-Cox2 or Ptgs2 (NFkB)	TLR4, CD11c+dendritic cells	[23]
	THRIL (hnRNP-L)	THP1/Macrophages	[24]
	PACER	Monocytes/DC	[25]
NK cells	KIR	NK cells	[26]

Table 2: List of LncRNA studies, associated with autoimmune diseases.

LncRNA	Diseases name	References
SAS-ZFAT	Thyroid disease	[19]
THRIL	Kawasaki disease	[24]
FNDC1, TAGP, SOD2, WTAP and ACAT2	SLE	[27]
LincRNA (total number 7.419)	RA	[28]
PRINS	Psoriasis	[29]

human diseases [30]. LncRNAs are classified on the basis of their genomic proximity to protein- coding genes as (1) sense or (2) antisense, when overlapping one or more exons for another transcript on the same or opposite strand, respectively, (3) bidirectional, when the expression of it and neighbouring coding transcript on the opposite strand is initiated in close genomic proximity, (4) intronic, a sequence which is derived entirely from within an intron of another transcript, and (5) intergenic, when it lies as independent unit within the genomic interval between two genes [7, 31]. Furthermore, at least three different groups can be categorized, namely, natural antisense transcripts (NATS), intronic RNA (IncRNAs), and long intergenic (intervening) noncoding RNA (lincRNAs) (Figure 1) [32]. These noncoding transcripts are often displayed as minimum or partial overlap with the coding sequence of the corresponding mRNAs regardless of protein coding potential but they may have an intrinsic function as mRNAs. Therefore, LncRNAs comprise a diverse class of transcripts that structurally resemble mRNAs but do not encode protein.

A study in human cell lines suggests that about 30% of lncRNAs are specifically expressed in the nucleus [33]. Several of them are involved in chromatin remodeling complexes and mediate genomic silencing [34]. Interestingly, the LncRNAs regulates the gene expression by interacting with its partner DNA, RNA and protein, which directly impacts upon human disease through various mechanisms, involved in epigenetic silencing, splicing regulation, translation control, regulating the apoptosis and cell cycle control. Every step of life cycle of gene from transcription to mRNA splicing and translations can be influenced by LncRNAs. However, lncRNAs might achieve regulatory specificity through modularity, collecting diverse combination of proteins, and possibly RNA and DNA interactions [35]. It also acts as evolutionary preserved transcripts of noncoding DNA succession, which have been implicated in the regulation of cellular differentiation [36] to genome rearrangement and inactivation of major tumor suppressor genes [32]. LncRNAs have been characterized to regulate the abundance of genomically neighbouring (*cis*-acting) or distal (*trans*-acting) gene products that were classified as *cis*-acting lncRNA-transcription dependent or *trans*-acting lncRNA-transcription dependent [37]. Moreover, the variety of molecular mechanism and biological roles of lncRNAs [38] are attributed but still limited to provide definitive understanding of its mechanisms. Recently, Marques and Ponting have described that most of *cis*-acting lncRNA to trait variation are low and *trans*-acting lncRNAs may be

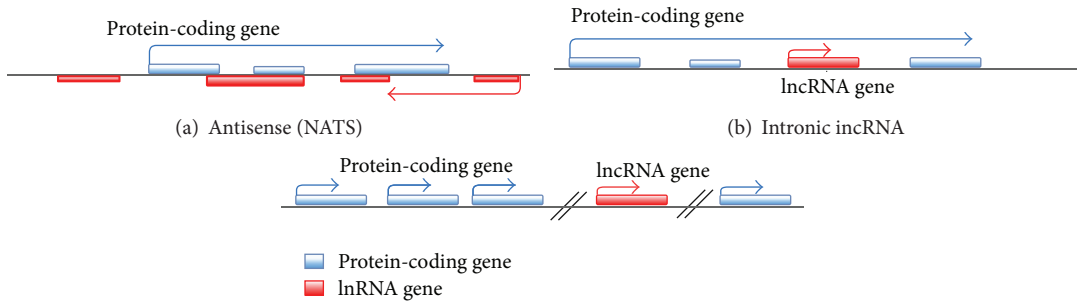

Figure 1: Classification and characterization of lncRNAs are based on their genomic localization with respect to the neighboring protein-coding gene. It is classified as overlapping lncRNAs including (a) natural antisense transcripts, or (b) intronic LncRNA and non-overlapping, (c) intergenic lncRNA; (lincRNAs), are transcribed from regions far away from protein-coding genes. The direction of arrow represents the different forms of transcription. Antisense lncRNA contains section of complementary sequences with the mature, spliced mRNA of the overlapping protein-coding gene. Intronic lncRNAs are transcribed within the intron of a protein-coding gene and therefore do not contain sequences complementary to the mature, spliced mRNA of the protein-coding gene [32].

higher although it was difficult to establish until the full set of their genome-wide targets in their trait appropriate cells and tissue [39]. Taken together, paradigms of lncRNAs are gene imprinting Xist [6], Tsix [40], and air [41]; chromatin modification, HOTAIR [42]; RNA processing, MALAT-1 [43]; cell apoptosis and cell cycle control, Gas5, lincRNAs-p21 [34, 44]; and so forth. Shortly, it was estimated that more than 8000 lncRNA exist in the human organism [32]. Therefore, deregulated reflection of lncRNA is associated with a variety of multigenetic human diseases ranging from different organs cancers [12] to noncancer such as Alzheimer disease (BACE1-AS) [10], coronary heart disease (ANRIL) [11], myocardial infarction (MIAT) [45], and membranous nephropathy Xist [46] and to some alternation of immune system both in innate and adaptive [13, 47] which may lead to better understanding of infectious and inflammatory diseases.

3. LncRNA in Immune Cells and Its Role in Autoimmunity

Development of autoimmune disease is associated with epigenetic mechanism that modulates the gene networks in response to complex profiles of environment [48]. The differentiation and activation of immune cells are dependent on synchronized set of transcriptional and posttranscriptional events. Chromatin-modifying complexes admeasure the regions of the genome which are accessible to transcription factors and regulate the transcription of immune genes [49, 50]. Importantly, the essentiality of micro-RNAs for normal immune functions, immune cell development, and prevention of autoimmune diseases had been studied [51, 52]. However, LncRNA study is emerging as important regulators of immune cells differentiation and activation in recent years [47]. Here, we are going to summarize the functions of long noncoding RNAs associated with immune cells (Figure 2).

3.1. Long Noncoding RNA in T Cells. The study of LncRNAs function in the immune system found them as important regulators of the various biological processes recently. Th1 helper cells are crucial for organizing for adaptive immune responses to variety of pathogens; they are involved in various pathogeneses of different types of immunological diseases including autoimmune diseases, allergy, and asthma [53]. LincRNAs, TMEVPG1 (also termed, LincR-*Ifng*-3′AS), recognized in human and mouse CD8+ T cells, has been displayed to be located within a cluster of cytokines genes, controlled Theiler's virus load in infection of CNS [54]. Th1 cells specific and selective transcription factor T-bet/Stat with TMEVPG1 controls the expression of interferon gamma (IFN-γ) [14]. Gomez et al. [15] described lncRNAs, also called NeST, which interact with WDR5, a core submits of the MLL H3K4 methyltransferases, and facilitate the histone methylation at the *Ifng* locus in CD8+/Th1 cells. Genome-wide expression analyses revealed that presence of hundreds of lncRNAs in CD8+ T cells from human and mouse spleen by using custom array suggests an essential role of lncRNAs in the differentiation and activation of lymphocytes [16]. Hu et al. [17] have performed an experiment in RNA-Seq of 42 subsets of thymocytes and mature peripheral T cells and identified 1,524 genomic regions that generate lincRNAs; key transcription factors including T-bet and STAT4 for the CD8+ and GATA-3 and STAT6 for the CD4+ lineages were largely accountable for the lineage-specific expression of T cell lincRNAs (LincR-Ccr2-5′AS), for the better understanding of lncRNAs in the development and differentiation of T cells. Currently, a novel study has appeared in the immunology; STAT3- binding lncRNAs lnc-DC, which is exclusively expressed in human conventional dendritic cells, bound directly to STAT3 signaling molecule in the cytoplasm, suggested that lncRNAs can affect cellular differentiation (monocyte into dendritic cells identified, lnc-DC) and function by directly interacting with signaling molecules in the cytoplasm and upregulate their posttranslational modification [18]. Interestingly, Lnc-DC, a specific regulator of DC differentiation and function, may have potential role to clinical diseases involving DC dysfunction and may have influence for the activation of CD4+ T cells response. In

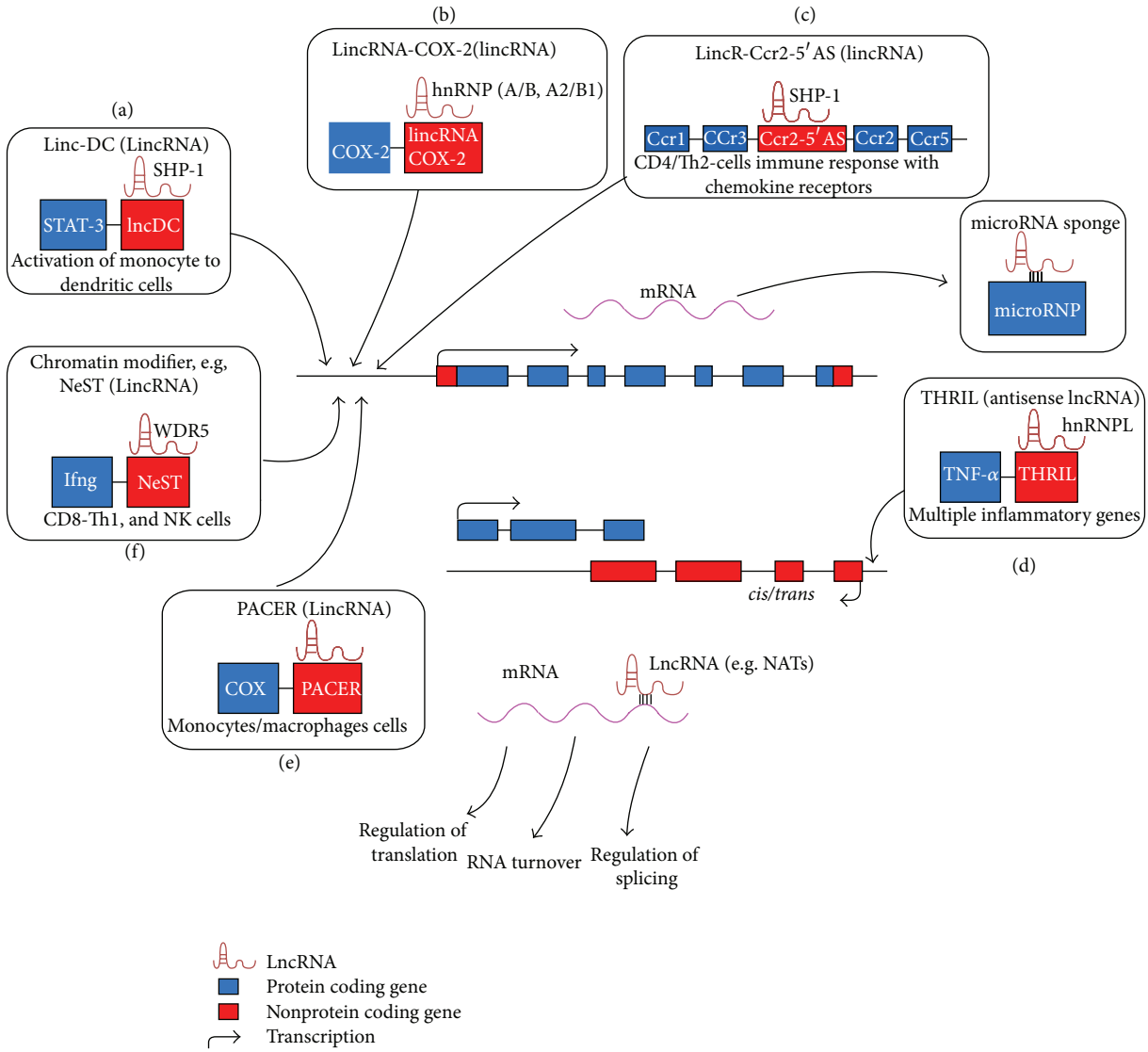

Figure 2: The functions of lncRNAs in autoimmune cells, T cells, B cells, macrophages, NK, and dendritic cells. (a) Lnc-DC expression is needed for differentiation of human monocytes into dendritic cells. Lnc-DC promotes STAT3 phosphorylation through inhibiting the action of Src homology region 2 domain-containing phosphatase-1 (SHP-1) [18]. (b) LincRNA-COX-2 is located in COX2 gene in mouse bone marrow-derived macrophages. It has extensive effects on inflammatory gene expression, repressing the transcription of anti-inflammatory genes in nonstimulated cells and promoting the expression of proinflammatory genes following Pam3Csk4 exposure via an interaction with hnRNP-A/B and A2/B1 [13]. (c) LincR-Ccr2-5′AS positively regulates the expression of genes involved in immunity particularly; lincR-Ccr2-5′AS regulates the transcription of several chemokine receptor genes in mouse, CD4+ TH2 cells, STAT-6 pathway [17]; (d) LincRNA (THRIL), as a key player in regulating the TNF-α and its expression was obviously lower during the acute phase of immune response. It was identified as an antisense lncRNA through a RNA-protein complex with hnRNPL and promotes TNF transcription [24]. (e) PACER is located upstream of the Cox2 transcriptional start site and is expressed in the antisense direction for innate immune response helping in gene expression, production of inflammatory mediators, and finally differentiation of monocytes/macrophages and dendritic cells [25]. (f) Chromatin modifier, for example, NeST, a lincRNA located downstream of Ifng which promotes the transcription of Ifng, WDR5, core submits of the MLL H3K4 methyltransferases, and facilitate the histone methylation at the Ifng locus, which promotes the transcription of Ifng in Th1 CD4+/CD8+ T cells. lncRNA also typically interacts with other transcripts and regulates miRNAs pathway, translation splicing, and RNA turnover [15]. LncRNAs characterized to regulate the abundance of genomically neighbouring (*cis- and trans*-acting) gene products [37].

another study, LncRNA, small antisense transcript of ZFAT gene expression, has been equally detected in CD4+ T cells, CD8+ T cells, CD19+ B cells, and CD14+ monocytes in autoimmune thyroid disease (AITD) [19]. Mycosis fungoides (MF) and Sezary syndrome are common form of cutaneous T cell lymphoma (CTCL); Sezary cell associated to LncRNAs (SeCATs) has been identified in a broad spectrum of normal human [20].

3.2. Long Noncoding RNA in B Cells. B lymphocytes are formed in human bone marrow. Principle functions of B cells are to make antibodies against antigen, to perform the role of APCs, and to develop into memory cells after activation by antigen interaction [55]. Interestingly, Gomez et al. [15] have demonstrated that the lncRNAs can regulate the immune response in animal model of infection with the help of T cells. In comparison to T cells, very little knowledge of B cells functions of lncRNAs is known; so far Bolland et al. [56] have explained a role of lncRNAs in the chromatin remodeling associated with the variable, diversity, and joining (V/D/J) genes recombination required to produce antigen receptors (Ig or TCR). Additionally, another study has shown that transcription of these antisense and sense lncRNAs is linked to looping of VH regions into close proximity with DJH region during recombination of pro-B cells; consequently it has been identified as the full transcriptome of sense and antisense transcripts throughout the Igh locus [21]. These processes occurred within transcription factors but the mechanism has not been broadly defined yet. A current study has revealed that profiling of lncRNAs expression during differentiation of monocyte into dendritic cells (Mo-DC) identified lnc-DC, which was exclusively upregulated [18]. Furthermore, this study showed significant characteristics of lnc-DC gene loci, which revealed that low or absent Lnc-DC expression in human blood CD20+ cells, B cells; CD14+, monocytes; CD34 cells, mobilized hematopoietic progenitor cells in human and H1-hESC (human embryonic stem cells) in periphery blood. Thus, lnc-DC is more exclusively expressed in human cDC (conventional dendritic cells) of the hematopoietic system than other DC markers. Functionally, Lnc-DC knockdown impacted protein coding genes, resulting in an antigen uptake, impaired allogenic CD4+ T cell proliferation, and reduced the strength of cytokine release. In LncRNAs, SAS-ZFAT gene was exclusively and specifically expressed in CD19+ B cells in peripheral blood lymphocytes and could have crucial roles in B cell function and determines the etiology of AITD [19].

3.3. Long Noncoding RNA in Macrophages. It has already been cleared through various studies that dead and dying cells in healthy persons are removed by macrophages in an anti-inflammatory environment. As antigen presenting cells, macrophages have an additional role optimizing the function of both the innate and acquired immune response [57]. The recognition and function of lncRNAs in monocytes/macrophages have not been studied abundantly. Not surprisingly, macrophages express the highest basal level of ptprj/CD148 (a tyrosine phosphatase that has tumor suppressor-like activity); its level gets changed by the treatments of LPS, TLR, and CSF-1 in different models. In this study they identified a 1,006 nucleotide long noncoding RNA species, ptprj-as1, that is transcribed antisense to ptprj. Transcribed ptprj-as1 is significantly expressed in macrophage enriched tissue, which was transiently induced by TLR ligands parallel to ptprj [22]. Thus, ptprj coding transcript may lead to modulation of inflammation directly linked with microphages. Myeloid (mDCs), CD11c+ dendritic cells (DCs, Antigen Presenting Cells) express lncRNAs-*COX2(Ptgs2)* when they are stimulated with lipopolysaccharide, an activator of TLR4 signaling through NFkB [23]. Similarly, Li et al. [24] have analyzed the change in expression of lincRNAs upon activation of innate immune signaling in THP1 macrophages and identified an unannotated LincRNAs, termed THRIL, as a key player in regulating the TNF-α and its expression was obviously lower during the acute phase of Kawasaki disease. LincRNA has shown that functions through a RNA-protein complex with hnRNPL [44] have critical role as regulator of physiological and pathological inflammatory immune responses. Many lncRNAs regulate transcription with hnRNPs (multifunctional nuclear RNA), have been identified as specific binding partners for lincRNA-cox2 in macrophages in both nuclear and cytoplasm, and are a major regulator of immune genes [13]. Likewise, LncRNAs PCAER, as a new potential target for Cox-2 modulation in inflammation and cancer, mediate Cox2 expression in human monocytes, as its direct upstream of the Cox2 transcriptional start side and expressed in the antisense directions. By this phenomenon widespread change in lncRNAs expression following the activation of innate immune response helps in gene expression, production of inflammatory mediators, and finally differentiation of monocytes into macrophages and dendritic cells [25].

3.4. Long Noncoding RNA in Natural Killer Cells. NK cells are a type of cytotoxic lymphocyte which is important to the innate immune system. The cytolytic activity of NK cells is modulated by the presence or absence of class I MHC molecules on target cells. NK cells use cell-surface receptors for class I MHC to assess the condition of target cells [58]. KIR (killer cell immunoglobulin-like receptor) is the major type of class I receptor expressed by NK cells. Wright et al. [26] reported the presence of an intron 2 promoter in several KIR genes that produce a spliced antisense transcript LncRNAs. The KIR antisense lncRNA is detected in progenitor cell lines and its overexpression in NK cells leads to decreased expression of KIR protein coding genes. KIR antisense lncRNA overlaps with KIR-coding exons 1 and 2, as well as the proximal promoter that is upstream of KIR. Transcription of KIR antisense lncRNA seems to be regulated by myeloid zinc finger one (MZF-1) that leads to silencing of KIR through an anonymous mechanism.

4. Long Noncoding RNA and Autoimmune Diseases

LncRNAs are coming into existence as new hopes in the autoimmune diseases paradigm demonstrating their potential roles in innate and adaptive immune system, which may be crucial players of autoimmunity. Here are few autoimmune diseases which have some association with lncRNAs expression though its role is not well established in the literature.

4.1. LncRNA in Systemic Lupus Erythematosus (SLE). Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease which involves a complicated interaction between the innate and the adaptive immune system,

loss of immunological tolerance to self-nuclear antigen, and antibody production [59]. The production of autoantibodies targeting double stranded DNA (dsDNA) and other nuclear autoantigens is the main characteristic of this disease [48]. Despite the huge numbers of research, the etiology of SLE remains subtle and it is thought that genetic and epigenetic predisposition joined with familiar and unfamiliar environmental factors play pivotal role in the development of SLE [60]. T cells, B cells, and dendritic cells are critical cells for SLE pathogenesis [61, 62]. Not surprisingly, the cellular and molecular role of small noncoding RNAs (miR-21, miR25, miR125, miR146a, and miR186, etc.) in the regulation and pathogenesis of SLE have been reported [52]. However, lncRNAs cellular and molecular mechanisms are still unexplored. Recently, Shi et al. [27] in their small cohort studies have compared the whole transcriptome analysis of purified monocytes from 9 female patients with SLE to the gene expression of 8 healthy controls; while doing so they found SLE specific alternative splicing, alternative polyadenylation, and novel loci transcription, an effect replicated by LPS treatment of control monocyte. In addition, the study identified the decrease expression of noncoding RNAs in Aicardi Goutieres syndrome, an infantile-onset disorder with features of lupus, in murine model to drive type I interferon. Pri-miRNA was clearly induced in SLE patients. These small noncoding RNAs are processed to suppress the translation and regulated the several messenger RNAs. Two specific pri-miRNAs were significantly upregulated in SLE monocyte compared with healthy controls, whereas two miRNAs showed decreased message levels, which shows that the pri-miRNA levels in SLE monocytes are functionally pertinent. Additionally, LncRNAs are less likely to get changed in SLE compared to other RNA classes, while the locations of some significantly changed lncRNA have suggested their involvement in SLE. For example, both *HIVEP2* itself and a lncRNAs about (800–1500 base) upstream of its TSS were significantly upregulated in SLE. Interestingly, LncRNAs which were located on chromosome 6q25.3 had commonly dysregulated in SLE monocytes. Moreover, protein coding genes TAGP, SOD2, WTAP, and ACAT2 transcription levels were all upregulated in SLE, while the other coding gene, FNDC1 transcription level, was low and downregulated in SLE.

4.2. LncRNA in Rheumatoid Arthritis (RA). Rheumatoid arthritis is a systemic autoimmune disorder characterized by chronic inflammation of synovial tissue that results in irreversible destruction of small to medium size joints [63]. The major cellular contributors in RA are T and B lymphocytes, neutrophils, macrophages, and proliferating fibroblast-like cells. Alteration of the synovial microenvironment by proinflammatory cytokines and chemokines attracts T, B, and APCs cells and encourages secretion of protease that promotes joint destruction [64]. Most of the studies in cultured cells and mammals have proved that micro-RNAs (miRNAs) play a critical role in the pathogenesis of RA (miR-124a, miR-146a, and miR-155) [51]. In human, within the past few years molecular studies exhibited a functional role of lncRNA in the cancer development. However, lncRNAs role in autoimmune disease like in RA is not well established. Recently, Müller et al. [28] have investigated ten patients, who were suffering from RA, and applied two biological treatments adalimumab (anti-TNF-α) and tocilizumab (anti-IL-6R); in the mean time they measured the serum level of cytokines TNF-α and IL-6, respectively, where they found that lincRNA (used total number, 7.419 lincRNA) has been regulated by TNF-α and superior to IL-6 in CD14 monocytes in vivo in human subjects with rheumatoid arthritis. These cytokines have a specific correlation with lincRNA transcription. Therefore, the interregulation of lincRNA may be important intracellular molecular effectors of different cytokines in cells of innate immune system in human in vivo in the context of rheumatoid arthritis.

4.3. LncRNA in Psoriasis. It is a hyperproliferative inflammatory skin diseases, PRINS (psoriasis—associated RNA induced by stress), a lncRNA that harbour two ALu elements, which is upregulated in the skin of patients with psoriasis, which contributes to psoriasis via the downregulation of G1P3, a gene coding protein with antiapoptotic effects in keratinocytes [29]. *In silico* structural homology studies have recommended that PRINS act as a noncoding RNA. PRINS is transcribed by RNA polymerase II and is expressed at different levels in various human tissues. Real time reverse transcription-PCR analysis exhibited that PRINS has higher expression in the uninvolved epidermis of psoriatic patients than in both psoriatic lesion and healthy epidermis, suggesting that PRINS has a role in psoriatic susceptibility. Furthermore, downregulating the RNA level of PRINS by RNA interference can impair cell viability after serum starvation but not under normal serum conditions. It was ascertained that PRINS may also function as a "riboregulator" to govern the expression of other genes involved in the proliferation and survival of cells revealed to stress.

4.4. LncRNA with Other Autoimmune Diseases

4.4.1. Autoimmune Thyroid Disease. Autoimmune thyroid disease (AITD) is caused by an immune response to self-thyroid antigens and has a significant genetic component. It includes Graves disease (GD) and Hashimoto's thyroiditis (HT) [65]. LncRNAs play crucial role in AITD. A study has revealed the importance of genetic component in AITD. Shirasawa et al. enrolled 515 affected individuals and 526 controls in a study and found the correlation of T-allele of SNP Ex9b-SNP10 to the increased risk for autoimmune thyroid disease [19]. The EX9b-SNP10 dwells in intron 9 of protein coding gene ZAFT (zinc-finger gene in ATD) and in the promoter region of LncRNA, SAS-ZFAT, which is the small antisense transcript of ZAFT gene. Being with the SNP (EX9b-SNP10), SAS-ZFAT expression gets exclusively upregulated, while it decreases the expression level of truncated-ZAFT (TR-ZFAT).

4.4.2. Sjögren's Syndrome (SS). Sjögren's syndrome is an autoimmune disease characterized by inflammation of exocrine glands mainly salivary and lachrymal glands, leading to

dry mouth and dry eye symptoms [66]. Typically age of onset is 40–50 and there is female predominance. The hallmark of the SS is B cell hyperactivity that is revealed by hypergammaglobulinemia, circulating immune complexes, and anti-Ro/SSA and anti-La/SSB autoantibodies [67]. A study of MSG (minor salivary gland) RNA samples, primary SS yielded 94 bp fragments of coxsackievirus B4 (CVB4) p2A genes, which may elucidate the possible role of CVB4 in induction and maintenance of primary Sjögren's syndrome [68]. However, in Pubmed and MeSH database the study of lncRNA in Sjögren's syndrome is not accessible yet.

5. Conclusion

In a nutshell, the LncRNAs are RNA transcript more than 200 nucleotides in length, which do not encode proteins and play a crucial role in autoimmune diseases such as SLE, RA, and psoriasis AITD, including various multigenetic human diseases. Activation, differentiation, and imbalance expression of immune cells, T cells, B cells, macrophages, and NK cells alter the autoimmunity which may have direct link to lncRNAs. However, the identification of lncRNAs expression in autoimmune diseases is largely unexplored. Majority of transcribed DNA encode noncoding RNAs. The relative proportion of noncoding genomic DNAs increases the developmental complexity which signifies that ncRNAs may serve more critical biological functions in autoimmune diseases that may resemble different organ cancers. A better understanding of long noncoding RNAs is emerging as key regulators of diverse biological process especially by immune cells and the molecular mechanism of autoimmunity. Based on aforementioned studies (Table 2) we can conclude that determining individual role of lncRNAs in autoimmune diseases remains a challenge, additional studies of lncRNAs are very essential for the better understanding of autoimmune diseases.

Abbreviations

Air:	Antisense Igf2r
AITD:	Autoimmune thyroid disease
ANRIL:	Antisense noncoding RNA in the INK4 locus
BACE1-AS:	β-site amyloid precursor protein- (APP-) cleaving enzyme
CNS:	Central nervous system
Gas5:	Growth-arrest-specific 5
HIVEP2:	Human immunodeficiency virus type I enhancer binding protein-2
hnRNPL:	Heterogeneous nuclear ribonucleoprotein L
HOTAIR:	HOX antisense intergenic RNA
Ifng:	Interferon gamma
KIR:	Killer cell immunoglobulin-like receptor
lincRNA:	Long intergenic noncoding RNA
lncRNA:	Long noncoding RNA
lnc-DC:	Long noncoding dendritic cells
LPS:	Lipopolysaccharide
MALAT1:	Metastasis-associated lung adenocarcinoma transcript 1
MIAT:	Myocardial infarction-associated transcript
miRNA:	microRNA
MLLH3k4:	Mixed lineage leukemias histone H3 at lysine 4
NATs:	Natural antisense transcripts
NeST:	Nettoie Salmonella pas Theiler's
pri-miRNAs:	Primary miRNAs
SLE:	Systemic lupus erythematosus
STAT3:	Signal transducers and activator of transcription 3
TLR:	Toll-like receptor
THRIL:	TNF-α and hnRNPL related immunoregulatory lincRNA
TSS:	Transcription start site
XIST:	X-inactive-specific transcript.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Authors' Contribution

Keshav Raj Sigdel and Ao Cheng contributed equally to this work.

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Research Article

Umbilical Cord-Derived Mesenchymal Stem Cells Inhibit Cadherin-11 Expression by Fibroblast-Like Synoviocytes in Rheumatoid Arthritis

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This study aimed to determine whether umbilical cord-derived mesenchymal stem cells (UCMSC) regulate Cadherin-11 (CDH11) expression by fibroblast-like synoviocytes (FLS) in rheumatoid arthritis (RA). FLS were isolated from the synovium of RA and osteoarthritis (OA) patients. FLS from RA patients were cocultured with UCMSC in a transwell system. CDH11 mRNA levels in FLS were tested, and levels of soluble factors expressed by UCMSC, such as indoleamine 2,3-dioxygenase (IDO), hepatocyte growth factor (HGF), and interleukin- (IL-) 10, were determined. IDO, HGF, and IL-10 were upregulated in cocultures, so that appropriate inhibitors were added before determination of CDH11 expression. The effects of UCMSC on arthritis were investigated in the collagen-induced arthritis (CIA) model in Wistar rats. FLS from RA patients expressed higher CDH11 levels than those from OA patients, and this effect was suppressed by UCMSC. The inhibitory effect of UCMSC on CDH11 expression by FLS was abolished by suppression of IL-10 activity. CDH11 expression in synovial tissues was higher in the context of CIA than under basal conditions, and this effect was prevented by UCMSC administration. IL-10 mediates the inhibitory effect of UCMSC on CDH11 expression by FLS, and this mechanism might be targeted to ameliorate arthritis.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by progressive destruction of joint. The primary site of inflammation in RA is the synovium, and hyperplasia of the synovial intimal lining is a hallmark of this disease. The synovial intimal lining is a loosely organized collection of cells that forms an interface between the synovium and the synovial fluid space. Macrophage-like cells and fibroblast-like synoviocytes (FLS) are the two major cell types in the lining. The intimal lining cells lack tight junctions and a definite basement membrane. Cadherins are single-pass transmembrane glycoproteins that mediate homophilic adhesion between cells [1]. Cadherin-11 (CDH11) is a type II cadherin predominantly expressed by FLS but not by macrophages or other cells of hematopoietic origin residing in the synovium. CDH11 plays a prominent role in the formation and organization of the synovial lining layer [2]. Recent research showed that CDH11 could regulate inflammation mediated by FLS [3] and promote migration of FLS and erosion of cartilages and bones [4]. This evidence indicates that CDH11 expressed by FLS plays an important role in RA pathogenesis.

Umbilical cord-derived mesenchymal stem cells (UCMSC) are multipotent stem cells that exhibit immune regulatory functions. UCMSC were reported to decrease the levels of proinflammatory cytokines and inhibit joint swelling and cartilage erosion. In the past, our team has carried out UCMSC transplants in RA patients, a treatment that improved symptoms of the disease [5]. However, the mechanisms that mediated the beneficial effects of UCMSC in RA patients, such as prevention of cartilage erosion, remain unclear.

In this study, we explored the effects of UCMSC transplantation on the expression of CDH11 in FLS from RA patients, and we investigated the mechanism whereby UCMSC ameliorate RA symptoms.

2. Materials and Methods

2.1. Harvesting of the Synovium and Umbilical Cord. Synovium samples were obtained from thirteen patients undergoing total knee arthroplasty at Drum Tower Clinical Medical College of Nanjing Medical University. Eight patients fulfilled the American College of Rheumatology criteria for the classification of RA and they had no other autoimmune or systemic diseases. One of the patients was male and seven were females, with the average age of 56.1 ± 11.1 years. Their average disease duration was 10.5 ± 5.8 years. Synovial tissues were also obtained from five osteoarthritis (OA) patients, 2 males and 3 females, with the average age of 56.8 ± 7.2 years. Their average disease duration was 8.0 ± 3.4 years. Umbilical cords were resected under sterile conditions during two natural deliveries in Drum Tower Clinical Medical College of Nanjing Medical University. The study protocol was approved by the ethics committee of the Drum Tower Clinical Medical College of Nanjing Medical University. Written informed consent was obtained from all donors.

2.2. Isolation and Culture of FLS and UCMSC. Synovial tissues were obtained from RA and OA patients and minced under sterile conditions. Synovial tissues were digested with collagenase I (Sigma-Aldrich, Saint Louis, Missouri, USA) at a concentration of 1 mg/mL for 4 hours (37°C, 5% CO_2), collected, and washed twice with phosphate buffered saline (PBS). Subsequently, cells were obtained by centrifugation and cultured in DMEM/F12 with 10% fetal bovine serum (FBS) (Gibco, Australia), which was changed every three days. Upon reaching 80% confluence, cells were detached from the culture substrate by exposure to 0.25% Trypsin-EDTA (Gibco, USA) and seeded on a surface 3 times larger than the original culture substrate. After 3 passages, expression of FLS markers was documented, and cells were used in the described studies.

Wharton jelly was obtained from umbilical cords following removal of the vessels and subsequently minced. Fragments were transferred to a culture flask in the presence of DMEM/F12 with 10% FBS. Every 7 days, half of the culture medium was changed. Adherent cells at the bottom of the culture flask were digested by 0.25% Trypsin-EDTA and passaged. Subsequently, the culture medium was changed every 3 days. After 3 passages, flow cytometry was carried out to identify UCMSC with selected mesenchymal stem cells surface markers, such as CD14, CD29, CD34, CD44, CD45, CD73, CD90, and HLA-G (eBioscience, USA). Cells from passages 3 to 8 were used in the reported studies.

2.3. Flow Cytometry. After 3 passages in culture, FLS and UCMSC were detached from the culture substrate and washed in PBS. FLS and UCMSC were incubated with appropriate antibodies, such as fluorescein isothiocyanate-(FITC-) conjugated anti-HLA-G, CD4, CD14, CD29, CD34, CD44, CD45, CD55, CD73, and CD90 (eBioscience, USA) for surface marker staining and then were maintained in the dark at 4°C for 30 min before washing and resuspension in PBS. These surface markers were detected by flow cytometry.

2.4. Coculture of FLS and UCMSC. UCMSC were seeded on transwells with a 0.4 μm pore size, whereas FLS from RA patients were seeded on 6-well plates. After reaching confluence, FLS were transferred to the transwell system and cocultured with UCMSC for 72 hours. Subsequently, cells were harvested to measure CDH11 expression in FLS and levels of soluble factors produced by UCMSC. Selected soluble factors inhibitors such as 1-MT, or an anti-IL-10 or anti-HGF antibodies (R&D Systems, Minneapolis, USA) were added to the coculture system.

2.5. Collagen-Induced Arthritis (CIA) and Cell Transplantation. Wistar rats were purchased from Vital River Laboratory Animal Technology Co. Ltd. The CIA procedure entailed emulsification of collagen type II (CII) with Freund's complete adjuvant (Sigma-Aldrich, Saint Louis, Missouri, USA) and administration of the emulsion to 8-week-old rats by intracutaneous injection. After 14 days, rats were administered a second injection of CII emulsified with Freund's incomplete adjuvant. Based on clinical scores, rats were monitored for signs of arthritis onset. Clinical arthritis was scored on a scale of 0 to 3, where 0 = no swelling, 1 = slight swelling and erythema, 2 = pronounced edema, and 3 = joint rigidity. Each limb was graded, and the grades were summed to yield the arthritis score for each animal (maximum possible score 12 per animal) [6]. After 17 days, 1×10^6 UCMSC or FLS or 1 mL PBS was administered by tail vein injection [7]. Rats were sacrificed after 42 days, and synovial tissues were collected.

2.6. Histologic Analysis. Formalin-fixed limbs were decalcified and paraffin-embedded using standard histologic techniques. Serial 4 μm sections were cut and stained with hematoxylin and eosin to examine morphologic features.

Formalin-fixed, paraffin-embedded tissue sections of 3 μm were placed on adhesive-coated slides. In the process of a heated antigen retrieval process, the slides were immersed in EDTA buffer (pH 8.0) and heated for 2 min in the steamer. The slides were incubated overnight at 4°C with monoclonal antibodies to CDH11 (P707, Abcam) with 1 : 50 dilution in bovine serum albumin before incubating with Envision Detection System (k5007, Dakocytomation, Glostrup, Denmark) at room temperature for 20 min. Color was developed by reaction with DAB solution for 10 min followed by counterstaining with Harris hematoxylin, dehydrated, coverslipped, and reviewed under light microscope.

2.7. RNA Isolation and Real-Time PCR. Cocultured cells, FLS from OA patients, and synovium from rats subjected to CIA were harvested in 500 μL of Trizol (Invitrogen, Van Allen Way, Carlsbad, California); 70 μL chloroform was added, and the solution was mixed. Subsequently, the sample and

Table 1: Primers for real-time PCR.

Gene	Forward	Reverse
h-GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
h-CDH11	GTGCATGCCAAAGACCCTGA	CTGCTGCAAAGACAGTGATGTTGA
IDO	GAATGGCACACGCTATGGAA	CAGACTCTATGAGATCAGGCAGATG
COX2	TGACCAGAGCAGGCAGATGAA	CCACAGCATCGATGTCACCATAG
HGF	GTCAGCCCTGGAGTTCCATGATA	AGCGTACCTCTGGATTGCTTGTG
IL-10	GAGATGCCTTCAGCAGAGTGAAGA	AGTTCACATGCGCCTTGATGTC
TGF-β	AGCGACTCGCCAGAGTGGTTA	GCAGTGTGTTATCCCTGCTGTCA
HLA-G	CCTTGCAGCTGTAGTCACTGGA	CACACAGGGCAGCTGTTTCA
r-GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA
r-CDH11	TGCTGCCAACAGCCCAATAA	GAGATGTTGAGCCAGGCAGTTTC

h: human; r: rat.

Trizol/chloroform mix were centrifuged at 11440 r/min for 15 min, the upper layer was collected, and an equal volume of isopropanol was added before incubation at room temperature for 10 min. Samples were centrifuged at 11440 r/min for 15 min and the supernatant was discarded. The total RNA pellets were washed with 75% ethanol and dissolved with water treated with diethyl pyrocarbonate (DEPC) 0.05 μg/μL. Reverse transcription of mRNA was carried out with Reverse transcription kits.

The real-time PCR reaction contained SYBR Premix Ex Taq 10 μL, ROX Reference Dye (50x) (Takara, DaLian, China) 0.4 μL, cDNA 2 μL, and dH_2O 6.8 μL, for a total volume of 20 μL. All reactions were conducted in duplicate. The $2^{-\Delta\Delta Ct}$ method was used to normalize expression of target genes mRNA to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression [8]. Primers for real-time PCR were shown in Table 1.

2.8. Western Blot. Cells were washed with ice-cold PBS and lysed in RIPA lysis buffer for 30 min on ice. Equal amounts of protein of cell lysates were separated on 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and analysed using anti-CDH11 (P707) antibody (Cell Signaling Technology). Horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulin G (Jackson ImmunoResearch) was used as secondary antibody. The target proteins were detected using an enhanced chemiluminescence detection kit (Millipore).

2.9. Statistical Analysis. Data are expressed as means ± SD. SPSS 13.0 was used to analyze data and GraphPad Prism 5 to draw graphs. Two independent groups were compared by unpaired t-test and multiple independent groups by one-way analysis of variance. $P < 0.05$ was considered significant.

3. Results

3.1. Identification of UCMSC and FLS. To ensure the MSC nature of UCMSC, we harvested umbilical cord-derived cells after 3 passages in culture and detected selected cell surface markers, such as HLA-G, CD14, CD29, CD34, CD44, CD45, CD73, and CD90 by flow cytometry. Cells isolated from umbilical cords were positive for CD29, CD44, CD73, and CD90 but negative for HLA-G, CD14, CD34, and CD45 (Figure 1(a)).

In the joints of RA patients, synoviocytes could be classified in scavenger synovial cells and FLS. Furthermore, inflammatory T cells were observed in the joint. Therefore, presence of the T cell marker CD4, of the macrophage marker CD14, and of HLA-G, CD44, CD55, and CD90 was tested in synoviocytes after 3 passages in culture. Neither CD4 nor CD14 was detected, whereas HLA-G was expressed marginally, indicating low immunogenicity. Cells were positive for the fibroblast markers CD44 and CD90. More than half of the synoviocytes expressed CD55, meaning that the majority of FLS originated from the lining layer (Figure 1(b)).

3.2. UCMSC Inhibit CDH11 Expression in FLS from RA Patients In Vitro. Since CDH11 is critical for cartilage destruction in RA, we compared CDH11 expression by FLS from RA and OA patients. CDH11 expression was higher in FLS from RA patients than in those from OA patients at the mRNA (1.76 ± 0.51 versus 1.02 ± 0.20, $P < 0.01$) and protein level (Figures 2(a)–2(c)).

Since we reported that UCMSC transplantation could ameliorate RA symptoms, we hypothesized that UCMSC might inhibit cartilage erosion by decreasing CDH11 expression in FLS. To test this hypothesis, we cocultured RA FLS with UCMSC and measured changes in CDH11 expression. UCMSC downregulated CDH11 expression in FLS at the mRNA (1.34 ± 1.24 versus 0.73 ± 0.30, $P < 0.05$) and protein level (Figures 2(b) and 2(c)).

3.3. Changes in Levels of Soluble Factors Expressed by UCMSC. UCMSC and FLS cocultured in the transwell systems were not in direct contact. Therefore, soluble factors mediated the inhibitory effects of UCMSC on CDH11 expression in FLS. In previous studies, multiple factors such as IDO, cyclooxygenase2 (COX2), HGF, IL-10, transforming growth factor- (TGF-) β, and human leukocyte antigen- (HLA-) G were reported to facilitate the inhibitory effects of MSC on inflammation. Expression of these soluble factors by UCMSC was tested, and we observed that IDO (1.27 ± 1.00 versus

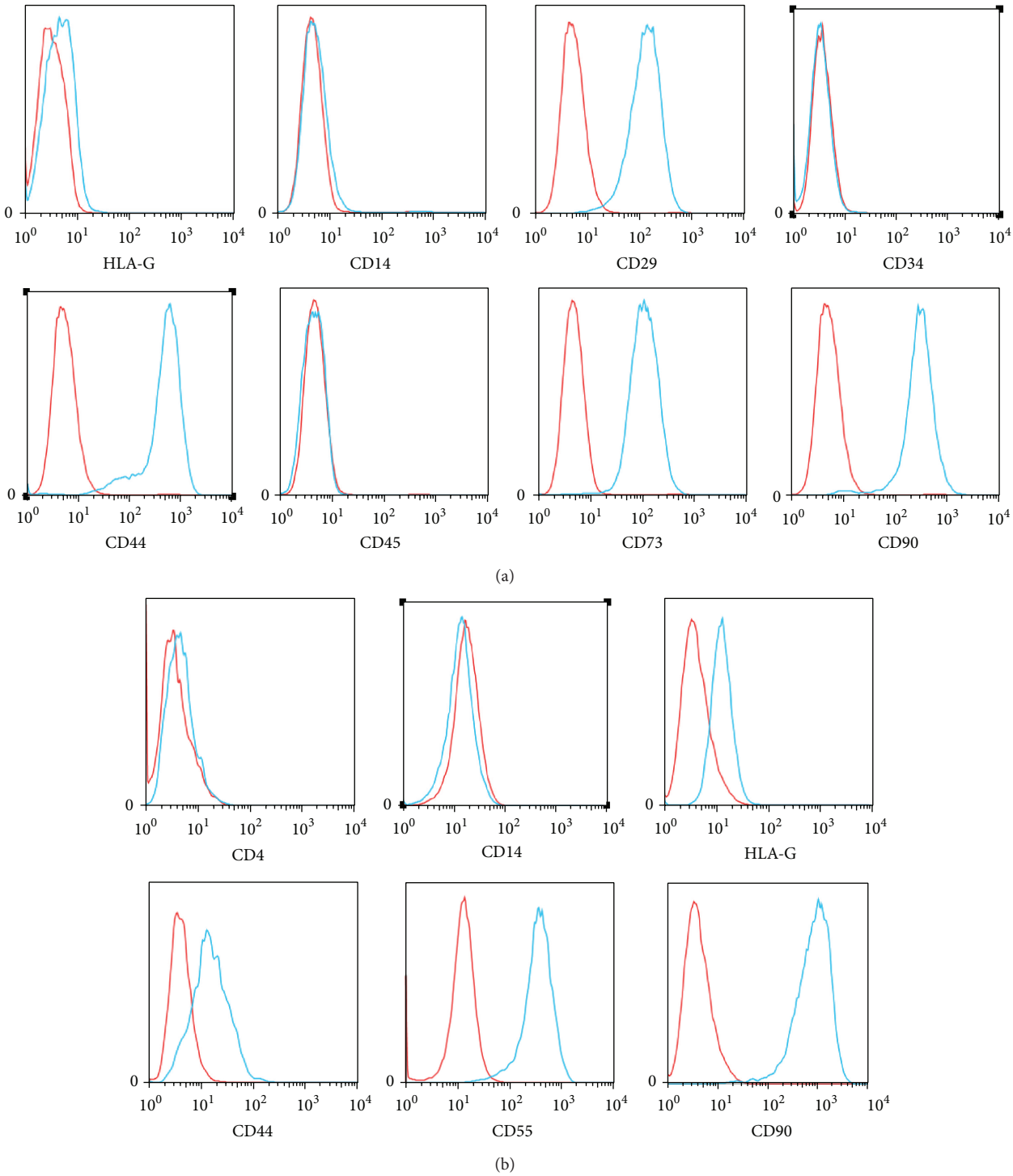


Figure 1: Identification of UCMSC and FLS. (a) Expression of the UCMSC markers: HLA-G, CD14, CD29, CD34, CD44, CD45, CD73, and CD90. (b) Expression of FLS markers: HLA-G, CD4, CD14, CD55, CD44, and CD90.

48.90 ± 44.79, $P < 0.05$), HGF (1.03 ± 0.26 versus 52.85 ± 55.69, $P < 0.05$), and IL-10 (1.99 ± 2.00 versus 12.38 ± 20.58, $P < 0.05$) were upregulated in cocultures with FLS. Conversely, changes in COX2 (1.23 ± 0.77 versus 0.91 ± 1.10, $P > 0.05$), TGF-β (1.11 ± 0.57 versus 1.03 ± 1.04, $P > 0.05$), or HLA-G (1.19 ± 0.73 versus 0.71 ± 0.71, $P > 0.05$) expression were modest (Figure 3).

3.4. Suppression of IL-10 Prevents the Inhibitory Effects of UCMSC on CDH11 Expression. We reported that UCMSC

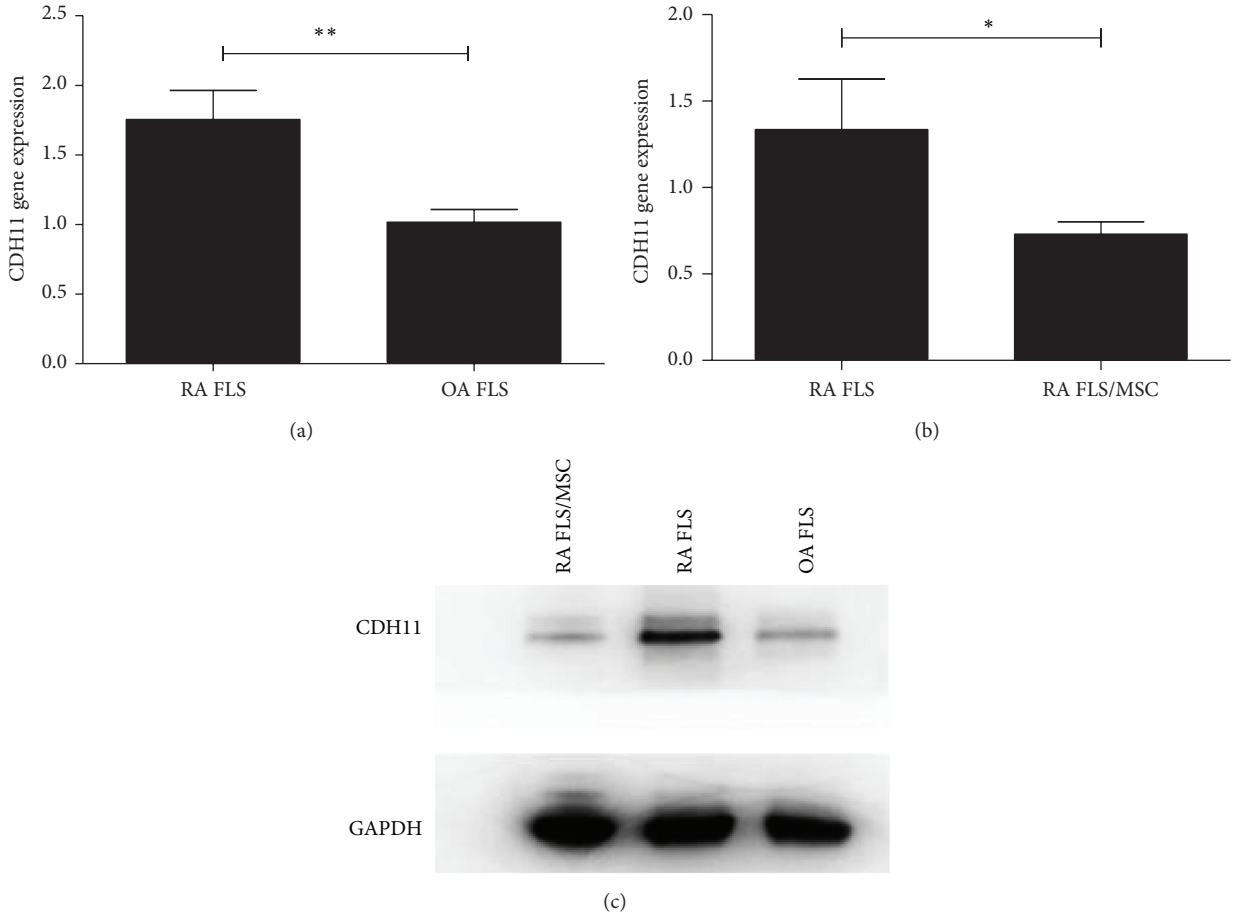


Figure 2: Effects of UCMSC on CDH11 expression by FLS from RA patients (a)–(c). Expression of CDH11 mRNA (a) and protein (c) by FLS from RA patients. RA: rheumatoid arthritis; OA: osteoarthritis; MSC: mesenchymal stem cells; CDH11: cadherin-11. $^{*}P < 0.05$; $^{**}P < 0.01$ (b)-(c). Expression of CDH11 mRNA (b) and protein (c) by FLS from RA patients, cocultured with UCMSC. RA: rheumatoid arthritis; OA: osteoarthritis; MSC: mesenchymal stem cells; CDH11: cadherin-11. $^{*}P < 0.05$; $^{**}P < 0.01$.

cocultured with FLS from RA patients expressed higher levels of IDO, HGF, and IL-10, and these molecules might mediate the inhibitory effect of UCMSC on CDH11 expression by FLS. Therefore, UCSMC and FLS were cocultured in the presence of IDO, HGF, and IL-10 inhibitors to determine the contribution of each factor to the effect of UCMSC on CDH11 expression by FLS. An anti-IL-10 antibody opposed the inhibitory effect of UCMSC on CDH11 expression (1.28 ± 0.60 versus 0.52 ± 0.10, $P < 0.01$). Conversely, an anti-HGF antibody (1.32 ± 1.44 versus 0.52 ± 0.10) or the IDO inhibitor 1-MT (1.28 ± 1.58 versus 0.52 ± 0.10) had no impact on the effect of UCMSC on CDH11 expression (Figure 4).

3.5. UCMSC Transplantation Prevented Tissue Damage in CIA. As shown in Figure 5(a), the severity of CIA was progressively attenuated in UCMSC treated rats, as compared with FLS and PBS treated rats. The therapeutic effects of UCMSC on CIA in rats were further verified by histological examination at the endpoint of clinical study. We observed that control rats exhibited a marked severe synovitis, cartilage erosion. In contrast, the majority of joints from rats injected with UCMSC had normal morphology with a smooth articulation cartilage surface (Figure 5(b)).

3.6. UCMSC Transplantation Decreased CDH11 Levels in the Synovial Tissue of Rats Subjected to CIA. UCMSC have negligible immunogenic potential, and as a consequence these cells have been used in therapies for autoimmune diseases in both humans and animals. UCMSC were transplanted in rats subjected to CIA, and PBS or fibroblasts were used as control. Following transplant, CDH11 mRNA levels in synovial tissues of rats subjected to CIA and administered UCMSC were similar to those in synovial tissues of normal rats (0.65 ± 0.91 versus 0.94 ± 0.46). Accordingly, CDH11 mRNA levels in rats administered UCMSC were lower than which in synovial tissues of rats subjected to CIA, either in the context of fibroblast or PBS administration (0.65 ± 0.91 versus 6.29 ± 6.45 versus 5.92 ± 7.51) (Figure 6(a)). Our immunohistochemical data showed that the expression of CDH11 increased remarkably in synovial tissues of rats subjected to CIA, whereas it decreased significantly after UCMSC transplantation (Figure 6(b)).

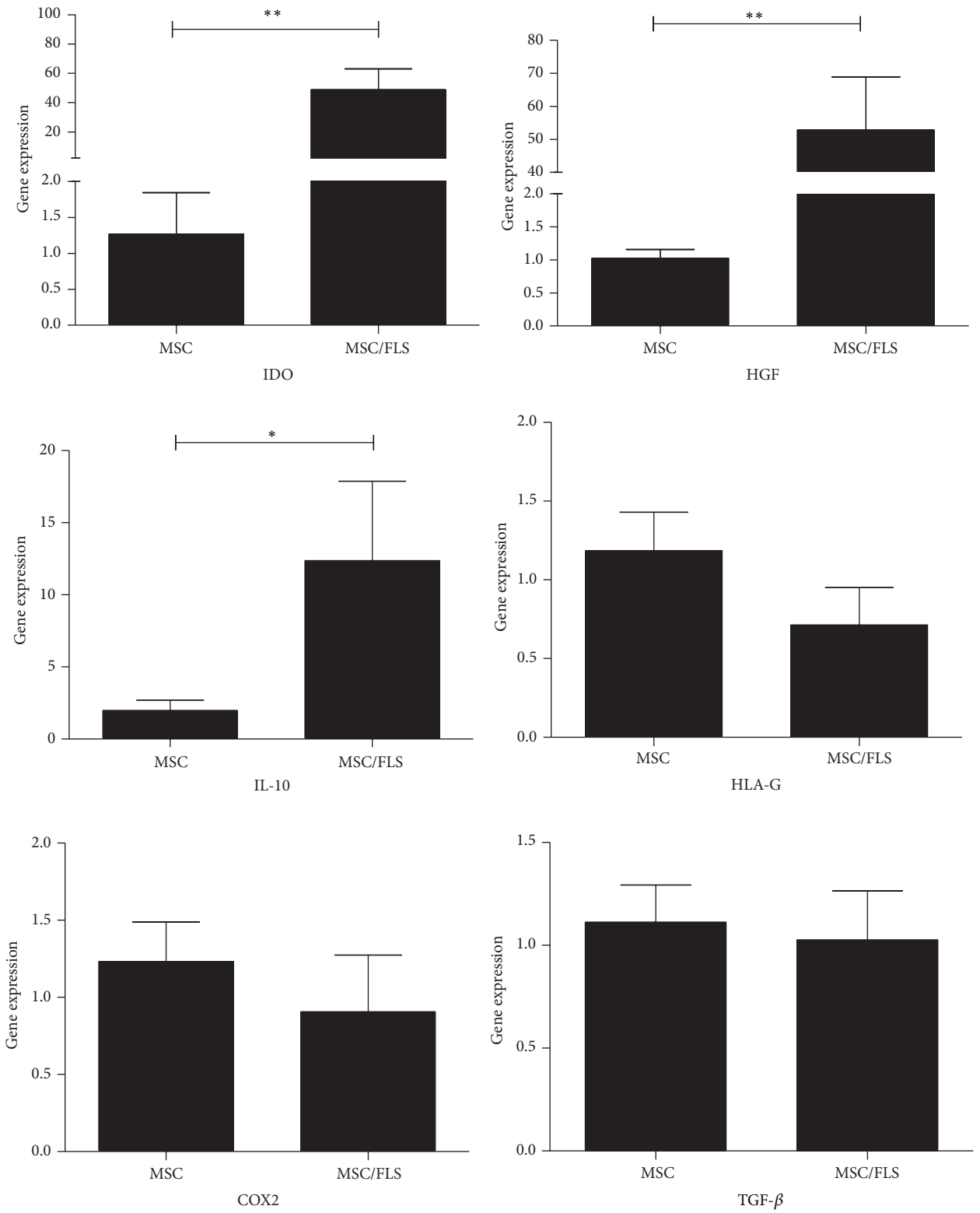


Figure 3: Soluble factors expressed by UCMSC. Expression of COX-2, HGF, HLA-G, IDO, TGF-β, and IL-10 by UCMSC cocultured with FLS. MSC: mesenchymal stem cells; FLS: fibroblast-like synoviocytes. $^{*}P < 0.05$; $^{**}P < 0.01$.

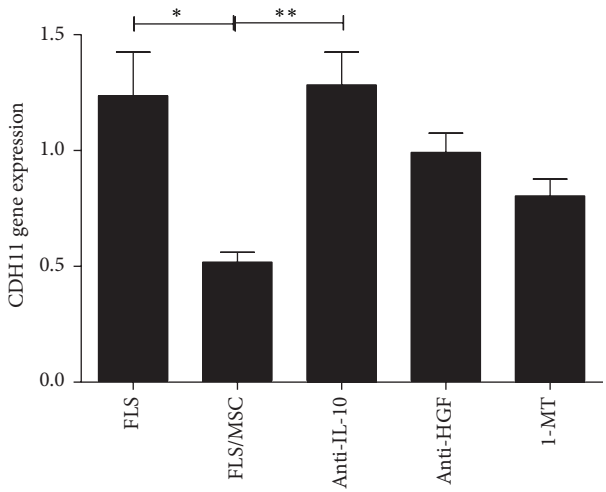


Figure 4: Suppression of soluble factors prevented the inhibitory effect of UCMSC on CDH11 expression by FLS from RA patients. Expression of CDH11 by FLS from RA patients, cocultured with UCMSC in the presence of anti-IL-10, anti-HGF antibody, or 1-MT. MSC: mesenchymal stem cells; FLS: fibroblast-like synoviocytes; CDH11: cadherin-11. $^{*}P < 0.05$; $^{**}P < 0.01$.

4. Discussion

RA is an autoimmune disease characterized by chronic proliferation of synovial cells and progressive joint damage. In the synovium of RA patients, proliferation of FLS, the most prominent cells in the lining layer, plays an important role in thickening of the synovium. Previous research demonstrated that activated FLS cause arthritis and cartilage degradation [9, 10]. In addition, it was reported that FLS act as cancer-like cells and are able to migrate and cause inflammation and degradation of other joints [11].

FLS express CDH11, an adhesion molecule that might mediate formation of the lining layer and preserve the integrity of the synovial tissue by mediating FLS-to-FLS interactions. A previous study demonstrated that the normal synovial lining layer was absent in *CDH11*$^{-/-}$ mice [12]. CDH11 might promote the secretion of interleukin- (IL-) 6, a critical inflammatory cytokine in the pathogenesis of RA [2]. Alternatively, CDH11 may induce the expression of IL-8, macrophage migration inhibitory factor (MIF) and monocyte chemotactic protein- (MCP-) 1 [3]. IL-8 and MCP-1 are chemokines that contribute to macrophage migration, an event that plays an important role in arthritis. Furthermore, IL-8 could promote the aggregation of neutrophils and angiogenesis [13]. *MIF*$^{-/-}$ mice are protected from inflammation observed in the CIA model [14], indicating that MIF is involved in cell migration, infiltration, and inflammation processes. CDH11 induces a robust MIF expression, an effect that was not mediated by tumor necrosis factor- (TNF-) α [15]. *CDH11* inactivation in mice prevents infiltration of inflammatory cells and erosion of cartilage by the pannus that are observed in the context of CIA mice [12]. These findings demonstrate that CDH11 is critical for the development of joint inflammation in RA. CDH11 could favor migration of FLS, thereby promoting the ability of these cells to erode cartilage [4]. CDH11 is expressed by multiple tumor cells of epithelial origin and correlated with poor differentiation and cancer aggressiveness [16]. This evidence illustrated that CDH11 is correlated to bone erosion and contributes to joint damage of RA. In addition, CDH11 activates MAPK and NF-κB in FLS, thereby promoting secretion of matrix metalloproteinases (MMP) [17]. OA is a disease of joint degeneration characterized by joint inflammation and cartilage degradation, although these effects are milder than in RA. In vitro studies reported that levels of secreted proinflammatory cytokines and MMP are higher in FLS from RA than in those from OA patients [18]. In our study, we demonstrated higher levels of CDH11 in RA FLS both in vitro and in vivo. This event possibly leads to a positive feedback loop where FLS activation by CDH11 increases expression of inflammatory factors and MMP, thereby exacerbating inflammation and cartilage degradation in RA.

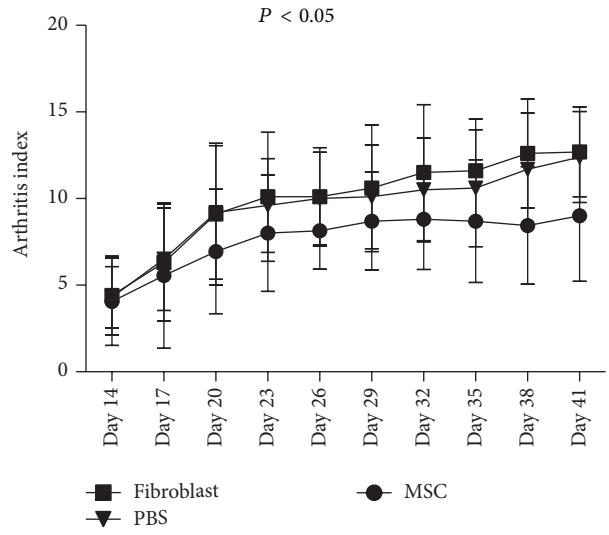
UCMSC are characterized by their self-renewal and multilineage differentiation potential, immunoregulatory properties, and low immunogenicity, making them promising candidates in therapeutic approaches in the field of cell therapy and tissue engineering [19]. Previous researches showed that UCMSC ameliorated symptoms of systemic lupus erythematosus (SLE) [20] and Crohn's disease [21] by secreting soluble factors that mitigate lymphocytes dysfunction. IL-10 secreted by Th2 cytokine inhibits Th1 cells and the activity of inflammatory cytokines, such as interferon-γ, IL-2, IL-12, and TNF-α [22]. *IL-10*$^{-/-}$ mice are sensitized to the joint inflammation caused by CIA. Accordingly, inhibition of IL-10 expression in the synovium induces IL-1 and TNF-α levels, two major dominant proinflammatory cytokines in RA [23], indicating that IL-10 has a protective function in RA. We report that CDH11 expressed by FLS from RA patients is downregulated following coculture with UCMSC, an effect mediated by soluble factors. IDO, HGF, and IL-10 are regulatory molecules secreted by UCMSC [24, 25], and we observed that levels of these factors were upregulated following coculture with FLS. Suppression of IL-10, but not of IDO or HGF, activity precluded the inhibitory function of UCMSC on CDH11 expression. Accordingly, synovium from rats subjected to CIA expressed higher levels of CDH11, an event observed in RA patients. UCMSC transplantation downregulated CDH11 expression levels in the synovium, suggesting that UCMSC inhibit CDH11 expression in RA FLS by secreting IL-10. This event precludes the ability of FLS from RA patients to migrate and erode cartilage, thereby improving arthritis.

5. Conclusion

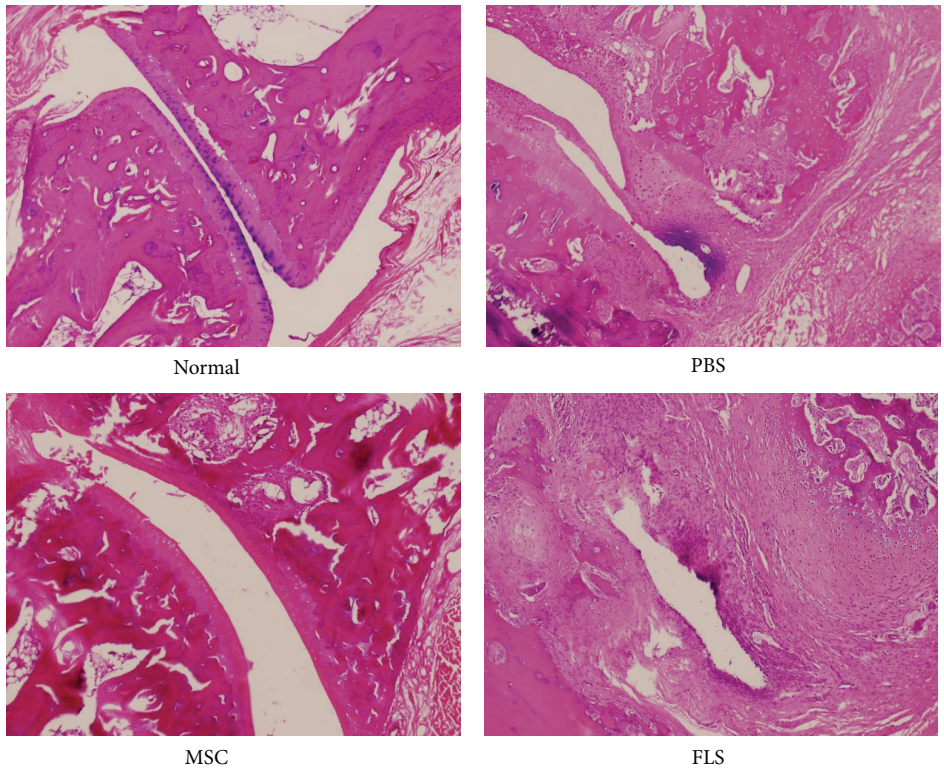
IL-10 mediates the inhibitory effect of UCMSC on CDH11 expression by FLS from RA patients, and this mechanism might be targeted to ameliorate arthritis.

Conflict of Interests

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



(a)



(b)

Figure 5: UCMSC prevented tissue damage in CIA. (a) The severity of CIA was progressively attenuated in UCMSC treated rats, as compared with PBS and FLS treated rats. $P < 0.05$. (b) H&E-stained sagittal sections of joints from CIA rats. PBS and FLS treated rats showed a marked severe synovitis and cartilage erosion. However, the majority of joints from mice injected with UCMSC had normal morphology with a smooth articulation cartilage surface. Magnification, 100x. UCMSC: umbilical cord-derived mesenchymal stem cell; FLS: fibroblast-like synoviocytes; CIA: collagen-induced arthritis.

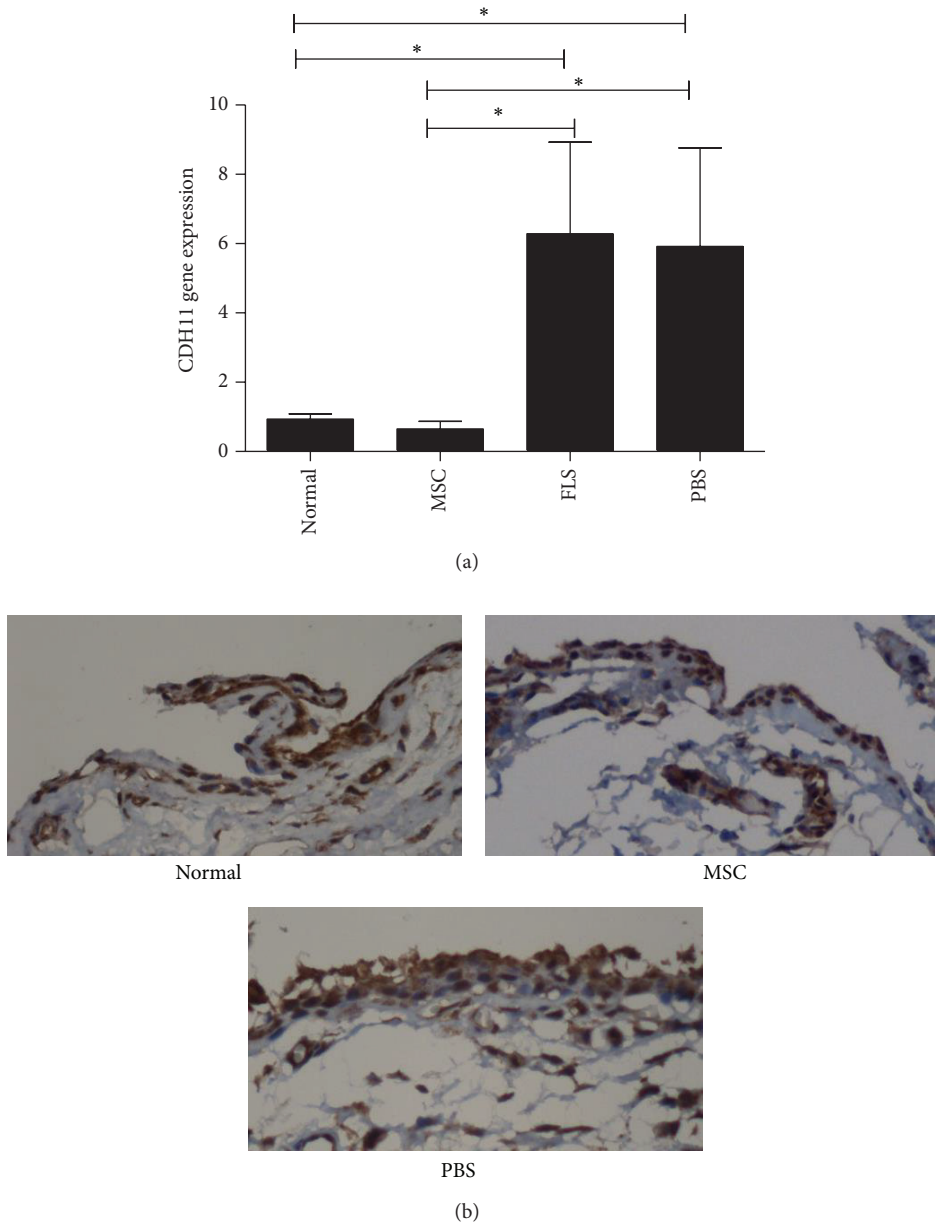


Figure 6: UCMSC transplantation inhibited expression of CDH11 induced by CIA in synovial tissue of rats. (a) Rats subjected to CIA were administered PBS, UCMSC, or fibroblasts. CDH11 mRNA levels in synovial tissues of rats subjected to CIA and administered UCMSC were similar to those in synovial tissues of normal rats, whereas which were lower than in the context of fibroblast or PBS administration. $^{*}P < 0.05$. (b) The expression of CDH11 increased remarkably in synovial tissues of rats subjected to CIA, whereas which decreased significantly after UCMSC transplantation. Magnification, 400x. UCMSC: umbilical cord-derived mesenchymal stem cell; CIA: collagen-induced arthritis; CDH11: cadherin-11.

Authors' Contribution

Cheng Zhao and Lu Zhang are co-first authors.

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Research Article

Decreased Frequency of Circulating Myelin Oligodendrocyte Glycoprotein B Lymphocytes in Patients with Relapsing-Remitting Multiple Sclerosis

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Although there is no evidence for a role of anti-MOG antibodies in adult MS, no information on B lymphocytes with MOG-committed BCR is available. We report here on the frequency of anti-MOG B cells forming rosettes with polystyrene beads (BBR) covalently bound to the extracellular domain of rhMOG in 38 relapsing-remitting patients (RRMS) and 50 healthy individuals (HI). We show a substantial proportion of circulating anti-MOG-BBR in both RRMS and HI. Strikingly, MOG-specific B cells frequencies were lower in MS than in HI. Anti-MOG antibodies measured by a cell-based assay were not different between MS patients and controls, suggesting a specific alteration of anti-MOG B cells in MS. Although anti-MOG-BBR were higher in CNS fluid than in blood, no difference was observed between MS and controls. Lower frequency of MOG-BBR in MS was not explained by an increased apoptosis, but a trend for lower proliferative capacity was noted. Despite an efficient B cell transmigration across brain derived endothelial cells, total and anti-MOG B cells transmigration was similar between MS and HI. The striking alteration in MOG-specific B cells, independent of anti-MOG antibody titers, challenges our view on the role of MOG-specific B cells in MS.

1. Introduction

Multiple sclerosis is a chronic inflammatory disease characterized by leukocyte infiltration and white matter demyelination [1]. Among lymphocytes, T cells are prevalent in inflammatory lesions [2] and anti-myelin T cell frequency was found increased in MS patient blood [3]. Myelin antigen-specific T lymphocytes have attracted a great deal of attention due to their potential for induction by adoptive transfer experimental allergic encephalomyelitis (EAE), an animal model of the immune component of the disease [4–6]. However, myelin reactive T cells are also found in healthy individuals (HI) [3, 7]. Antibodies against myelin derived possible autoantigens, and particularly anti-MOG, have been widely studied with no clear evidence of linkage with the disease prevalence or severity in adult MS disease [8]. However, several recent studies indicated that antibodies to MOG are present in a subset of predominantly pediatric inflammatory demyelinating diseases different from MS such as ADEM or AQP4-IgG seronegative NMO (see [9] for review). A possible role of B cells in MS has been more recently highlighted through the beneficial clinical effect of anti-CD20 monoclonal antibodies which, before modifying antibodies titers, deplete B cells [10] and modify B cell functions [11]. Indeed, B cells present

Table 1: Summary of patients and sample characteristics.

	n	Age	% females	% relapses	EDSS
RR MS	38	36.74 ± 9.8	71%	24%	1.75 ± 0.33
SP MS	8	53.25 ± 7.86	75%	—	—
HI	50	38.18 ± 11.48	62%	—	—
CIS	9	36.44 ± 14.82	67%	—	—
Controls	8	40.13 ± 21.70	50%	—	—

38 patients with relapsing-remitting form, 50 healthy individuals, and eight patients with secondary progressive form were included in this study. Forintrathecal analysis, nine patients with clinically isolated syndrome and eight controls were included. Among the eight controls without neurological disease, there was four hydrocephalus, one anti-N-methyl-D-aspartate (NMDA) receptors' encephalitis, one leukopathy, one tetrapyramidal syndrome, and one idiopathic high pressure hydrocephalus.

several functions thought to play a role in MS autoimmune processes [8]. Harp et al. have shown that, in MS, B cells committed to myelin proteins are efficient as antigen presenting cells [12]. B cells can also exhibit a regulatory function in autoimmune diseases [13, 14] or in a transplantation setting [15, 16]. Yet, an alteration of this function in MS [17] remains controversial [18]. B cell tolerance to autologous determinants depends on a first checkpoint which occurs in bone marrow [19]. Autoreactive B cells are nevertheless released in the periphery [20] where a T cell dependant second checkpoint operates. However, a substantial proportion of circulating B cells still remains poly- or cross-reactive despite these processes.

In this paper, using a novel approach [21] to detect MOG committed B cells in comparison with circulating anti-MOG antibodies, we show that (i) as for anti-MOG T cells [7, 22] normal individuals present a substantial high level of circulating anti-MOG B cells and (ii) although there were no differences in circulating anti-MOG antibodies, MS patients have a significantly lower circulating anti-MOG B cell frequency than healthy individuals.

2. Materials and Methods

2.1. Patients and Healthy Controls. Patients included in this study were diagnosed with MS using revised Mac Donald criteria [23]. 38 Relapsing-Remitting patients (RRMS), listed in Table 1, were recruited. RRMS group was composed of 27 females and 11 males ranging from 23 to 60 years old (mean age: 36.74). All patients were scored on the Kurtzke Expanded Disability Status Scale (EDSS) and were without immunomodulatory treatment for at least three months and immunosuppressive treatment for at least six months before testing. Another group of eight patients with secondary progressive MS, from 40 to 64 years old (mean age: 53.25) was also included with 6 females and 2 males. 50 healthy individuals (HI) participated in the study, 31 females and 19 males ranging from 22 to 61 years old (mean age: 38.24).

Nine patients with clinically isolated syndrome (CIS) and eight with other noninflammatory or inflammatory neurological disease (OND or OIND) were also enrolled in the intrathecal study. There were six females and three males ranging from 20 to 61 years old in the CIS group (mean age: 36.44). The control group (Table 1) was composed of four females and four males ranging from 15 to 77 years old (mean age: 40.13). Our study complies with the Nantes University Hospital Ethical Committee guidelines and all participants signed an informed consent for the study.

2.2. Obtention of Protein-Coupled Beads. Extracellular domain of recombinant human $MOG_{1\text{-}125}$ (rMOG) (Eurogentec, France) expressed in *E. coli*, human albumin (LFB, France), and nontoxic tetanus toxin C-fragment (Sigma, France) proteins were coupled to fluorescent Bio-Plex COOH beads (Bio-Rad, France) as described [21]. The carboxyl groups of fluorescent COOH beads were activated by EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) (Fisher Scientific, France) and S-NHS (sulfo-N-hydroxysulfosuccinimide) (Fisher Scientific); then the Bio-Plex amine coupling kit (Bio-Rad) was used to couple proteins to the activated COOH beads. The coupling reaction was systematically checked through flow cytometry using the appropriate antibodies. For MOG, we checked that the anti-MOG 8.18C5 mouse antibody, shown to react with the folded MOG pattern [24], effectively recognizes the $MOG_{1\text{-}125}$ covalently coated beads. In addition, we checked that these $MOG_{1\text{-}125}$ coated beads are also recognized by B cells from transgenic 8.18C5 antibody mice [21].

2.3. Frequencies and Phenotype of Blood MOG-Specific B Cells. Plasmas were collected from blood samples and mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation (PAA, France) and frozen in serum-DMSO 10%. Purified B cells were obtained by negative magnetic selection (Miltenyi, France) from cryopreserved PBMC. Briefly, 3×10^5 B cells were stained with CD19-PE (Clone HIB19) (BD, France), CD27-QDot 605 (CLB-27/1) (Invitrogen, France), IgD-FITC (IA6-2) (BD), CD5-FITC (53-7.3) (BD), CD38-PECy5 (Clone LS198-4-3) (Beckman Coulter, France), CD40 (5C3) (BD), KI-67-FITC (B56) and IgG1, κ isotype control (BD) at 4°C for 30 minutes. The cells were then washed twice with PBS/2%FCS/2 mM EDTA buffer and incubated with protein-coated beads for 1 hour at 4°C and DAPI was added before FACS assay to select only the live cells. Specificity was tested through competitive incubation of the B cells with soluble MOG or albumin for 30 minutes prior to the addition of MOG-coated beads. The B cells were also preincubated with anti-IgG+IgA+IgM polyclonal Fab'2 fragments. Polyreactivity was also tested by the same approach. Briefly, soluble dsDNA (Sigma, France), LPS (Sigma), and insulin (Sigma) were added during 30 minutes on B cells prior to the incubation with MOG-coated beads. The frequency of antigen-specific B cells was evaluated on $3 \cdot 10^4$ live $CD19^+$ cells recorded for each sample through flow cytometry, using a LSR II (BD). All analyses were performed with FlowJo software, version 7.6.12 [21].

The absolute B-lymphocyte count was determined by combining a precise volume of blood with appropriate antibodies (CD45-V500—Clone HI30—and CD19-PE) in TruCount tubes (BD) for 15 minutes at room temperature. Then, the red blood cells were lysed with FACS lysing solution (BD). The TruCount tubes were analyzed through flow cytometry and the quantity of B cells/μL of blood was obtained.

2.4. Apoptosis Assay. An annexin V-FITC apoptosis detection kit (BD) was used to evaluate the apoptosis in the BBR fraction. Briefly, $3 \cdot 10^5$ purified B cells were stained with CD19-PE and incubated with protein-coupled beads for 1 hour. Annexin V-FITC was added and incubated with the cells for 15 minutes at room temperature (RT). Then, binding buffer and DAPI were added before analyzing by flow cytometry. Live B cells (annexin V^- $DAPI^-$), early apoptotic B cells (annexin V^+ $DAPI^-$), and late apoptotic B cells (annexin V^+ $DAPI^+$) were selected.

2.5. Quantification of Intrathecal MOG-Specific B Cells. After lumbar puncture, 10 mL of CSF was immediately added to a RPMI supplemented medium. The CSF samples were centrifuged at 1500 rpm for 7 minutes to collect the cells and supernatants were collected. Cell pellets were resuspended in PBS/2%FCS/2 mM EDTA buffer and stained with CD19-PE antibody for 30 minutes at 4°C. The CSF cells were then washed and incubated with either albumin or MOG-coupled beads as indicated above.

Out of 29 CSF samples obtained during the study period, only 17 CSF (58%) patients contained more than 50 live CD-19 reactive B cells per sample, the minimal amount considered required for data interpretation. The total cell numbers for these CSF ranged from 50 to 2710 live B cells. Blood samples were obtained from each CIS and control patient, for comparison (9 from CIS and 8 from non-MS).

2.6. B Cell Transmigration Assay through Endothelial Cell Line. The human blood-brain-barrier (BBB) endothelial cell line HCMEC/D3 is an immortalized endothelial cell line derived from a primary cell culture coexpressing hTERT and the SV40 large T antigen via a lentiviral vector system [25]. Transmigration assays were performed using the Transwell system (8.0 μm pore filters; BD Falcon, France) as previously described [26]. Cell concentration was tested and concentrations which allowed more than 5% human albumin diffusion in the lower chamber after 6 hours were discarded. Two days before the migration assay, 1×10^6 HCMEC/D3 cells were cultured on the apical side of the filter insert. All cells were grown in endothelial basal medium-2 (EBM-2, Lonza) supplemented with human serum (PAA). 5×10^5 B cells were added on top of the BBB layer. After 18 h at 37°C with 5% CO_2, the contents of the bottom chamber were collected with EDTA to detach any adherent cell, and B cells were counted. The transmigrated cells were washed, stained with CD19-PE, and incubated with albumin or MOG coupled beads as indicated previously.

2.7. Assessment of Anti-MOG and EBNA1 Reactive Circulating Antibodies. Anti-MOG antibodies were assessed with an immunofluorescent *cell-based assay* (CBA) as described in detail elsewhere [27]. Briefly, all plasma and CSF samples were tested for reactivity against the human MOG expressed in HEK293 cells using an immunofluorescence live cell assay. The plasma samples were tested at a 1/20 dilution and CSF samples were undiluted. Titers were considered positive above a cut-off of 1 : 160 [27]; correlations were also performed using direct OD values.

The detection of anti-EBNA1 IgG in plasma samples was performed routinely using the DiaSorin kit (Liaison EBNA1 IgG) on an automat (Liaison XL).

2.8. Statistical Analysis. All data are expressed as mean ± SEM. Mann-Whitney tests were used to compare pairs of different groups and Wilcoxon test to compare two variables in the same group. *t*-test was used to compare pairs of groups with more than 30 individuals. One way ANOVA or Kruskall Wallis tests were used to compare more than 2 groups. All statistical analyses were performed with GraphPad Prism 5. Results were considered significant when $P < 0.05$.

3. Results

3.1. Validation of the Method and Reactivity of the 8.18C5 Antibody. Mouse monoclonal antibody 8.18C5 recognizes conformational $MOG_{1\text{-}125}$ (Ig-like domain) but not the linear MOG epitopes in mice [24]. 8.18C5 antibody was used to both check the efficiency of the MOG coupling to the beads and the covalently bound MOG reactivity to the antibody. The reactivity of the MOG coated beads with the 8.18C5 antibody was potent (91%) as shown in Supplementary Figure 1a available online at http://dx.doi.org/10.1155/2015/673503. The MOG covalently coated beads were also recognized by the transgenic IgH-MOG B cells (corresponding to the 8.18C5 heavy chain) [28]. Spleen B cells of these mice exhibited 59% of MOG-BBR, with a drop of 90% when the B cells were preincubated with soluble rMOG but not with albumin (Supplementary Figure 1b).

3.2. Quantification of MOG-Specific B Cell Frequencies in MS Patients. MOG is a myelin antigen only expressed in the CNS, which induces EAE, and is suspected to play a role in MS [29]. In this study, MOG-coated polystyrene beads were used to identify *in vitro* $CD19^+$ cells able to make rosettes with the MOG coated beads (*referred to as BBR for bead/B cell rosettes*) as described elsewhere [21]. Several negative controls were used: uncoated beads, albumin coated beads, or T cells instead of B cells. Tetanus toxin (TT) coated beads were used as the positive controls [30]. Using this approach, we observed substantially high frequencies of circulating B cells rosetting with rMOG coated beads in both cohorts (MS patients and HI) (Figure 1). However, unexpectedly, the frequency of anti-MOG B cells was statistically lower in RRMS ($n = 38$) than in the cohort of 50 healthy individuals (HI) tested in parallel (0.86 ± 0.12 and 1.33 ± 0.14%, resp., $P = 0.0188$, Figure 1). In contrast, the frequency of B cells which recognized albumin coated beads was not statistically different in MS (0.40 ± 0.09%) and HI (0.42 ± 0.07%) (Figure 1). RRMS patients and HI show a similar and high frequency of B cells recognizing TT coated beads, 6.64 ± 0.74 and 5.84 ± 0.66%, $P = 0.37$ (Figure 1). The specificity and the polyreactivity of the MOG-coated beads were assessed by addition of 20 μg of soluble rMOG, albumin, dsDNA, LPS, and insulin. The addition of soluble rMOG resulted in a dose dependent inhibition of MOG-specific B cell frequency (from 1.12 ± 0.17 to 0.11 ± 0.03) whereas soluble albumin did not provide competition (Figure 2(a)). Soluble LPS dropped by

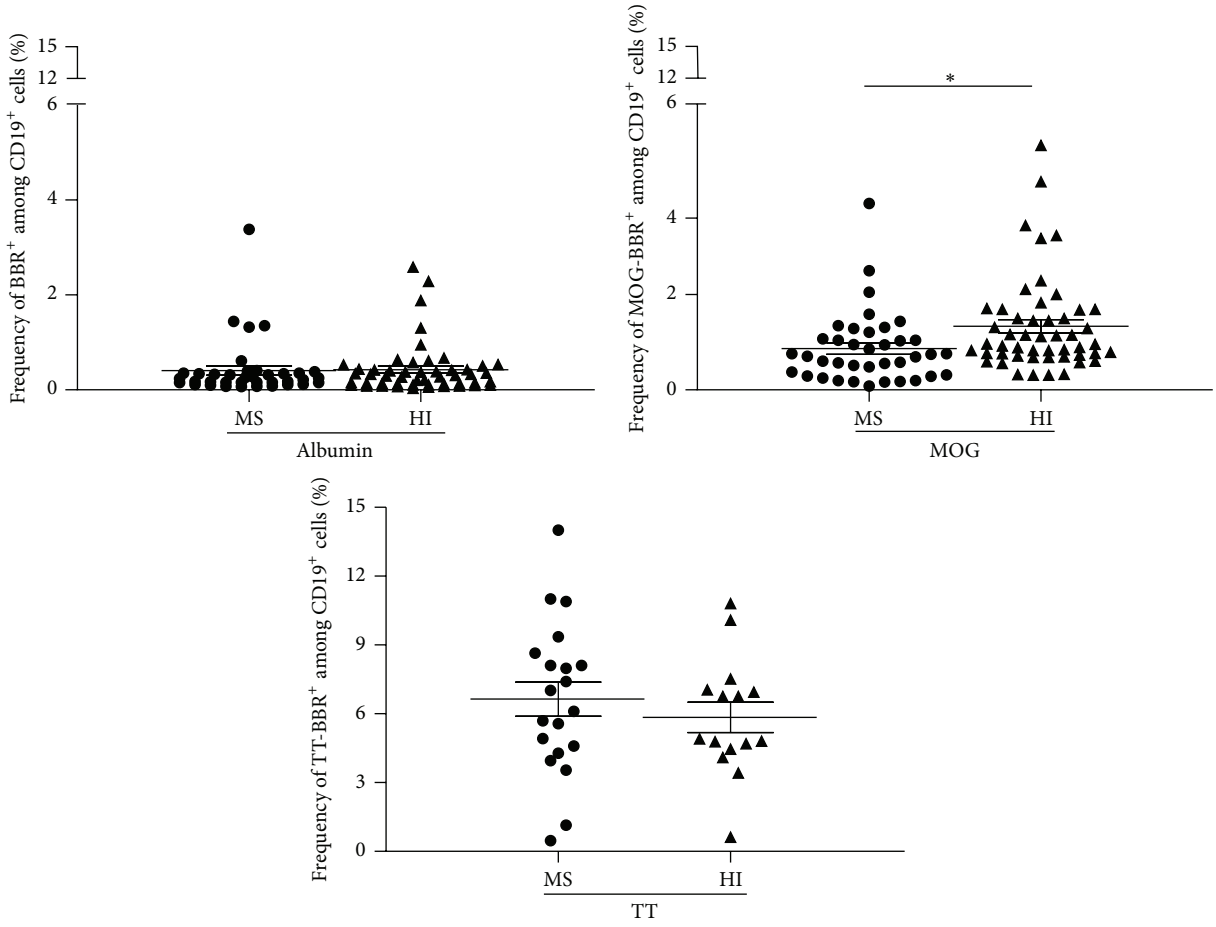

Figure 1: Quantification of MOG-specific B cell frequencies in MS patients and HI. Purified B cells, stained with CD19 antibody, were incubated with human albumin, rMOG, or tetanus toxin (TT) coated beads. After exclusion of dead cells (DAPI$^+$), the frequency of B cells which recognized albumin or rMOG was obtained in MS patients ($n = 38$) and HI ($n = 50$) $^*P = 0.0188$, *unpaired t-test*. The frequency of B cells bound to TT coated beads was also assessed in MS patients ($n = 20$) and in HI ($n = 15$) (ns, $P > 0.05$, *Mann-Whitney test*).

73% the recognition of MOG-specific B cells (from 2.24 ± 0.22 to 0.59 ± 0.11). However, dsDNA (from 2.24 ± 0.22 to 1.99 ± 0.12) and insulin (from 2.24 ± 0.22 to 1.45 ± 0.63) have a smaller competing effect on the MOG-specific B cell frequency (Figure 2(b)). Polyreactivity of MOG-specific B cell in MS patients and HI was similar. MOG-specific frequencies after dsDNA and insulin incubation were, respectively, 1.30 ± 0.03 (11% of inhibition) and 0.99 ± 0.07 (32%) in MS group (Figure 2(b)).

The Fab'2 fragment anti-human Ig (A, G, and M isotypes) was also able to decrease the MOG-specific B cell frequency (nearly 50%) (Figure 2(c)), further suggesting that the interaction of the tested B cells with MOG-coated beads involved the surface membrane BCR.

The frequency of MOG-BBR cells in MS was then classified according to the state of the disease and to the clinical form. No significant difference between the frequency of MOG-BBR of patients in relapse ($n = 9$, 0.72 ± 0.15%) and in clinical remission ($n = 29$, 0.92 ± 0.16%) was observed (Figure 3). Both groups had a lower frequency of MOG-BBR compared to HI ($P = 0.01$ and $P = 0.02$, resp.). In the same condition to detect MOG-specific B cells, a small group ($n = 8$) of patients with a secondary progressive form of MS (SPMS) showed a similar MOG-BBR frequency to the HI (1.61 ± 0.21 and 1.33 ± 0.14%, resp., *ns*). The difference between the values of MOG-BBR for the SPMS and RRMS groups was statistically significant ($P = 0.005$).

3.3. Phenotype of MOG-BBR Cells

3.3.1. Naïve, Memory Phenotype and Activation State. CD27 is a marker of human memory B cells [31]. The absolute value of B cells was assessed in RRMS patients and HI and no significant difference was observed (data not shown). Moreover, we find the same proportion of memory B cells in both groups (Supplementary Figure 2a). Assessment of frequency of memory (CD27$^+$) and naïve (CD27$^-$) B cells recognizing MOG-coated beads indicates 32.6 ± 3.16% CD27$^+$ MOG-BBR and 64.86 ± 2.88% of CD27$^-$ MOG-BBR in MS patients versus 37.31 ± 3.84% of CD27$^+$ MOG-BBR and 59.77 ± 4.02% of CD27$^-$ MOG-BBR in HI (Supplementary Figure 2b). The frequency of switched and unswitched memory MOG-BBR, as approached by CD27$^+$IgD$^-$ and CD27$^+$IgD$^+$ distribution, did not differ between MS and HI (Supplementary Figure 2c).

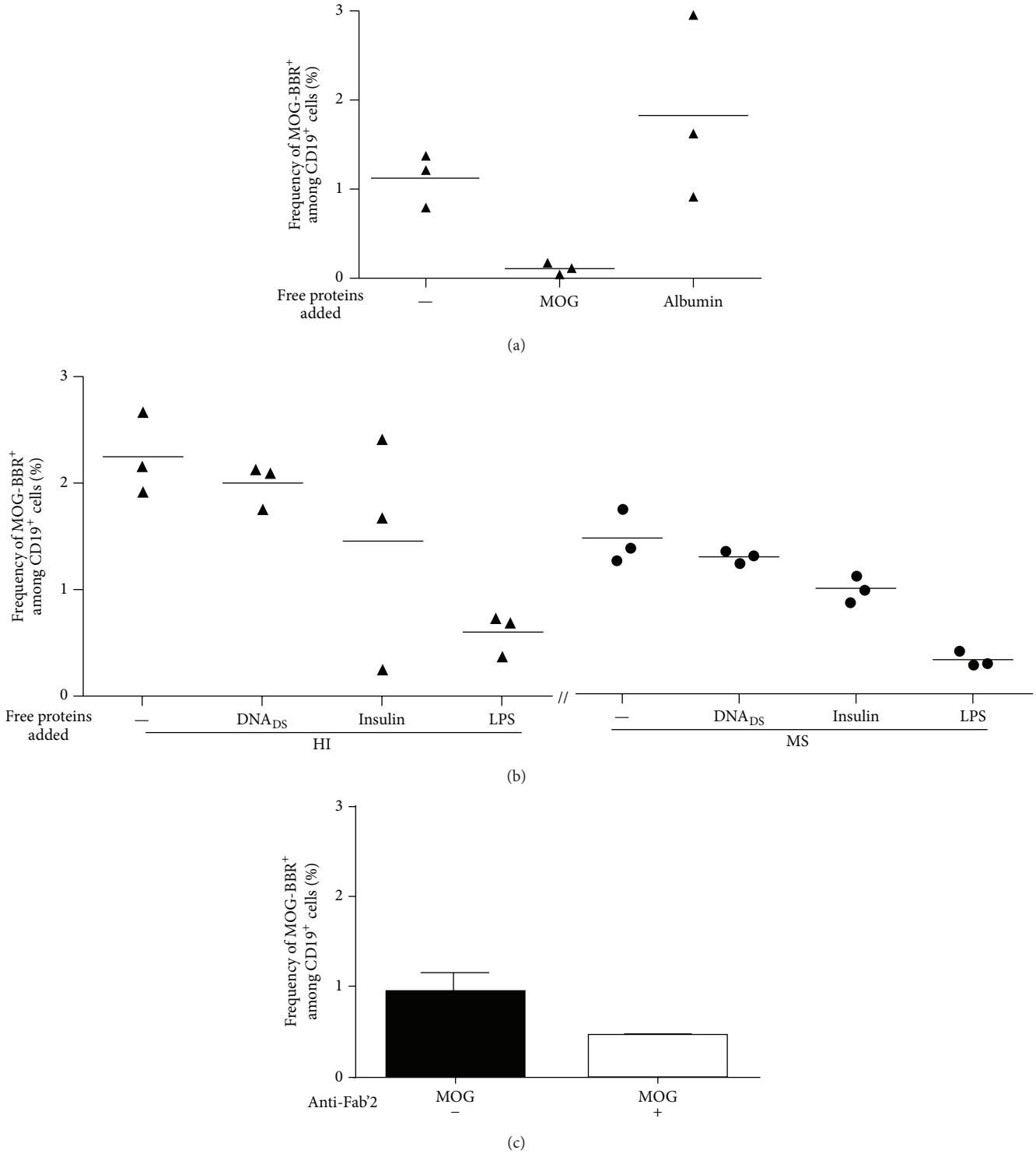

Figure 2: Reactivity of MOG-specific B cells. B cells were preincubated with 20 μg of soluble MOG, albumin, dsDNA, insulin, or LPS before the addition of MOG-coated beads ($n = 3$). (a) The frequency of MOG-BBR drops with the soluble MOG preincubation but not with albumin preincubation. (b) The recognition of MOG-coated beads decreased after the addition of soluble LPS (% of inhibition: 73%), dsDNA (11%), and insulin (35%). Competitive assay was tested in MS ($n = 3$); the percentage of inhibition after soluble antigen addition was the same proportion as HI: LPS (78%), dsDNA (11%), and insulin (32%). (c) B cells were preincubated with Fab'2 anti-human IgG+IgA+IgM. The frequency of B cells recognizing MOG-coated beads was assessed on 3 HI. Fab'2 antibody modified the frequency of MOG-specific B cells (ns, $P > 0.05$, *Wilcoxon test*).

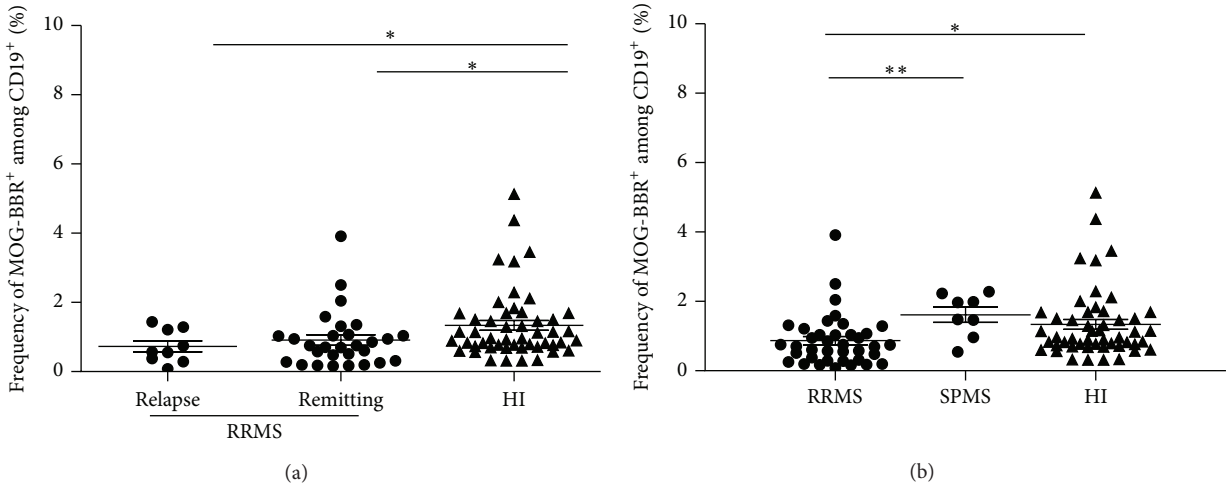

Figure 3: Quantification of MOG-specific B cell frequencies in MS patients according to disease activity and MS forms. (a) RR patients were classified according to disease state, for example, relapse or remission. We analysed the difference in MOG-BBR frequencies for patients in relapse ($n = 9$) and patients in clinical remission ($n = 29$) versus HI ($n = 50$) ($1P < 0.05$), *Kruskal-Wallis test*. No difference between patients in relapse and patients with clinical remission was observed (ns, $P > 0.05$). (b) Eight patients with secondary progressive (SP) forms were included and compared to RR patients and HI. There was a statistically significant difference between SPMS patients and RR MS patients ($P < 0.05$) but not between SPMS patients and HI ($P > 0.05$), *Kruskal-Wallis test*.

Naïve MOG-BBR were more frequent than the memory phenotype in both groups ($P = 0.01$). In this compartment, activated naïve B cells ($CD27^{-}CD38^{+}$) were more represented than mature naïve B cells ($CD27^{-}CD38^{-}$) in MOG-BBR subsets (Supplementary Figure 2d).

The B cells were also stained for CD40 activation marker, a candidate for contributing to autoimmune processes in which B cell activation may play a role [32]. We assessed the CD40 MFI of MOG-specific B cells in MS and HI. No significant difference was observed between RRMS ($n = 12$) and HI ($n = 11$) in the MOG-BBR subset (Supplementary Figure 2e).

3.3.2. Proliferation State. Ki-67, expressed during all active phases of the cell cycle [33], was used to assess the proliferative status of MOG-specific B cells in RRMS patients and HI. Although the detection of KI-67 was difficult in unstimulated B cells, a trend ($P = 0.06$) was found for the two cohorts: $1.44 \pm 0.37\%$ MOG-BBR positive for KI-67 in MS patients ($n = 16$) and $3.49 \pm 0.86\%$ in HI ($n = 17$) (Figure 4). MOG-specific B cells were stained more strongly by KI-67 than B cells in MS and HI ($P < 0.05$, data not shown).

3.4. Apoptosis of Anti-MOG-BBR Cells. Among several possible explanations for the decreased frequency of MOG-BBR in MS patients compared to HI, apoptosis was considered. A combination of annexin V (recognizing phosphatidylserine (PS) on the cell surface) and DAPI was used to detect early (annexin V^{+}, $DAPI^{-}$) and late (annexin V^{+}, $DAPI^{+}$) apoptotic cells (Figure 5(a)). Ten patients with RR-MS and 10 HI were tested. Early and late apoptosis markers of MOG-BBR showed no difference in MS and HI (for early apoptosis: 6.56 ± 1.38 and $7.29 \pm 1.61\%$, $P = 0.88$; for late apoptosis: 16.20 ± 2.75 and $17.14 \pm 3.53\%$, resp., Figure 5(b)).

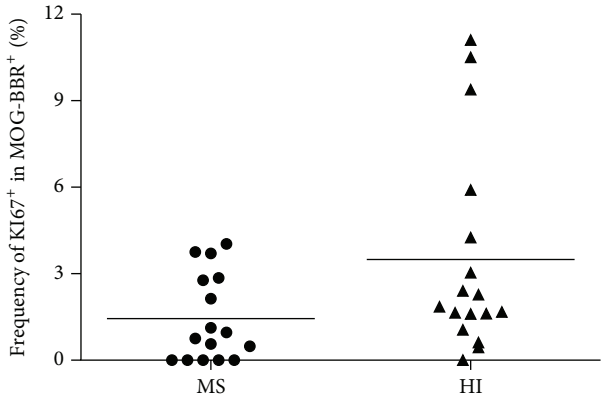

Figure 4: KI-67 expression in MOG-specific B cells: KI-67 marker was used to determine the proliferating states of MOG-BBR. The frequency of MOG-BBR positive for KI67 in MS ($n = 16$) was not different to HI ($n = 17$), (ns, $P = 0.06$) *Mann-Whitney test*.

3.5. Intrathecal MOG-Specific B Cells. Although cell numbers in CSF samples reduced intrathecal study, we then checked if MOG-specific B cells accumulated in the spinal fluid of 8 patients with a clinically isolated syndrome (CIS), in whom there was a clinical need for CSF analysis and in 8 patients with other neurological disorders, both inflammatory and noninflammatory, as controls. All analyses were performed with CSF immediately processed after harvesting. As mentioned in the M&M, only 58% of the samples contained enough cells to perform the test. There was an average of 525 ± 105 B cells ($CD19^{+}DAPI^{-}$) in fresh CSF samples (ranging from 50 to 2710 B cells) in the tested samples. An example of the gating strategy is represented in Figure 6(a). Cells in CSF samples were selected according to the SSC and

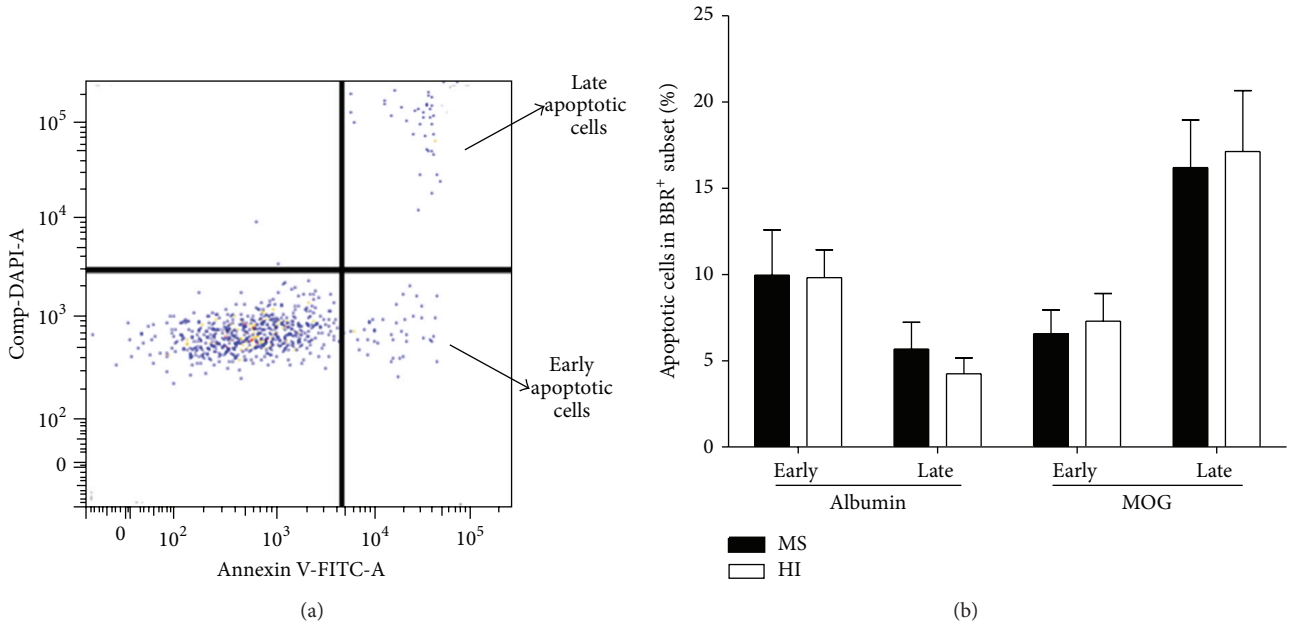

Figure 5: Analysis of apoptosis in MOG-specific B cells. (a) We used annexin V and DAPI to characterize MOG-BBR apoptosis cells in MS ($n = 10$) and HI ($n = 10$). We analysed annexin V^+ $DAPI^-$ (early apoptotic cells) and annexin V^+ $DAPI^+$ (late apoptotic cells) in the MOG-BBR subset. The gating strategy was represented. (b) We compared the frequency of MOG-BBR apoptotic cells in MS and HI in early (ns, $P > 0.05$) and late states (ns, $P > 0.05$), *Mann-Whitney test*.

FSC parameters as beads and rosettes which had a small size (FSC) and high SSC. DAPI and CD19 markers were first used to analyze only live B cells and exclude dead rosettes. The costaining of APC and PE determines MOG-specific B cells (beads-APC/CD19-PE). Then, the Boolean gate function was used to determine the % of rosettes selected in CD19 live cells, giving the frequency of MOG-specific B cells.

We found an identical intrathecal frequency of MOG-specific B cells in CIS (10.28 ± 4.48%) and control individuals (8.62 ± 3.98%) ($P = 0.6$) (Figure 6(b)). The CSF frequency of MOG-BBR was higher in both groups compared to the albumin-BBR frequency in CIS (Figure 6(b)) and circulating MOG-BBR (1.28 ± 0.11 and 0.57 ± 0.10%) in CIS patients and in the control group, respectively ($P < 0.05$) (Figure 6(c)).

3.6. Anti-MOG-BBR Transfer through Brain-Derived Endothelial Cells. We first tested whether B cells from RRMS patients transmigrated more efficiently than B cells from HI across the endothelial cell layer *in vitro*. In MS, 8750 ± 1505 B cells transmigrated overnight compared to 10309 ± 1622 B cells in HI out of 5×10^5 $CD19^+$ cells initially placed in the double chamber device (about 2% of transmigrating B cells). Despite confirming that human B cells migrate efficiently across the blood-brain barrier [34], there was no statistical difference between the MS and HI values ($P = 0.49$, Figure 7(a)). We then checked whether MOG-BBR accumulated more efficiently in MS patients compared to HI by assessing the capacity of the B cells to make MOG-BBR after a transmigration assay. We observed no statistical difference in the transmigration rate of MOG-specific B cells in MS (24.85 ± 6.32%) and in HI (25.11 ± 6.50%) (Figure 7(b)).

3.7. Plasma Anti-MOG IgG Reactivity. The presence of anti-MOG antibodies was tested with a cell-based assay (CBA). A cut-off of 1 : 160 was chosen and used for discrimination of ADEM and other groups, as in previous studies [27]. Above this threshold, we found only two seropositive samples in a single RR MS patient (Supplementary Figure 3a). Surprisingly, one normal individual also exhibited a strong reaction. The frequency of BBR-MOG for this patient was 0.32%, which is not within the highest frequency values of RR MS patients. Similar observation can be done for the anti-MOG seropositive HI. We did not observe a significant correlation between MOG-specific BBR and CBA reactive soluble antibodies ($r^2 = 0.032$, $P > 0.05$) (Supplementary Figure 3b). Since beads were coupled to the extracellular domain of MOG, reactivity to this antigen was also analyzed by ELISA, but again no differences were observed between MS patients and controls and no significant correlation between MOG-specific BBR and MOG reactive soluble antibodies was found (data not shown).

The presence of anti-EBNA1 IgG, reported to be associated with MS [35, 36], was tested to see whether the frequency of MOG-specific B cells correlates with EBNA-1 titers. Anti-EBNA1 IgG titers were higher in MS patients than HI ($P = 0.03$) (Supplementary Figure 4) but no correlation was evidenced between the frequency of MOG-specific B cells and anti-EBNA1 titers (data not shown).

4. Discussion

Despite the beneficial effect of anti-CD20 in treating the disease, the potential role of B cells in multiple sclerosis (MS) has been less well documented than that of T cells. In this

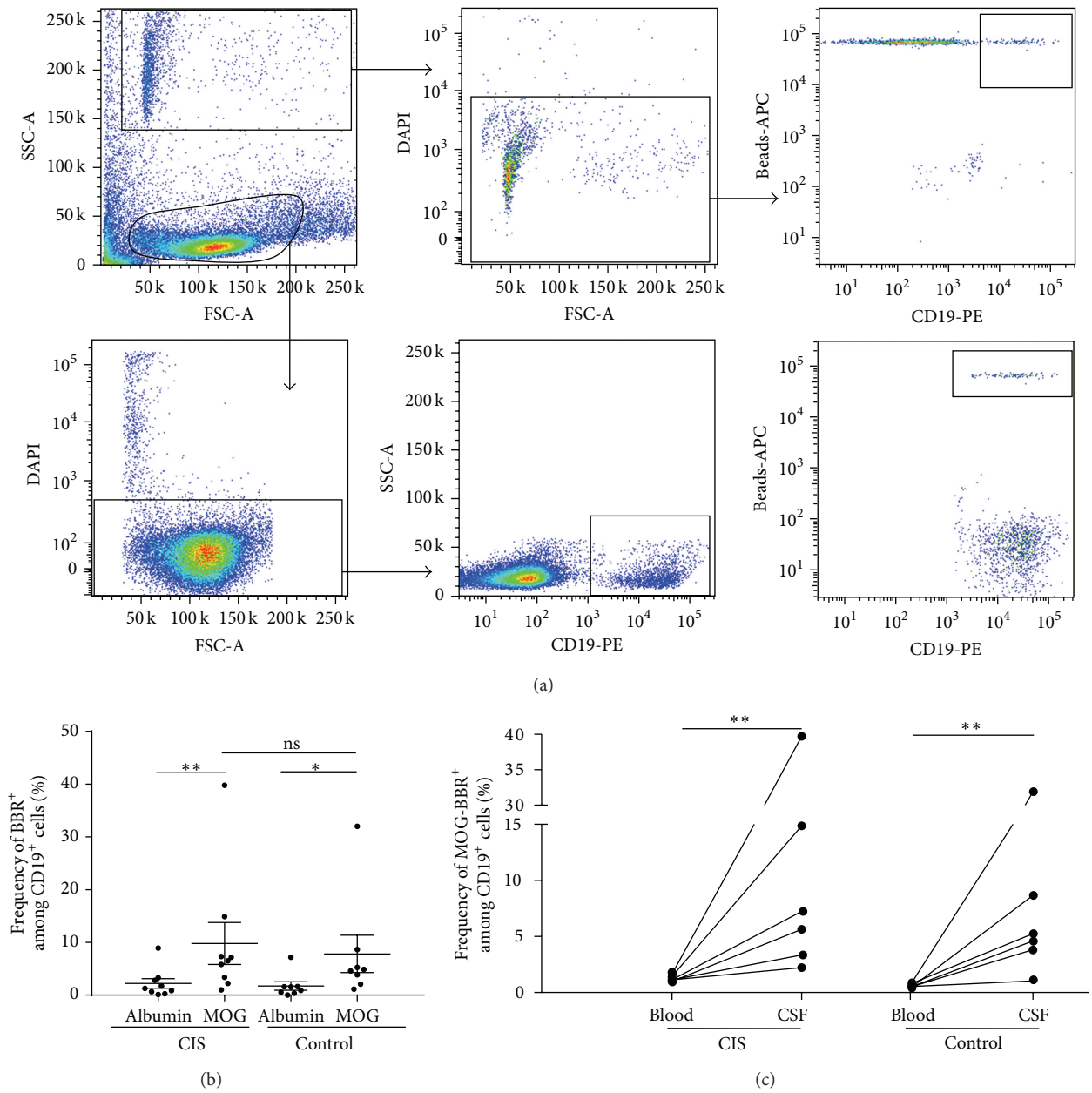

Figure 6: Intrathecal MOG-specific B cells in CIS and patients with non-MS-related pathologies. (a) The gating strategy of CSF analysis is represented. The number of B cells in this CIS sample was 1771 cells. The BBR frequency was obtained by FlowJo analysis which calculated the percentage of MOG-specific B cells among B cell subsets: 107 BBR corresponds to 5.69%. (b) Cells from fresh CSF samples were stained with CD19 antibody and incubated with rMOG and human albumin coated beads. After exclusion of dead cells (DAPI$^+$), the frequency of BBR recognizing rMOG and albumin was assessed in CIS ($n = 9$) and control patients ($n = 8$) (ns, $P > 0.05$). *Mann-Whitney test and Wilcoxon test* were used to compare albumin-BBR and MOG-BBR in CIS ($P < 0.05$) and control patients ($P < 0.05$) (c). The frequency of circulating and intrathecal MOG-BBR was assessed in CIS ($n = 6$) and control patients ($n = 6$). Intrathecal frequency of MOG-BBR was higher than circulating frequency (ns, $P > 0.005$, *Mann-Whitney test*).

paper, we explore the frequency of MOG-specific B cells in the blood of patients with MS and in the spinal fluid of CIS patients, using a novel method based on the measurement of a direct interaction between a B lymphocyte and a fluorescent polystyrene bead to which human MOG is covalently bound [21].

The extracellular Ig-like MOG domain is unique in that it is the only protein structure known to induce both demyelinating autoantibody and encephalitogenic T cell responses in EAE [37]. MOG is a quantitatively minor component of myelin in CNS [38]. Antibodies directed toward the conformational $MOG_{1\text{-}125}$ and not directed toward the linear MOG,

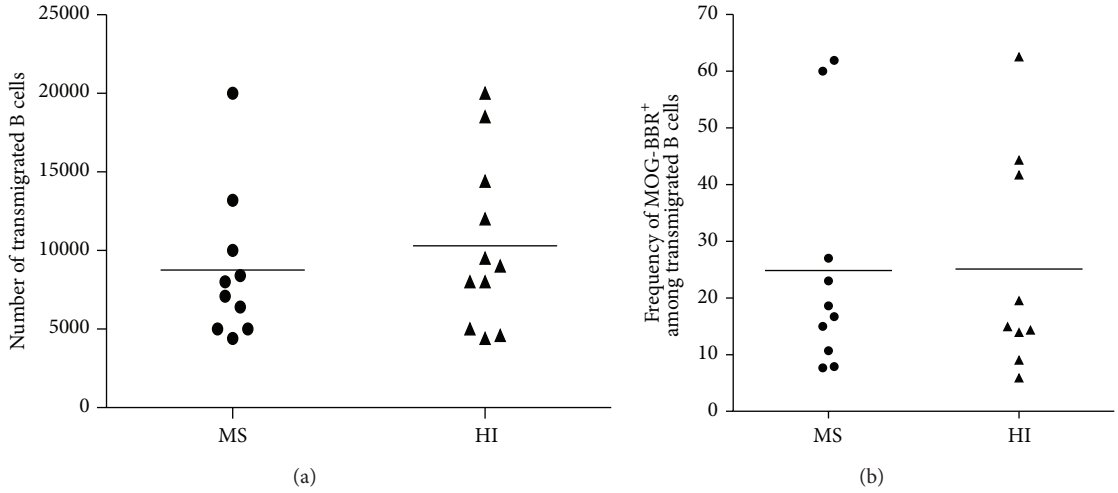

Figure 7: Transmigration of B cells across brain vessel derived endothelial cells. (a) B cells were negatively selected and a transmigration assay across HCMEC/D3 cell line was performed. Transmigrated cells in MS ($n = 10$) and HI ($n = 11$) were counted; no difference was observed (ns, $P > 0.05$, *Mann-Whitney test*). (b) Transmigrated B cells were stained and incubated with MOG coated beads. The frequency of MOG-BBR was assessed in MS patients ($n = 10$) and HI ($n = 9$) (ns, $P > 0.05$, *Mann-Whitney test*).

such as the mouse antibody 8.18C5 [24], are instrumental in inducing anti-MOG antibody mediated EAE [39]. Several studies suggest that they are present in a subset of predominantly pediatric inflammatory demyelinating diseases which are distinct from MS [9]. While a significantly higher level of anti-EBNA1 titers was found in MS patients, no correlation with anti-MOG-BBR frequency was observed. Regarding the rMOG used, it is important to note that the 8.18C5 antibody recognizes 91% of the $MOG_{1\text{-}125}$ after covalent binding to the beads, suggesting that a good proportion of the bound $MOG_{1\text{-}125}$ exhibit the correct folding. However, to what extent the folding of $MOG_{1\text{-}125}$ affects the frequency of B cells with a membrane antigen receptor interacting with MOG-coated beads could not be precisely assessed in this test, which differs from the cell-based assay used to measure soluble anti-MOG antibodies. Finally, since we did not test antibody secretion of MOG-BBR, we cannot exclude that this subset is able to produce some MOG antibodies. Nevertheless, it is important to note that we observed a significant difference in anti-$MOG_{1\text{-}125}$ committed B cells between the MS patients and the controls ($P = 0.018$), whereas no difference in anti-MOG antibody titers against conformational and linear MOG has been found [40] in adult MS patients, clearly suggesting a B cell specific alteration in this disease.

A first finding was that the frequency of B cells engaging with MOG-coated beads was unexpectedly high, both in the MS patients and in healthy individuals (HI). This observation has been in parallel with the well documented description of high frequency of MOG-specific T cells in HI [7]. It is likely that B cell specific maturation processes are involved in the high anti-MOG B cell frequencies.

BCR cross-reactivity and/or polyreactivity studies of blood B cells [19] have indeed ambiguously shown a substantial percentage of circulating B cells (20% of immature B cells remain autoreactive, with 4% of them being polyreactive cells) having escaped the first BCR-dependant bone marrow selection checkpoint, which triggers apoptosis or reediting for autoreactive BCR [19, 20]. However in these seminal studies, the end point was the production of Ig against a panel of antigens in limiting conditions. A study of the direct binding of the B cell to its antigen may be a more sensitive tool for frequency calculation. We also tested the polyreactivity of MOG-BBR to dsDNA, LPS, and insulin antigens [19]. However, unexpectedly high values of competition for anti-MOG BCR were found for LPS, a major component of the outer membrane of Gram negative bacteria, and a toll-like receptor agonist. Cross-reactivity of surface BCR of MOG-BBR for dsDNA was roughly of the same magnitude as the cross-reactivity/polyreactivity of secreted antibodies anti-dsDNA after the first repertoire checkpoint of B cells [19]. Higher competition was noted for insulin. These differences may be due to a difference in maturation of surface B cell receptor that we explored versus secreted antibodies [19]. This polyreactivity normally downregulated in the last phase of B cell development has been proposed as the link between infection and autoimmune diseases leading to a breakdown of the immunological tolerance as documented in clinical settings and in animal models [41, 42]. In line with this, a study has shown an increase of immune reactivity to bacterial LPS in NMO patients [43]. However, our study was not designed to specifically address this issue and this observation requires confirmation and caution in interpretation.

In addition, our method used Luminex beads which, when used for testing specific antibodies such as anti-HLA, exhibits a gain of sensitivity of an order of magnitude compared to classical methods [44]. The case that an assay using the same readout (FACS) and material (Luminex) also displays a gain in sensitivity is conceivable.

Strikingly, we found a significant decreased MOG-specific B cell frequency in the blood of MS compared to age/gender matched control individuals. Importantly, the samples from HI and MS were routinely processed back to

back in the tests. Furthermore, no difference was exhibited in the frequencies of B cells recognizing tetanus toxin and albumin coated beads between patients with MS and HI. Finally, the anti-MOG B cell frequency was decreased neither in a small group of MS patients with secondary progressive form (SPMS) nor in the CIS patients suggesting that this alteration occurs in the course of the relapse-remitting disease rather than at its onset. We did not find evidence for a specific phenotype bias of the anti-MOG B cells, with normal distribution (both for MS and HI) of naïve, memory cell phenotypes.

Recently, a subset of potentially autoreactive B cells, B1 cells, defined as $CD3^{-}CD20^{+}CD27^{+}CD43^{+}$ [45], was found to be lower in the blood of MS patients versus HI [46]. Although no test for the MOG specificity of the B1 cells was carried out in this study, the magnitude of the decrease in B1 cells (29% of the B cells) in the referred study was however too small (1.2% in MS patients versus 1.7% in normal individuals) to account significantly for our observation (RRMS had a 36% decrease in MOG-BBR). Furthermore, the B cell selection kit that we used did not select $CD43^{+}$ cells, which accounted for most of the B1 cells. To the best of our knowledge, only one study is available on anti-MOG B cell frequency, using biotinylated MOG in a small cohort ($n = 9$) of patients with RRMS [12]. This interesting study suggests that circulating memory anti-MOG B cells of MS patients could provide a greater help to $CD4^{+}$ T cells than memory anti-MOG B cells of HI. However, no significant difference of frequency in anti-MOG B cells between MS patients and HI was noted in this study. The reason for this discrepancy with our study is not clear, although our cohort was 4 times bigger (38 MS and 50 HI). Recently, another study reported that autoreactive B cells in MS were more prone to produce polyreactive antibodies to an extract of white matter [47] than in HI. However, no frequency of myelin or MOG-specific B cells was analyzed. Finally, our data do not support that idea that normal individual B cells have no reactivity against brain extracts, as was recently suggested [48]. However, it must be said that Kuerten et al. used *in vitro* polyclonally stimulated B cells and CNS extract.

Reasons for the significant decrease in anti-MOG circulating B cells remain so far undefined. Particularly, we could not find evidence for an increase in early or late apoptosis of anti-MOG B cells. However, the growth potential of MOG-specific B cells, as assessed by expression of the KI-67 marker, shows a trend ($P = 0.06$) for a lower expression in MS patients that may contribute to the lower frequency of anti-MOG-BBR in MS. The possibility that the "missing" MOG-specific circulating B cell population could be in the CNS was suggested by a documented increase of anti-MOG IgG in spinal fluid from MS patients [49] and by the shared BCR clonotypes between blood and CSF [50]. We could only measure the level of anti-MOG B cells in the CSF of CIS patients. However, we could not document a possible increased homing of MOG-specific B cells in CSF of our RR-MS patients since there is no clinical indication for CSF analysis in overt MS disease, making the study unethical.

Connections between the periphery and CNS allowing trafficking of antigens and presenting or effector cells have been well documented [51, 52] and may offer a substratum for transfer and homing of anti-MOG B cells in the brain. More recently evidenced connecting pathway may also be involved [53, 54]. Nevertheless, there was no difference of anti-MOG-BBR in transmigration assays although B cells (2%) showed efficient transmigration through a brain-derived endothelial cell layer, in concordance with another study [34].

In conclusion, our study gives the initial evidence of an abnormal circulating pool of anti-MOG B cells. *In vitro* analyses were unable to detect discrete changes in surface molecules involved in B cell homing in noncirculating sites of the immune system. It is possible that the mechanisms that govern this decreased MOG-specific frequency are no longer active at the time of the assay. Significantly, our observation involved a specific B cell alteration which is independent of the pattern of anti-MOG antibodies and which is not restricted to the optimal MOG conformation as is the case for soluble antibodies detected in the cell-based assay [9]. Recently, a cellular involvement of B cells, independent of their humoral function, was shown in pathogenesis of EAE [55]. Finally, whether MOG-specific B cells are actually involved in the disease pathology is not directly confirmed by our observations, considering that a high percentage of these specific B cells are also observed in HI blood, mimicking the situation described for T cells with myelin autoreactivity [3, 7]. In this respect, the positive effect of anti-CD20 on the disease outcome might be misleading in terms of the actual role of MOG-specific B cells in the disease. Indeed, besides the destruction of a fraction of potentially pathogenic B cells (partial depletion in lymph nodes [11]), such reagents also affect the properties of bystander B cells and may induce these cells to produce more immunoregulatory cytokines or to exhibit modified functions, as recently suggested [11, 56].

5. Conclusion

In this paper, we detected MOG-specific B cells in MS by a novel approach using fluorescent beads covalently bound to extracellular domain of MOG. We show for the first time that, as for T cells, healthy individual blood harbors autoreactive MOG B cells. In addition, we showed a significantly lower frequency of MOG-specific B cells in patients with relapsing-remitting MS compared to HI. The decrease of this subset suggests their implication in MS and stresses further studies.

Abbreviations

BBR: B cell beads rosettes
CBA: Cell-based assay
CSF: Cerebrospinal fluid
HI: Healthy individuals
MOG: Myelin oligodendrocyte glycoprotein
MS: Multiple sclerosis
RR: Relapsing-remitting
SP: Secondary progressive.

Conflict of Interests

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

Authors' Contribution

Annie Elong Ngono and Maud Lepetit have equally contributed as co-first authors and Sophie Brouard, David-Axel Laplaud, and Jean-Paul Soulillou have equally contributed as co-senior authors and are listed by alphabetical order.

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Research Article

GDF15(MIC1) H6D Polymorphism Does Not Influence Cardiovascular Disease in a Latin American Population with Rheumatoid Arthritis

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Objective. Rheumatoid arthritis (RA) is the most common autoimmune arthropathy worldwide. The increased prevalence of cardiovascular disease (CVD) in RA is not fully explained by classic risk factors. The aim of this study was to determine the influence of rs1058587 SNP within *GDF15(MIC1)* gene on the risk of CVD in a Colombian RA population. *Methods*. This was a cross-sectional analytical study in which 310 consecutive Colombian patients with RA and 228 age- and sex-matched controls were included and assessed for variables associated with CVD. The mixed cluster methodology based on multivariate descriptive methods such as principal components analysis and multiple correspondence analyses and regression tree (CART) predictive model were performed. *Results*. Of the 310 patients, 87.4% were women and CVD was reported in 69.5%. Significant differences concerning *GDF15* polymorphism were not observed between patients and controls. Mean arterial pressure, current smoking, and some clusters were significantly associated with CVD. *Conclusion*. *GDF15* (rs1058587) does not influence the development of CVD in the population studied.

1. Introduction

Rheumatoid arthritis (RA) is the most common autoimmune arthropathy worldwide. It is a chronic, multifactorial, and systemic disease characterized by the presence of long-standing inflammation [1, 2]. As with most autoimmune diseases, RA predominantly affects women [2, 3], and its prevalence had been reported to be between 0.3 and 1.6% [4]. In addition to diarthrodial joints, RA can damage virtually any organ thus leading to potential extra-articular manifestations (EAMs), including cardiovascular disease (CVD) [5, 6]. CVD is the major predictor of poor prognosis and represents the main cause of death in this population [7, 8]. It accounts for 30–50% of all deaths in RA patients [9] and the prevalence of CVD in RA Latin American population has been estimated to be around 35% [8].

In the etiological and pathogenic mechanisms of CVD in RA the inflammatory activity plays an important role. Several studies assessing traditional risk factors for CVD in RA have been published [10]. Nevertheless, the increased prevalence of cardiovascular events in RA is not fully explained by these classic risk factors. Nontraditional risk factors have also been identified and categorized into three groups: genetic, RA-related, and others [7]. Recently, an association between a nonsynonymous single nucleotide polymorphism (SNP) at growth differentiation factor 15 (*GDF15*) gene (i.e., rs1058587) and CVD in RA patients was reported [11].

GDF15, also known as macrophage inhibitory cytokine-1 (*MIC1*), depending on the tissue that was described, is a growth and differentiation factor which belongs to the superfamily of transforming growth factor-beta (TGF-β). *GDF15*

plays multiple roles in various pathologies such as CVD, obesity, inflammation, and cancer due to its anti-inflammatory, antiproliferative, and antitumoral properties [11–13].

Its mechanism of action is not fully elucidated but it is believed that *MIC1* activates the TGF-β receptors. Specifically in inflammation, it can limit the later phases of macrophage activation, inhibit the production of tumoral necrosis factor-α (TNF-α) induced by lipopolysaccharides, and also regulate the role of the proinflammatory cytokine interleukin-6 (IL-6) [10]. Additionally, *MIC1* influences metabolism of carbohydrates and lipids. For instance, patients with obesity and type 2 diabetes mellitus (T2DM) have the highest serum concentrations of this factor [14].

Previous studies have found that some *GDF15(MIC1)* polymorphisms are associated with different atherothrombotic manifestations such as stroke, transient ischemic attack, deep vein thrombosis, and pulmonary embolism. *GDF15(MIC1)* had also been associated with RA *per se,* proposing that it acts as a proinflammatory cytokine as well as a common etiologic factor for RA and CVD [11, 12]. The aim of this study was to determine the influence of rs1058587 SNP within *GDF15(MIC1)* gene on the risk of CVD in a Colombian RA population.

2. Methods

2.1. Study Population. This was a cross-sectional analytical study in which 310 consecutive Colombian patients with RA and 228 age- and sex-matched controls were included. The sample size was not calculated. This was a nonprobability sample. The subjects were seen at the Center for Autoimmune Diseases Research (CREA) in Medellín and Bogotá, Colombia. Patients fulfilled the 1987 American College of Rheumatology (ACR) classification criteria for RA [15]. This study was undertaken between February 2008 and April 2010 and done in compliance with Act 008430/1993 by Ministry of Health of the Republic of Colombia. The institutional review board of the Universidad del Rosario approved the study design.

Each patient was evaluated by a rheumatologist. The information on patient sociodemographic and cumulative clinical and laboratory data was obtained by interview, physical examination, and chart review. Household description by questionnaire and clinical evaluation of the affected family members was done with the same methodology as above. All data were collected in an electronic and secure database.

Sociodemographic variables included age at RA onset, disease duration, educational status, socioeconomic status (SES), current occupation, smoking habits, and physical activity. Definitions of these variables are as follows. Age at onset (AOD) is the age at which patients began to suffer from pain, typical morning stiffness (more than 1 hour), and symmetrical inflammation of hand and/or foot joints. Disease duration is the difference between AOD and the date of first participation in the study. Educational level was recorded as the number of years of education and was also divided into two groups—more or less than 9 years of education based on the General Law of Education in Colombia laws [16, 17]. SES was categorized on the basis of national legislation and was divided into high status, medium status, and low status. Information on current occupational status was also requested. Familial autoimmunity (FA) was defined as the presence of any AD in first degree relatives (FDRs) of the proband [18].

Erosions were defined as having at least one unequivocal cortical bone defect evaluated by a rheumatologist [19]. EAMs were defined as the presence of at least one of the following: skin and lung nodules, skin ulcerations, episcleritis, vasculitis, neuropathy, pleural effusion, pulmonary hypertension, or embolism. CVD was categorized as positive if any of the following variables were present: hypertension, coronary artery disease (CAD), occlusive arterial disease, carotid disease, or thrombosis [20]. Regarding medical treatment, current or past use of methotrexate and other conventional disease modifying antirheumatic drugs (DMARDs) such as sulfasalazine, D-penicillamine, gold salts, and leflunomide; biological therapy (etanercept, infliximab, adalimumab, abatacept, tocilizumab, and rituximab); and azathioprine and cyclosporine were also assessed. In addition, steroid therapy (prednisolone, methylprednisolone, and deflazacort) and antimalarials (chloroquine and hydroxychloroquine) were taken into account.

Autoantibodies including rheumatoid factor (RF), anti-citrullinated protein (ACPA), and anti-nuclear antibodies (ANA) were extracted from the patient's clinical record.

2.2. Assessment of Traditional Risk Factors for CVD. Patients were assessed for traditional CVD risk factors including current age ($\geq$45 and $\geq$55 years for men and women, resp.) [21] and history of premature CAD in FDR [22], and all individuals were asked about smoking and physical activity [23]. T2DM was defined as having a fasting plasma glucose level $\geq$7 mmol/L (126 mg/dL) or taking any antidiabetic agents at the time of assessment [24]. A diagnosis of dyslipidemia was given if the patient had (a) hypercholesterolemia, defined as taking lipid-lowering medication as a surrogate or having a fasting plasma total cholesterol $\geq$200 mg/dL, (b) HDL $\leq$40 mg/dL, (c) hypertriglyceridemia (triglycerides $\geq$150 mg/dL), or (d) elevated LDL ($\geq$100 mg/dL) [25] and hypertension (defined as having a blood pressure $\geq$140/90 mmHg or using any antihypertensive medication). Systolic and diastolic blood pressures were measured twice with at least a 15-minute interval in between and the averages were recorded [26].

2.3. Anthropometric Measurements. A body mass index (BMI) $\geq$25 kg/m^2 (overweight and obesity) was considered abnormal [27]. Values of waist circumference ($\geq$102 cm for men and $\geq$88 cm for women) and waist-to-hip ratio (WHR) $\geq$0.9 for men and $\geq$0.85 for women were considered indicators of abdominal obesity. Waist circumference was measured around the midpoint between the lowest rib and the iliac crest after exhaling and viewed from the front. Hip circumference was measured at the point of maximum extension of the buttocks when viewed from the side [28]. Abnormal WHR values are consistent with National Cholesterol Education Program Adult Treatment Panel III and World Health Organization

definitions [29, 30]. The above measurements were used to find the most reliable predictors of vascular involvement.

2.4. CVD and Assessment of Carotid Intima-Media Thickness (IMT). CVD was categorized as positive if any of the following subphenotypes were present: hypertension, coronary artery disease (CAD), occlusive arterial disease, carotid disease, or thrombosis [20]. In addition, an expert blind to the patients' clinical records performed ultrasound measurements of the IMT from the common carotid artery. High-resolution, 2-dimensional images were obtained using an ultrasound machine (Agilent-Hewlett-Packard, Santa Clara, CA). A 7.5 MHz linear-array transducer with an axial resolution of 0.15 mm and a penetration depth from 1.0 to 5.0 cm was used [31]. The patient rested on the examination table for 15 minutes before the initial carotid ultrasound scan. The measurement was applied to the far wall of the right and left carotid arteries. Following R. Salonen and J. T. Salonen [32], a B-mode screening method was used with electronic calipers within 10 mm proximal to the common carotid bifurcation in a temperature-controlled room (22°C to 24°C) [33]. Ultrasound images were recorded on videotape (Sony MD385). IMT was measured at the site of the greatest thickness and at 2 additional points: 1 cm upstream and 1 cm downstream from this site. The average of these 3 values was computed. Since carotid IMT greater than 0.90 mm is included among the definitions of subclinical organ damage [34], we established this point as having severe subclinical atherosclerosis (AT). The reproducibility of the IMT was evaluated in 12 volunteers, by taking 2 measurements one month apart; obtaining an intraclass correlation of 0.98.

2.5. Genotyping. Genomic DNA was extracted from buffy-coat cells using standard methods. All the identified genomic DNA samples were genotyped for *GDF15(MIC1)* rs1058587 at the Oklahoma Medical Research Foundation using the TaqMan allelic discrimination assay (Applied Biosystems). The *GDF15(MIC1)* gene, located at band p13.11 on chromosome 19, has two exons that encode the 308-amino acid *GDF15(MIC1)* polypeptide, consisting of a 29-amino acid signal peptide, a 167-amino acid propeptide, and a 112-amino acid mature protein. Cleavage of the propeptide allows the mature protein to be secreted as a disulfide-linked homodimer. rs1058587 SNP is at codon 202 (**C**AC to **G**AC) and results in a histidine to aspartic acid substitution at position 6 of the mature protein (H6D) (http://www.ncbi.nlm.nih.gov/gene/9518).

2.6. Statistical Analysis. Hardy-Weinberg equilibrium was checked in each genotypic marker. Univariate analyses were performed as follows: the categorical variables were analyzed by the frequencies. The quantitative continuous variables were expressed as the mean and standard deviation, as well as the median and range. Bivariate analysis of CVD outcome (subphenotypes and IMT) versus genetic, clinical, and demographic variables was assessed by means of chi square and Kruskal-Wallis test. The mixed cluster methodology proposed by Morineau et al. [35] based on multivariate descriptive methods such as principal components analysis (PCA) and multiple correspondence analysis (MCA) was performed to resume some sets of variables that have strong associations. For example, AOD and duration of disease were analyzed in this setting to derive three groups that resemble the associations of these variables (i.e., time cluster). The same was done for SES and educational level deriving four groups (i.e., sod-cluster) and, for Sjögren's syndrome (SS), EAM, and comorbidity three groups were determined (i.e., clinical cluster). This allows the integration of these sets of variables that are confounded, via the new clusters, in association models like logistic regression and classification and regression trees (CART).

CART were used to find predictive factors for CVD in RA. The CART model was adjusted in the Salford Predictive Modeler software v7 using different splitting rules and cross-validation samples to accurately estimate the relative errors of the classification tree. As the independent factors, the model included the variables that were statistically significant in bivariate analyses and those variables that were biologically plausible, as well as the new clusters derived. Bivariate and multivariate analyses were performed in R 3.0.2 [36].

3. Results

Of the 310 patients, 87.4% were women and CVD was reported in 69.5%. The SNP rs1058587 was in Hardy-Weinberg equilibrium ($P > 0.05$). Allelic frequencies corresponding to rs1058587 SNP C and G were 82.7% and 17.5%, respectively. The most frequent genotype was CC (68.4%). The frequencies of RF and ACPA were 68.3.% and 80.4%, respectively. Characteristics of the cohort are illustrated in Table 1. Significant differences concerning *GDF15* polymorphism (rs1058587) were not observed between patients and controls (Table 2).

Data analysed by PCA and MCA were resumed in clusters under clinical, time, and sociodemographic categories. The clinical clusters was gathered into 3 groups: (1) the highest frequency comorbidities (e.g., T2DM, dyslipidemia, osteoporosis, acid peptic disease, kidney disease, depression, periodontal disease, and fibromyalgia), not having polyautoimmunity with SS and moderate frequency of EAM; (2) presence of comorbidities and polyautoimmunity with SS and the highest frequency of EAM; (3) absence of comorbidities, not having polyautoimmunity with SS and lower frequency of EAM. The time clusters was categorized into 3 groups: (1) later AOD and shorter duration of the disease; (2) earlier AOD and longer duration of the disease; (3) earlier AOD and shorter duration of the disease. Finally, the sod-clusters was classified into 4 groups: (1) medium SES and the highest educational level; (2) medium SES and lower educational level; (3) the highest SES and higher educational level; and (4) the lowest SES and higher educational level.

The patients with CVD were significantly older and had a later AOD of RA. Duration of EAM, mean arterial pressure, current smoking, clinical cluster number 1, and time cluster number 1 were all risk factors significantly associated with CVD. Associations between *GDF15* rs1058587 SNP and CVD

Table 1: Demographic and clinical characteristics of 310 patients with RA.

Variable	Median (range)
Age (y)	37 (20–54)
Age at onset (y)	50 (26–67)
Duration of the disease (y)	12 (1–20)
BMI	29 (19–37)
EAM duration (m; media ± SD)	11.7 ± 3.3
Genetics	*n*/*N* (%)
rs1058587 CC	212/310 (68.4)
rs1058587 CG	88/310 (28.4)
rs1058587 GG	10/310 (3.22)
Allele C	513/620 (82.7)
Allele G	109/620 (17.5)
Sociodemographic	*n*/*N* (%)
Female	271/310 (87.4)
Civil status: married	174/282 (61.7)
Occupation: household duties	109/287 (38)
Socioeconomic status: medium	195/278 (70.1)
High educational level	169/212 (79.7)
Current smoking	49/272 (18)
RA-related	*n*/*N* (%)
Polyautoimmunity: SS	16/310 (5.1)
Familial autoimmunity	47/272 (17.3)
EAM[&]	117/303 (38.6)
Comorbidity[#]	225/305 (73.7)
Rheumatoid factor (+)	162/237 (68.3)
ACPA (+)	107/133 (80.4)
ANA (+)	31/75 (41.3)
Erosive (+)	99/110 (90)
Methotrexate	248/273 (90.8)
DMARDs, ever[¥]	83/117 (71)
Antimalarials, ever[£]	158/256 (61.7)
Steroids, ever[æ]	250/269 (92.9)
Biological therapy, ever[€]	37/256 (14.4)
Cardiovascular	*n*/*N* (%)
Cardiovascular disease[₳]	130/187 (69.5)
cIMT abnormal	66/118 (55.9)
Familial cardiovascular disease	19/245 (7.7)
BMI: obesity	18/118 (15.2)
BMI: overweight	42/118 (35.6)
Physical activity	20/121 (16.5)
Abnormal waist-hip ratio	90/117 (76.9)

ACPA: anti-citrullinated protein antibodies; ANA: anti-nuclear antibodies; BMI: body mass index; cIMT: carotid intima-media thickness; DMARDs: disease modifying antirheumatic drugs; EAMs: extra-articular manifestations; HLA: human leukocyte antigen; m: months; *MICI*: macrophage inhibitory cytokine-1; RA: rheumatoid arthritis; SD: standard deviation; SS: Sjögren's syndrome; y: years.

[&]EAM is defined as the presence of at least one of the following: nodules, skin ulcerations, episcleritis, scleritis, vasculitis, neuropathy, pleural effusion, pulmonary nodules, or pulmonary hypertension.

[#]It is defined as the presence of at least one of the following: type 2 diabetes mellitus, dyslipidemia, kidney disease, anemia, osteoporosis, depression, fibromyalgia, acid peptic disease, epilepsy, or infections (e.g., hepatitises A, B, and C, tuberculosis, or malaria).

[¥]If patient had used at least one of the following: sulfasalazine, D-penicillamine, gold salts, and leflunomide.

[£]If patient had used at least one of the following: chloroquine and hydroxychloroquine.

[æ]If patient had used at least one of the following: prednisolone, methylprednisolone, and deflazacort.

[€]If patient had used at least one of the following: etanercept, infliximab, adalimumab, abatacept, tocilizumab, and rituximab.

[₳]It was categorized as positive if any of the following variables were present: hypertension, coronary artery disease, occlusive arterial disease, carotid disease, or thrombosis.

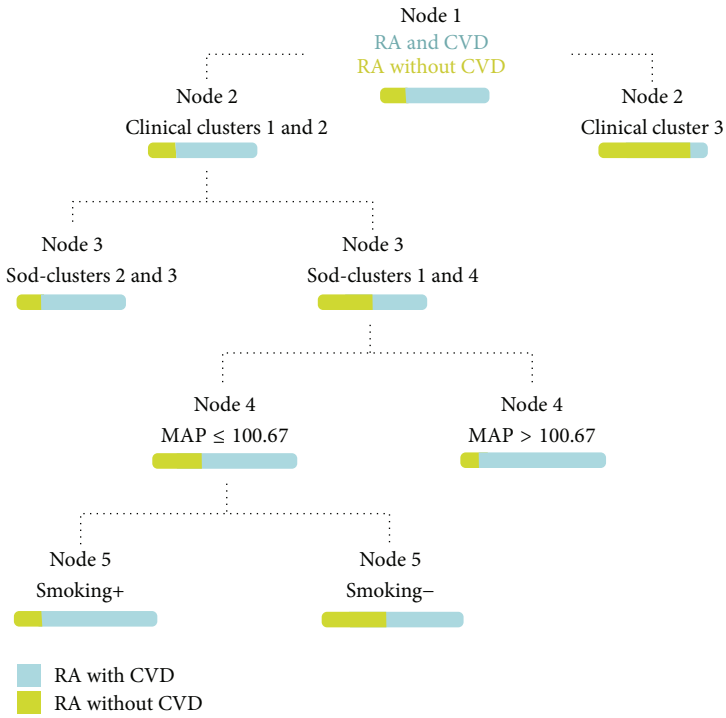

Figure 1: CART predictive model: the main predictive factors significantly associated with the development of CVD in RA are clinical and sociodemographic cluster, mean arterial pressure (MAP), and current smoking, with variable importance scores higher than 0.73.

Table 2: *GDF15* polymorphism (rs1058587) in Colombian patients with RA.

Genotype	RA $N = 310$ (%)	CTR* $N = 228$ (%)
CC	212 (68.4)	151 (66.2)
CG	88 (28.4)	70 (30.7)
GG	10 (3.2)	7 (3.1)
Allele	$2N = 620$	$2N = 456$
C	512 (82.6)	372 (81.6)
G	108 (17.4)	84 (18.4)

*Significant differences were not observed.

(subphenotypes) in RA patients (Table 3) or with IMT were not found.

In Tables 4 and 5 are mentioned the variables associated with the presence of allele C and allele G of the *MIC1* rs1058587 SNP, respectively. Once again, we did not find associations between this SNP and CVD in those patients.

In the genotype analysis, the homozygotes G/G were significantly older (median 54, IQR 11.7 years), had later AOD (median 43.7, IQR 9.92 years), and had the highest diastolic blood pressure (median 88.33, IQR 8.66 mmHg). On the other hand, the genotype CC was found as protective factor to have positive ANA (OR 0.27; 95% C.I. 0.11–0.85, $P = 0.019$).

3.1. CART Predictive Model. The CART model displayed a high proportion of patients with RA through clinical clusters 1 and 2 who also develop CVD (nodes 1 and 2). However, there was association between CVD and sod-cluster, mean arterial pressure (MAP), and current smoking; in fact the major association with CVD is in sod-clusters 2 and 3. However, sod-clusters 1 and 4 show a better discrimination for CVD (node 3). Moreover, in the same group of patients, CVD was higher in those with high MAP (i.e., more than 100 mmHg) (node 4). Finally, a greater proportion of CVD was observed in patients in node 4 with low MAP but presenting with current tobacco exposure (node 5) (Figure 1). This model had an AUC of 0.73 (cross-validated AUC), which represents an adequate predictive performance.

4. Discussion

Several previous studies of gene polymorphisms in patients with RA who had CVD had demonstrated genetic factors implicated in the development of this comorbidity, such as HLA-DRB1 shared epitope alleles [37, 38]. Moreover, the choice of genes for analysis in those studies was based on a relationship with RA *per se* or with inflammation. Herein, we were unable to replicate the influence of *GDF15(MIC1)* rs1058587 SNP in Colombian patients with RA and CVD as was previously observed in Swedish and Australian patients [11, 14]. Differences in admixture patterns and allele frequencies among populations may account for the observed differences in the influence of genetic factors on disease. Our population is highly admixed with Amerindian, African, and Caucasian ancestries as compared with Australians and Swedish who are mainly Caucasians. The rs1058587 G allele

Table 3: Characteristics associated with CVD in RA patients.

Variable	CVD 130/187 (69.5) Median (IQR)	Non-CVD 57/187 (30.5) Median (IQR)		*P* value
Duration of the disease (y)	14.6 (9.038)	9 (5.565)		<0.001
EAM (m)	17.115 (37.6)	4.962 (9.559)		0.028
Mean arterial pressure	99.709 (12.992)	95.491 (11.605)		0.0361
Variable	ECV *n*/*N* (%)	Non-ECV *n*/*N* (%)	OR (95% CI)	*P*
Clinical cluster 1[&]	112/130 (86.1)	32/57 (56.1)	33.93 (8.6–259.7)	<0.001
Time cluster 1[#]	62/130 (47.6)	24/57 (42.1)	1.99 (1.07–4.16)	0.0274
Current smoking	29/130 (22.3)	4/57 (7.4)	2.54 (1.01–8.45)	0.0312
GDF15 rs1058587 CC	89/130 (68.4)	36/57 (63.1)	—	NS
GDF15 rs1058587 CG	35/130 (26.9)	21/57 (36.8)	—	NS
GDF15 rs1058587 GG	6/130 (4.6)	0/57 (0)	—	NS
Allele C	213/260 (81.9)	93/114 (81.5)	—	NS
Allele G	41/260 (15.7)	21/114 (18.4)	—	NS

CVD: cardiovascular disease; EAMs: extra-articular manifestations; *GDF15*: growth differentiation factor 15; IQR: interquartile range; y: years; m: months; NS: not significant; RA: rheumatoid arthritis.
[&]Clinical cluster 1 corresponds to patients with the highest frequency of comorbidity, with moderate frequency of EAM, and without Sjögren's syndrome.
[#]Time cluster 1 corresponds to patients with later age at onset and shorter duration of the disease.

Table 4: Characteristics associated with allele C of the *GDF15* rs1058587 SNP in RA patients.

Variable	Allele C (CC/CG) 300/310 (96.7) Median (IQR)	Allele G (GG) 10/310 (3.22) Median (IQR)		*P* value
Diastolic blood pressure	80.662 (10.437)	88.333 (8.66)		0.0286
Body mass index	25.326 (4.691)	29.322 (4.177)		0.0457
Variable	Allele C *n*/*N* (%)	Allele G *n*/*N* (%)	OR (95% CI)	*P* value
Current smoking	45/300 (15)	4/10 (40)	0.14 (0.02–0.80)	0.016
Obesity	15/300 (5)	3/10 (30)	0.09 (0.009–0.97)	0.0175
CVD	124/300 (41.3)	6/10 (60)		NS

CVD: cardiovascular disease; IQR: interquartile range; RA: rheumatoid arthritis.

Table 5: Characteristics associated with allele G of the *GDF15* rs1058587 SNP in RA patients.

Variable	Allele G (GG/CG) 98/310 (31.6) Median (IQR)	Allele C (CC) 212/310 (68.3) Median (IQR)		*P* value
Age (y)	49.398 (12.46)	53.986 (11.976)		0.001
Age at onset (y)	37.469 (11.513)	41.344 (12.59)		0.008
Variable	Allele G *n*/*N* (%)	Allele C *n*/*N* (%)	OR (95% CI)	*P* value
Clinical cluster 1[&]	72/98 (73.46)	129/212 (60.84)	2.88 (1.16–9.32)	0,0145
ANA (+)	15/98 (15.30)	16/212 (7.54)	2.72 (1.15–8.21)	0,0203
Sjögren's syndrome	4/98 (4.08)	26/212 (12.26)	0.28 (0.11–0.91)	0,0209
Time cluster 1[#]	32/98 (32.63)	98/212 (46.22)	0.52 (0.31–0.92)	0,0228
CVD	41/98 (41.88)	89/212 (41.98)		NS

ANA: anti-nuclear antibodies; CVD: cardiovascular disease; IQR: interquartile range; RA: rheumatoid arthritis.
[&]Clinical cluster 1 corresponds to patients with the highest frequency of comorbidity, with moderate frequency of EAM, and without Sjögren's syndrome.
[#]Time cluster 1 corresponds to patients with later age at onset and shorter duration of the disease.

frequency was significantly lower in Colombians than in Australians and Swedish [11, 14].

The general prevalence of CVD in our cohort was 69.5%. Some associations between RA patients with CVD were found. Variables that were significantly associated include smoking, higher MAP, longer duration of disease with EAM, clinical cluster number 1, and time cluster number 1. It is important to highlight that these associations encompass only clinical variables that include modifiable traditional risk factors for CVD like smoking and nontraditional risk factors including duration of disease and EAM [5].

GDF15(MIC1) rs1058587 C allele was nevertheless associated with minor frequency of smoking and obesity, lower BMI, and lower measurements of diastolic blood pressure (Table 4), whereas G allele was associated with earlier AOD, clinical cluster number 1, and positive ANA and it was inversely associated with SS and time cluster number 1. Although modest, these findings contrast with reports showing a specific interaction between the G allele and smoking, with an increase of risk for stroke [11].

At the genotype level a statistically significant association was found between G/G genotype and high diastolic blood pressure, while C/C genotype was inversely associated with positive ANA, suggesting a protective role for polyautoimmunity. We were not able to find other studies reporting associations between the rs1058587 homozygote forms and CVD or RA *per se*. As it is shown, these associations do not confirm a direct relationship between *MIC1* and CVD.

Many reasons exist why no significant association was elicited within the cohort and the gen evaluated with the presence of CVD. One of them is the sample size calculation since it was a nonprobability sample and all RA patients from this region were not included in the study. Besides, the heterogeneity of the cohort may prevent us from finding a direct association. A group of patients studied constitute a closed subpopulation into the admixed Colombian community (i.e., Medellín) and another group represents a major variation in ancestry. Otherwise, previous publications addressing the influence of *GDF15(MIC1)* in CVD were made in general population, not in RA patients. Therefore, the interaction among autoimmunity, admixture population, and genetic, epigenetic, and environmental factors could influence the behavior of *GDF15(MIC1)* in our study.

5. Limitations of the Study

The aim of this study was to determine the influence of rs1058587 SNP within *GDF15(MIC1)* gene on the risk of CVD in a Colombian RA population and we are aware of our study limitations. First of all, selection bias could be present in our analysis as not all patients from this region with RA were systematically included. Secondly, although the study sample size is not negligible, it would have been more valuable to have had an appropriate follow-up to establish valid associations between CVD, *GDF15(MIC1),* and RA. This, in turn, could have improved both internal and external validities. Finally, the cross-sectional nature of the study does not allow us to infer causality. Although we were able to evaluate some associations between *GDF15(MIC1)* and risk factors for CVD, it would have been more valuable to have a follow-up, as it is possible in cohort multicenter studies, to validate and establish more associations between this gene, CVD, and RA.

6. Conclusions

Contrary to prior reports, we could not find an association between the SNP rs1058587 of the gene *GDF15* and the development of CVD (measured through subphenotypes or IMT) in the population studied. There was an association between this SNP and traditional and environmental factors for CVD (i.e., age, obesity, and smoking) and some nontraditional risk factors (i.e., ANA and SS). However, we had not found direct association between the polymorphism and CVD. In fact, MAP, EAM, smoking, duration of the disease, and AOD are highlighting the pivotal role of these factors in our population. CVD in RA patients should be approached following the 5P rule: (1) predicting patients at risk of developing CVD; (2) preventing CVD in susceptible patients; (3) personalizing the approach of CVD based on the premise that susceptibility and severity of the disease are unique in each patient; (4) allowing the patient to participate in the decisions made in order to prevent and treat CVD; and (5) incorporating all data and policies according to the population, since the characteristics and natural history of diseases are population-specific [39].

Conflict of Interests

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

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Review Article

The Role of Posttranslational Protein Modifications in Rheumatological Diseases: Focus on Rheumatoid Arthritis

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The definition of posttranslational modification (PTM) encompasses a wide group of chemical reactions that allow modification and modulation of protein functions. The regulation of PTMs is crucial for the activity and survival of the cells. Dysregulation of PTMs has been observed in several pathological conditions, including rheumatoid arthritis (RA). RA is a systemic autoimmune disease primarily targeting the joints. The three PTMs mainly involved in this disease are glycosylation, citrullination, and carbamylation. Glycosylation is essential for antigen processing and presentation and can modulate immunoglobulin activity. Citrullination of self-antigens is strongly associated with RA, as demonstrated by the presence of antibodies directed to anti-citrullinated proteins in patients' sera. Carbamylation and its dysregulation have been recently associated with RA. Aim of this review is to illustrate the most significant alterations of these PTMs in RA and to evaluate their possible involvement in the pathogenesis of the disease.

1. Introduction

Human cells are able to maintain high levels of efficiency and organization thanks to a complex and finely regulated network of numerous processes (e.g., DNA transcription, protein synthesis). The proper managing of these processes strictly depends on a large group of chemical reactions named posttranslational modifications (PTMs). A PTM consists in every change of chemical structure or property of a protein that occurs after or at the same time of its translation. Nowadays, over 300 PTMs are known [1] and over 200 of them are enzyme-mediated [2], highlighting the attempt of the cell to exploit these modifications for surviving. PTMs are crucial for the development and evolution of every living organism, and it is universally accepted that the more the species are advanced, the better they use and organize PTMs [3]. PTMs and their dysregulations in pathological conditions gained great interest, due to the continuous improvement in biotechnologies that allow better investigation of and definition of these processes.

Rheumatoid arthritis (RA) is an autoimmune disease affecting about 1% of the general population and it is characterized by polyarticular, symmetric involvement of synovial joints, as well as several extra-articular manifestations, such as rheumatoid nodes, pulmonary fibrosis, and accelerated atherosclerosis [4]. The pathogenesis of RA has not been completely elucidated yet and although in these last decades the use of new therapeutic agents has improved the prognosis of the disease, RA is still an important cause of morbidity and disability.

The aim of this review is to give an overview on the role of PTMs in the pathogenesis of RA, focusing on the modifications that have been strongly associated with the disease: glycosylation, citrullination, and carbamylation (Table 1).

2. Glycosylation in Rheumatoid Arthritis

Glycosylation consists in the addition of sugars on nitrogen (N-glycosylation) or oxygen (O-glycosylation) atoms of the side chain of the protein amino acids. This reaction is

Table 1: Summary of some posttranslational modifications in rheumatoid arthritis.

PTM	Effect(s)	Reference
Glycosylation: PTM that consists in the addiction of glucides on an atom of nitrogen (N-glycosylation) or oxygen (O-glycosylation) of the lateral chain of the amino acids that forms a protein.	**Epitopes presentation**: -Protein glycosylation interacts with the processes of antigen presentation. -These interactions regard both antigen processing pathways in APCs and TCR-MHC II complex formation.	Haurum et al., 1999 [6] Jefferis et al., 1995 [22]
	Igs properties: -Fc fragment of immunoglobulins has an important site of N-glycosylation, the asparagine 297 (Asn297). -It has been shown that IgG can have both proinflammatory and anti-inflammatory activity, depending on which Fcγ receptor they preferentially bind to: those different affinities for receptors are strictly dependent on the composition of the saccharine lateral chain linked to Asn297.	Goulabchand et al., 2014 [23]
Citrullination: PTM consisting in the switch of the imine nitrogen of an arginine to an atom of oxygen, linked to the backbone structure as a ketone. It is mediated by a family of enzymes called PAD (or PADI).	-It is associated with histone modification, genomic regulation and NET formation. -PAD activation can be an intracellular or extracellular event, due to various conditions. -This activation can lead to the creation of altered self-epitopes and ACPA formation.	Yamada et al., 2005 [49] Khandpur et al., 2013 [60]
Carbamylation: Nonenzymatic PTM that consists in the addiction of a cyanate group on proteins.	-Loss of the native proteic structure. This event can lead to a break of tolerance and finally results in the formation of anti-CarP autoantibodies.	Shi et al., 2011 [90]

ACPA: anti-citrullinated protein antibodies; anti-CarP: anti-carbamylated protein; APC: antigen presenting cell; NET: neutrophil extracellular trap; PAD: peptidylarginine deiminase; PTM: posttranslational modification.

mediated by hundreds of glycosyl-transferases (GTs), regulated basically at transcriptional level. Human cells modify simple sugars, such as glucose or galactose, in more complex ones, in order to create a larger number of precursors for glycosylation; subsequently, the linking of uridine diphosphate or, rarely, cytosine monophosphate activates the sugars. Glycosylation occurs on both extracellular and intracellular proteins and it is crucial for several vital processes such as proteins folding or cell-to-cell interactions [5]. Glycosylation is involved in two physiopathological processes—epitope presentation to immune system and immunoglobulins- (Igs-) mediated regulation of immune response—suggesting a possible association of aberrant glycosylation with RA (Figure 1). Furthermore, aberrant glycosylation of different plasma proteins was demonstrated in RA patients.

2.1. Glycosylation and Epitope Presentation. Glycosylation affects the antigen presentation acting both on antigen processing pathways in antigen presenting cells (APCs) and on T cell receptor- (TCR-) MHC II complex formation.

Glycosylation plays an important role in antigen presentation by modifying the cleavage of proteins in both proteasome (MHC I-associated pathway) and endosome (MHC II-associated pathway). A considerable number of cytosolic peptides presented on MHC I molecules carry an O-glycosylation with a molecule of N-acetyl-glucosamine (GlcNAc) [6]. The O-GlcNAcylation is a PTM that interacts with phosphorylation [7] to regulate several intracellular processes, such as the activation of nuclear transcription factors and the responses to nutrient deprivation. Abolishing or modifying the physiological O-GlcNAcylation processes, as observed during inflammation or other responses to stressful stimuli [8], could determine the presentation of new antigenic glycopeptides on MHC I molecules that can bind to autoreactive T cells.

Regarding MHC II-associated pathway, it was demonstrated that different glycoforms of the same protein are processed in different ways, generating different peptides linked on MHC II molecules; this process can lead to the presentation of cryptic self-antigens or to an enhanced presentation of normally nonimmunodominant self-antigens [9].

During the antigen presentation, APCs are not capable of cleaving the side sugar chains; so, if a certain peptide carries a glycan side chain, the whole glycan-peptide complex will be presented on MHC II molecule. This process may improve or diminish TCR affinity for the MHC-peptide complex by steric interference [10].

Glycosylation influences T lymphocyte activation. Many studies investigated T cell responses to subtypes of collagen II-derived peptides in mice with collagen induced arthritis (CIA) [11, 12]. Corthay and coworkers pointed out that the response to these peptides is strictly dependent on the PTM carried on lysine 264 (K264). B10.Q mice showed a strong response to galactosylated K264 and a very weak response to unmodified or hydroxylated K264 collagen derived peptides [13]. As expected, mice immunization with galactosylated K264/MHC II complex reduced the severity of arthritis and the extent of the humoral [anti-type II collagen (anti-CII) antibodies] immune response [14]. Recently, Batsalova et al.

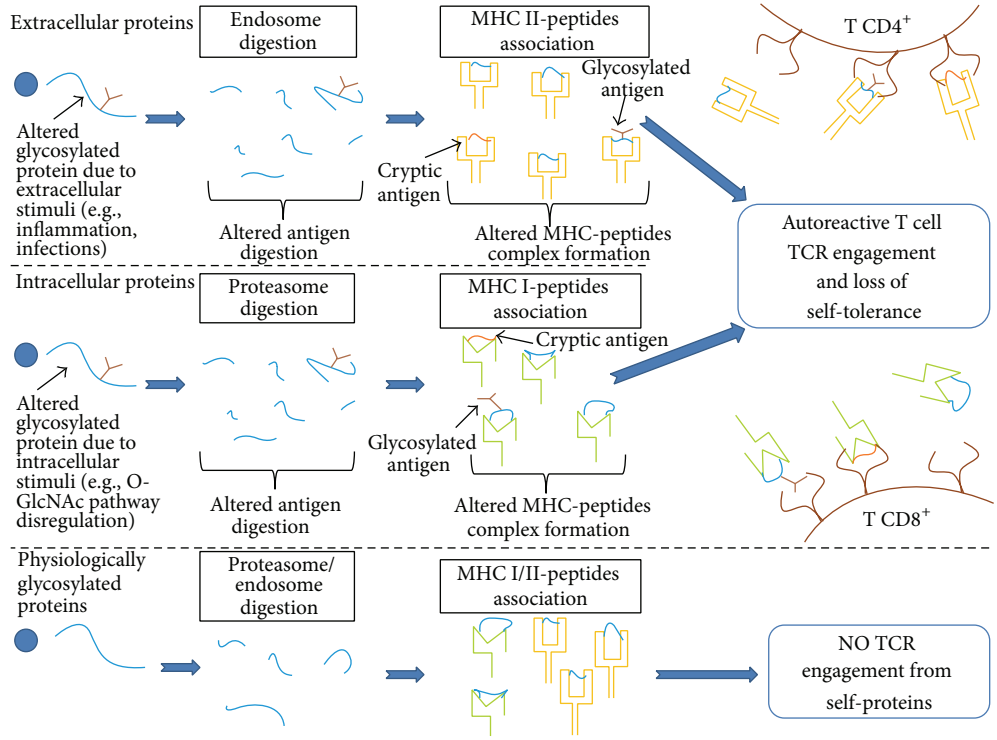

Figure 1: Aberrant glycosylation affects antigen presentation and can induce breaking of tolerance. (Of note, due to the cross-presentation pathway, intracellular proteins with altered glycosylation could be presented on MHC II molecules to T CD4$^+$).

demonstrated that DR4 transgenic mice (expressing human HLA-DR4 allele) are more responsive to the unmodified or hydroxylated K264 compared to the galactosylated one [15]. These results are in accordance with the previous observation that collagen from human and rats with active arthritis contains glycosylated and unglycosylated zones; on the contrary, in healthy subjects collagen is uniformly glycosylated [16]. Besides collagen, other proteins—for example, p68—show a glycosylation-dependent T cell recognition [17]; hence, an aberrant glycosylation might be one of the triggers for the onset and progression of RA.

Infections as well lead to an alteration of self-protein glycosylation. *E. coli* is able to modify glycosylation of self-proteins, due to its own GTs expression [18]. *H. pylori* CagA toxin could also alter the glycosylation processes in B lymphocytes [19]. Moreover, the cytokine secretion induced by these (and other) pathogens is able to modify the cellular pattern of GTs [20, 21]. All together, these events could be a rational explanation for the theory of "infection-triggered autoimmunity."

2.2. Glycosylation and Immunoglobulin Properties. As previously mentioned, glycosylation may affect the immune system also by modifying IgG properties. Fc fragment of IgGs has an important site of N-glycosylation, the 297 asparagine (N297). IgGs can exert both proinflammatory and anti-inflammatory activities depending on which Fcγ receptor they preferentially bind: the different receptor affinity strictly depends on the composition of the sugar side chain linked to N297 [22, 23]. The phenotype associated with a higher proinflammatory activity displays low levels of galactose and sialic acid [24]; differently, the anti-inflammatory phenotype is characterized by normal galactose and sialic acid and reduced GlcNAc levels [25, 26]. IgGs can exert their anti-inflammatory effects also by binding to other receptors: whether this interaction is dependent or not on Fc fragment glycosylation is still unclear [27–29]. Different factors, such as interleukins or lipopolysaccharide, can induce a proinflammatory pattern of the sugars linked to IgGs [30, 31]. This whole process finally leads to a vicious circle of self-sustaining immune activation.

In active RA, anti-citrullinated peptide antibodies (ACPA) and rheumatoid factor (RF) display the proinflammatory N297 glycosylation pattern, with low levels of galactose and sialic acid [32–34]. These autoantibodies acquire this glycosylation pattern before the clinical onset of the disease [35]; interestingly, changes in IgGs glycosylation were associated with RA remission observed during pregnancy [36, 37]. Taken together, these observations remark the crucial role of IgG glycosylation in the pathogenesis of the disease.

Many circulating proteins can also display an altered glycosylation in RA [38–40]. For example, lubricin isolated by synovial fluid of RA patients expresses an aberrant glycan determinant and shows an L-selectin ligand activity that may induce the activation of neutrophils and polymorphonuclear cells [40].

A good response to therapy turns the IgGs glycosylation back to a noninflammatory phenotype [41]. In order to stop the vicious circle triggered by the altered IgGs glycosylation,

Nandakumar et al. proposed a treatment with endostreptosin (EndoS)—a bacteric glycosidase able to trim the whole N297 side chain. *In vitro* incubation of anti-CII monoclonal antibodies with EndoS prevents the onset of arthritis in mice; this effect persists even if antibodies-EndoS are injected together with anti-CII antibodies [42].

Some authors hypothesized a possible treatment of severe RA and, in general, of autoimmune diseases with sialic acid enriched-intravenous Igs; however, the preliminary results are not conclusive and call for further researches [43, 44].

3. Citrullination and Rheumatoid Arthritis

Citrullination is a protein modification consisting in the switch of the iminic nitrogen of arginine to oxygen, linked to the backbone structure as a ketone; this process results in the production of an amino acid called citrulline. The reaction is mediated by a family of enzymes called peptidylarginine deiminase (PAD or PADI) [45] (Figure 2). Nowadays, 5 subtypes of PAD (PAD1-6), variously distributed in human cells, are known [46, 47]. The activity of these enzymes is regulated by intracellular Ca^{2+} concentration. The subtypes of PAD expressed by the immune cells are PAD4 and PAD2, markedly present in neutrophils and mast cells [48]. The switch from arginine to citrulline has several different consequences on protein structure: the oxygen of citrulline is a noncharged atom, differently from the positive-charged nitrogen of arginine; since side chain charge of the amino acid sequence is crucial for the protein folding, an alteration in the electronic milieu modifies the tertiary structure of the peptide [49]. So far, citrullination is associated with histone modifications, neutrophils extracellular trap (NET) formation, and epidermal, central nervous system and skeletal muscle tropism regulation [47].

Immunogenicity of citrullinated proteins has been studied, especially in autoimmune diseases such as RA, leading to the identification of ACPA. Nowadays, ACPA are essential in RA diagnosis: the presence of these autoantibodies, revealed with the ACPA assays, has a specificity of 85–95% and a sensitivity of about 80% [50].

ACPA can be identified in about 50% of RA patients approximately 1 year before the onset of arthritis [51]. Higher titre of ACPA at diagnosis is considered a negative prognostic factor and ACPA titre seems to be reduced after treatment [52, 53]. Furthermore, citrullinated antigens recognized by ACPA are abundant in inflamed synovia; among the others, alpha-1 antitrypsin, fibrinogen, apolipoproteins, histones, immunoglobulins, and vimentin had been characterized [54].

All these findings depose even for a pathological role of these autoantibodies in the development of RA.

Two questions arising from these data still remain partially unsolved: why do RA patient antibodies recognize as "non-self" the citrullinated proteins? And how citrullinated antigens are produced?

The first question found a partial answer in the strong association between ACPA-positive RA and specific HLA polymorphisms, especially the conserved region of HLA-DRB1*0104 allele: subjects who carry this allele have an higher risk of developing an erosive ACPA-positive RA, due to the ability of the HLA molecule coded by this specific haplotype to bind and present citrullinated antigens [55]. Nevertheless, this haplotype is also diffused in healthy population. Other susceptibility genes have been identified, but altogether they represent only 50% of genetic variance associated with ACPA-positive arthritis [56]. Clearly, more studies are needed to elucidate the physiopathological pathway leading to ACPA formation.

The latter question is much more complex. In fact, even if the final step of citrullination always consists in the activation of the PAD-family enzymes, the modulation of this pathway is variable and still not completely understood.

Summarizing, the processes leading to the formation of citrullinated epitopes can result in an accumulation of citrullinated proteins at extracellular and/or intracellular levels, which will be subsequentely described.

3.1. Extracellular Accumulation. Extracellular accumulation of citrullinated proteins is related to two main factors: NET formation and *P. gingivalis* infection. NETosis is a powerful defensive mechanism developed by immune system in order to trap and destroy extracellular bacteria. NETs are constituted by loose chromatin complexed with many antimicrobial enzymes stored in neutrophils granules; this whole complex is expelled by the cell in the extracellular space. The negative charge of chromatin traps the bacteria, allowing the enzymes to kill the microorganisms [57]. To undergo NETosis, neutrophils need PAD4 activation: the histone citrullination mediated by this enzyme causes chromatin relaxation that is crucial for the subsequent processes [58]. NETosis has been associated with several pathological conditions, like thrombosis, multiple sclerosis, and RA [59]. In RA, several alterations in NET formation have been noticed. When compared to healthy subjects, neutrophils isolated by the peripheral blood of RA patients undergo NETosis more easily; NETosis can be induced by RF or ACPA and, finally, the enzyme load of the chromatin shows differences between NETs of RA patients and healthy subjects [60].

In an inflammatory contest, such as the rheumatoid synovia, NETs release citrullinated antigens [61]: in RA predisposed individuals, synovial citrullinated antigens may be caught and presented to T lymphocytes, promoting a local immune response.

P. gingivalis, a Gram negative, nonmotile, anaerobic bacterium, is considered the major causative agent of periodontitis in humans [62]. Periodontitis is an inflammation of the structures surrounding the teeth, affecting about 50% of the adult population [63]. It is also a well-studied risk factor for the development of RA [64], since *P. gingivalis* is the only bacterium able to synthesize its own PAD [65]. This bacterium can lead to an increase in citrullination of host periodontium proteins, creating new citrullinated epitopes [66].

Arandjelovic et al. hypothesized a new pathway for extracellular citrullination: *in vitro*, ATP from dying cells can bind an ATP-receptor on mast cells and promote the activation and the release in the extracellular space of PAD2 [67].

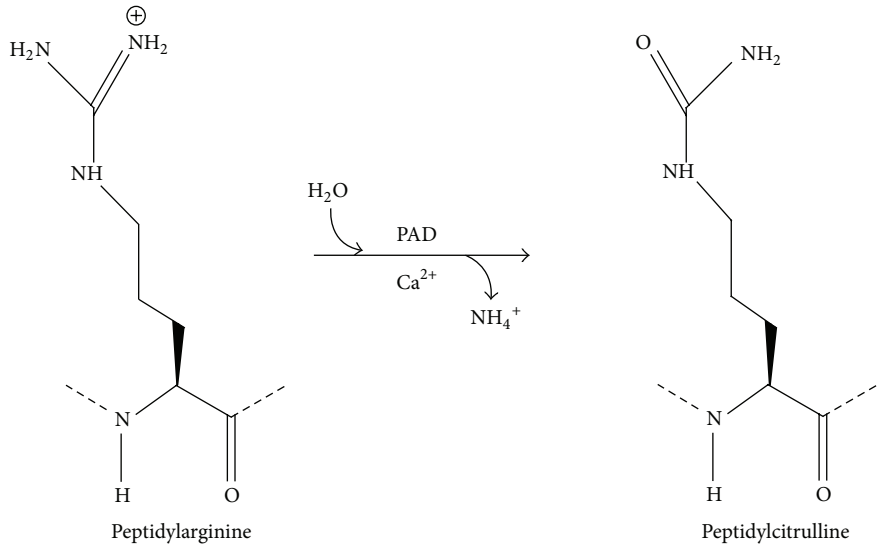

Figure 2: Biochemical process that occurs in protein citrullination event.

3.2. Intracellular Accumulation. Several mechanisms of intracellular accumulation of citrullinated proteins have been demonstrated *in vitro* and they represent an interesting field of research.

Autophagy is a cellular process consisting in the fusion of lysosomal vacuoles with vacuoles coming from intracellular elements, in order to digest them and restore energetic and nutrient reserve. Autophagy plays an important role in the regulation of intracellular organelle turnover [68]. Physiologically, this process occurs in starvation or under other stressful conditions for the cell and it has been also associated with several pathological conditions [69], including RA. During amino acid deprivation in B lymphocyte cell cultures, the induction of autophagy pathways leads to the intracellular activation of PAD and to the citrullination of self-peptides occurring during the MHC-peptide complex formation; moreover, treatment of B lymphocytes with both anti-IgG and anti-IgM results in an enhanced presentation of citrullinated antigens, abrogated by the addition of autophagy inhibitors; *in vivo*, the same mechanism may be mediated by RF [70].

Romero et al. demonstrated that membrane disruption can cause intracellular citrullination. This group showed that neutrophils treated with granzyme/perforin B or complement membrane attack complex undergo necrosis; the loss of membrane integrity led to a massive influx of Ca^{2+} and to an uncontrolled activation of PAD enzymes. The so-called hypercitrullination deriving from these events produces an elevated number of citrullinated proteins. Therefore, it can be hypothesized that *in vivo* an immune response directed to neutrophils could result in the formation of self-citrullinated antigens with a subsequent breaking of tolerance [71].

3.3. Smoking and Citrullination. Smoking is a risk factor for the development of ACPA-positive RA, especially in people who present HLA-DRB∗0104 allele [72]. This habit can increase the formation of citrullinated proteins and promote an activation of the immune system. Nanoparticles inbreathed with tobacco smoke can lead to the activation of PAD2 and PAD4 inside the cells [73]. Smoke effects on the cells include upregulation of PAD2 expression and subsequent citrullination [74].

To point out the crucial role of other predisposing factors in the onset of RA, Bongartz et al. revealed that lung biopsies of smokers that have developed lung cancer did not show any intracellular protein citrullination, in contrast with the strong intracellular protein citrullination detected in RA affected smokers [75].

3.4. PAD Inhibitors. Recently, several authors studied inhibitors of PAD for the treatment of RA. Cl-amidine, a pan-PAD inhibitor, showed an efficacy in mice with CIA in reducing severity of disease, synovial citrullination, and histological joint damage [76]. Another PAD inhibitor, the compound named YW3-56, showed a 5-fold increase in PAD4 activity inhibition, if compared with Cl-amidine. YW3-56 has been tested as anticancer drug, but its ability in blocking PAD4 could make it a good candidate also for the treatment of RA [77].

4. Carbamylation and Rheumatoid Arthritis

Carbamylation is a nonenzymatic PTM that consists in the addition of a cyanate group ($O=C=N^-$) on self-proteins [78]. This reaction usually affects the atoms of nitrogen, although a certain degree of carbamylation has also been demonstrated on sulphur atoms [79]. Free nitrogen atoms in proteins can be found at the N-terminus or in the side chain of lysine and arginine. The most important carbamylation detected in patient with RA is the lysine carbamylation: this chemical process leads to the formation of homocitrulline, a noncanonical amino acid, through the link of the carbon atom of cyanate with the nitrogen atom of the lysine [80].

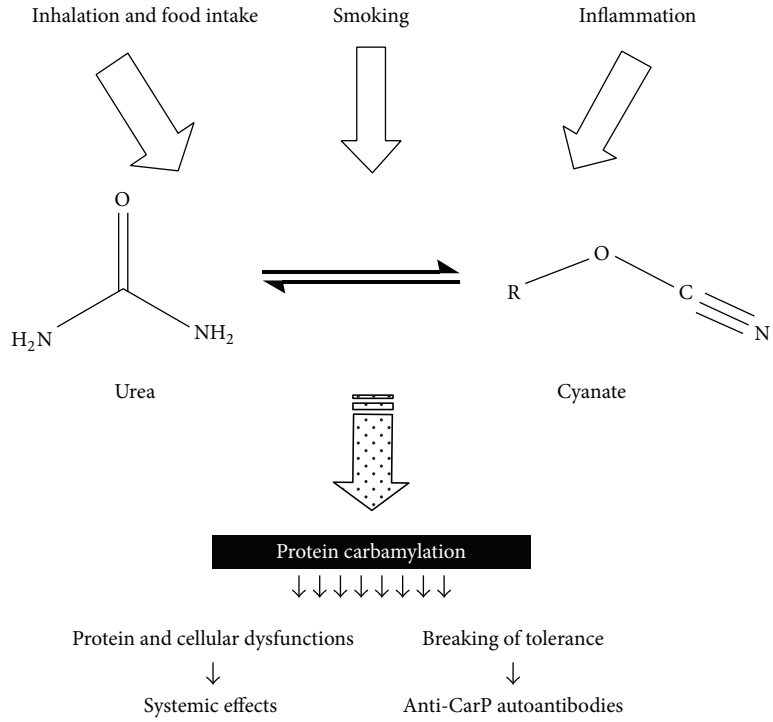

Figure 3: Protein carbamylation: causes and consequences. Several conditions can shift the balance between urea and cyanate towards cyanate production, enhancing in this way the process of protein carbamylation. This event results in systemic effects and production of anti-CarP autoantibodies.

Cyanate required for carbamylation can be produced in two different ways.

The first way is the spontaneous degradation of blood urea. In fact, urea is in equilibrium with cyanate [81], and the equilibrium in cyanate-urea ratio *in vivo* seems to oscillates around 1 : 200 [82].

The second way consists in cyanate intake from the external environment. Cyanate is found at a concentration of about 200 parts-per-trillion by volume in urban air and can be detected in both tobacco and biomass smoke [83]; potassium and sodium cyanate are used in several herbicides [84]; finally, many foods (e.g., broccoli) possess a moderate quantity of thiocyanate that, reacting with reactive oxygen species, can be converted in cyanate [85, 86].

In healthy individuals, the concentration of blood cyanate is about 50 nmol/L, an amount that is 1000 times lower than the expected one, according to urea kinetic equilibrium [87]. For some authors, this alteration can be explained with a sort of "physiological carbamylation" of N-terminus of blood proteins [88].

Carbamylation is time dependent: structural proteins, due to their slow turnover, are indeed much more likely to be carbamylated, if compared to proteins with shorter half-life [89].

Carbamylation of self-proteins causes a loss of the native proteic structure and can lead to a break of tolerance, finally resulting in the formation of anti-carbamylated proteins (anti-CarP) autoantibodies (Figure 3). These autoantibodies can be detected in RA patients. Inhibition assays demonstrated that ACPA and anti-CarP are 2 different families of autoantibodies [90].

Immunization with homocitrullinated peptides can cause T and B cell mediated immune response against the synovial membrane, and the intrarticular injection of homocitrulline-containing peptides can result in the development of a mild arthritis even in nonimmunized mice. Interestingly, in the study of Mydel and coworkers it was demonstrated that mice immunization with homocitrulline followed by the intrarticular injection of citrullinated or homocitrullinated peptides determined a severe arthritis in a higher percentage of the first group (more than 90%), suggesting this an unexpected response as a final linkage between alteration in citrullination and severe arthritis development [91].

Anti-CarP autoantibodies can be detected in about 16% of seronegative RA patients; their presence is correlated to a more severe and erosive disease [90] and represents a risk factor for developing RA in patients with inflammatory arthralgia [92]. Anti-CarP autoantibodies have been detected in RA patients' sera before the clinical manifestation of disease [93]. So, they appear to be a new useful tool in the diagnosis and follow-up of RA.

5. Conclusion

PTMs are certainly implicated in the development of RA and in several other autoimmune and nonautoimmune diseases.

Nowadays, many aspects of RA pathogenesis have been understood, thanks to the large number of studies focusing on the role of PTMs in the induction of this disease. However, none of the theories proposed to explain the exact aetiology of RA is completely exhaustive. Every single PTM alteration alone is not sufficient to ignite the disease, so overlap of multiple alterations seems to be needed for the onset of RA in predisposed patients.

The challenge for the future studies is to find a linkage between the various PTM alterations that occur during the development of RA, in order to identify possible steps leading to the breaking of self-tolerance and to the clinical onset of the disease. Improving knowledge in the pathogenesis of RA could offer new suggestions for the development of more effective drugs.

Conflict of Interests

The authors have no conflict of interests to declare. The authors alone are responsible for the content and writing of the paper.

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Review Article

Treatment of Bullous Systemic Lupus Erythematosus

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Bullous systemic lupus erythematosus (BSLE) is an autoantibody-mediated vesiculobullous disease in patients with SLE. Autoimmunity in BSLE is characterized by the presence of circulating anti-type VII collagen antibodies. BSLE patients often present with multiple, tense, clear fluid-filled vesicles and bullae overlying erythematous edematous plaques. Skin biopsy from BSLE patients shows subepidermal bullae with numerous neutrophils and only occasional eosinophils. Furthermore, immunofluorescence examination showed linear deposition of lgG, lgA, C3, and C1q along the basement membrane zone. BSLE patients with corticosteroids treatment constantly do not receive a marked improvement, while dapsone generally dramatically improved the skin condition. Recently, it has been reported that quite a few cases of BSLE were successfully treated with other immune suppressive drugs. Therefore, a comprehensive review of the treatment of BSLE would be beneficial to cure the disease.

1. Introduction

Bullous systemic lupus erythematosus (BSLE) is a subepidermal blistering disease that occurs in a subset of patients with systemic lupus erythematosus (SLE) [1, 2]. Cutaneous lesions are reported during the course of SLE in 76% of patients; however, it has been reported that BSLE is very rare and occurs in less than 1% of patients with SLE [3–5]. Clinically, in addition to the features of SLE, the BSLE patients especially present with a rapid, widespread development of tense fluid-filled vesicles and bullae. Moreover, this blistering disease may vary from a small group of vesicles to large tense blisters with urticarial eruptions, erosions, itching, and crustations. Histologically, BSLE is characterized by a subepidermal blister, with a predominantly neutrophilic dermal infiltrate and only occasional eosinophils. Furthermore, immunofluorescence examination showed linear deposition of lgG, lgA, C3, and C1q along the basement membrane zone [5, 6]. Because of the particularly clinical and histological presentation of BSLE, Camisa and Sharma proposed diagnostic criteria for BSLE; these include a diagnosis of SLE based on the following criteria of the ACR; vesicles and bullae mainly located on sun-exposed areas; the histopathology is characterised by subepidermal bullae with microabscesses of neutrophils in the dermal papillae, similar to those found in dermatitis herpetiformis and deposition of IgG, IgM, or both and often IgA in the basement membrane zone [7].

Although BLSE may exhibit any of the symptoms associated with SLE, the onset and course of blistering eruption do not necessarily parallel the activity of the systemic involvement [8]. Furthermore, the therapeutic options for SLE are not usually fit for BSLE [9, 10]. In some cases, the eruption flared after systemic corticosteroid administration for SLE [11, 12]. However, most of the patients have a striking therapeutic response to dapsone [13–16]. A response may be seen with very small doses of dapsone [1]. In the case of the present paper, we report a significantly and clinically meaningful improvement of BSLE following dapsone administration. Other drugs such as cyclophosphamide, azathioprine, and mycophenolate mofetil and biologic drugs may also be effective for BSLE treatment [17]. In the part of literature review, we provide a review of all the available treatment options for BSLE.

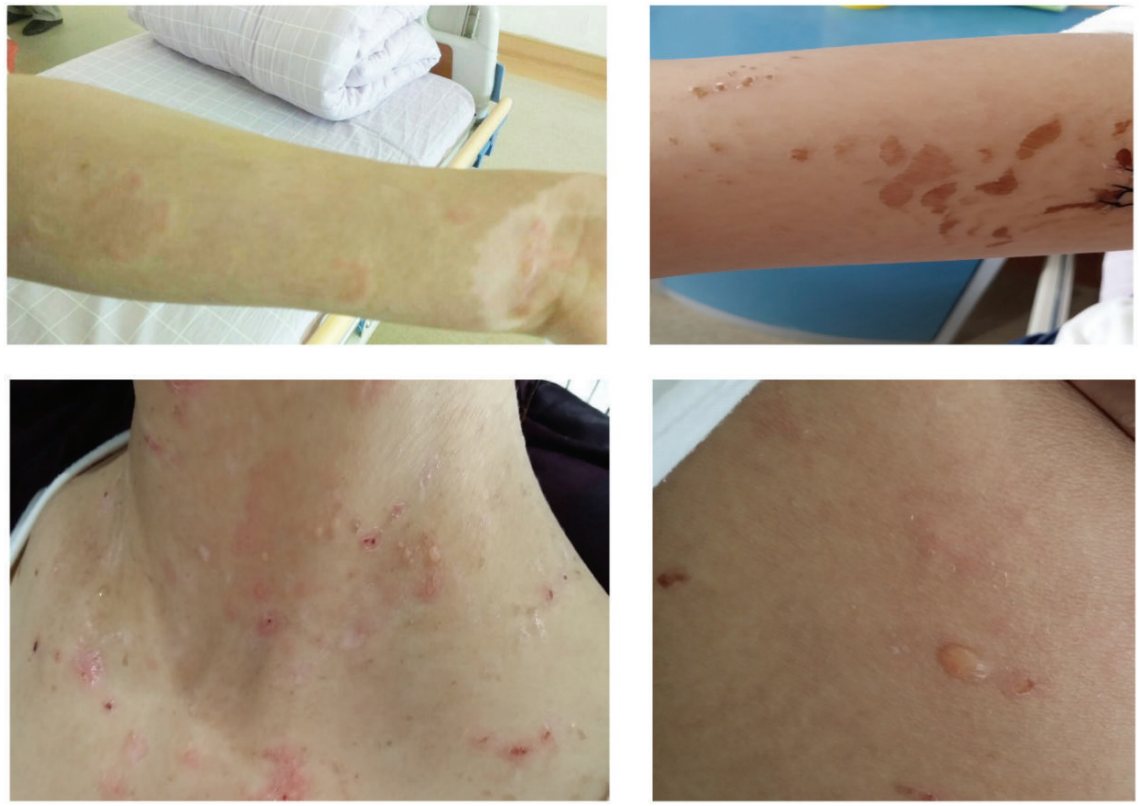

Figure 1: Gross view of the skin lesion. Presence of tense vesicles (marked with an arrow) filled with clear fluid on the arm, neck, and back.

2. Literature Review

Steroids and antimalarials are the standard treatments for the cutaneous manifestations of SLE. In unresponsive patients, azathioprine and high dose or pulse steroids, cyclosporin, and pulse cyclophosphamide are the most commonly used alternative therapies [18–20]. Dapsone is less used in the control of the SLE rash but has a dramatic improvement in the eruption of BSLE patients [15, 16]. A relatively low dose has also been shown to be an efficacious response. We also found that a 22-year-old woman with BSLE had multiple tense vesiculobullous lesions on the face, trunk, and limb (Figure 1). A biopsy from the upper limb showed a subepidermal blister with a predominantly neutrophilic dermal infiltrate and only occasional eosinophils (Figure 2(a)). Immunofluorescence showed a granular band of C1q, C3, and IgG at the basement membrane; less IgA and IgM were observed (Figures 2(b)–2(f)). The skin condition showed no response in the methylprednisolone, while a considerable improvement after dapsone administration was observed. Regarding the special clinical feature and the discriminative therapies from the SLE treatments, we review all the available treatment for BSLE.

2.1. Dapsone. Dapsone is a sulfone that has played a critical role in the eradication of leprosy [21]. Besides, a number of cutaneous eruptions are effectively controlled by dapsone [22]. Due to these eruptions that are largely characterized by the presence of cutaneous neutrophilic dermal infiltrate [22], such as dermatitis herpetiformis and the inflammatory variant of epidermolysis bullosa acquisita, the mechanism of its anti-inflammatory action mainly relies upon its inhibition of the functions of polymorphonuclearleukocytes and of complement activation via the alternative pathway that has been postulated [15, 23]. Although a new or recurrent rash was considered a factor of SLE disease activity index, the eruption of BSLE was not constantly associated with a flare of SLE [8]. Consequently, because of being unparallel with the disease activity, the eruption of BSLE patients is often unresponsive to corticosteroid therapy. Due to the striking histologic resemblance to dermatitis herpetiformis, the patients who were treated with dapsone (2 mg/kg/day) usually obtained a dramatic improvement in the eruption. Patients tend to have an efficacious response with cessation of new blister formation in 1-2 days and healing of existing lesions within several days. A relatively low dose (25–50 mg) has also been shown to have a response [1, 15, 24]. Interestingly, improvement of the eruption did not correlate with amelioration of the systemic manifestations [25]. The dramatic response to dapsone therapy demonstrated that dapsone is useful in treating bullous lesions of SLE [5, 15, 16, 26]. Notably, the blistering eruption was not improved by dapsone in some cases, and even worsening has been noted after its administration. It has been reported that patients with BSLE, who initially presented with lesions clinically resembling erythema multiform, experienced exacerbation of their disease with dapsone [27–29]. Furthermore, dapsone

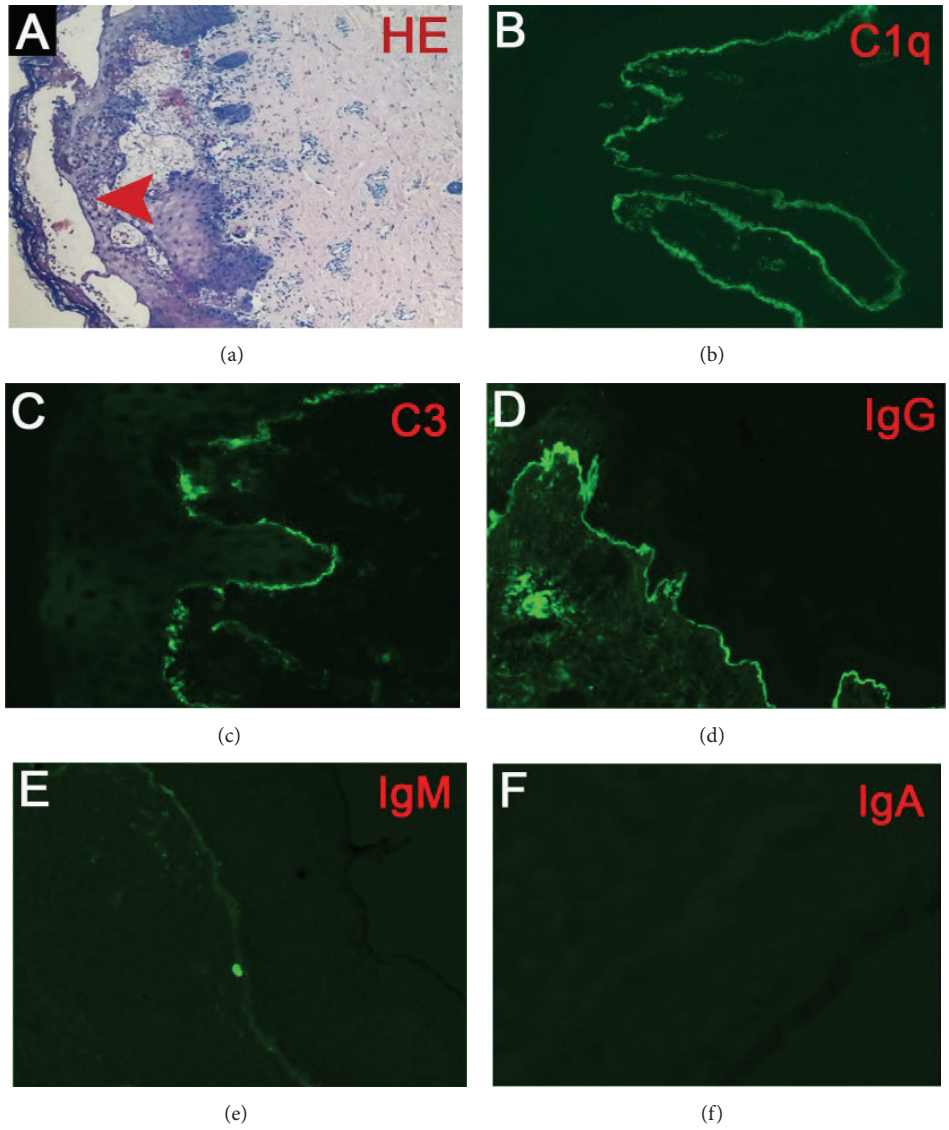

Figure 2: Histopathology of the skin lesion. (a) Histopathologic examination of the skin biopsy specimen showed a subepidermal blister (arrow indicated) with abundant neutrophils infiltration and only occasional eosinophils (H&E stain, 100x). (b) Direct immunofluorescence examination showed linear, granular deposition of C1q (b), C3 (c), and IgG (d) at the dermoepidermal junction (400x); less IgM and IgA were detected.

has been assigned to pregnancy category C; BSLE patients with pregnancy might not be fit for administration of this drug [30]. Hemolysis and hepatic and renal toxicity usually accompany administration of the drug in a dose related fashion [31–34]; therefore its clinic use was confined and a careful monitoring of its toxicity is required.

2.2. Corticosteroids. Corticosteroids are usually required to improve clinical symptoms and laboratory abnormalities and are still a mainstay for inducing remission in SLE patients [18, 20, 35]. Topical corticosteroids may be helpful in the treatment of cutaneous SLE. Unexpectedly, many bullous SLE patients tend to be unresponsive to systemic corticosteroid therapy that has been described [36]. Furthermore, in some cases of SLE patients, the eruption flared a few days after systemic corticosteroid administration [11, 12]. Interestingly, some patients responded effectively to corticosteroids, although they required relatively high doses [37]. A patient with SLE who presented with vesiculobullous lesions during the third trimester of pregnancy has been presented. A skin biopsy of this patient was performed, and it showed significant necrosis of keratinocytes in the epidermis and granular, dense, and continuous deposits of moderate IgG positivity in the basement membrane zone. Dapsone has been assigned to pregnancy category C. The pregnant woman treated with high-dose corticosteroids obtained a satisfactory response [30]. As the dapsone administration often causes hepatic and renal toxicity [32, 33, 38–40], prednisone alone or in combination with low doses dapsone might be the treatment of choice for these BSLE patients. These observations

demonstrated that corticosteroids may act as an alternative treatment for BSLE when patients are unresponsive or unfit for other drugs [37].

2.3. Rituximab. Biologic agents, such as infliximab, rituximab, and anakinra, have emerged as effective therapies for treating a wide spectrum of diseases which includes various rheumatic, gastrointestinal, and cutaneous diseases [41–44]. The involvement of all of the key components (especially cytokines and immune cells) of the immune system in the pathogenesis of SLE offers many potential targets for therapeutic management of this disease. B cells, a critical immune cell, which can act as antigen-presenting cells, differentiate into plasma cell to produce pathogenic autoantibodies and secrete various cytokines and chemokines in the immune response [45, 46]. These functions of the B cell support the fact that it plays an important role in the development of pathogenesis of SLE. Therefore, use of B cell depletion therapy in SLE has emerged as a novel and promising therapeutic alternative for SLE patients [47–49]. Rituximab which is a chimeric monoclonal antibody that reacts with CD20, an antigen that is present on immature, naive, and memory B cells but not on mature plasma cells, has been approved in the treatment of SLE [50]. Up to now, there is only a case report about rituximab in BSLE. The patient was treated with hydroxychloroquine (HCQ) twice daily, mycophenolate mofetil 1000 mg/d, and varying doses of corticosteroids, while the eruption was not improved. Dapsone and azathioprine were added but had to be stopped because of elevated liver enzymes and leukopenia. Mycophenolate mofetil was increased to 2000 mg/d, but her skin disease remained active. Then the patient was treated with intravenous infusions of rituximab. The skin lesions improved within 10 days after the first dose and cleared by day 15 after the second dose. Furthermore, prednisone was successfully tapered, and the patient has remained free of recurrence of cutaneous and oral blistering lesions [34]. The results of this case suggest a potential role for treatment of refractory BSLE with rituximab.

2.4. MTX. Methotrexate (MTX) has been widely proved to be an effective agent in control of the rheumatoid arthritis [51]. It has proved that MTX was beneficial in sporadic cases of SCLE refractory to therapy with conventional therapy [52–54], such as antimalarials and corticosteroids. Furthermore, in a randomized and double-blind trial in 41 patients with SLE, MTX reveals a role for controlling the skin lesions in 75% of cases, with a mean reduction of prednisone dose of 44% [55]. These reports demonstrated that MTX could represent a valid therapeutic option in controlling the cutaneous SLE and in sparing the steroid dose. However, BSLE is a subepidermal blistering disease that occurs in a subset of patients with systemic lupus erythematosus (SLE). Cutaneous lesions of BSLE are reported during the course of SLE in less than 1% of patients, while lesions are not in line with the disease activity [9]. Unexpectedly, BSLE is often unresponsive to antimalarials and corticosteroids [56]. In a recent report, a rapid and full resolution of cutaneous lesions was obtained with methotrexate alone. A case of 40-year-old female with systemic lupus erythematosus (SLE) developed a severe bullous eruption on sun-exposed areas, while the previous manifestations of the disease were quiescent. In consideration of prior intolerance to many drugs, methotrexate was administrated. The drug administration was followed by a rapid and full resolution of cutaneous lesions. Therefore, MTX might be an alternative therapeutic choice to dapsone [57].

2.5. Other Therapies. HCQ is a commonly used drug for controlling the cutaneous lesions and the disease activity of the SLE [58, 59], while it does not act effectively in the eruption of BSLE [10]. The conventional treatment for SLE only revealed modest improvement in steroids and antimalarials [9]. Although cyclophosphamide has been shown to produce moderate improvement of skin lesions of SLE, the beneficial role in BSLE has not been demonstrated [60]. Mycophenolate mofetil (MMF) has been widely used for suppressing the lupus activity, while it also was not valid for BSLE, even at a high dose [10, 37]. In cases nonresponsive to dapsone, the eruption has been controlled by prednisone or with combination therapy of prednisone and azathioprine [8].

3. Conclusions

In the treatment of bullous SLE, dapsone is the effective basic therapy, and it often induces a dramatic response. In some cases, where an adequate response is not achieved with dapsone or the SLE disease activity index is high, other immunosuppressants, such as prednisolone, methotrexate, and azathioprine, can be used for controlling the eruption and suppressing the systemic symptoms. Moreover, in some special case, where dapsone administration or other chemical drugs (MTX, azathioprine, etc.) induce serious side effects, biologic agents might be an alternative choice for BSLE.

Conflict of Interests

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

Authors' Contribution

Lihua Duan and Liying Chen contributed equally to this work.

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Research Article

Endothelin Receptors Expressed by Immune Cells Are Involved in Modulation of Inflammation and in Fibrosis: Relevance to the Pathogenesis of Systemic Sclerosis

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Endothelin-1 (ET-1) plays a pivotal role in vasoconstriction, fibrosis, and inflammation, the key features of systemic sclerosis (SSc). ET-1 receptors (ET_A and ET_B) are expressed on endothelial cells, smooth muscle cells, and fibroblasts, but their presence on immune cells has not been deeply investigated so far. Endothelin receptors antagonists such as bosentan have beneficial effects on vasoconstriction and fibrosis, but less is known about their potential anti-inflammatory effects. We studied the expression of ET-1 receptors on immune cells (T and B lymphocytes, monocytes, and neutrophils) and the link between ET-1 and inflammation in patients with SSc. We show here that ET-1 exerts a proinflammatory effect in CD4+ T cells, since it induces an increased IFN-γ production; preincubation with antagonists of both receptors reduces IFN-γ production. Moreover, following ET-1 stimulation, neutrophils produce proinflammatory mediators, thus amplifying the effects of activated CD4+ T cells. Our data indicate that ET-1 system is involved in the pathogenesis of inflammation and fibrosis typical of SSc, through the activation of T lymphocytes and neutrophils and the consequent release of proinflammatory and profibrotic cytokines. These findings suggest that dual ET-1 receptors antagonist therapy, besides its effect on vasculopathy, has a profound impact on the immune system favouring antiinflammatory and antifibrogenic effects.

1. Introduction

Systemic sclerosis (SSc) is an autoimmune disease that involves the connective tissue of skin and internal organs with a remarkable heterogeneity in the disease course and affected organs, resulting in high morbidity and mortality. The disease is characterized by vascular dysfunction and injury and by overproduction and accumulation of collagen and other extracellular matrix proteins, resulting in the thickening of the skin and fibrosis of the affected organs [1, 2]. The pathogenetic mechanisms involve three interactive components represented by severe and diffuse endothelial cell damage, immune system dysfunction, and fibroblasts activation.

Endothelin-1 (ET-1) has been described to play a role in fibrosis, angiogenesis, and inflammation, all major features of SSc [3, 4]. Indeed ET-1 level is elevated in the serum and tissues of SSc patients, especially in diffuse SSc patients, and serum levels have been shown to correlate with the extent of vascular damage and cutaneous fibrosis [3–6].

ET-1 is the major isoform of three endothelin isoforms and is a soluble mediator that exerts a potent vasoconstrictor

effect [7]. ET-1 has been firstly described in endothelial cells and in vascular smooth muscle cells, where hypoxia, cold exposure, low shear stress, angiotensin II, cytokines, and growth factors may facilitate its production [7, 8]. Many cells can produce ET-1 including fibroblasts and myofibroblasts, mast cells, monocytes/macrophages, polymorphonuclear leukocytes, and dendritic cells [9–14]. Transforming growth factor- (TGF-) β and ET-1 itself, with an autocrine loop, are able to induce ET-1 production in fibroblasts and myofibroblasts [9, 15].

There are at least three ET-1 receptors: ET_A, ET_B, and ET_C [8, 16, 17]; however, the function of ET_C is poorly known; ET_A and ET_B are expressed on the majority of cells that actively contribute to SSc pathogenesis, such as fibroblasts, myofibroblasts, vascular smooth muscle cells, and platelets, while endothelial cells selectively express ET_B [6–8, 16]. Upon binding receptors on vascular smooth muscle cells, ET-1 is able to induce vasoconstriction, cell growth, and proliferation, leading to lumen narrowing at arterial and arteriolar level [18–20]. Moreover, ET-1 facilitates fibroblasts transdifferentiation into myofibroblasts and induces the production of both collagen and ET-1 probably through an autocrine mechanism [9, 15, 21–26].

There is increasing evidence that ET-1 may play a pivotal role in inflammation in several human diseases including chronic renal disease, asthma [27–30], and sepsis (reviewed in [44]); however, the mechanisms by which ET-1 induces the activation of the innate and adaptive immune systems have not been fully elucidated so far. Saleh and Pollock suggested that ET-1 can directly activate neutrophils and can induce the production of chemoattractant factors, such as monocyte chemoattractant factor-1 (MCP-1), and the synthesis of cell adhesion molecules, such as soluble intercellular adhesion molecule-1 (ICAM-1) [27]. Moreover ET-1 seems to be associated with the activation of transcription factors such as NF-κB and the production of proinflammatory cytokines including tumor necrosis factor-alpha (TNF-α), interleukin- (IL-) 1, and IL-6 [31]. Little is known on ET-1 receptors expression on immune cells with the exception of a few data on cells of the innate immune system, such as dendritic cells and monocytes [10–14, 32]. The expression of ET-1 receptors on adaptive immune effectors cells (T and B lymphocytes) has not been investigated so far and therefore little is known on the possible role of ET-1 as a mediator of inflammatory responses.

In the last decade, orally active ET-1 receptor antagonists (ERAs) were developed and approved for clinical use. Two orally active ERAs are currently approved, the dual receptor antagonist, bosentan, and the selective ET_A receptor antagonist, ambrisentan [33–35]. Both ERAs are used in the treatment of pulmonary arterial hypertension (PAH) whereas only bosentan has been shown to be effective in the prevention of new scleroderma-related digital ulcers (DUs) [36]. In addition to the effects on vasculature and fibrosis [36–38], it has been recently reported that ET-1 blockade using bosentan may also have some anti-inflammatory effects ([39], reviewed in [44]). In particular, bosentan seems to be able to suppress the ET-1-induced production of TNF-α and other proinflammatory mediators by monocytes *in vitro* [11]. *In vivo*, bosentan significantly reduces IL-6, ICAM-1, and pro-brain natriuretic peptide (pro-BNP) serum levels in patients with PAH [40] and leads to the normalization of soluble adhesion molecules in SSc-associated PAH [40, 41].

Inflammation is deeply involved both in the early phase of SSc pathogenesis and in the progression of vascular damage and fibrosis. Therefore, we aimed at investigating the role of ET-1 as possible mediator of inflammatory damage in SSc. Since immune effectors cells, such as T and B lymphocytes, monocytes, and neutrophils, are important players of inflammation in SSc, we aimed at clarifying the possible role played by ET-1 receptors in immune cells activation.

In this paper, we studied the presence of ET-1 receptors on T and B lymphocytes, monocytes, and neutrophils by FACS analysis. We also analysed the effects of ET-1 receptors engagement in order to verify the proinflammatory activity of ET-1 and the potential anti-inflammatory effects of ERAs.

2. Materials and Methods

2.1. Patients and Controls. We studied a cohort of 41 patients (5 males and 36 females, mean age: 57 ± 14 years) affected by SSc, attending the Unit of Autoimmunity Diseases at the University Hospital of Verona, Italy. SSc diagnosis was performed in accordance with the American College of Rheumatology/European League against Rheumatism classification criteria for systemic sclerosis [42, 43].

Patients were classified according to the following clinical features: limited (lSSc) or diffuse (dSSc) cutaneous form of SSc (32 patients with lSSc and 9 with dSSc) and presence or absence of ischemic digital ulcers, PAH, and interstitial lung disease (ILD). Ten patients were on bosentan therapy because of digital ulcers or PAH. Twenty age and sex matched healthy subjects were used as control group.

Blood samples (20 mL) were collected in heparinized Falcon tubes (Becton Dickinson, NJ, USA) from both patients and control subjects. A written informed consent was obtained from all the participants to the study and the study was approved by the local ethical committee. All clinical investigations have been conducted according to the principles expressed in the Helsinki declaration.

2.2. Isolation of Peripheral Blood Mononuclear Cells and Flow-Cytometry. Blood samples obtained from patients and controls were diluted with 20 mL of phosphate buffered saline (PBS) solution. Mononuclear cells isolated by density gradient centrifugation using lymphoprep Ficoll-Isopaque (Axis-Shield, Oslo, Norway) were washed twice with PBS and suspended in tubes containing 1 million cells for flow-cytometry (FACS) analysis. Analysis of monocytes and lymphocytes was carried out in different tubes; cells used for monocytes staining were preincubated with mouse serum (DAKO, Glostrup, Denmark) for 10 minutes at room temperature. Each sample was incubated for 1 hour at 4°C with eitherrabbit polyclonal anti-ET_A (Acris Antibodies GmbH, Herford, Germany) or sheep polyclonal anti-ET_B (Lifespan Biosciences, Seattle, WA, USA) antibodies. Phycoerythrin- (PE-) conjugated goat anti-rabbit IgG monoclonal (0.25 mg/mL) was used as a secondary antibody for ET_A (R&D Systems, Minneapolis, MN, USA)

and PE-conjugated donkey anti-sheep IgG monoclonal (0.2 mg/mL) was used as a secondary antibody for ET_B (R&D Systems) and incubated for 30 minutes at 4°C. Samples were also stained for 20 minutes at room temperature in a dark room with allophycocyanin- (APC-) conjugated anti-CD3 or anti-CD14 or anti-CD19 antibodies (BD Biosciences, San Jose, CA, USA). After labeling, samples were acquired in a FACSCanto cytometer (Becton Dickinson). The sensitivity of fluorescence detectors was set and monitored using Calibrite Beads (Becton Dickinson) according to the manufacturer's recommendations; 20.000 CD3+, CD14+, or CD19 cells per sample were, respectively, acquired in live gating. FlowJo 8.8.2 software (Tree Star, Ashland, OR) was used to analyze the data. Expression of ET_A or ET_B was calculated as the difference between mean fluorescence intensity (MFI) of cells stained with primary plus secondary antibodies and MFI of their negative control (cells stained with secondary antibodies): ΔMFI.

2.3. T Cells Stimulation. In order to assess receptors expression on activated CD4+ and CD8+ T cells, PBMC isolated from 4 patients and 4 controls were stimulated for 24 hours with anti-CD3/CD28 antibodies coated microbeads-Dynabeads Human T-Activator (Dynal, Oslo, Norway), according to the manufacturer's recommendations. Cells were cultured in RPMI-1640-GlutaMAX-I, supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 microg/mL streptomycin (all purchased from Life Technologies, Carlsbad, CA). In order to identify CD4+ and CD8+ T lymphocytes, we incubated cells with a mixture of the following antibodies: PerCp-conjugated anti-CD3, APC-H7-conjugated anti-CD4, and APC-conjugated anti-CD8 antibodies. Activated cells were detected by incubating cells with FITC-conjugated anti-CD25 antibodies; all reagents were purchased from BD Biosciences. Cells were previously stained with anti-ET_A and anti-ET_B primary and secondary antibodies as previously described and samples were acquired on a FACSCanto cytometer FlowJo 8.8.2 software was used to analyse data. The variation in receptors surface exposure was expressed as the difference between activated cells MFI and unstimulated cells MFI (ΔΔMFI).

2.4. Isolation of T CD4+ Cells. Mononuclear cells from healthy donors buffy coats were isolated by density gradient centrifugation using lymphoprep Ficoll-Isopaque. CD4+ T cells were obtained through negative selection using CD4+ T Cell Isolation Kit II (Miltenyi Biotec) and MidiMACS Starting Kit, including MACS LD column and MACS Separator (Miltenyi Biotec), following manufacturer's instructions.

2.5. RNA Extraction and RT-PCR from CD4+ T Cells. Total RNA was extracted from CD4+ T cells using TRIzol Reagent (Gibco BRL, Billings, MT, USA) following the manufacturer's protocol. RNA was previously treated with DNAse I (Invitrogen).

First-strand cDNA was carried out using the Super Script III System (Invitrogen, Carlsbad, CA, USA), with random hexamers, according to the manufacturer's recommendations. Fibroblasts cDNA was used as positive control for the detection of ET_A- and ET_B-coding mRNA.

CD4+ T cells and fibroblasts cDNA were amplified with ET_A and ET_B specific primers:

ET_A forward 5′-ATGCACAACTATTGCCCACA-3′,
ET_A reverse 5′-GGACAGGATCCAGATGGAGA-3′;

ET_B forward 5′-GCACATCGTCATTGACATCC-3′,
ET_B reverse 5′-CAGAGGGCAAAGACAAGGAC-3′
(Sigma-Aldrich, Saint Louis, MO, USA).

Vimentin was used as PCR reaction-control. Amplification was performed using the AmpliTaq Gold PCR MasterMix system (Applied Biosystems, Foster City, CA, USA). cDNA was amplified using the primers specific for ET_A and ET_B receptors and for vimentin using the GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) and the amplification reaction was carried out as follows: 10 minutes at 95°C followed by 40 cycles of denaturation (45 seconds at 94°C), annealing (30 seconds at 53°C for ET_A and 55°C for ET_B and for vimentin), and extension (1 minute at 72°C and 7 minutes at 72°C to stop reaction). Amplicons (length: 447 bp for ET_A, 558 bp for ET_B, and 266 bp for vimentin) were run on agarose gel (1.5%) and revealed using VersaDoc video documentation system (Bio Rad, Hercules, CA, USA).

2.6. Evaluation of Cytokine Secretion by CD4+ T Lymphocytes following ET_A and ET_B Stimulation. In order to study the cytokine production in response to ET_A and ET_B stimulation by ET-1 in CD4+ T cells, we seeded CD4+ cells in microplates: 1 million CD4+ T cells per well were seeded in 24-well plates and different conditions were carried out in duplicate. Cells were incubated (a) without ET-1 and receptors antagonists (control sample); (b) with ET-1 alone; (c) with ET_A antagonist (BQ123) and ET-1; (d) with ET_B antagonist (BQ788) and ET-1; (e) with BQ123 plus BQ788 and ET-1. Cells were incubated with BQ123 and BQ788 at the concentration of 10^{-6} M for 45 minutes and with ET-1 at concentration of 10^{-7} M for 24 hours. All reagents were purchased from Sigma-Aldrich.

We then measured interferon- (IFN-) γ, IL-4, and IL-17 concentrations in cell culture supernatants by enzyme-linked immunosorbent assay (ELISA) (Quantikine Human IFN-γ Immunoassay, Quantikine Human IL-4 Immunoassay, and Quantikine Human IL-17 Immunoassay, resp., obtained from R&D Systems), following the manufacturer's instructions. Sunrise absorbance reader for microplates (Tecan, Salzburg, Austria) was used to determine optical density for each sample.

2.7. Isolation of Neutrophils, Flow-Cytometry, and RT-PCR. We isolated neutrophils from healthy donors buffy coat in order to study surface expression of ET_A and ET_B by flow-cytometry and the transcripts for ET_A and ET_B by RT-PCR. Highly purified granulocytes (neutrophils > 96.5%) were isolated and prepared under endotoxin-free conditions using lymphoprep Ficoll-Isopaque. Neutrophils were further enriched by positively removing all contaminating cells with mAb against CD3, CD56, CD19, CD36, CD49d, and Gly-A

Table 1: ET_A and ET_B expression on T and B cells, monocytes, and neutrophils in healthy controls and SSc patients.

ET-1 receptors	T lymphocytes		B lymphocytes		Monocytes		Neutrophils	
	ET_A	ET_B	ET_A	ET_B	ET_A	ET_B	ET_A	ET_B
Healthy controls ($n = 20$)	110.45 ± 35.89	49.23 ± 29.16	269.75 ± 37.14	150.75 ± 26.42	188.4 ± 35.61	98.74 ± 54.66	191.65 ± 42.61	92.54 ± 50.89
SSc patients ($n = 41$)	100.61 ± 45.21	46.85 ± 29.78	253.5 ± 40.54	161.33 ± 43.97	212.24 ± 64.27	91.14 ± 29.16	205.74 ± 59.67	88.34 ± 36.78

Data are expressed as mean ± standard deviation of ΔMFI determined by FACS analysis.

using a custom-made Easy-Sep kit (StemCell Technologies, Vancouver, BC, Canada) to reach more than 99,7% purity. One million neutrophils were suspended in tubes for FACS analysis. Staining for ET_A- and ET_B was carried out as already described. RNA extraction and RT-PCR were performed as previously described.

2.8. Analysis of Cytokines and MMP-9 in the Supernatants of Neutrophils Stimulated with ET-1. Neutrophils were seeded in microplates and incubated with or without 100 ng/mL Ultrapure *E. coli* lipopolysaccharide (LPS) (Invivogen, San Diego, CA) and with or without ET-1. Therefore we used 4 different conditions: (a) neutrophils without any stimulus as negative control, (b) neutrophils incubated with ET-1, (c) neutrophils incubated with LPS alone, (d) and neutrophils incubated with both ET-1 and LPS. Cells were cultured in RPMI-1640-GlutaMAX-I, supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 microg/mL streptomycin. Interleukin-8, TNF-α, vascular endothelial growth factor (VEGF), IFN-γ, IL-17, and matrix metallopeptidase 9 (MMP-9) released in the supernatants of cultured neutrophils were assessed by ELISA at two different time points (3 and 10 hours). Quantikine Human Immunoassay for the selected molecules was purchased from R&D Systems. Sunrise absorbance reader for microplates was used to determine optical density for each sample.

2.9. Statistical Analysis. All the calculations were performed with SPSS 21.0 statistical package (SPSS Inc., Chicago, IL, USA). All the results are expressed as ΔMFI mean ± standard deviation. Quantitative data were assessed using Student's *t*-test. Correlations between ET-A and ET-B cell surface expression and clinical features were assessed with nonparametric test and multivariate analysis. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. ET-1 Receptors Expressed by Immune Effector Cells. T and B lymphocytes as well as monocytes and neutrophils express ET_A and ET_B on their surface, using FACS analysis; the data were obtained as a difference of mean fluorescence intensity between samples incubated with primary and secondary antibodies and their negative controls incubated with secondary antibody alone (Figure 1). In addition some of the data were validated by reverse transcription-PCR in CD4+ T cells and neutrophils (Figure 2).

In both patients and controls, T lymphocytes and monocytes showed a higher surface expression of ET_A (patients: ΔMFI = 100.61 ± 45.21 and 212.24 ± 64.27, resp.; controls: ΔMFI = 110.45 ± 35.89 and 188.4 ± 35.61, resp.) when compared to ET_B (patients: 46.85 ± 29.78 and 91.14 ± 27.44, resp.; controls: ΔMFI = 49.23 ± 29.16 and 98.74 ± 54.66, resp.) ($P < 0.001$) (Table 1).

These data indicate that surface ET-1 receptors distribution on T cells and monocytes of SSc patients is similar to the one observed in healthy donors.

Patients affected by dSSc showed a lower ET_B surface expression on T cells when compared to patients affected by lSSc (28.6 ± 17.9 versus 51.9 ± 31.1) ($P < 0.01$); a similar pattern of surface expression was observed on monocytes (74.4 ± 29.6 versus 97.2 ± 24.5) ($P < 0.05$). No significant difference in ET_A expression by T lymphocytes and monocytes was observed in the diffuse or limited form of disease (94.8 ± 48.2 versus 99.1 ± 42.1 and 251.2 ± 116.3 versus 199.3 ± 34.5, resp.).

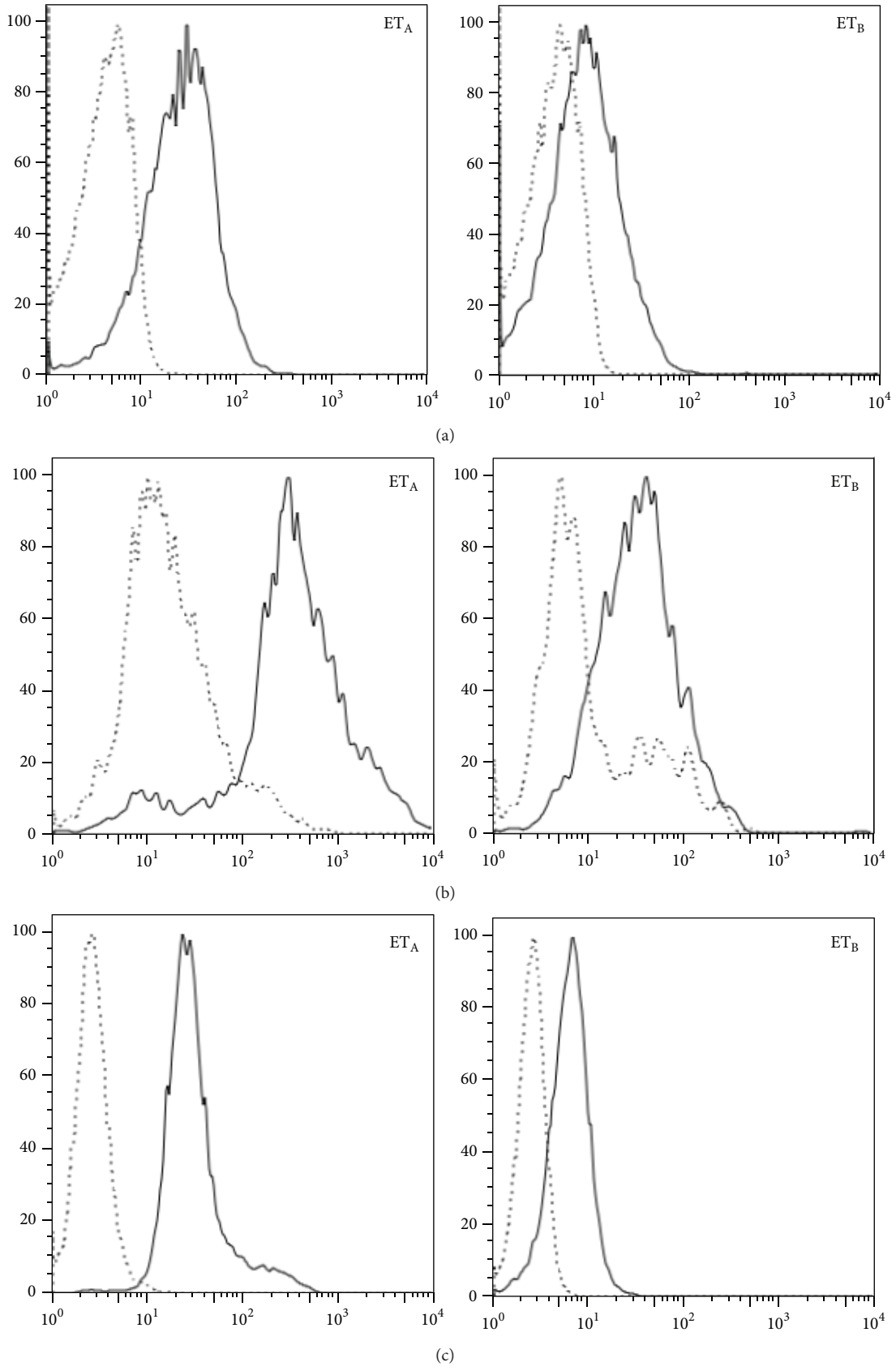
ET_A and ET_B surface expression were not modified by bosentan treatment, both on T cells (ET_A: 97.9 ± 52 versus 102.6 ± 44.3; ET_B: 47.9 ± 17.7 versus 47.6 ± 32.3) and on monocytes (ET_A: 240.7 ± 130.6 versus 205.1 ± 34.9; ET_B: 89.6 ± 22.9 versus 93.1 ± 27.7), suggesting that bosentan therapy does not induce an increased ET-1 receptors expression.

ET_A and ET_B surface expression on T cells and monocytes did not correlate with the presence or absence of DUs (T cells: 121.4 ± 69 versus 98.8 ± 41.6 and 40.8 ± 20.1 versus 48.6 ± 31, resp.; monocytes: ET_A: 221 ± 4.3 versus 211.3 ± 69.6; ET_B: 80.4 ± 25.3 versus 93.6 ± 27.5) (Table 2).

Patients with PAH had a lower ET_B surface expression on monocytes when compared to patients without PAH, although the difference was not statistically significant (77.2 ± 23.4 versus 96.9 ± 27.3); this difference was significant when considering patients with the limited subset of the disease (77.6 ± 17.6 versus 102.3 ± 24.4; $P < 0.05$) (Table 2).

Furthermore, ET_A expression was lower on T cells of lSSc patients with ILD when compared to T cells of patients without ILD (77.8 ± 34.2 versus 111.6 ± 43.9; $P < 0.05$) (Table 2).

ET_A and ET_B expression on B lymphocytes were similar in patients and healthy donors (Table 1). In SSc patients ET_A surface expression was higher than ET_B and was not influenced by the treatment with bosentan (ET_A: 281.33 ± 43.47 and ET_B: 161.33 ± 43.97; $P < 0.05$ versus ET_A: 270.00 ± 28.16 and ET_B: 171.33 ± 35.47; $P < 0.05$). ET_A and ET_B surface expression were similar in the diffuse or limited form



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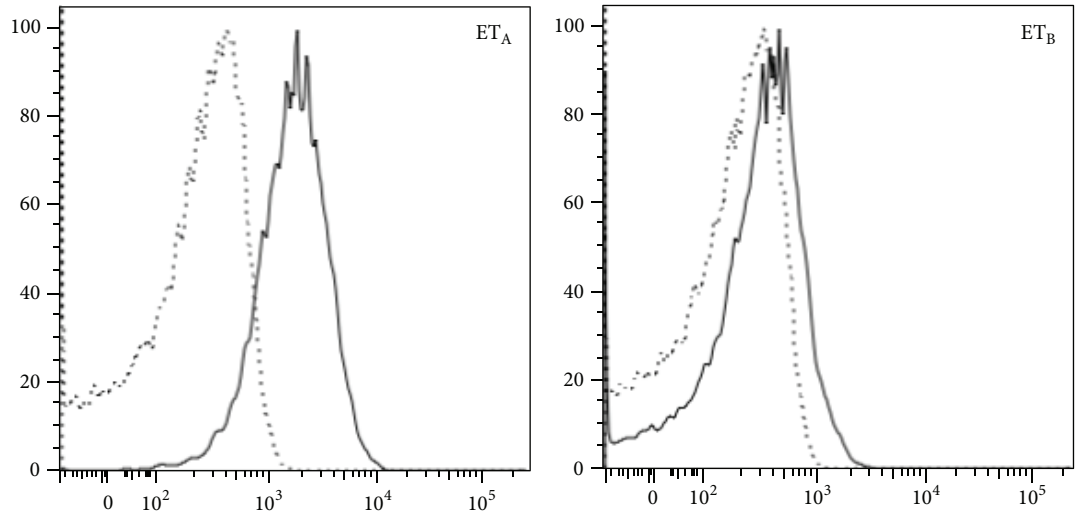


Figure 1: ET_A and ET_B expression by cells obtained from SSc patients. The quantification of receptors expression by T (a) and B lymphocytes (b), monocytes (c), and neutrophils (d) is represented by the difference of fluorescence intensity between the sample (continuous line) and its negative control (dotted line). The profile of one of 41 SSc patients is shown. All the other patients had a similar behaviour.

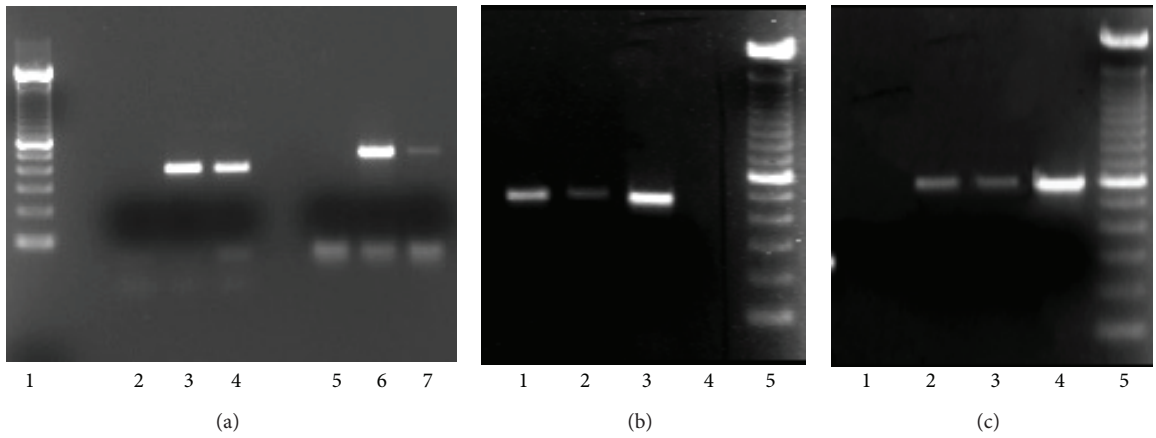


Figure 2: ET_A and ET_B transcripts amplified by RT-PCR in fibroblasts, CD4+ T lymphocytes, activated CD4+ T cells, and neutrophils. ET_A corresponds to a molecular weight of 446 bp and ET_B to a molecular weight of 558 bp. (a) ET_A and ET_B transcripts amplified by RT-PCR in fibroblasts and neutrophils. Lane 1: molecular weight ladder; lane 2: negative control; lane 3: fibroblasts (ET_A); lane 4: neutrophils (ET_A), lane 5: negative control; lane 6: fibroblasts (ET_B); lane 7: neutrophils (ET_B). (b) ET_A transcripts amplified by RT-PCR in T lymphocytes, activated T cells, and fibroblasts. Lane 1: T lymphocytes; lane 2: activated T cells; lane 3: fibroblasts; lane 4: negative control, lane 5: molecular weight ladder. (c) ET_B transcripts amplified by RT-PCR in T lymphocytes, activated T lymphocytes, and fibroblasts. Lane 1: negative control; lane 2: T lymphocytes; lane 3: activated T cells; lane 4: fibroblasts; lane 5: molecular weight ladder.

Table 2: ET_A and ET_B expression on T lymphocytes and monocytes in relation to the clinical features of the disease, such as cutaneous form and presence or absence of PAH, ILD, and DUs.

	T lymphocytes		Monocytes	
	ET_A	ET_B	ET_A	ET_B
lSSc ($n = 32$)/dSSc ($n = 9$)	99.1 ± 42.1/94.8 ± 48.2	51.9 ± 31.1/28.6 ± 17.9 ($P < 0.01$)	199.3 ± 34.5/251.2 ± 16.3	97.2 ± 24.5/74.4 ± 29.6 ($P < 0.05$)
PAH presence/absence	102.6 ± 45.3/104.7 ± 40.9	47.2 ± 26.8/44.9 ± 30.1	202.7 ± 31.4/200.8 ± 30.9	77.2 ± 23.4/96.9 ± 27.3
ILD presence/absence	111.6 ± 43.9/77.8 ± 34.2 ($P < 0.05$)	44.8 ± 27.3/45.3 ± 21.6	199.8 ± 56.5/211.2 ± 47.3	90.6 ± 26.5/89.7 ± 31.7
DUs presence/absence	121.4 ± 69/98.8 ± 41.6	40.8 ± 20.1/48.6 ± 31	221 ± 4.3/196.2 ± 69.6	80.4 ± 25.3/93.6 ± 27.5

Table 3: Detection of cytokines in the supernatants of CD4+ T lymphocytes after 24 hours of incubation with ET-1 alone or with selective or dual receptors blockade. One million cells were incubated in each cell culture condition.

	Control cells	Cells with ET-1	Cells with ET-1 and ET_A antagonist	Cells with ET-1 and ET_B antagonist	Cells with ET-1 and dual receptor blockade
INF-γ (pg/mL)	0.8 ± 0.2	7.6 ± 0.2	1.2 ± 0.45	1.6 ± 0.6	0 ± 0.1
IL-4 (pg/mL)	78.1 ± 74.3	60.8 ± 80.2	711.42 ± 102.2	694.47 ± 99.8	682 ± 100.6
IL-17 (pg/mL)	28.7 ± 10.2	36.1 ± 11.1	37.2 ± 9.8	35.6 ± 12.3	36.6 ± 10.8

Table 4: Molecules detected in neutrophils supernatants after 1, 3, or 10 hours of incubation with either no stimulus or ET-1, LPS, and ET-1 plus LPS, respectively.

	No stimulus			ET-1			LPS			ET-1 + LPS		
Time of incubation	1	3	10	1	3	10	1	3	10	1	3	10
IL8 (pg/mL)	5.38	6.92	16.92	7.69	7.69	16.92	31.54	59.23	93.85	18.46	67.69	134.6
TNFα (pg/mL)	0	0	0	0	0	23.4	0	0	0	0.7	0	2.1
MMP9 (ng/mL)	46.4	67.8	58.8	165.1	64.7	63.3	131.3	203.8	205.3	102.9	205.6	220.7
VEGF (pg/mL)	18.58	20.42	28.75	16.25	22.1	28.7	33.75	52.92	57.92	32.08	54.58	56.25
INFγ (pg/mL)	0.28	0	0	1.94	0	0.28	14.17	0	0	0	0	0
IL17 (pg/mL)	0	0	0	0	0	3.5	0	0	0	0	0	0

of disease and were not influenced by the presence of PAH, ILD, and DUs.

Neutrophils presented the same pattern of expression of ET-1 receptors in SSc patients and control subjects (Table 1).

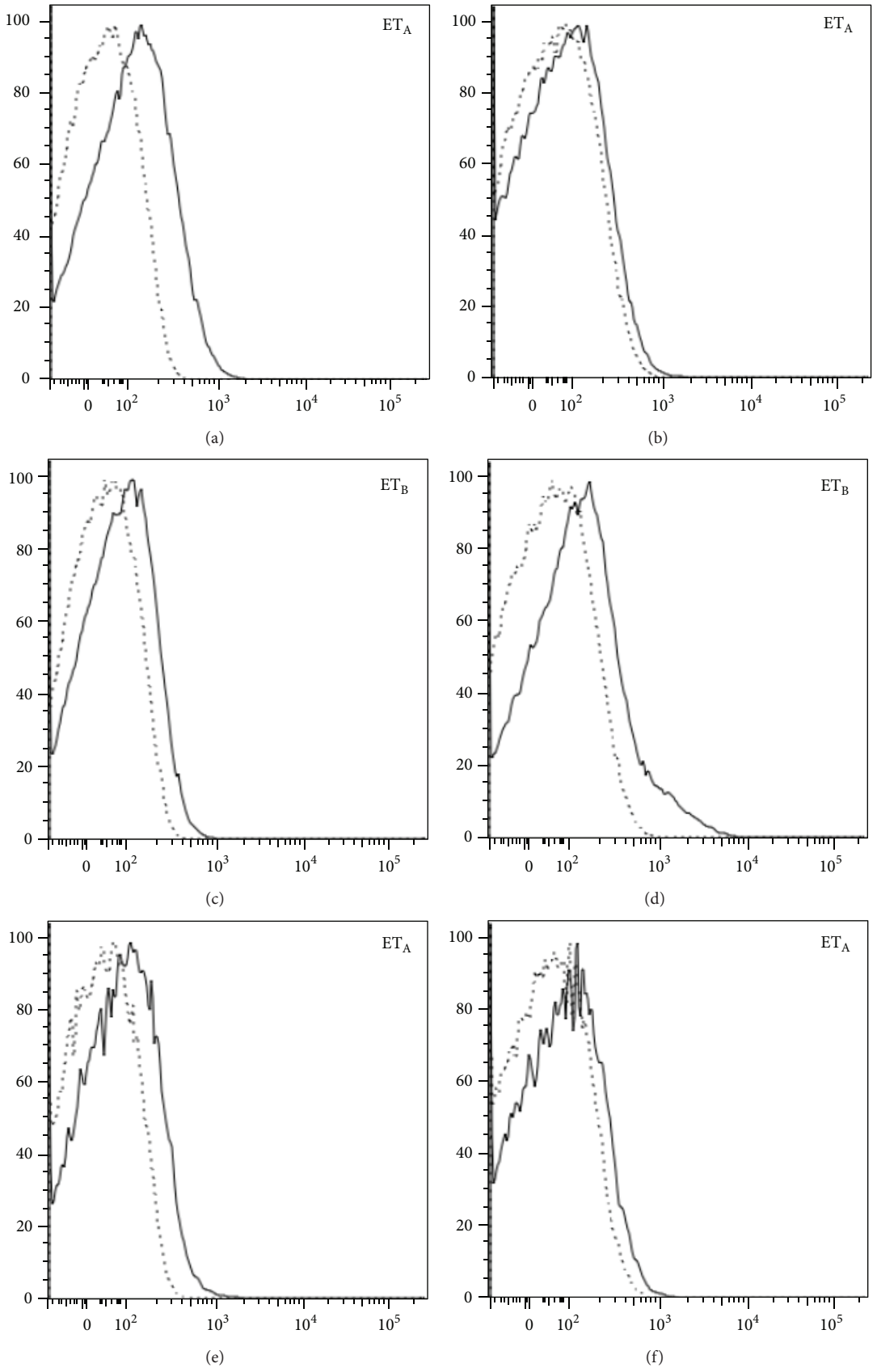
3.2. Quantification of ET_A and ET_B Expression on Activated T CD4+ and CD8+ Cells. As already shown on the entire T cell population, both CD4+ and CD8+ T cell subsets isolated from SSc patients and control healthy donors express ET_A and ET_B on their surface. A higher ET_A expression on resting CD4+ and CD8+ T cells was also confirmed. Upon activation, we found a decreased expression of ET_A and an increased expression of ET_B (Figure 3).

3.3. Cytokines Production by CD4+ T Cells following ET_A and ET_B Stimulation by ET-1. We tested the levels of INF-γ, IL-4, and IL-17 in the supernatants of CD4+ T cells obtained from SSc patients and controls. Cells were either incubated without any stimulus or incubated with ET-1 in the presence or absence of ET-1 receptors blockade. After 24 hours of incubation with ET-1, INF-γ concentration was 9.5 times higher than in the supernatants of cells cultured without ET-1 (7.6 pg/mL versus 0.8 pg/mL; $P < 0.05$). Following selective ET_A or ET_B blockade before ET-1 stimulation, INF-γ levels decreased to 1.2 and 1.6 pg/mL, respectively. Remarkably, the simultaneous dual ET-1 receptors blockade, which mimics *in vitro* the effects of bosentan treatment, caused a marked reduction of INF-γ concentrations in the cells culture supernatants. Interleukin-4 levels did not change in the supernatant of cells exposed to ET-1 for 24 hours. In the presence of selective inhibition of ET_A, IL-4 levels increased in a more significant manner than in the presence of ET_B blockade (711.42 ± 102.2 pg/mL and 694.47 ± 99.8 pg/mL, resp., versus 60.8 ± 80.2; $P = 0.018$ and $P < 0.01$). Also, the double receptors blockade induced the production of IL-4 (682 ± 100.6 pg/mL versus 60.8 ± 80.2 pg/mL; $P = 0.02$). Remarkably, we observed a slight increase in IL-17 level following the incubation with ET-1. However, this effect was not modified by partial or complete ET-1 receptor blockade (Table 2).

3.4. Molecules Released by Neutrophils after ET_A and ET_B Stimulation by ET-1. Levels of MMP-9, IL-8, TNF-α, VEGF, IFN-γ, and IL-17 were evaluated in the medium of neutrophils isolated from 4 healthy donors incubated with ET-1 or LPS or with ET-1 and LPS for 1, 3, or 10 hours (Table 3).

A 1-hour incubation with ET-1 induced a marked increase of MMP-9 (165.1 ng/mL versus 46.4 ng/mL; $P < 0.05$), whereas, at the same time point, we did not observe significant changes in the production of the other soluble molecules analysed, as shown in Table 4. After 3 hours of incubation with both ET-1 and LPS, neutrophils released a higher amount of IL-8 when compared to the concentration detected following the incubation with ET-1 alone or LPS alone (67.7 versus 7.7 versus 59.2 pg/mL, resp.), whereas the levels of the other soluble mediators remained unchanged.

The concentration of TNF-α released in the supernatants following a 10-hour incubation with ET-1 was higher than the levels detected in the medium of cells stimulated with ET-1 and LPS or LPS alone (23.4 pg/mL versus 2.1 pg/mL versus undetectable level; $P < 0.05$). At the same time point, incubation with ET-1 alone induced a higher production of IL-17 compared to the stimulation with both ET-1 and LPS or LPS alone (3.5 pg/mL versus undetectable level; $P < 0.05$). Finally, the simultaneous stimulation with ET-1 and LPS induced a higher secretion of IL-8 and MMP-9 in the supernatant (134.6 pg/mL and 220.7 ng/mL, resp.) when compared to the concentration reached with ET-1 (16.6 pg/mL and 63.7 ng/mL, resp.) or LPS alone (93.8 pg/mL and 205.3 ng/mL, resp.).



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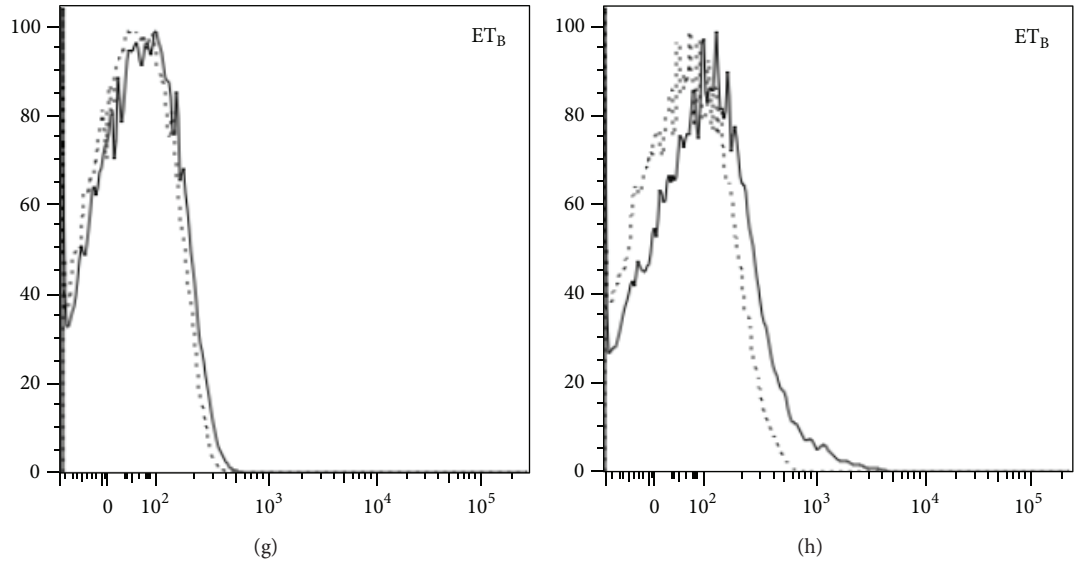

FIGURE 3: Change in ET_A and ET_B expression on activated T CD4+ and CD8+ cells. The stimulation of cells, performed with microbeads coated by anti-CD3/CD28 antibodies, leads to a reduction of ET_A and an increase of ET_B expression, both in CD4+ (a-b; c-d) and CD8+ cells (e-f; g-h), respectively. The profile of one of ten similar experiments is shown.

Taken together, these data indicate that ET-1 is able to induce neutrophils to release proinflammatory mediators.

4. Discussion

In the present study, we aimed firstly at analysing the cellular surface distribution of ET-1 receptors in the different immune cell subsets and secondly at dissecting the mechanisms by which the ET-1 signalling network may participate in the inflammatory responses in SSc.

ET-1 is a potent vasoconstrictor which plays a fundamental role in key pathogenetic aspects of SSc such as vascular damage and fibrosis and treatment with ERAs exerts beneficial effects on vasculopathy [36, 37]. More recently, amelioration of inflammatory parameters during ERA treatment has been reported (reviewed in [44]), thus implying an important role for ET-1 in inflammation, another important aspect of SSc pathology. Indeed inflammation plays a pivotal role in early SSc [45]; however, it may influence also different phases of SSc.

The presence of ET-1 receptors on dendritic cells and polymorphonuclear cells has been already reported [6–8, 16], often with conflicting results, whereas very little is known on ET_A and ET_B expression on T and B lymphocytes. Moreover, a proinflammatory role for ET_B expressed by monocytes/macrophages has been hypothesized on the basis of increased production of inflammatory mediators (TNF-α, prostaglandin E2, and IL-1β) upon ET-1 stimulation [11, 44].

We show here that all the immune cells studied (B and T lymphocytes, monocytes, and neutrophils) express ET-1 receptors both in normal subjects and in SSc patients with a difference in the relative expression of either ET_A or ET_B in the different cell types analysed. In particular, B lymphocytes and neutrophils show the same pattern of expression in healthy controls and in SSc patients, without any significant difference related to the clinical features of the disease. T lymphocytes and monocytes express a higher ET_A expression than ET_B on both subsets. Since ET-1 serum levels are higher in dSSc than lSSc patients and they correlate with the extent of vascular damage and cutaneous fibrosis, we may hypothesize that at least part of ET-1 profibrotic effects is preferentially mediated by the engagement of ET_A [3, 16].

Interestingly, we noticed that, in lSSc patients, a lower ET_B expression on monocytes correlates with the presence of PAH and a lower ET_A expression on T cells correlates with ILD. We can therefore hypothesize that a different pattern of receptor expression on immune cells is associated with a different functional activity that may contribute to the development of PAH or ILD.

A recent multicenter, placebo-controlled trial investigating new drug therapies for idiopathic pulmonary fibrosis compared the effects of ambrisentan, a selective ET_A antagonist, to placebo on disease progression. The study showed that the treatment was associated with an accelerated decline in pulmonary function tests, increased hospitalizations, and higher mortality [46]. Since a selective ET_A inhibition, such as the one obtained with ambrisentan, seems to accelerate pulmonary disease, we may suggest that an imbalanced expression of ET-1 receptors with a diminished expression of ET_A on immune cells may predispose SSc patients to develop ILD possibly through an increased ET_B-mediated stimulation on T cells. The results of this study are in accordance with our findings of a lower ET_A expression on T cells of SSc patients with ILD. However, the precise role played by ET_A stimulation and inhibition in the progression of pulmonary fibrosis remains unclear.

We next evaluated whether the presence of an inflammatory microenvironment could influence the relative expression and/or distribution of ET-1 receptors and, to this aim, we stimulated T cells with anti-CD3/CD28 antibody-coated

microbeads. Stimulation resulted in reduced expression of ET_A and increased expression of ET_B on CD4+ and CD8+ T cells, thus suggesting that these cells, once activated, modulate receptor surface expression by overexpressing ET_B and downregulating ET_A. These results support the hypothesis that ET_B signalling plays a major role in inflammation and, as a consequence, that dual ET-1 receptors blockade may represent a more suitable therapeutic strategy in SSc. We have then investigated the functional effects of ET-1 stimulation on CD4+ T cells and found that ET-1 is able to induce a proinflammatory response since the engagement of both ET_A and ET_B induced an IFN-γ secretion 9.5 times higher than the one observed in basal condition. IFN-γ production was markedly reduced following dual receptor blockade, a situation which resembles the effect of bosentan treatment. These findings suggest the need of ET_A-ET_B receptors cooperation to obtain an inflammatory response in CD4+ lymphocytes. These results are in agreement with the observation that simultaneous blockade of both ET_A and ET_B in scleroderma fibroblasts is necessary in order to suppress collagen production [24]. In addition, ET-1 stimulation of CD4+ T cells leads to a mild increase of IL-17 concentration in cell supernatants, suggesting again an important role for ET-1 in inducing the production of proinflammatory cytokines. Finally, the receptors blocking induces the production of the anti-inflammatory cytokine IL-4.

Finally, it is interesting to note that neutrophils activated with LPS are able to increase the production of proinflammatory molecules after stimulation with ET-1, thus giving further support to the proinflammatory effects of ET-1 also on cells of innate immunity.

All together, these data indicate that ET-1 behaves also as a proinflammatory molecule through a synergistic action on ET_A and ET_B. Therefore, a dual receptor blockade strategy is likely to better control inflammation and fibrosis than a selective receptor blockade. In conclusion, our results, besides generating useful insight in the understanding of ET-1 effects on immune cells in healthy donors and in SSc patients, provide a rationale for the use of dual receptor antagonist in the early stages of SSc, when inflammation is prominent.

Conflict of Interests

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

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Research Article

Autoantibodies Affect Brain Density Reduction in Nonneuropsychiatric Systemic Lupus Erythematosus Patients

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This study explores the relationship between autoantibodies and brain density reduction in SLE patients without major neuropsychiatric manifestation (NPSLE). Ninety-five NPSLE patients without obvious cerebral deficits, as determined by conventional MRI, as well as 89 control subjects, underwent high-resolution structural MRI. Whole-brain density of grey matter (GMD) and white matter (WMD) were calculated for each individual, and correlations between the brain density, symptom severity, immunosuppressive agent (ISA), and autoantibody levels were assessed. The GMD and WMD of the SLE group decreased compared to controls. GMD was negatively associated with SLE activity. The WMD of patients who received ISA treatment were higher than that in the patients who did not. The WMD of patients with anticardiolipin (ACL) or anti-SSB/La antibodies was lower than in patients without these antibodies, while the GMD was lower in patients with anti-SM or anti-U1RNP antibodies. Thus, obvious brain atrophy can occur very early even before the development of significant symptoms and specific autoantibodies might contribute to the reduction of GMD or WMD in NPSLE patients. However, ISAs showed protective effects in minimizing GMD and WMD reduction. The presence of these specific autoantibodies might help identify early brain damage in NPSLE patients.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease involving almost all of the organ systems. Central nervous system (CNS) involvement is typical during the course of SLE [1, 2]. Brain atrophy has long been reported in SLE using neuroimaging techniques [3] and often correlates with clinical manifestations, even in patients without clear CNS signs and symptoms [4]. Magnetic resonance imaging (MRI) is widely used to detect brain anatomy abnormalities, including cerebral atrophy [3, 5, 6]. Although MRIs are widely used to evaluate CNS involvement in SLE, conventional or anatomical MRI findings are sometimes nonspecific or negative [7] in patients with and without neuropsychiatric SLE (NPSLE). Many patients with mood or cognitive disorders have been identified as normal according to conventional MRI. There is evidence that abnormal WM microstructures may be found in non-NPSLE patients or in patients with an apparently normal brain structure [8], suggesting that there may be microstructural abnormalities before obvious CNS manifestations appear. Although important for clinical evaluation, the discrimination of mild structural abnormalities in these patients is difficult. If a subclinical involvement of the brain microstructure could be identified before the emergence of clear neuropsychiatric symptoms, earlier intervention could be initiated, potentially preventing progressive brain injury.

The pathogenesis of CNS involvement in SLE patients remains unclear. Various autoantibodies have been implicated in the pathogenesis of NPSLE, including anticardiolipin

antibodies (ACL) [9]. Because they are prothrombotic, ACL antibodies may cause cerebral infarctions and correlate with focal neurological syndromes [10]. Associations between ACL antibodies and nonfocal neuropsychiatric manifestations have also been reported [11]. Antiribosomal P-protein (P0) antibodies recognize specific proteins on ribosomes. P0 antibodies detected in blood have been associated with psychosis in some studies [12]. Although these autoantibodies are considered to play important roles in the etiology of SLE, few studies have focused on the relationship between the autoantibodies and structural brain damage. Only a VBM study reported that the presence of antiphospholipid antibodies was associated with white and gray matter loss [13]. However, the role of antibodies in the pathogenesis of neuropsychiatric symptoms in patients without conventional MRI abnormalities remains unclear.

Here, we evaluate whether there is microstructural brain atrophy in a relatively large sample of SLE patients without NPSLE who were diagnosed as normal by conventional MRI. Another objective of this study was to explore the potential association between these brain abnormalities and the presence of specific autoantibodies.

2. Material and Methods

2.1. Subjects. SLE patients treated in the in-patient or outpatient facilities of the Rheumatology and Immunology Department of the First Affiliated Hospital of Kunming Medical University (from September 2009 to November 2011) and from the Chinese SLE Treatment and Research Group (CSTAR) member units were recruited in this study. All of the participants were studied via a standardized protocol and were followed by the same investigator throughout the course of the study. Prior to entry into the study, each participant provided written informed consent after receiving a complete description of the study. This research was approved by the Institutional Review Board of Kunming Medical University, Yunnan Province, China (ClinicalTrials.gov: NCT00703742).

The following were the inclusion criteria: (1) patients diagnosed as having SLE by four or more criteria from the 1997 revised American College of Rheumatology (ACR) criteria for the classification of SLE [14]; (2) subjects between the ages of 16 and 50; and (3) subjects willing to attend this study and who gave written consent.

The exclusion criteria included the following: (1) patients fulfilling the ACR criteria for rheumatoid arthritis, systemic sclerosis, Sjögren syndrome (primary or secondary) or other connective tissue diseases, or drug-induced SLE; (2) patients with organic brain or neurological disorders that would disturb the structure or diffusion imaging of the brain (i.e., history of head trauma, Parkinson's disease, or seizures); (3) patients with major active CNS manifestations, such as an obviously disorganized behavior, psychiatric disorders, conscious disturbances, and neurological symptoms; (4) patients with a history of substance abuse; (5) patients who are pregnant or have any physical illness assessed by personal history; (6) patients unable to undergo MRI or with claustrophobia or a pacemaker; (7) patients with serious clinical conditions that could influence cerebral atrophy, such as a history of arterial hypertension, diabetes mellitus, stroke, or renal insufficiency; and (8) structural abnormities of the brain identified by a conventional T1 and T2 weighted MRI.

One-hundred three diagnosed SLE patients were interviewed. All 103 patients were tested using conventional and additional laboratory tests (thyroid function tests and renal function tests, etc.), disease activity scales, questionnaires, and MRI scans. Ninety-eight healthy controls (CTLs) were also recruited. Complete general physical and neurological examinations were given to all of the CTLs by an experienced rheumatologist and neurologist, respectively, to exclude major disorders or, especially, neurological problems. Psychiatric symptoms were screened by an experienced psychiatrist using the Structured Clinical Interview for DSM-IV, Nonpatient Version (SCID-NP). All of the participants were Han Chinese people and were right-handed.

2.2. Scales and Clinical Features of SLE Patients. Data on sex, age at disease onset, and disease duration were collected for each patient. Disease duration was defined as the period from the initial manifestation that was clearly attributable to SLE until the day of the MRI acquisition. All of the clinical manifestations and laboratory test findings were recorded according to the ACR criteria [14]. Disease activity was measured by the systemic lupus erythematosus disease activity index (SLEDAI), and cumulative SLE-related damage was determined by the Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index for Systemic Lupus Erythematosus (SLICC/ACRDI) [15] in all of the SLE patients at the time of the MRI. Active disease was defined as a SLEDAI score higher than 8 [16].

Data on the total dose of corticosteroids and immunosuppressive agents from the time of drug initiation until the study date (including previous and current treatment) were collected by a careful interview. The cumulative dose of the immunosuppressive agents was calculated by summing the daily dosages and multiplying by the number of treatment days. The total doses of oral and intravenous corticosteroids were calculated by conversion to equivalent doses of prednisone.

A complete neurological examination was given to all of the patients to exclude major neurological problems, such as stroke and seizure. Obviously disorganized behavior and psychiatric symptoms, such as illusion and delusion, might imply possible serious involvement of the brain. Therefore, the patients with these symptoms were also excluded. All of the participants were right-handed, as assessed by the Edinburgh Handed Inventory [17]. All of the clinical data were collected on MRI examination days by an experienced psychiatrist.

2.3. Autoantibody Detect. Ten autoantibodies from all of the patients were tested, including the SLE-characteristic antibodies antinuclear antibodies (ANA), anti-dsDNA, anti-SSA/Ro52 kD, anti-SSA/Ro60 kD, anti-SSB/La, anti-SM, and previously reported antibodies related to CNS damage, such

as ACL, antihistone, anti-P0, antinucleosome, and anti-U1RNP antibodies. The ANA tests were assessed by indirect immunofluorescence using Hep-2 cells as the substrate (SCIMEDX Corporation, New Jersey, USA); the anti-double-stranded DNA antibodies were determined using *Crithidia luciliae* as the substrate (H&J Novomed Ltd., Beijing, China); the ACL tests were assessed by conventional ELISA (Aesku Diagnostics, Wendelsheim, Germany). IMTEC-ANA-LIA (IMTEC, Wiesbaden, Germany) is a line immunoassay (LIA) that detects antinuclear antibodies (dsDNA, antinucleosome, SmD1, P0, antihistones, U1-RNP, SSA/Ro60 kD, SSA/Ro52 kD, and SSB/La). The assay was performed according to the manufacturer's instructions. All of the autoantibody samples were collected on the MRI examination day.

2.4. Image-Acquisition. Image-acquisition was performed by an experienced neuroradiologist. MRI sequences were performed on all of the subjects with a 1.5-T clinical GE MRI scanner (Twinspeed, Milwaukee, WI, USA) equipped with a birdcage head coil. Supportive foam pads were used to minimize head motion. A rapid sagittal localizer scan was used to confirm alignment. Normal T1 and T2 MRI scans were taken to exclude obvious structural abnormalities. Of all of the 103 SLE patients who received MRI scans, eight patients were excluded due to structural abnormities of the brain that were identified by common T1 and T2 weighted MRIs (three for local infarction, three for ischemia, and two for a WM hyperintense signal near the caudate nucleus). The data from the remaining 95 patients were included in this study. Nine subjects from the CTL group were also excluded due to local ischemia. A set of three-dimensional volumetric structural MRI scans were performed on each subject using a fast spoiled gradient echo sequence (FSPGR) with the following parameters: TR/TE = 10.5/2 ms, matrix size = 256 × 256, thickness = 1.8 mm with no interslice gap, field of view = 240 mm, and flip angle = 90°. Whole-brain data were acquired in axial planes parallel to the anterior commissure-posterior commissure line, yielding 172 continuous slices, with individual thicknesses of 0.9 mm.

2.5. Data Preprocessing and VBM Statistical Analysis. The DICOM image data were processed via MRIcro software (version 1.40; http://www.mricro.com/). All of the data were analyzed via statistical parametric mapping (SPM5, Wellcome Department of Cognitive Neurology, London, UK; http://www.fil.ion.ucl.ac.uk/) and VBM5 (http://dbm.neuro.uni-jena.de/vbm/vbm5-for-spm5/) software based on Matlab 7.1 (The MathWorks, Inc., Natick, MA, USA). Each individual image was normalized and transformed into the standardized Montreal Neurological Institute (MNI) template and then resampled in the 2 × 2 × 2 mm dimensional scale. The normalized images were then segmented into grey matter (GM), white matter (WM), and cerebrospinal fluid. The unmodulated GM and WM images were separately smoothed to remove noise using a filter with a half-width half-maximum of 8 mm.

2.6. Analysis of the Mean Whole Brain Grey Matter Density (GMD) and Mean Whole Brain White Matter Density (WMD). Initially, we used the standard GM and WM templates in SPM5 as the whole-brain GM and WM masks. Then, using the smoothed GM and WM images from each participant, the GMD and WMD were retrieved. Two-sample t-tests were performed to analyze the differences in GMD and WMD between the two groups, using SPSS version 17.0 (SPSS, Inc.). Correlation and partial correlation methods were used to analyze the correlations between the disease characteristics and the WM volume of clusters. Covariance analysis was performed to detect the effect of different therapies on the GMD and WMD. Finally, we used two-sample t-tests to determine whether there were any differences in the GMD and WMD between the patients with different autoantibodies.

3. Results

3.1. Demographic Data on SLE Patients and HCs. In total, 95 SLE and 89 CTL subjects were analyzed in this study. The mean age was 28.65 years [standard deviation (SD) = 7.51, range 16–48] for the SLE patients and 30.70 years (SD = 7.93, range 17–50) for HCs. There were no significant differences in age or sex between these two groups (Table 1).

3.2. Clinical, Laboratory, and Treatment Features. The disease duration in SLE patients ranged from 0.5 to 204 months (mean = 18.99 months, SD = 27.55). Forty-seven patients were newly diagnosed with SLE, and 56 patients had disease durations that were less than 12 months. The other 39 patients had disease durations 13–204 months. At the time of the MRI scanning, the mean SLEDAI score was 10.01 (SD = 6.45; range 0–30). Of the 95 SLE patients, 33 patients were positive for APL, 51 for antihistone antibody, 46 for anti-P0, 51 for anti-SSA/Ro52 kD, 61 for anti-SSA/Ro60 kD, 34 for anti-SSB/La, 38 for antinucleosome, 30 for anti-U1RNP, 45 for anti-SM, and 63 for anti-dsDNA. According to the SLICC, fifteen patients had a score of 1 (ten cases had proteinuria >3.5 gm/24 hours and five cases had cutaneous small vessel vasculitis in a terminal finger or minor tissue loss). The remaining 80 patients were without serious organic impairment; their SLICC score was 0. The mean SLICC score for all of the patients was 0.158 (SD = 0.367; range 0-1). Of the 95 patients, 55 were treated with immunosuppressive agents (ISA) [17 with cyclophosphamide (CTX), 32 with hydroxychloroquine (HCQ), and 6 with both]. The other 41 patients were never treated with immunosuppressive agents (Table 1).

3.3. GMD/WMD Differences between the SLE and CTL Groups. The GMD and WMD were compared between the SLE patients and CTLs. Both the GMD and WMD were significantly decreased in the SLE group compared with the CTL group (Table 1, Figure 1(a)).

3.4. Association between GMD/WMD and Symptomatic Severity. Comparison of the GMD/WMD between the active and inactive SLE patients showed a lower GMD in the active

Table 1: Demographic and clinical characteristics of SLE patients and healthy controls.

	Group		t	P
	SLE ($n = 95$)	CTL ($n = 89$)		
Age (year, mean ± SD)	28.65 ± 7.51	30.70 ± 7.93	−1.795	0.074
Female/male	79/16	64/25	3.357 (x^2)	0.067
Duration (month, mean ± SD)	18.99 ± 27.55	NA		
SLEDAI (mean ± SD)	10.01 ± 6.45	NA		
Total steroid (g, mean ± SD)	6.86 ± 12.05	NA		
Total CTX (g, mean ± SD)	0.96 ± 0.24	NA		
Total HCQ (g, mean ± SD)	23.19 ± 66.22	NA		
GMD	0.5450 ± 0.0253	0.5767 ± 0.0276	−8.132	0.000
WMD	0.5183 ± 0.0252	0.5405 ± 0.0213	−6.455	0.000
Manifestation (n (%))				
Seizure	0 (0)			
Psychosis	0 (0)			
Organic brain syndrome	0 (0)			
Visual disturbance	0 (0)			
Cranial nerve disorder	0 (0)			
Lupus headache	0 (0)			
Cerebrovascular accident (CVA)	0 (0)			
Neurological sign	0 (0)			
Vasculitis	5 (5.26)			
Arthritis	25 (26.32)			
Myositis	5 (5.26)			
Urinary casts	2 (2.11)			
Hematuria	30 (31.58)			
Proteinuria	24 (25.26)			
Pyuria	32 (33.68)			
Malar rash	25 (26.32)			
Discoid rash	8 (8.42)			
Photosensitivity	18 (18.95)			
Alopecia	18 (18.95)			
Mucosal ulcers	6 (6.32)			
Pleurisy	10 (10.53)			
Pericarditis	5 (5.26)			
Fever	4 (4.21)			
Low complement	79 (83.16)			
Thrombocytopenia	9 (9.47)			
Leukopenia	30 (31.58)			
Autoantibody positive (n (%))				
Antinuclear	95 (100)			
ACL	33 (34.74)			
Histone	51 (53.68)			
P0	46 (48.42)			
SM	45 (47.37)			
dsDNA	57 (60.00)			
SSA52	51 (53.68)			
SSA60	61 (64.21)			
SSB	34 (35.79)			
U1RNP	30 (31.58)			
Nucleosome	38 (40.00)			

SLEDAI: SLE disease activity index; GMD: mean whole brain grey matter density; WMD: mean whole brain white matter density; CTX: cyclophosphamide; HCQ: hydroxychloroquine; CTL: healthy control; ACL: anticardiolipin antibodies; histone: antihistone antibodies; P0: antiribosomal P antibodies; SM: Anti-Sm antibodies; ds-DNA: anti-dsDNA antibodies; SSA52: anti-Ro/SSA 52-KD antibodies; SSA60: anti-Ro/SSA 60-KD antibodies; SSB: anti-La/SSB antibodies; U1RNP: anti-U1 RNP antibodies; nucleosome: antinucleosome antibodies; NA, not applicable.

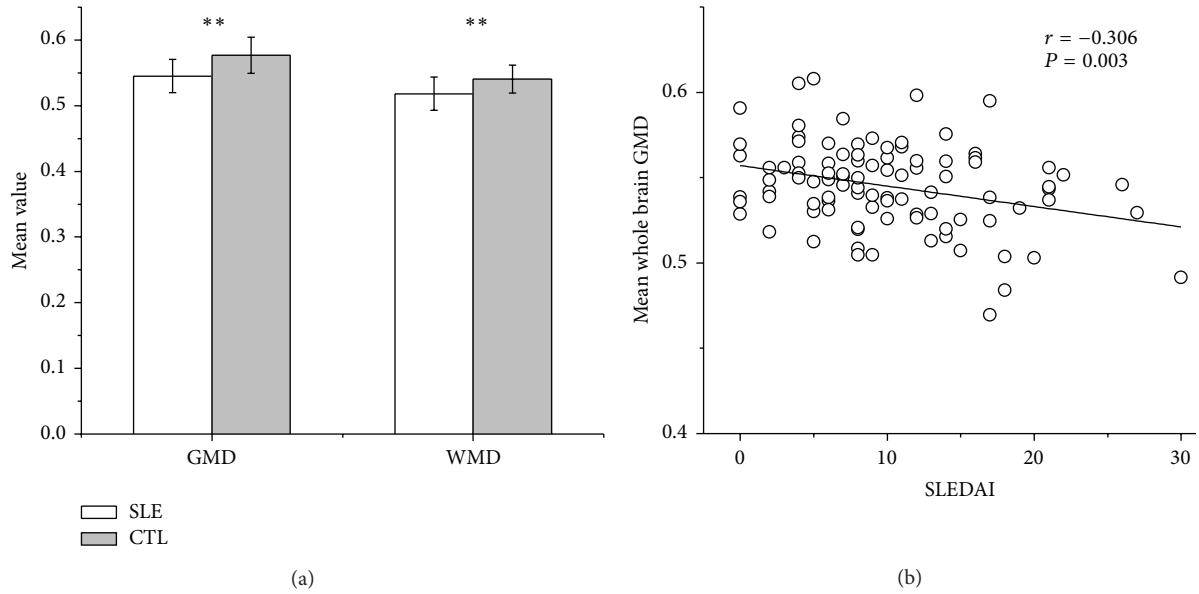

Figure 1: GMD/WMD reduction and the correlation between GMD with disease activity in non-NPSLE. (a) Significantly reduced GMD and WMD in SLE compared with CTLs; (b) negative correlation between GMD and the SLEDAI score of SLE. SLE: systemic lupus erythematosus; CTL: control; GMD: mean whole brain grey matter density; WMD: mean whole brain white matter density; SLEDAI: systemic lupus erythematosus disease activity index. $^{*}P < 0.05$, $^{**}P < 0.01$.

patients than the inactive patients. There was a negative correlation between the total SLEDAI score and GMD ($r = -0.306$, $P = 0.003$; Figure 1(b)). Considering the possible influence of age on grey matter, we carried out a partial correlation using age as the control variable to assess the correlation between the disease severity and GMD. The results demonstrated that the negative correlations between the total SLEDAI score and GMD persisted ($r = -0.338$, $P = 0.001$). No significant correlation was found between the SLEDAI score and WMD ($r = -0.081$, $P = 0.437$). There was also no significant correlation between the SLICC score and GMD ($r = -0.153$, $P = 0.139$) or WMD ($r = -0.002$, $P = 0.987$).

3.5. Association of GMD/WMD with Different Therapies. In this study, 47 patients were newly diagnosed with SLE. However, there was no significant difference in the GMD/WMD between the newly diagnosed and long-term patients. There was no association between the total corticosteroids and GMD or WMD ($r = -0.032$, $P = 0.759$ for GMD and $r = -0.099$, $P = 0.338$ for WMD). The possible effect of immunosuppressive agents on the brain structure was also considered. First, the 95 patients were divided into two groups. One group was treated with the immunosuppressive agents HCQ, CTX or both until the study day, including any current or previous treatment (treated group, $n = 55$). The other group was never treated with immunosuppressive agents (untreated group, $n = 40$). The treated group had a greater WMD ($t = 3.793$, $P < 0.0001$) than the untreated group (Table 2, Figure 2(a)). The GMD was not significantly difference between the two groups ($t = 1.286$, $P = 0.202$).

Of the 55 patients, 17 patients received CTX, 32 received HCQ, and 6 received CTX and HCQ. Thus, the 95 patients were then divided into four groups to further study the effect of the different therapies on the GMD and WMD. The four groups were the non-ISA treated (NI), CTX, HCQ, and CTX + HCQ groups. Considering the possible influence of age and severity of SLE on the GMD and WMD, we used age and the SLEDAI score as the control factors to perform covariance ANCOVA analysis. The results showed that there was a significant intergroup difference in the WMD (between-group P value = 0.003). The results of a pairwise-group comparison showed that the NI patients had the lowest WMD. The CTX-, HCQ-, and CTX+HCQ-treated patients had significantly higher WMD compared with the NI group (see Supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/920718, Figure 2(b)). There were no significant differences in the GMD between four groups.

3.6. GMD/WMD and Autoantibodies. Ten autoantibodies were detected in all 95 patients. Among all of the patients, 33 patients were ACL-positive (34.74%). The GMD of the anti-U1RNP antibody-positive patients was significantly reduced compared with the antibody-negative patients ($t = -2.095$, $P = 0.039$). A similar result was found for the anti-SM positive and negative patients, with the anti-SM positive patients showing a significantly reduced GMD compared to the antibody-negative patients ($t = -2.938$, $P = 0.004$) (Table 3, Figure 3). There was a possible trend toward a lower GMD in the antihistone antibody-positive patients ($t = -1.934$, $P = 0.056$).

We found a significant difference in the WMD between the ACL positive and negative patients groups ($t = -2.186$, $P = 0.032$). The anti-SSB/La-positive patients also had a greater reduction in the WMD than the anti-SSB/La-negative

Table 2: Comparison of GMD/WMD.

	GMD		t	P	WMD		t	P
	Mean	SD			Mean	SD		
SLE ($N = 95$)	0.5450	0.0253	−8.132	0.000**	0.5183	0.0252	−6.455	0.000**
CTL ($N = 89$)	0.5767	0.0276			0.5405	0.0213		
Treated ($N = 55$)	0.5496	0.0221	1.286	0.202	0.5262	0.0224	3.868	0.000**
Untreated ($N = 40$)	0.5388	0.0282			0.5074	0.0248		
Active ($N = 49$)	0.5399	0.0266	−2.069	0.041*	0.5169	0.0280	−0.555	0.580
Inactive ($N = 46$)	0.5505	0.0328			0.5198	0.0220		
First diagnosis ($N = 47$)	0.5413	0.0267	−1.425	0.158	0.5154	0.0274	−0.233	0.273
Long duration ($N = 48$)	0.5487	0.0236			0.5211	0.0226		

GMD: mean whole brain grey matter density; WMD: mean whole brain white matter density; * $P < 0.05$, ** $P < 0.01$.

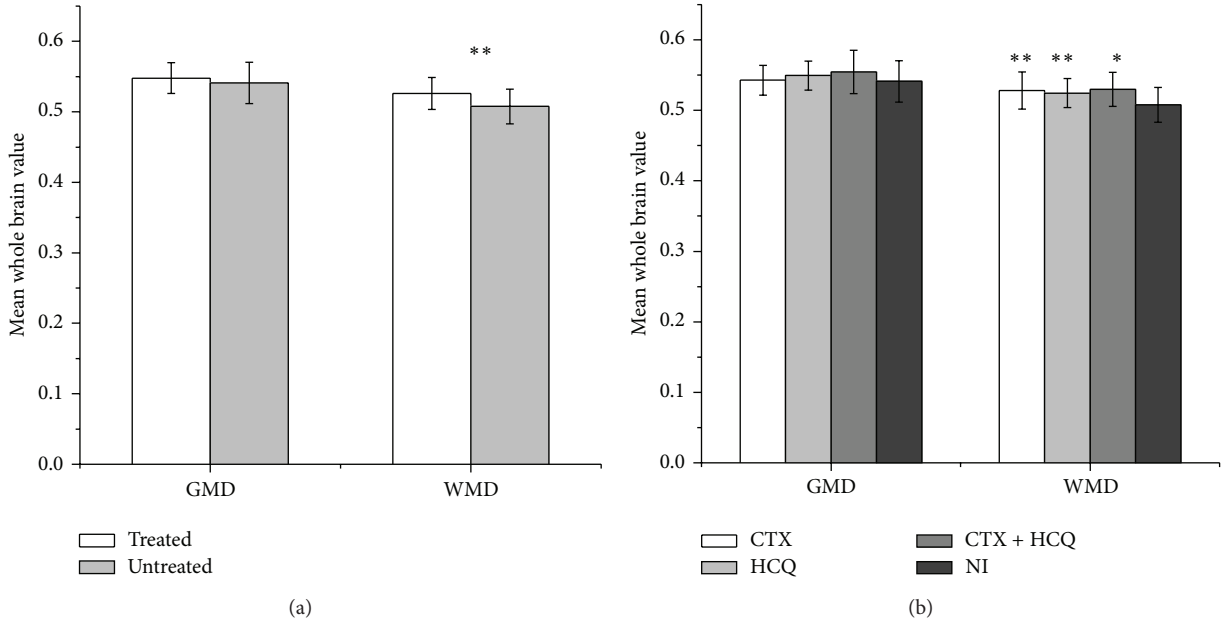

Figure 2: GMD/WMD and the relationships with different treatments in SLE patients. The patients receiving treatment with immunosuppressive agents had a greater WMD than the patients who were never treated with immunosuppressive agents. There was no significant difference in the GMD between the two groups (a). The CTX-, HCQ-, or CTX+HCQ- treated patients had significantly higher WMDs compared with the NI group. Pairwise-group comparisons showed that the NI patients had the lowest WMD (b). GMD: mean whole brain grey matter density; WMD: mean whole brain white matter density; CTX: cyclophosphamide; HCQ: hydroxychloroquine; NI: nonimmunosuppressive agents treated. * $P < 0.05$, ** $P < 0.01$.

patients ($t = -2.313$, $P = 0.023$). There was a possible trend of a lower WMD in the anti-SSA/Ro52 kD antibody-positive patients, but this was not significant ($t = -1.936$, $P = 0.056$). For the other three antibodies, anti-P0, anti-dsDNA, and anti-SSA/Ro60 kD, there were no significant differences in the GMD and WMD between the antibody-positive and -negative patients.

Negative correlations between the ANA and GMD and WMD were found ($r = -0.241$, $P = 0.019$ for both GMD and WMD). However, when we controlled for age, these trends disappeared ($r = -0.125$, $P = 0.302$ for GMD and $r = -0.205$, $P = 0.089$ for WMD).

4. Discussion

In this study, we found a clear reduction in the GMD and WMD in the NPSLE patients. These patients were previously identified as normal by conventional MRI. We also identified relationships between several autoantibodies and GM and WM reduction in the NPSLE patients. The presence of these specific autoantibodies might help identify early brain damage in NPSLE patients.

Although MRI is considered to be a good method to evaluate CNS manifestations in SLE, conventional or anatomical MRI findings are often nonspecific or negative in patients with and without NPSLE. The patients in this study did not

Table 3: GMD/WMD comparison between autoantibody-positive and -negative patients.

	AB-positive patient		AB-negative patient		t	P
	Mean	SD	Mean	SD		
U1RNP	$N = 30$		$N = 65$			
GMD	0.5372	0.0272	0.5487	0.0237	−2.095	0.039*
WMD	0.5152	0.0202	0.5197	0.0271	−0.805	0.423
Sm	$N = 45$		$N = 50$			
GMD	0.5373	0.0263	0.5520	0.0223	−2.938	0.004**
WMD	0.5147	0.0247	0.5215	0.0253	−1.321	0.190
ACL	$N = 33$		$N = 39$			
GMD	0.5372	0.0227	0.5483	0.0226	−1.894	0.062
WMD	0.5135	0.0301	0.5261	0.0184	−2.186	0.032*
SSB	$N = 34$		$N = 61$			
GMD	0.5452	0.0252	0.5449	0.0255	0.049	0.961
WMD	0.5105	0.0277	0.5226	0.0227	−2.313	0.023*
SSA52	$N = 51$		$N = 44$			
GMD	0.5464	0.0243	0.5434	0.0266	0.564	0.574
WMD	0.5137	0.0257	0.5236	0.0238	−1.936	0.056
SSA60	$N = 61$		$N = 34$			
GMD	0.5471	0.0235	0.5413	0.0281	1.070	0.288
WMD	0.5162	0.0246	0.5221	0.0261	−1.110	0.270
Histone	$N = 51$		$N = 44$			
GMD	0.5404	0.0213	0.5504	0.0228	−1.934	0.056
WMD	0.5134	0.0254	0.5225	0.0244	1.781	0.078
P0	$N = 46$		$N = 49$			
GMD	0.5456	0.0271	0.5445	0.0237	0.218	0.828
WMD	0.5169	0.0276	0.5196	0.0228	−0.510	0.611
Nucleosome	$N = 38$		$N = 57$			
GMD	0.5418	0.0279	0.5472	0.0234	−1.019	0.311
WMD	0.5200	0.0251	0.5171	0.0253	0.549	0.584
DsDNA	$N = 63$		$N = 32$			
GMD	0.5434	0.0274	0.5483	0.0206	0.899	0.371
WMD	0.5153	0.0233	0.5241	0.0279	1.613	0.110

AB: antibodies; ACL: anticardiolipin antibodies; histone: antihistone antibodies; P0: antiribosomal P antibodies; SM: Anti-Sm antibodies; ds-DNA: anti-dsDNA antibodies; SSA52: anti-Ro/SSA 52-KD antibodies; SSA60: anti-Ro/SSA 60-KD antibodies; SSB: anti-La/SSB antibodies; U1RNP: anti-U1 RNP antibodies; nucleosome: antinucleosome antibodies; $^{*}P < 0.05$, $^{**}P < 0.01$.

have major CNS manifestations or disease; therefore, the GM and WM loss implied that brain damage occurs before clear clinical neurological symptoms are present. Consistent with a previous magnetic resonance spectroscopy (MRS) study, our results supported the notion that abnormal microstructural changes may occur before the appearance of any clear CNS symptoms or conventional imaging signatures [8]. Here, we applied a more advanced method that can calculate the precise quantitative whole-brain GMD and WMD. Thus, the present findings also highlight the value of quantitative volumetric MRI techniques in detecting minor GM and WM reductions in NPSLE. This may aid in predicting NPSLE in patients and better reporting the cumulative injury inflicted by SLE.

Until now, the role of antibodies in the pathophysiology of brain damage in SLE was unclear. Our results are the first to identify the relationship between GMD/WMD and autoantibodies. Anti-U1RNP and anti-SM antibodies demonstrated greater effects on the GMD, while ACL and SSB showed greater effects on the WMD. Anti-U1RNP antibodies may be linked to central neuropsychiatric manifestations [18] and act as an inducer of proinflammatory cytokines. In a previous study, a correlation between the presence of anti-Sm antibodies in the serum and central NPSLE was observed [19]. The anti-Sm autoimmune response is a polyclonal humoral immune response against protein components of small nuclear ribonucleoprotein (snRNP) particles and is found in greater than 30% of the patients with SLE. This response is specific to SLE [20]. The association between anti-U1RNP and anti-SM antibodies with the GMD reduction suggests a possible diagnostic and prognostic value of these antibodies in determining CNS involvement in SLE. Various

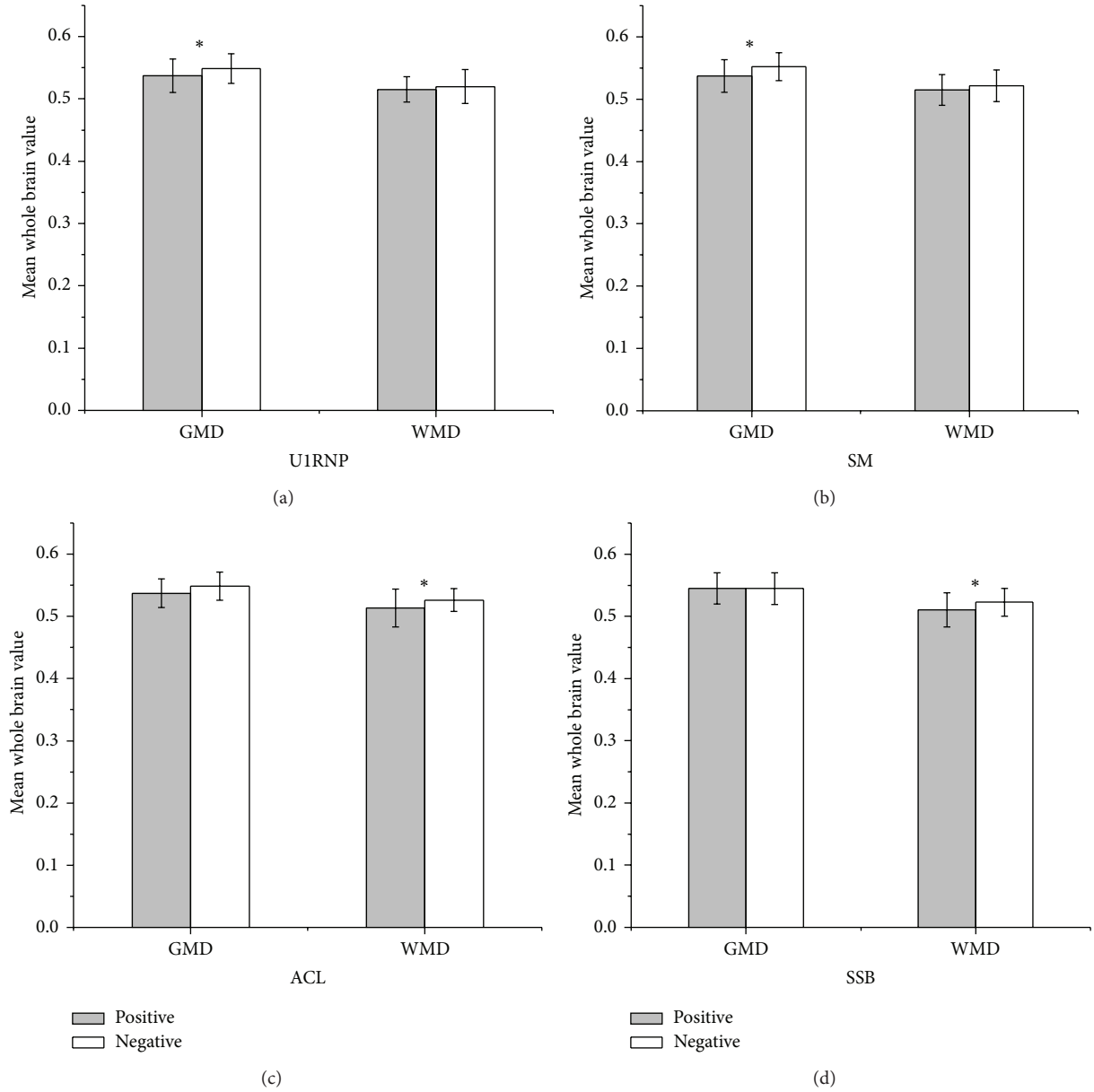

Figure 3: GMD/WMD difference and the relationships with different autoantibodies in SLE patients. The anti-U1RNP and anti-SM antibody-negative patients had higher GMDs than the antibody-positive patients (a, b). The ACL- and anti-SSB/La antibody-negative patients had higher WMDs than the antibody-positive patients (c, d). GMD: mean whole brain grey matter density; WMD: mean whole brain white matter density; ACL: anticardiolipin antibodies; SSB: anti-SSB/La antibodies; U1RNP: anti-U1 RNP antibodies; SM: anti-Sm antibodies. $^{*}P < 0.05$.

autoantibodies, including ACL, have been implicated in the pathogenesis of NPSLE [21]. ACL has been a focus in SLE research [22] and was reported to be associated with neuropsychiatric manifestations [10] and brain abnormalities [23]. As phospholipids are the main constituent of WM, we evaluated ACL and found a reduction of the WMD in ACL-positive patients. Another study using magnetization transfer imaging also found an association between the presence of ACL and cerebral damage in grey and white matter in NPSLE [24]. In combination with these studies, our results provide evidence that ACL might damage WM even before patients show obvious neuropsychiatric symptoms. Because of their prothrombotic tendency [25], ACLs may cause cerebral ischemia and result in white matter atrophy. The anti-SSB/La antibodies were also related to the observed WMD reduction. The exact role of anti-SSB/La pathology in WM damage is unclear. It has been reported that anti-SSB/La antibodies can cause increased neutrophil apoptosis and decreased phagocytosis and affect the inflammation process [26]. Thus, it is possible that the abnormal inflammatory reactions [27] or the secondary reactions in SLE can induce WM atrophy.

Our results implicate other antibodies, specifically the anti-P0, anti-SSA/Ro60 kD, antinucleosome or anti-dsDNA

antibodies, in CNS. The remaining tested antibodies showed only possible associations with the GMD and WMD reduction, such as antihistone antibodies with GMD and anti-SSA/Ro52 kD antibodies with WMD. The role of antibodies in the pathology of SLE may be complex and the results sometimes contradictory. For example, anti-P antibodies recognize specific proteins on ribosomes, and anti-P antibodies detected in blood have been associated with psychosis in some studies [28]; however, these possible associations have not been confirmed in other studies [29]. Precise and prospective cohort studies that discuss the association between these antibodies and brain damage, including both grey matter and white matter, are needed.

This study also found that the patients treated with immunosuppressive agents (CTX, HCQ, or both) had increased WMD compared with the patients who were never treated with immunosuppressive agents. This finding suggests a potential protective role of immunosuppressive agents in preventing WM atrophy. Several studies support using CTX in the treatment of NPSLE [30]. The potential neuroprotective effect of CTX has been identified in SLE [31] and other white matter demyelinating diseases, such as antiphospholipid syndrome [32] and experimental autoimmune gray matter disease [33]. A possible mechanism for the neuroprotective effect of immunosuppressive agents may be their ability to reduce demyelination due to vasculitis. However, further studies are needed to determine the advantages and disadvantages of long-term immunosuppressive therapy. On the other hand, the protective effect of immunosuppressive therapy was more obvious in the WMD. This result might imply that WM is more sensitive than GM to immunosuppressive agents. Early use of immunosuppressive agents may prevent the aggressive reduction of brain density.

The loss of GM and WM may originate from the brain atrophy previously described in SLE [5, 34, 35]. However, the exact mechanism of brain atrophy in SLE remains unclear. The WM hyperintensity in SLE that has been demonstrated in longitudinal research may become progressive over time in patients with severe SLE [36] and may be caused by the neurotoxic effect of the chronic disease. There are several possible explanations for the atrophy: (1) neurodegenerative changes due to axonal damage that is primary or secondary to the vasculopathy in SLE; (2) some antibodies, such as APL, affecting both the small vascular and brain cellular elements that lead to cerebral dysfunction [36] are reportedly related to nervous system damage similar to that seen in NPSLE [24]; (3) the activation of a cytokine network independent of the pathological process has been observed in SLE patients with CNS complications, which suggests a neurotoxic effect of cytokines in SLE [37]; (4) damage of the brain endothelium causes damage to the blood-brain barrier, which normally restricts the entry of plasma constituents, including proteins [38]; (5) demyelination originates from decreased serum brain-derived neurotrophic factor (BDNF) levels in patients [39].

As an invasive technique, MRI is considered to be a useful tool in evaluating the involvement of the CNS in SLE [4, 40]. This study has provided evidence for microstructural brain atrophy preceding the emergence of a clear neurological manifestation in NPSLE. Because the patients in our study were all without obvious neuropsychiatric symptoms or obvious structural abnormalities detected by conventional MRI, the results presented here support brain involvement as a primary deficit in SLE and suggest the neuroprotective effect of immunosuppressive therapy in managing white matter atrophy. We believe that these results might have significant value in the early diagnosis of CNS involvement in SLE. On the other hand, the tight relationship between autoantibodies and microstructural brain damage reveals the value of brain microstructural damage as one of the sensitive indicators for the CNS involvement in SLE. There are several limitations of this study. Although we used a quantitative method to calculate the GMD/WMD, the segmentation of grey matter and white matter based on the MRI signal does not reflect the pathology of the delineation of grey matter and white matter or the exact pathological mechanisms of the GMD/WMD reduction, such as atrophy, apoptosis, or demyelination. Future studies are needed to help us understand the underlying complex mechanisms of CNS deficit in SLE.

Conflict of Interests

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

Authors' Contribution

Jian Xu and Yuqi Cheng equally contributed to this study.

Acknowledgments

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