Clinical and Developmental Immunology

Systemic Lupus Erythematosus

Guest Editors: Chaim Putterman, Roberto Caricchio, Anne Davidson, and Harris Perlman

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Editorial

Systemic Lupus Erythematosus

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1. Introduction

Systemic lupus erythematosus is a systemic autoimmune disease with a worldwide distribution. Although both men and women of all age groups can be affected, women outnumber men almost 10 fold and the typical lupus patient is a young woman during her reproductive years. Clinically, lupus is a disease with an unpredictable course involving flares and remissions, where cumulative damage over time significantly interferes with the quality of life and adversely affects organ function. Multiple cells, tissues, and organs can be affected in this disease, and the clinical picture can vary greatly between patients. Indeed, even in the same patient the clinical picture may not be consistent over time. Organ systems most commonly involved in lupus patients include joints, skin and mucous membranes, blood cells, brain, and kidney.

Although the prognosis of lupus patients has dramatically improved with the widespread introduction of potent immunosuppressive therapies and better medical management of acute disease exacerbations, a diagnosis of SLE remains associated with an appreciably shortened life span. Moreover, the mortality rates are still significant among patients with active disease. With more lupus patients living with chronic, intermittently active disease, it has become evident that there is significantly accelerated atherosclerotic cardiovascular disease that is insufficiently explained by traditional risk factors.

A second major cause of mortality in SLE is infection. Lupus patients have an inherent susceptibility to infections due to their disease. In addition, the major side effect of the large majority of medications currently used for treatment of lupus is immunosuppression, which confers a greatly increased risk for infections with typical and atypical organisms. For this reason, the use of more aggressive approaches is usually restricted to patients with active disease, with lower doses of immunosuppressive treatment being used for chronic maintenance.

The universal belief and expectation among investigators and physicians involved in SLE is that a more comprehensive and accurate understanding of the underlying mechanisms of disease will lead to the development of more targeted therapies. Such novel approaches to treatment would presumably result in improved patient response rates, decreased numbers of flares, attenuated cumulative damage, and enhanced preservation of organ function over time. Moreover, even if newer therapies have a similar efficacy profile to medications in current use, the employment of more targeted and specific therapeutic modalities could reasonably result in less unintended side effects. In this special issue, we have gathered contributions from physicians and researchers from North America, South America, Europe, and Asia that highlight several important and/or novel aspects of the molecular pathogenesis, clinical organ involvement, and experimental therapies in this prototypical systemic autoimmune disease.

2. Disease Pathogenesis

Gender and hormones play a crucial role in SLE: the disease is much more common in females, and its presentation often correlates with changes in estrogen and/or progesterone levels. Nonhormonal, X-chromosome-related contributions

may be important as well. The contribution of gender to the prevalence of disease, types of clinical manifestations, and pathogenesis are summarized in this issue by J. Schwartzman et al. The genetics of SLE are being unraveled by the use of genomewide association studies (GWAS) that have uncovered the role of multiple genetic polymorphisms, each of which confers a modest increase (<2 fold) in an individual's risk for lupus. Nevertheless, in all these studies an association with particular MHC alleles remains the major genetic contributor, conferring a 3-4 fold risk for lupus. M. Relle and A. Schwarting review the role of MHC-linked susceptibility genes in experimental and human disease. A. H. Draborg et al. remind us of the importance of infection in triggering lupus autoimmunity in the genetically susceptible host, particularly the Epstein-Barr virus, which has been epidemiologically linked to SLE onset.

The immune pathogenesis of SLE is complex and remains a matter of considerable study and debate. Although initially lupus was believed to be a disease of the adaptive immune response, a growing recognition of the crosstalk between the innate and adaptive arms of the immune system in recent years, the discovery of several families of pattern recognition receptors, and the suggestive role of type I IFN pathway uncovered by GWAS studies, have led to an increasing realization that innate immune cells and effectors are central contributors to the pathogenesis of SLE. Moreover, there is abundant evidence from animal models that experimental lupus can be induced by individual aberrations in a multitude of cell types and cellular pathways, suggesting that the pathogenesis of lupus probably varies between individuals. Furthermore, the operative mechanisms may be different during the triggering, amplification, persistence, and flare phases of the disease.

Lupus research has benefited tremendously from the rapid pace of advances in the field of immunology at large. Considering the intricate and multifaceted pathogenesis of SLE, it is not surprising that advances in basic immunology are often followed by studies investigating the relevance of this particular mechanism in the pathogenesis of lupus. In this issue, the role of some of these new players in SLE is critically examined. Y.-P. Chuang et al. investigate invariant NK T cells and their modulatory function, while A. Alunno et al. provide new insights into the balance between T_H17 and regulatory T cells. Finally, A. P. Alegretti et al. demonstrate decreased complement regulatory proteins on lupus peripheral blood mononuclear cells, a finding which may be relevant to pathogenesis and may also serve as a disease biomarker.

3. Organ Damage

Kidney disease is a particularly important manifestation of lupus. Renal manifestations appear in a high percentage of patients, and even if treated with aggressive therapy can lead to progressive renal failure and end-stage renal disease. Indeed, renal involvement and its associated complications stubbornly remain one of the major causes of morbidity and mortality in lupus patients. Although T cells, macrophages, cytokines, and chemokines, among many other immune mediators, are important in nephritis initiation and/or progression, B cells and autoantibodies are known to play an instrumental role in the pathogenesis of lupus nephritis. S. Yung and T. M. Chan review several of the main types of lupus autoantibodies closely associated with nephritis and the results of the interactions of these pathogenic antibodies with resident kidney cells, while Y. Shen et al. demonstrate that the presence of intrarenal B cell infiltrates may be a significant prognostic factor in human lupus nephritis.

Two other specific (albeit less common) types of disease manifestations which are receiving increasing attention, namely, pulmonary hypertension and gastrointestinal involvement, are also addressed in this issue by A. Dahla and Y. Yang et al., respectively.

4. Therapies

There has been much recent interest in therapies that enhance immune regulation as a means of normalizing tolerance defects in autoimmunity. Autologous mesenchymal stem cell transplantation is engendering interest as a potential treatment for several types of immunologically mediated disease. Its potential role as a treatment for lupus is reviewed in this issue by D. Wang et al. and A. Darmont. One caveat of this approach is that lupus mesenchymal stem cells have signaling abnormalities, as demonstrated by Y. Tang et al.

Murine lupus models have many similarities to human disease, and serve as valuable in vivo laboratories for proof-of-concept therapeutic studies. One such new approach is tested by Y. W. Jiang et al. in the lupus prone MRL/lpr mouse strain.

5. Summary

Lupus remains a puzzling disease with protean manifestations that has so far been disappointingly resistant to new forays into biologic therapies based on rational immune approaches. Clearly, much headway has been made in our understanding of pathogenic mechanisms and many promising targeted approaches are being tested both in animal models of disease and in human trials. Nevertheless, the heterogeneity of disease mechanisms, clinical manifestations, and pathologic findings makes the design of clinical trials particularly challenging. The contributors to this issue have identified a number of expanding research areas that continue to yield new insights into pathogenesis and treatment of lupus. An increase in this knowledge will be required to develop therapies that can prevent and treat disease without the excessive toxicities of our current armamentarium.

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Review Article

Hematopoietic Stem Cell Transplantation for Systemic Lupus Erythematosus

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Two streams of research are at the origin of the utilization of hematopoietic stem cell transplantation (HSCT) for severe autoimmune diseases (SADs). The allogeneic approach came from experimental studies on lupus mice, besides clinical results in coincidental diseases. The autologous procedure was encouraged by researches on experimental neurological and rheumatic disorders. At present the number of allogeneic HSCT performed for human SADs can be estimated to not over 100 patients, and the results are not greatly encouraging, considering the significant transplant-related mortality (TRM) and the occasional development of a new autoimmune disorder and/or relapses notwithstanding full donor chimerism. Autologous HSCT for refractory SLE has become a major target. Severe cases have been salvaged, TRM is low and diminishing, and prolonged clinical remissions are obtainable. Two types of immune resetting have been established, "re-education" and regulatory T cell (Tregs) normalization. Allogeneic HSCT for SLE seems best indicated for patients with disease complicated by an oncohematologic malignancy. Autologous HSCT is a powerful salvage therapy for otherwise intractable SLE. The duration of remission in uncertain, but a favorable response to previously inactive treatments is a generally constant feature. The comparison with new biological agents, or the combination of both, are to be ascertained.

1. Introduction

Hematopoietic stem cell transplantation (HSCT), especially in its autologous form, has become a significant treatment modality for severe autoimmune diseases [1–9] (SADs), and more specifically for systemic lupus erythematosus (SLE) and the antiphospholipid syndrome [10–14]. Most of the evidence concerns the hematopoietic lineage. However, the utilization of another distinct lineage, consisting of mesenchymal stromal cells (MSC), is also becoming a promising sector in the field of regenerative medicine and immune disorders [15, 16]. Bone mesenchymal stem cells (BMSC) are not transplanted along with hematopoiesis in standard marrow and blood transplantation [17]. However there are 2 important studies in which allogeneic MSC were transplanted in patients with severe-refractory SLE. In both, no pretransplant conditioning was utilized because of the well-known low MSC immunogenicity. Fifteen lupus patients received 1 intravenous infusion of 1×10^6 MSC/Kg, and both the clinical (by SLEDAI score) and the laboratory (DNA, ANA) results were clearly favorable [18]. Another study by the same investigators was performed with umbilical MSC, utilizing low-dose cyclophosphamide (CY) conditioning in about half of them, in 16 lupus patients, again with significant amelioration in SLEDAI and laboratory results [19], which were accompanied by an increase in peripheral Treg cells, a feature that was also found in other SLE patients treated with conventional autologous HSCT [20]. However, notwithstanding these recent and encouraging results, the bulk of classical evidence provenes from the two traditional procedures of hematopoietic stem cell transplantation, allogeneic (allo-HSCT) and overwhelmingly autologous (auto-HSCT).

2. Historical Perspective and Rationale

Two streams of research, experimental and clinical, are at the origin of the increasing utilization of HSCT, autologous and allogeneic, for SADs. Somewhat unexpectedly, although the

initial evidence was in favor of the allogeneic procedure, it was the autologous one that attained greater consensus and much greater utilization. The history of these earlier studies has been described in detail elsewhere [21]. It all started with animal experiments.

In memorable studies it was shown that the transfer of spleen cells or whole bone marrow cells from New Zealand Black (NZB; H-2^d) mice to antilymphocyte globulin treated BALB-L, H-2^d id irradiated mice was capable of reproducing the donors' murine lupus [22, 23]. These original experiments have been considerably enlarged by recent studies by Smith-Berdan et al. [24], who obtained the reversal of murine lupus by nonmyeloablative transplantation of purified allogeneic HSC, a procedure which they advocated also for human SADs. Other studies demonstrated that the B lymphoid precursors from B/W F_1 bone marrow (BM) cultures reproduced the disease in SCID mice [25]. In a series of consecutive experimental investigations Ikehara came to the conclusion that animal ADs were stem cell diseases [26, 27].

Allogeneic HSCT received a vigorous impulse also from the clinic. There is a series of reports of patients harboring an AD and having developed a hematological malignancy, who were cured of both diseases following an allogeneic HSCT. Such patients go under the definition of coincidental diseases, and a detailed review has been published [28]. These results were encouraging, but in other ones the AD persisted in spite of cure of the malignancy. Thus the initial enthusiasm for the allogeneic procedure has decreased considerably [29].

The apparent paradox of treating patients with ADs with autotransplantation, that is giving them back, with or without T cell depletion, their own HSC originated with the pioneering experimental investigations by van Bekkum and his group, who treated successfully experimental ADs, such as experimental autoimmune encephalitis (EAE: the experimental model of multiple sclerosis, MS) and adjuvant arthritis (AA: the experimental model of rheumatoid arthritis, RA) with first syngeneic and then autologous BM transplants [30]. However, these results were obtained in the induced rather than in the spontaneous animal ADs [26, 27]. These apparently paradoxical but encouraging results considerably strengthened the philosophy of auto-HSCT for human SADs, which has grown almost exponentially in the last 2 decades. Systemic lupus erythematosus (SLE) is widely considered as the paradigm of ADs and autologous HSCT for patients with severe refractory lupus disease was first proposed by myself in 1993 [31]. This proposal was soon after extended to all SADs [32] and updated guidelines have been published recently by Sullivan et al. [8].

3. Allogeneic Transplantation

Two important conferences have analyzed the possible indications for allo-HSCT in ADs [33, 34]. In the Position Paper of 2005 [34] the potential results and the attending risks of allo-HSCT for SADs have been discussed in detail. The capability of a 1-time delivery of a curative therapeutic strategy was considered as "appealing." A comprehensive recent review of clinical results has been published by Gratwohl [35]. However the number of SLE patients having undergone allo-HSCT is minimal and reference must be made to the greater experience in SADs in general.

A retrospective EBMT study [36] identified 35 patients having received 38 allogeneic transplants for various SADs, including 2 cases of SLE (one died and the disease progressed in the other). The transplant-related mortality (TRM) was 22.1% at 2 years and 30% at 5 years, while death during to progression of disease was 3.2% at 2 years and 8.7% at 5 years. Of the 29 surviving patients, 55% achieved complete clinical and laboratory remission and 24% partial remission. The consensus is that nonmyeloablative reduced intensity conditioning regimens should be utilized [37], as will be further discussed dealing with auto-HSCT.

A safe and effective conditioning protocol has been developed in Israel [38], but no lupus patients were transplanted. A large number of SLE patients were allotransplanted in Ahmedabad according to a complicated conditioning protocol [39], but they all relapsed after a mean of 7.35 months of disease-free interval.

A series of mechanisms were considered for the effects of allo-HSCT in ADs, including immunomodulation, tolerization by T regulatory cells and, most importantly immune-mediated destruction of autoreactive cells [40]. By analogy with well-known Graft-versus-Leukemia (GVL) effect [41], this last was defined as a Graft-versus-Autoimmunity (GVA) effect [42]. It was originally found to be more evident when associated with Graft-versus-Host disease (GVHD) [43], but it was not found in the review by Daikeler et al. [36]. Contrarywise, evidence for a GVA effect was demonstrated in models of experimental encephalomyelitis [44]. Mixed chimerism has been thought to be capable of controlling ADs, both in experimental and clinical studies, [45, 46] but in other cases it was accompanied by relapse. The concept that complete remission of ADs depends upon full donor chimerism has been supported by the favorable effect of donor lymphocyte infusions (DLI) for posttransplant relapses, designed to obtain full chimerism.

Single case reports of SLE and RA patients having undergone allo-HSCT for coincidental diseases are often contradictory. Along with a 20-year complete remission in 2 patients with RA [47] and in 1 with SLE [48], there are also patients with RA who relapsed notwithstanding allo-SCT [49–51].

Donor lymphocyte infusions have been efficacious in controlling incipient relapse [51, 52], but the most disquieting reports are those of patients with SADs having relapsed notwithstanding full donor chimerism [53]. A recent case report concerns a female patient with severe Sjogren's syndrome with associated lupus features [54] complicated by chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) and total inability to walk, who was treated with success for the neurological complication with an auto-HSCT, subsequently developed severe aplastic anemia (SAA), was successfully transplanted from her HLA-identical sister and achieved cure of SAA, but still maintains positive ANA of the speckled type after 5 years [54].

The causes of this almost paradoxical behavior are unclear. The persistence of autoantibodies after Auto-HSCT is not an infrequent phenomenon, as will be discussed in the following section. However the relapse of AD notwithstanding the acquisition of a new, healthy immune system is much more intriguing. The persistence of long-lived plasmacells in marrow survival niches [55, 56] has been considered as a possible mechanism of relapse, but their pathogenesis may be even more complex, and the relentless stimulation by self-antigens in genetically autoimmune prone subjects must also be considered [57]. More specifically in SLE, the importance of nucleosome challenge is well ascertained [58].

Still another complication is the occurrence of secondary ADs following HSCT, both autologous and allogeneic [59–61]. Given the absolute preponderance of the autologous versus the allogeneic procedure, it is obvious that most cases have been found in the first category. In the EBMT study 3 patients developed 4 secondary ADs after allo-HSCT, and 13 did not [61]. This number is too small to state that SLE is the disease most liable to develop ADs following allo-HSCT, but this has been confirmed in the autologous setting. Multiple sequential pathogenetic mechanisms have been proposed, but the common features of genetic factors and immune dysregulation are most probably at the origin of this complication. Finally it has been reported that in 5 cases of lupus patients having developed malignant B lymphomas, high-dose chemotherapy (HDCT) was able to eradicate the malignancies, but not SLE [62].

Concluding this section on allo-HSCT for SADs, it must be considered that new clinical studies are under way, in order to explore its efficacy and tolerance. However there can be no doubt that only obtaining a cure can justify its performance. As recently stated by Tyndall [63], "the jury is still out" for a definite judgment. The philosophy of our Center is to offer allo-HSCT to patients with ADs having developed complications such as oncohematological diseases; SAA and others, all requiring the allogeneic procedure.

4. Autologous Transplantation

The first two reports of patients with severe, refractory SLE having undergone auto-HSCT were published in 1997 [64, 65]. The patient transplanted in Genoa had a long history of lupus with many severe complications, and has been followed to the time of this writing, making it the longest followup of a single patient (16 years; see Table 1). There followed a series of single case reports, all of them characterized by an extremely severe condition associated with complete refractoriness to conventional therapy. They included patients with refractory SLE in general [66], with severe pulmonary involvement [67], and with complicating Evans' syndrome [68]. Of special interest are two cases of neuropsychiatric SLE (NP-SLE) that were salvaged by auto-HSCT [69, 70]. These case reports paved the way to single center retrospective clinical studies, and subsequently to more extended cooperative ones. They are not to be disregarded, since patients in desperate conditions were rescued by means of the bold and knowledgeable utilization of a procedure until then mostly ignored in this specific area. Single-case reports are known to be classified at the lowest degree of strength in observational studies, but they are considered of interest when reporting "newly recognized or uncommon observations" [71], and this is the case of these pioneering interventions. More extensive clinical trials by dedicated teams were to follow worldwide. They have been resumed in 2 tables, one in the recent summation by Illei et al. [13] and another published in a former contribution by us [72]. Two fundamental findings emerged from these clinical observations, namely the powerful therapeutic effect reported by all centers, and the greatly inferior transplant-related mortality (TRM) as compared to the allogeneic procedure. As discussed with greater detail elsewhere [6], there are three basic questions to be addressed.

4.1. Mobilization and Conditioning: Which Are the Best Procedures? Hematopoietic stem and early progenitor cells, initially obtained from the bone marrow, and now almost universally from peripheral blood ("mobilized HSC"), are utilized for this procedure. At first there was the suspicion that, in patients with SADs, and even more specifically in SLE, abnormalities of the hematopoietic system, primary or secondary to prolonged immunosuppressive therapy, might have affected their engraftment potential. Accelerated telomeric loss and functional exhaustion have been found in the HSC of rheumatoid arthritis (RA) and of SLE [73, 74], However, recent research in another AD, multiple sclerosis (MS), has shown normal HSC reserves in the bone marrow, largely capable to support hematopoiesis in the autologous transplant setting [75], and this notion has been extended to the majority of SADs, in which the collection of SC is routinary, and their hematopoietic capability is apparently normal, as evaluated by hematological reconstitution.

The main reason for the shift in the collection of HSC from marrow to blood is the larger number that can be harvested, resulting in a faster and stable engraftment [76–78]. T-cell depletion may be performed by *ex vivo* manipulations, but is performed infrequently and only in special cases [79], Cyclophosphamide (CY), with subsequent utilization of granulocyte colony stimulating factors (G-CSF), is the most used drug for mobilization, at the dosage of 2–4 g/m^2 [79]. Its utilization often allows to achieve a partial remission [78], which in most cases is a favorable prognostic indicator [6]. This observation is in line with the well-known strategy of high dose CY alone performed at John Hopkins University [80, 81], USA.

Conditioning is the conventional term used to indicate the immunosuppressive treatment (combinations of chemo- and radiotherapy) utilized both in allo- and auto-HSCT [82]. While in oncohematological disease there is the double target of reducing to a minimum residue the malignant cells later to be eradicated by the graft's immune activity [40], and to abrogate allogeneic reactivity, in the autologous setting the purpose is the elimination of the autoreactive lymphoid system thought to be at the origin of the AD. This effect practically coincides with the purpose of resetting the immune system, as will be discussed later. When evaluating

Table 1: A synthesis of the first case of SE performed in Genoa, with a followup of 16 years.

Year	Age	Clinical symptoms	Laboratory tests	Therapy
1983	33	Arthralgias, fever	ANA+ Wasserman test+	NSAIDs
1985	35	Exudative pleuritis pericarditis		Prednisone bolus plus tapered doses
1995	36	Nephropathy proteinuria >10 g/day	ANA 1:160, ds-DNA pos, LE phenomenon pos, CH50 620, proteinuria, hematuria	CY, prednisone bolus, AZA, auto-HSCT
2000	50	Asthenia, proteinuria 2 g/24 h	ANA 1:320 homogeneous, ds-DNA neg, complement normal, proteinuria, hematuria	Mycophenolate mofetil 2 g/day, prednisone 1 mg/kg
2005	55	Tendinitis	ANA 1:320 homogeneous, ds-DNA neg, LE phenomenon neg, complement normal, proteinuria 0.5 g/24 h	Mycophenolate mofetil 2 g/day, prednisone 2 mg/kg plus tapered doses
2008	58	Facial erythema	ANA 1:320 homogeneous, ds-DNA neg, LE phenomenon neg, complement normal, proteinuria 0.5 g/24 h	Mycophenolate mofetil 2 g/day, prednisone 0.5 mg/kg, hydroxichloroquine
2012	62	Disease quiescent, the patient is well	ANA 1:680 homogeneous, proteinuria 0.18 g/24 h, complement normal, ds-DNA neg	Mycophenolate mofetil 1 g/day, prednisone 0.5 mg/kg/every other day

the most appropriate conditioning regimen for SLE and most other SADs, there are two main aspects to be examined. The first is the clear demonstration that the intensity of the conditioning regimen is usually proportional to its toxicity, but can be inversely proportional to the incidence of relapses. In a retrospective analysis of 450 patients having undergone auto-HSCT for SADs, the different conditioning regimens were divided in high, intermediate, and low intensity, and a significant association was found with intensity and TRM, while an inverse relationship was shown with the incidence of relapse [83]. The second consists in the strategy of utilizing lymphoablative regimens specifically targeting the self-reactive immune system [84, 85].

There should not be a real competition between immunosuppressive monoclonals and transplantation in this area. A combination of both strategies, in which 500 mg of the anti-CD20 cell monoclonal Rituximab are administered before and after the usual 200 mg/kg of CY ("sandwich technique") is currently being utilized for SLE with impressive results, at Northwestern University, Chicago, USA [86]. Anti-CD20 immunotherapy for the control of relapse following auto-HSCT in rheumatoid arthritis has been utilized with success [87], and the strategy of using an additional agent to the transplantation procedure is attractive. However, a devastating complication, progressive multifocal leukoencephalopathy (PML), caused by the activation of the John Cunningham virus, has been reported in a disquieting proportion of patients having been immunosuppressed with biological agents. The first cases were reported in SLE [88], and a recent review reported 52 patients having developed PML, 7 of which following auto-HSCT (3 allogeneic and 4 autologous) for lymphoproliferative disease [89]. This demonstrates that, once again, maximal immunosuppression may lead to unforeseen severe infectious complications.

4.2. Is the Procedure Safe, and What Benefits Does It Confer? At the time of this writing there are little more than 300 patients having undergone auto-HSCT worldwide. Two tables specifying Centers, results and TRM have been published [12, 13]. TRM varied considerably from center to center. A center effect, similar to the one demonstrated in leukemias, could not be clearly confirmed, but there is evidence of a learning curve. This favorable trend is confirmed in the much greater clinical material composed by SADs in general, in which TRM reached 12% in the first EBMT Registry [83], decreased to $7^{+/-}$ 3% in 2005, and attained 4% in the Northwestern University's study [86] in SLE. Guidelines and recommendations have been published in detail regarding the choice of the conditioning regimen and the selection of patients [9]. It is obvious that patients with very severe organ damage make poor candidates, and that a patient with end-stage lupus nephropathy is in need more of a kidney than a HSC transplant. However two patients who were already in dialytic treatment recovered renal function following auto-HSCT sufficient to forego dialysis [84]. Although the selection of patients within approved and/or investigational protocols is the best policy, it must be realized that, in selected patients with advanced refractory SLE, the decision to perform auto-HSCT will ultimately rely on a combination of clinical acumen, experienced teams and good patient-doctor relationship.

Coming to the question of what type of benefit does auto-HSCT confer to severe, progressive, refractory-relapsing SLE, more often than not it may be dramatic. In a recent, provocative editorial commenting its utilization in SADs, and more specifically in the rheumatic diseases, Illei has posed the question, whether "the glass is half full or half empty" [90]. We have already given a tentative answer to this question [91], but I shall try to be more specific here.

The idea of obtaining stable complete remissions, if by this term, in analogy with oncohematological diseases, we intend clinical remission, abrogation of all autoimmune markers, and definitive freedom from drug therapy, is not realistic [6, 13, 31, 85, 86]. Independently from the heterogeneity of the clinical material, progression-free survival (PFS), which may be considered as the most accurate estimated outcome of a therapeutic procedure, was 43% at three years in the EBMT study [7]. However very good remissions occur, transplantation may be a salvage treatment in many cases, and in most relapses, often of a milder form than the original disease, the utilization of conventional therapies, to which the patients were formerly refractory, is generally possible. The effects of auto-HSCT may be divided in two phases: the early suppression of ongoing immune-inflammatory events, and the later resetting of the autoimmune clock, which is closely related to the length and grade of remission. The first effect is clearly due to the immunosuppressive conditioning regimens, and is proportional to the dose intensity [83]. No complicated immune dynamics occur here, besides the well-known combination of immunosuppression and abrogation of the attending inflammation. This effect is responsible for the dramatic disease-arresting ("nosostatic") effects which have been observed in practically all actively aggressive SADs, and most demonstratively in SLE [84, 92]. In the aggressive refractory phases of disease, Auto-HSCT may well be the most potent salvage therapy available. A clear distinction of the diverse sensitivity to auto-HSCT according to the phases of disease has been recently made in multiple sclerosis (MS) by Schevchenko et al. [93], who have divided the transplant phases in MS in "early," "conventional," and "salvage-late" procedures. Among the many examples of this early, dramatic therapeutic effect there are, besides the cancellation of systemic symptoms, the almost immediate clearance of inflammatory urinary sediments in lupus nephritis [94], the rapid improvement of nailfold capillaroscopy in SSc [95], and the early abrogation of Gadolinium-enhancing lesions in MS [96]. Intermediate changes may be considered the striking disappearance of diffuse calcinosis in a child with overlap connective disease [97], and the early regression of dermal fibrosis in patients with severe scleroderma [98].

4.3. What Significant Changes in the Immune System Take Place following HSCT ? Are We Really Curing Autoimmunity? No other aspect of the Auto-HSCT-based procedures has been the object of so much research, enthusiasm and controversy. A prolonged depression of CD4+ CD45RA cells is a general finding [99], and takes place following both auto-HSCT and high-dose immunosuppressive therapy (HDIS) alone [80, 81]. What type of immunomodulation then follows had been called a "black box" by Muraro and Douek [99], but thanks to their own [100] and others' investigations [101, 102] is becoming increasingly clear. High-dose immunosuppression (HDI) reduces the population of autoimmune cells to a condition which may be considered as minimal residual autoimmune disease (MRAD). While the cure of oncohematological disease requires the eradication of cancer SC, a different view may be entertained for ADs. Two types of immune resetting are now considered, and have been divided in Type I and Type II, according to the modulations of the T/B repertoire and off immune regulation [100].

The first has been defined as a "reeducation" [103] of the faulty immune system, obtained by restoring a diverse antigen-specific repertoire through reactivation of the thymic output ("thymic rebound"), which has been shown to persist, albeit in lesser measure, also in adults. In an immunological study of auto-HSCT in 7 SLE patients the Berlin group has found evidence for an overwhelming regeneration of the B cell lineage, that apparently become tolerant to self-antigens [104]. The recurrence of lupus activity observed in three of these patients was accompanied by the development of antinuclear antibodies with new specificities, a finding they considered as *de novo* development of SLE [105]. Be that as it may, the development of secondary ADs following auto-HSCT has been found to be maximal precisely in SLE [81]. The switch from one to another abnormal balance has been described by Shoenfeld as the kaleidoscope of the autoimmune mosaic [106]. The Type II modality has received a powerful impulse by the recent demonstration that, in 15 post-transplant lupus patients, both $CD4^+$ CD25 $FoxP3^+$ and an unusual $CD8^+$ $Fox3^+$ Treg subset return to levels seen in normal subjects [20], accompanied by almost complete inhibition of pathogenic T cell response to critical peptide autoepitopes from histones and nucleosomes. This was not observed in patients in drug mediated remissions, in which CD4T cell autoreactivity to nucleosomal epitopes persisted. Former investigations have also highlighted the role of Tregs in restoring tolerance following auto-HSCT [107].

There are also, however, some controversial results, mostly in other ADs, reporting that autoreactivity did return. In a study of autotransplanted MS patients the T cells recognizing myelin basic protein were indeed initially depleted by immunoablation, but then rapidly expanded from the reconstituted T-cell repertoire in 12 months [108]. An early recovery of $CD4^+$T cell receptor diversity was found after Auto-HSCT [109]. In a comprehensive study analyzing original and pooled data from autotransplanted MS patients Mondria et al. [110] found not only the persistence of CSF oligoclonal bands in 88% of the reported cases, but also the persistence of the soluble lymphocyte activator CD27, concluding that complete eradication of activated lymphocytes from the CNS had not been established notwithstanding auto-HSCT and radiation.

Finally, although all these therapies are addressed to eradicate, or just to control, an aberrant, autodestructive immune system, little has been done on the side of the antigens. Available data suggest that the autoimmune response is antigen driven [111], and the consequences of the neo-antigenicity of the "altered self" [112] in genetically disease-prone individuals [113] must be taken into account, especially in patients relapsing after allo-HSCT. A treatment founded on gene therapy-assisted autologous HSC transplantation, with the object of achieving antigen-specific tolerance, is being actively pursued by Alderuccio et al. [114].

5. Conclusions and Perspectives

Allogeneic HSCT seemed, at the start of the transplantation saga for SLE, to possess the ability of delivering a 1-hit cure for SLE. Unfortunately this has not been so, and, unless ongoing and future clinical investigations will bring about overwhelmingly solid data, it should be reserved, as in our institution, to patients with so called double trouble [115], that is lupus patients having developed malignant lymphomas and/or other transplant-requiring diseases.

Autologous HSCT has become a promising treatment for severe SLE, and for SADs in general, worldwide. It may be a salvage therapy as well as a disease-controlling procedure. Its effects are both immediate and gradually progressive ("reeducation"). It may turn out to be a robust bridge for more and better biological therapies in the future, similarly to discovery of the tyrosine-kinase inhibitors that have cancelled most allogeneic transplants for chronic myelogenous leukemia (CML).

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Research Article

Gene Expression Profile Reveals Abnormalities of Multiple Signaling Pathways in Mesenchymal Stem Cell Derived from Patients with Systemic Lupus Erythematosus

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We aimed to compare bone-marrow-derived mesenchymal stem cells (BMMSCs) between systemic lupus erythematosus (SLE) and normal controls by means of cDNA microarray, immunohistochemistry, immunofluorescence, and immunoblotting. Our results showed there were a total of 1, 905 genes which were differentially expressed by BMMSCs derived from SLE patients, of which, 652 genes were upregulated and 1, 253 were downregulated. Gene ontology (GO) analysis showed that the majority of these genes were related to cell cycle and protein binding. Pathway analysis exhibited that differentially regulated signal pathways involved actin cytoskeleton, focal adhesion, tight junction, and TGF-β pathway. The high protein level of BMP-5 and low expression of Id-1 indicated that there might be dysregulation in BMP/TGF-β signaling pathway. The expression of Id-1 in SLE BMMSCs was reversely correlated with serum TNF-α levels. The protein level of cyclin E decreased in the cell cycling regulation pathway. Moreover, the MAPK signaling pathway was activated in BMMSCs from SLE patients via phosphorylation of ERK1/2 and SAPK/JNK. The actin distribution pattern of BMMSCs from SLE patients was also found disordered. Our results suggested that there were distinguished differences of BMMSCs between SLE patients and normal controls.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by multiorgan involvement including renal, cardiovascular, neural, musculoskeletal, and cutaneous systems and remarkable variability in clinical presentation and the etiopathogenesis of SLE remains unclear [1]. In recent years, several studies suggest that SLE may be identified as a stem cell disorder, the etiopathogenesis of this autoimmune disease is attributable to defects in the bone marrow microenvironment, mainly in the hematopoietic stem cells (HSCs) [2], and the bone marrow transplantation (BMT) has a curative effect on systemic autoimmune disease in (NZB × NZW) F1, BXSB, and (NZW × BXSB) F1 mice [3, 4].

Stromal cells in bone marrow, also called bone-marrow-derived mesenchymal stem cells (BMMSCs), are one of important components of bone marrow microenvironment, which play a crucial role in the growth, differentiation, and function of HSCs [5]. In addition, BMMSCs can differentiate into a variety of cell types including osteoblasts, chondrocytes, adipocytes, and myoblasts [6–9] and possess immuno-modulatory properties such as inhibiting T-cell proliferation in vitro [10, 11]. Studies on animal models showed BMMSCs from lupus BXSB mice were slower to grow, less proliferative, and harder to differentiate into

Table 1: Demographic data and clinical features of SLE patients for cDNA microarray analysis.

Patient no.	Sex/Age (yrs)	Disease duration (months)	SLEDAI	Clinical manifestations	Therapy
1	F/20	84	14	Nephritis, arthralgia, vasculitis	Pred, HCQ, CYC
2	F/44	12	10	Nephritis, arthralgia, cytopenia	Pred, HCQ, CYC
3	F/43	240	19	Nephritis, cytopenia, interstitial pneumonia	Pred, HCQ, CYC
4	F/42	6	20	Nephritis, cytopenia, interstitial pneumonia, polyserositis	Pred, HCQ, CYC

Pred: Prednisone, CYC: Cyclophosphamide, HCQ: Hydroxychloroquine.

osteoblasts compared with those from healthy C57/Bl6 mice, and the deficiencies were associated with structural alterations in the gap junction protein Cx43 [12]. BMMSCs from SLE patients have impaired hematopoietic function [13], demonstrating early signs of senescence [14]. In our previous study, we reported BMMSCs derived from SLE patients showed significantly decreased bone-forming capacity and impaired reconstruction of bone marrow osteoblastic niche in vivo [15]. Moreover, the mRNA level of IL-6 and IL-7 were downregulated in BMMSCs from SLE patients [16]. So we hypothesize that SLE might not only be a stem cell disease, but also a BMMSCs disorder. Based on this hypothesis, in the clinical setting, we intravenously infused allogenic BMMSC or umbilical cord mesenchymal stem cell (UCMSC) to SLE patients, the majority of recipients experienced rapid improvement postinfusion [15, 17–19]. Those studies indicated that, as one of components in the bone marrow microenvironment, BMMSCs dysfunction probably partook in the pathogenesis of SLE and correction of the abnormalities might contribute to the disorder improvement.

Nonetheless, relatively little is known about the cellular and molecular mechanisms underlying the control of mesenchymal stem cell (MSC) proliferation, differentiation, and survival. Recent results have demonstrated multiple signaling pathways involved in the functions of MSCs. For example, the osteogenic differentiation of MSCs induced by bone morphogenetic proteins-2 (BMP-2) may be mediated by coordinated activation of Notch, Wnt, and transforming growth factor-β (TGF-β) signaling pathways [20]; MSCs were activated by TLR ligands leading to modulation of the differentiation, migration, proliferation, survival, and immunosuppression capacities [21–23]. But the studies concerning pathways involved in the deficiency of BMMSCs from SLE patients are almost blank.

In this study, using the microarray assay, we firstly found that there were significant differences in gene expression profile (GEP) of BMMSCs between SLE patients and normal controls. And in the further investigation, we confirmed that there were abnormalities in actin cytoskeleton, cell cycling regulation, BMP/TGF-β, and MAPK signaling pathways in BMMSCs from SLE patients.

2. Materials and Methods

2.1. Patients and Controls. Bone marrow (BM) was obtained for cDNA microarray from 4 SLE patients according to the American College of Rheumatology criteria [24]. All were female, and the mean age was 37 ± 11 years (range 20∼44). The demographic data and clinical features of SLE patients were listed in Table 1. The normal controls were 1 male and 3 females, with a mean age of 39 ± 7 years (range 29∼45). Further qRT-PCR was performed from 10 female patients (mean 40 ± 14 years, range 15∼60 years) and 10 female normal controls (mean 41 ± 14 years, range 24∼65 years). All SLE patients had active disease with a SELENA-SLEDAI (Systemic Lupus Erythematosus Disease Activity Index) [25] score of more than 10 at the time of bone marrow aspiration. All participants gave written consent to the study which was approved by the Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School.

2.2. Cell Culture and Flow Cytometry. BM was taken from the iliac crest of SLE patients and normal controls, resuspended by phosphate-buffered saline (PBS), and then layered over 1.077 g/mL Ficoll (TBD, Tianjin, China) solution before being centrifuged at 600 ×g for 20 minutes at room temperature. The mononuclear cells were collected and resuspended in low glucose Dulbecco Modified Eagle Medium (L-DMEM, Gibco) supplemented with 10% heat inactivated fetal bovine serum (FBS, Invitrogen, USA) and 1% antibiotic-antimycotic solution and plated at a density of 2×10^7 cells per 25 cm-dish. The cultures were maintained at 37°C in a 5% CO_2 incubator, and the medium was changed after 48 hours and then every three days. When the MSCs were confluent, the cells were recovered by the addition of 0.25% trypsin-EDTA (Gibcoth) and then replated at a density of 1×10^6 cells per 25 cm dish. Cells at passage 3 were consequently analyzed by flow cytometry as described previously [16].

2.3. Microarray Hybridization. BMMSCs were placed in Trizol (Invitrogen, USA) and processed for RNA extraction using the RNeasy kit according to the instructions of the manufacture (Qiagen, Valencia, CA). The universal human reference RNA samples which comprised of 10 different cell lines of humans (Stratagene Corporation, USA) were used as a common reference in the two channel microarray. Total RNA was reverse transcribed, and the cDNA of BMMSCs from SLE patients and normal controls was added with Cy3-dCTP while the cDNA of human reference was added with Cy5-dCTP in the present with Klenow enzyme (GE Healthcare Cat. Nos. PA 55021/PA 53021) [26]. Microarray analysis was performed in CapitalBio Corp (Beijing, China)

using 22 K Human Genome Array. The slide contains gene-specific 70-mer oligonucleotides representing 21, 329 human genes including four human housekeeping genes as positive controls and twelve random negative controls that are designed to have no significant homology with known human DNA sequences as negative controls. Labeled samples were quantitatively adjusted based on the efficiency of Cy-dye incorporation and mixed into 80 μL hybridization solution (3 × SSC, 0.2% SDS, 25% formamide, and 5 × Denhart's). DNA in hybridization solution was denatured at 95°C for 3 min prior loading on a microarray. The array was hybridized at 42°C overnight and washed with two consecutive washing solutions (0.2% SDS, 2 × SSC at 42°C for 5 min, and 0.2% SSC) for 5 min at room temperature. Finally, arrays were scanned with a confocal LuxScan 10 KA scanner (CapitalBio). The data of obtained images were extracted with LuxScan 3.0 software (CapitalBio). Genes with the signal intensity more than 800 (Cy3 or Cy5) were regarded as the expressed ones. In every two channel slides, the intensity ratio of the Cy3 to Cy5 of each spot was calculated after normalization with LOWESS regression. Statistical data and differential analysis files were generated by using SAM software 3.0 (Stanford University, Stanford, CA, USA). The significant changed genes were selected based on P value < 0.05 and >2-fold as criteria. All the differentially expressed genes were analyzed using a free web-based Molecular Annotation System 2.0 (MAS 2.0, http://bioinfo.capitalbio.com/mas3/) [27, 28].

All data is MIAME compliant and that the raw data has been deposited in a MIAME compliant database (GEO). The raw data can be seen http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21649. The accession number is GSE 21649.

2.4. Quantitative Reverse Transcription-Polymerase Chain Reaction. Gene expressions were examined by real time RT-PCR performed by ABI 7500 FAST real-time PCR detection system (Applied Biosystems, USA) using SYBR Green detection mix (TaKaRa, Japan) [16]. The expressions of Id (inhibitor of differentiation or inhibitor of DNA binding)-1, Id-2, Id-3, cyclin D, and cyclin E2 were analyzed from 5~10 samples from other SLE patients and normal controls. The following primers were used in this study:

GAPDH (sense): 5′-TGACTTCAACAGCGACAC CCA-3′

(antisense): 5′-CACCCTGTTGCTGTAGCCAAA-3′;

Id-1 (sense): 5′-ACGACATGAACGGCTGTTACT CAC-3′

(antisense): 5′-CTCCAACTGAAGGTCCCTGATG TAG-3′;

Id-2 (sense): 5′-TGTCAGCCTGCATCACCAGA-3′

(antisense): 5′-CCACACAGTGCTTTGCTGTCA-3′;

Id-3 (sense): 5′-TCAGCTTAGCCAGGTGGAAATC -3′

(antisense): 5′-GGCTGTCTGGATGGGAAGGT-3′;

Cyclin D: (sense) 5′-TGATGCTGGGCACTTCAT CTG-3′

(antisense): 5′-TCCAATCATCCCGAATGAGAGTC -3′;

Cyclin E2 (sense): 5′-GCCGTTTACAAGCTAAG CAGCAG-3′

(antisense): 5′-CCAGATAATACAGGTGGCCAA CAA-3′.

2.5. Immunofluorescence Staining. Cells were washed three times with PBS, fixed for 10 min 3.7% formaldehyde in PBS, and permeabilized for 5 min with 0.2% triton X-100 3.7% formaldehyde. The fixed cells were rehydrated with Tris buffered saline (TBS) and incubated for 1 h in blocking solution (3% BSA in TBS), then they were incubated with Alexa Flour 594 conjucted phalloidin (Invitrogen, USA) or phalloidin-FITC (Sigma, USA) antibodies for 1 h at 37°C. Nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole). Finally, cells were rinsed in TBS, mounted in DABCO/mowiol. Images were acquired using a TCS SP2 confocal microscope (Leica, Germany) or fluorescence microscope (Olympus, Japan).

2.6. Immunocytochemistry Staining. Cells were seeded on poly-L-lysine-coated 6-well chamber slides (BD, Bioscience), cultivated for another 3 days. Samples were then fixed with cold acetone for ten minutes followed by incubation in 3% hydrogen peroxide to block the endogenous peroxide activity. To prevent nonspecific antibody binding, slides were preincubated for 30 min in normal goat serum. Slides were then incubated with primary monoclonal antibody against human BMP-5 (Bioword Technology, USA) at 37°C for 1 h, followed by incubated with second antibody MaxVision kit (Maxim Inc., China) for 15 min at room temperature. After a 15-min wash, slides were treated with 3,3′-diaminobenzidine (DAB) for 5 min and finally counterstained with hematoxylin.

2.7. Immunoblotting. Cells were lysed with sodium dodecyl sulfate- (SDS-) sample buffer containing 0.1 M Tris-HCl, 4% SDS, 0.2% Bromophenol Blue, and 5% β-mercaptoethanol. Cell lysates were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). Blots were probed by anti-phospho- and anti-total-extracellular signal-regulated kinase (ERK)1/2 MAPK antibodies (Cell Signaling Technology Inc.), anti-phospho- and anti-total-P38 MAPK antibodies (Cell Signaling Technology Inc.), anti-phospho- and anti-total-stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) MAPK antibody (Cell Signaling Technology Inc.), anti-cyclin D antibodies (Epitomics Inc.), and anti-cyclinE (Epitomics Inc.) before visualizing with HRP-conjugated secondary antibodies followed by development with FluorChem FC2 System (Alpha Innotech Corporation, USA).

2.8. ELISA Analysis. Serum from 10 SLE patients and 20 normal controls were collected, and the concentrations of

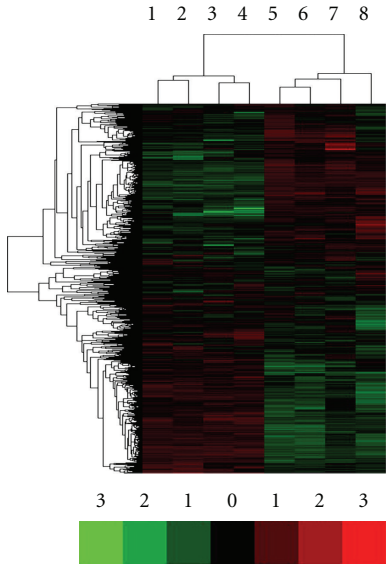

Figure 1: Genes differentially expressed in BMMSCs between SLE patients and normal controls. Genes were shown by the ratio of hybridization intensity between normal control and SLE BMMSCs. The ratio ≥2 or ≤0.5 was considered significant. Genes highly expressed in BMMSCs from normal controls were highlighted in green, while those highly expressed in BMMSCs from SLE patients were highlighted in red.

tumor necrosis factor-α (TNF-α) of each individual were measured using commercial ELISA kit (R&D) according to the manufactory introduction.

2.9. Statistical Analysis. Statistical analyses were performed using SPSS for 16.0. All data were expressed as mean ± SEM. The relative expression of the target genes in SLE samples as compared with that in normal controls was examined using $2^{-\Delta\Delta Ct}$ method [29]. Briefly, for each sample, a value for the cycle threshold (Ct) was determined, defined as the mean cycle at which the fluorescence curve reached an arbitrary threshold. The ΔCt for each sample was then calculated according to the formula Ct target gene-Ct GAPDH; ΔΔCt values were then obtained by subtracting the ΔCt of a reference sample (average ΔCt of the control group) from the ΔCt of the studied samples. Finally, the levels of expression of the target genes in the studied samples as compared with the reference sample were calculated as $2^{-\Delta\Delta Ct}$. A P value of 0.05 or less ($P < 0.05$) by independent Student's t test or nonparametric test was considered statistically significant.

3. Results

3.1. Unsupervised Hierarchical Clustering in the BMMSCs from SLE Patients and Normal Controls. Firstly, we sought to investigate whether the gene expression profiles were globally different between BMMSCs from patients with SLE and normal controls. We used the total number of 8, 769 genes detected to perform the unsupervised hierarchical clustering after faint spots were removed. As expected, hierarchical

Table 2: Differentially expressed genes in regulation of actin cytoskeleton and TGF-β signaling between SLE patients and normal controls.

Upregulated genes in actin cytoskeleton pathway	Fold	P value
ACTN4	2.30	$\leq 1.0E-6$
ACTB	2.41	$\leq 1.0E-6$
VAV1	4.08	$\leq 1.0E-6$
MATK	2.85	$\leq 1.0E-6$
ITGB5	8.46	$\leq 1.0E-6$
ITGB4	2.43	$\leq 1.0E-6$
Downregulated genes in actin cytoskeleton pathway		
KRAS	5.62	$\leq 1.0E-6$
ARPC3	2.17	$\leq 1.0E-6$
ARPC4	2.10	$\leq 1.0E-6$
ARPC5	2.53	$\leq 1.0E-6$
NRAS	2.01	$\leq 1.0E-6$
GNG12	2.17	$\leq 1.0E-6$
NCKAP1	3.16	$\leq 1.0E-6$
ITGA1	3.63	$\leq 1.0E-6$
CRKL	2.71	$\leq 1.0E-6$
ITGB5	2.58	$\leq 1.0E-6$
PPP1CC	3.36	$\leq 1.0E-6$
CFL2	2.06	$\leq 1.0E-6$
ROCK2	2.38	$\leq 1.0E-6$
PDGFRA	2.77	$\leq 1.0E-6$
F2R	2.57	$\leq 1.0E-6$
RDX	2.23	$\leq 1.0E-6$
PPP1R12A	2.99	$\leq 1.0E-6$
ARHGEF6	2.31	$\leq 1.0E-6$
ITGAV	7.84	$\leq 1.0E-6$
CRK	2.71	$\leq 1.0E-6$
Upregulated genes TGF-β signaling		
BMP5	4.23	$\leq 1.0E-6$
Downregulated genes TGF-β signaling		
SMAD1	2.08	$\leq 1.0E-6$
SMAD5	2.60	$\leq 1.0E-6$
SMURF2	2.66	$\leq 1.0E-6$
ID1	3.81	$\leq 1.0E-6$
BMPR1A	3.03	$\leq 1.0E-6$
TGFBR1	4.50	$\leq 1.0E-6$
TGFBR2	2.01	$\leq 1.0E-6$
ACVR1	3.12	$\leq 1.0E-6$
CREBBP	2.06	$\leq 1.0E-6$
ROCK2	2.38	$\leq 1.0E-6$
RPS6KB1	2.64	$\leq 1.0E-6$
CDKN2B	2.03	$\leq 1.0E-6$
THBS1	3.14	$\leq 1.0E-6$
THBS3	2.11	$\leq 1.0E-6$
THBS2	3.05	$\leq 1.0E-6$
LTBP1	2.27	$\leq 1.0E-6$
COMP	4.89	$\leq 1.0E-6$
FST	2.23	$\leq 1.0E-6$

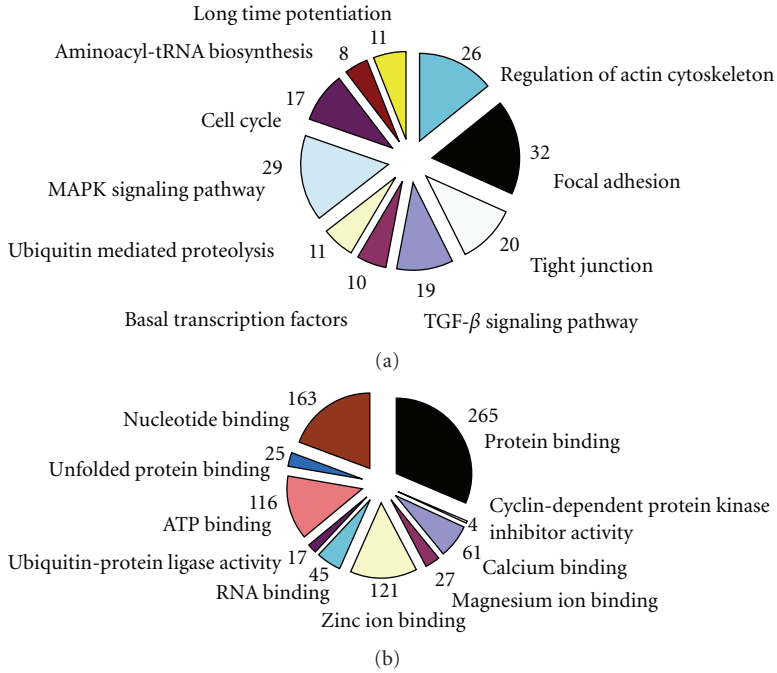

Figure 2: Classification of the differentially expressed genes according to the pathway and GO analysis by MAS software. (a) The top 10 statistically significant ($P < 0.001$) pathways. (b) The top 10 statistically significant ($P < 0.001$) molecular functions by GO analysis. The numbers indicated the differentially expressed genes in the specific pathway or function.

clustering of the 8 BMMSCs samples fell into 2 groups displaying different expression patterns of those 8, 769 genes. One cluster consisted of samples from SLE patients (samples 1~4) and the other cluster consisted of those from normal controls (samples 5~8, Figure 1). These data suggested the existence of different gene expression patterns of BMMSCs between SLE patients and normal controls.

3.2. Gene Ontology (GO) and Pathway Analysis of BMMSC. There were 1, 905 genes found to be differently expressed between the SLE patients and the normal controls using SAM software combined P-value < 0.05 and >2-fold criteria. Of those genes, 652 were upregulated in the BMMSCs of SLE patients, while other 1, 253 were downregulated. The functions of differentially expressed pathways included actin cytoskeleton, focal adhesion, TGF-β signaling, and tight junction. The altered expression of 26 genes was found to be involved in regulating actin cytoskeleton pathway, among which 6 genes were up-regulated while 20 down-regulated in BMMSCs from SLE patients. Interestingly, most genes in TGF-β signaling pathway were downregulated except for BMP5. Moreover, GO analysis found that genes involved in the control of the cell cycle, protein binding, and calcium ion binding showed the most significant differences among gene expression profiles (all $P < 0.0001$; Figure 2). The differentially expressed genes in regulation of actin cytoskeleton and TGF-β signaling were listed in Table 2. The expressions of SMAD1, BMPR1A, ACTB, and ARPC5 by microarray assay were confirmed by qRT-PCR analysis. The four selected genes were initially validated by qRT-PCR in the RNA samples used for the microarrays. As expected, the qRT-PCR data showed significant differences between SLE patients and normal controls and confirmed the direction of the fold changes (supplementary Figure 1; see Supplementary material available online at doi:10.1155/2012/826182).

3.3. Abnormal Actin Cytoskeleton Distribution Pattern in BMMSCs from SLE Patients. Consistent with our previous findings, flow cytometric analysis showed CD29, CD44, and CD105 expression of >95%, in parallel with CD45, CD34, CD14, and HLA-DR expression of <5% (supplementary Figure 2). Although BMMSCs from SLE patients and normal controls showed similarly fibroblast-like morphology as observed by light microscopy [16], the actin distribution pattern in BMMSCs from SLE patients, distinct from that from normal controls (Figure 3(a)), exhibited an irregular and twisted pattern under fluorescence microscope (Figure 3(b)). Under confocal microscopy, BMMSCs from normal controls displayed a pattern of parallel actin stress fibers extending across the entire cytoplasm as revealed by phalloidin staining (Figure 3(c)), while F-actin in BMMSCs from SLE patients was disorganized and condensed on the edge of cytoplasm (Figure 3(d)).

3.4. Altered Protein Expression in Regulating Cell Cycle. Since microarray analysis showed altered expression profile of genes involved in cell cycle, we evaluated the mRNA and protein levels of cyclin D and cyclin E in samples from 5 SLE patients. No difference was found in the levels of cyclin D and cyclin E2 transcripts between BMMSCs from SLE

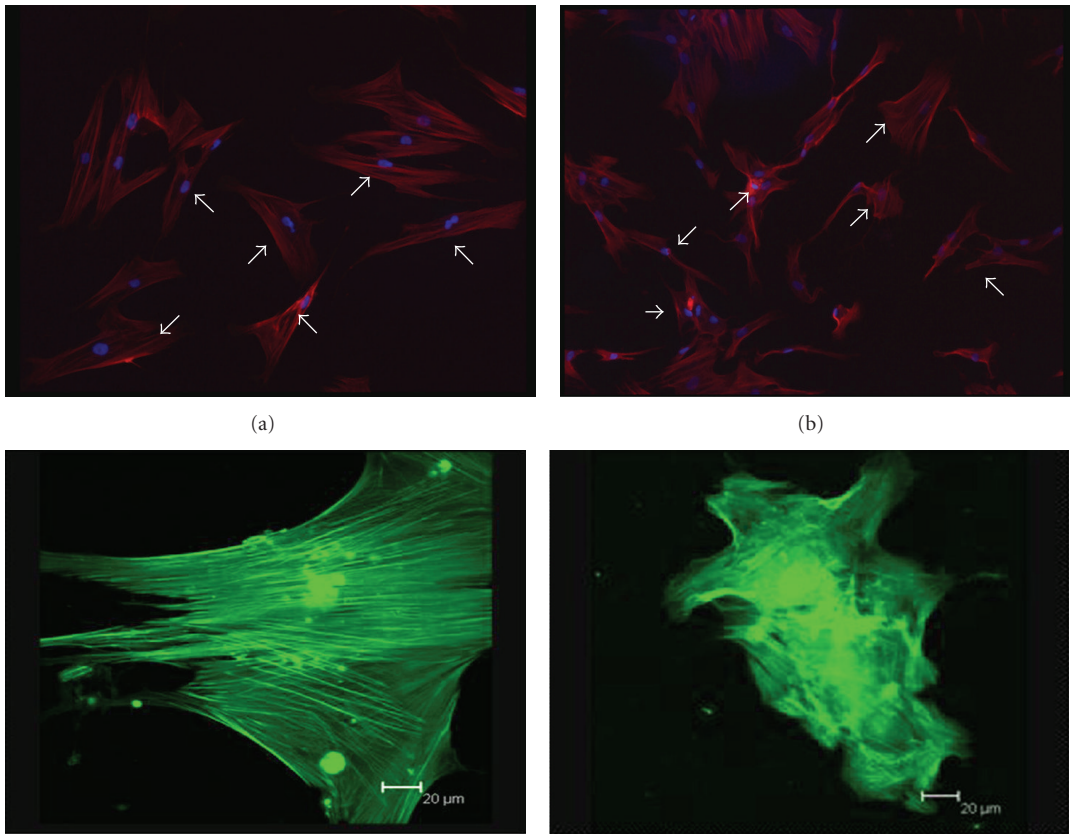

Figure 3: Actin distribution patterns in BMMSCs from SLE patients or the normal controls. (a, b) Actin filaments were stained with Alexa Flour 594 conjucted phalloidin (red) and nuclei were counterstained with DAPI (blue). Cells (white arrows) were observed by fluorescence microscope. (a): BMMSCs from normal controls, (b): BMMSCs from SLE patients. (magnification ×200) (c, d) Actin filaments was stained with phalloidin-FITC and observed under confocal microscopy. (c): BMMSC from one normal control, (d): BMMSC from one SLE patient (Bar = 20).

patients and normal controls. However, immunoblotting analysis further revealed reduced protein level of cyclin E in BMMSCs from SLE patients ($n = 3$, $P = 0.003$) (Figure 4).

3.5. Abnormal Gene and Protein Expressions in BMP/TGF-β Signaling Pathway. In addition, we performed immunostaining experiments to detect the protein level of BMP-5, which was the only upregulated gene in BMP/TGF-β signaling pathway in microarray. Moreover, the expressions of target gene of BMP signaling pathway, including Id-1, Id-2, and Id-3, from 10 samples of SLE patients and normal controls were analyzed by qRT-PCR. Most of BMMSCs from both normal controls and SLE patients were positively stained cells. However, BMMSCs from normal controls showed light brown staining in cytoplasm while BMMSCs from SLE patients were dark brown stained in both nuclei and cytoplasma, suggesting that BMP-5 protein expression was upregulated in BMMSCs from SLE patients. Among the target genes, only the expression of Id-1 was lower in SLE (0.89 ± 0.51) compared with normal controls (1.86 ± 1.26) ($n = 10$, $P = 0.037$). The results indicated that the BMP signaling pathway appeared to be dysregulated in BMMSCs from SLE patients (Figure 5).

3.6. Activated MAPK Pathway in BMMSCs from SLE Patients. Another cascade that appeared to be disordered was the MAPK pathway. As shown in Figure 6, the phosphorylation of ERK1/2 ($n = 3$, $P = 0.03$) and SAPK/JNK ($n = 3$, $P = 0.03$) were higher in BMMSCs from SLE patients, as compared with normal controls ($n = 4$), while the phosphorylation of P38 showed similar levels in BMMSCs between SLE patients and normal controls, suggesting a partially activated MAPK pathway in BMMSCs from SLE patients.

3.7. Id-1 Associated with Serum TNF-α Level in SLE Patients. In order to identify the relationship between the differentially expressed genes and the clinical outcome, correlation analysis was used between Id-1 mRNA levels and serum levels of antinuclear antibodies (ANAs), TNF-α, and SLEDAI in SLE patients. The level of TNF-α in the serum of SLE patients ($n = 10$) was higher than that in normal controls ($n = 20$) ($P = 0.006$). Id-1 mRNA levels had no correlation with ANA,

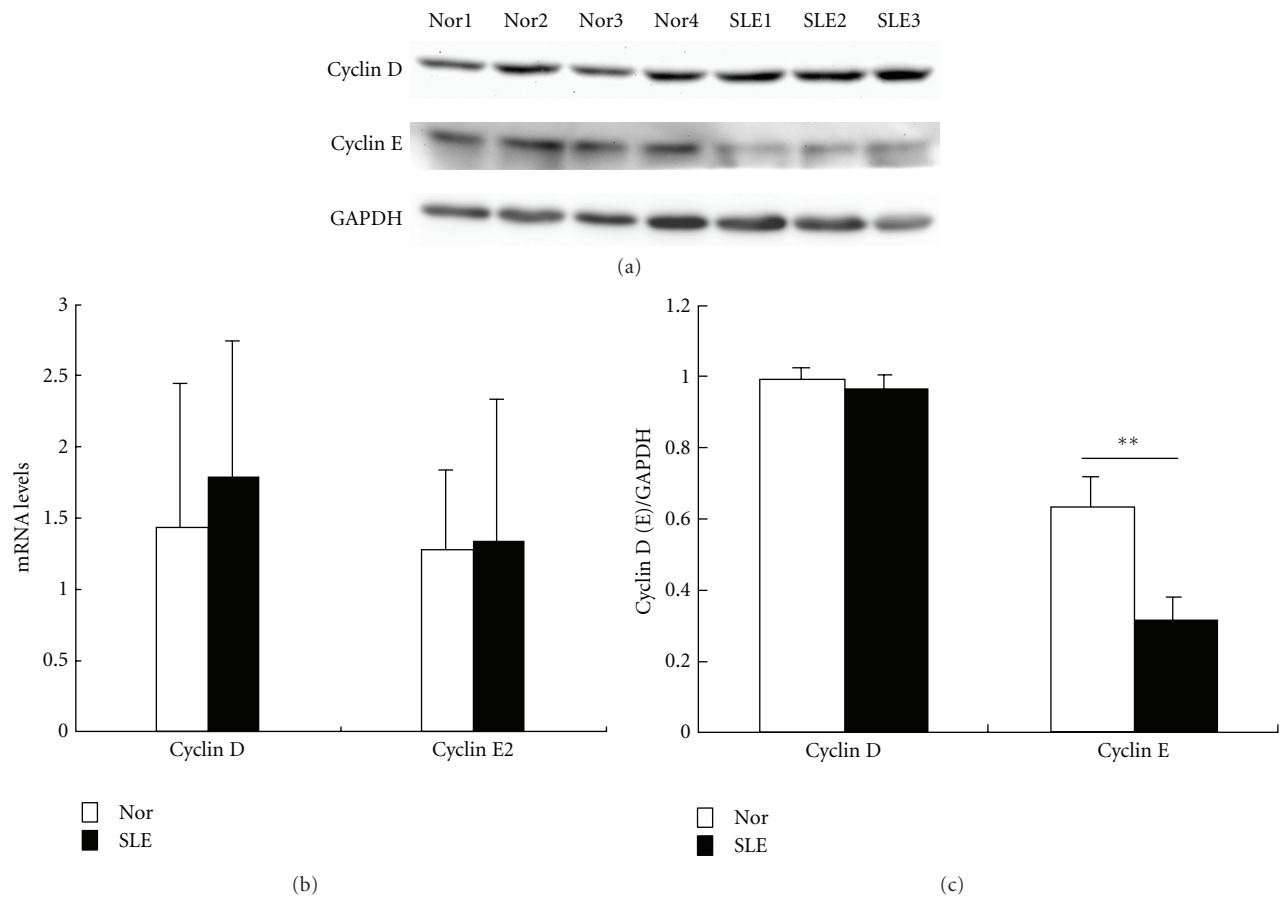

Figure 4: mRNA and protein levels of cyclin D and cyclin E in BMMSCs from SLE patients. (a) Immunoblotting analysis of cyclin D and cyclin E. (b) qRT-PCR studies of the expression of cyclin D and cyclin E2 in BMMSCs from SLE and normal controls ($n = 5$, $P > 0.05$). Results were shown as mean ± SEM, each performed with triplicate samples. (c) Quantity analysis showed low protein level of cyclin E in cells from SLE patients, $^{**}P < 0.01$ by Student's t-test. SLE: systemic lupus erythematosus, Nor: normal controls.

SLEDAI, but it was reversely correlated with serum level of TNF-α in SLE patients (Figure 7). In addition, Id-1 mRNA levels of BMMSCs from normal controls had no correlation with their serum levels of TNF-α (see supplementary Figure 3, $n = 10$, $P = 0.76$).

4. Discussion

Previous studies using microarray in SLE examined gene expression in peripheral blood mononuclear cells (PBMCs) and showed interferon- (IFN-) inducible and granulopoiesis signatures correlating with both disease severity and disease activity [30, 31]. IFN-related genes and genes involved in extra-cellular matrix (ECM) homeostasis were also found differentially expressed in target organs, such as lupus glomeruli and synovium of SLE patients in some studies [29, 32]. One study differentiated active SLE from inactive by the microarray analysis of the bone marrow mononuclear cells (BMMCs), and the upregulated genes in SLE patients were involved in cell death and granulopoiesis [33]. In our study, genes in regulation of cell cycle, actin cytoskeleton regulation, TGF-β, focal adhesion, and MAPK pathways, rather than type I interferon signature were found to be differentially expressed in the BMMSCs from SLE patients, suggesting the distinct role of bone marrow, especially the stromal cells in regulating the immune response.

Although the morphological characteristics of BMMSCs from SLE patients was the same as the normal controls [16], we observed under confocal and fluorescence microscopes in this study that the F-actin of BMMSCs from SLE patients was confused and condensed on the edge of cytoplasm, which was absolutely different from normal controls. This actin distribution of BMMSCs from SLE patients supported the notion that MSCs from SLE patients tended to be senescent [8]. Actin filaments form the cytoskeleton with microtubules and their prokaryotic cousins play central roles in cell shape, motility, and chromosome segregation control [34, 35]. Moreover, recent studies found actin filaments were closely related to the apoptosis, aging, and malignant transformation of cells [36, 37]. Those studies in combination with present results indicated that BMMSCs

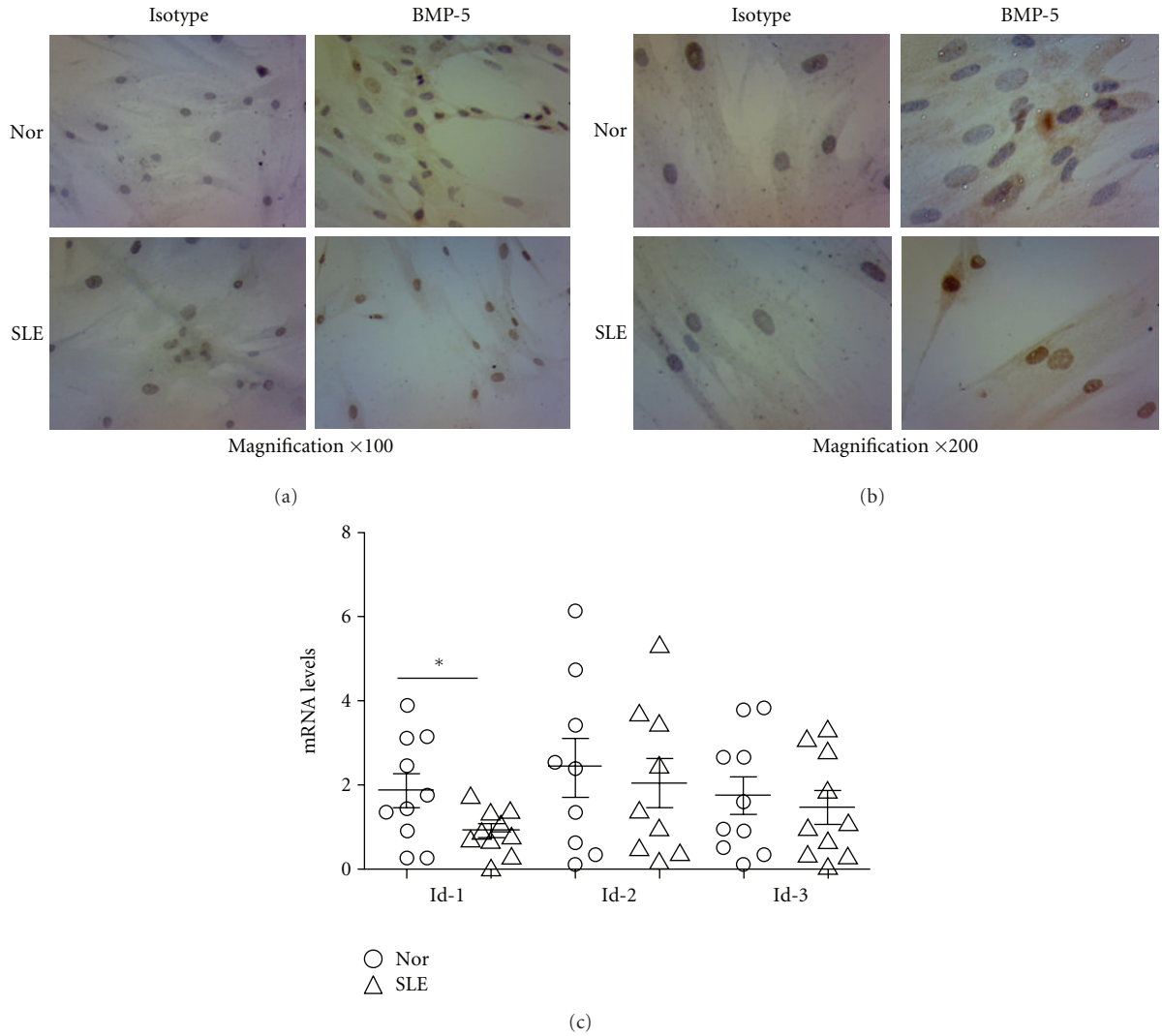

Figure 5: Immunohistochemical detection of BMP-5 protein and mRNA levels of Id-1, Id-2, Id-3 in BMMSCs. (a) magnification ×100, (b) magnification ×200. The left pictures are isotype controls, the right are BMP-5 immunohistochemical staining. (c) qRT-PCR studies of the expression of Id-1, Id-2, and Id-3 in BMMSCs from SLE patients and normal controls ($n = 10$). $^*P < 0.05$ by nonparametric test of SPSS 16.0 software. SLE: systemic lupus erythematosus, Nor: normal controls.

from SLE patients might be abnormal in such functions as migration and aging, which attributes to the disordered actin cytoskeleton.

According to the gene ontology analysis, the most differentially expressed genes were those involved in regulating the cell cycle, which is consistent with the reports that BMMSCs from SLE patients showed lower proliferative capacity compared with normal controls [14, 16]. In this study, we examined the mRNA and protein levels of only two members of cyclin family, cyclin D and cyclin E2. Although no difference was found in the mRNA level in BMMSCs between SLE patients and normal control, immunoblotting analysis demonstrated that the cyclin E expression was lower in the BMMCs from SLE patients, suggesting a deficiency at protein level. This deficiency in regulation of cell cycle might result in the decreased cell proliferative capacity of BMMSCs in SLE patients.

BMPs are multifunctional growth factors that belong to the TGF-β superfamily. Studies have shown that BMP signaling plays critical roles in bone formation and cartilage development [38]. Specific BMPs such as BMP-2, BMP-6, and BMP-9 promote the differentiation of MSCs into osteoblasts in vitro [39]. The BMP signaling cascade initiate from the binding with BMP receptors. Binding of an extracellular ligand promotes the dimerization of the two serine/threonine protein kinases. The type-II kinase phosphorylates the type-I receptor. Activation of the type-I receptor initiates phosphorylation of downstream effector proteins, such as receptor-regulated Smads (R-Smads), including Smad-1, 2, 3, 5, and 8, leading to signal transduction. Following activation, the R-Smad protein forms a heterooligomeric complex with a common mediator Smad (Co-Smad; Smad4), which translocates into the nucleus and regulates the transcription of target genes, such as

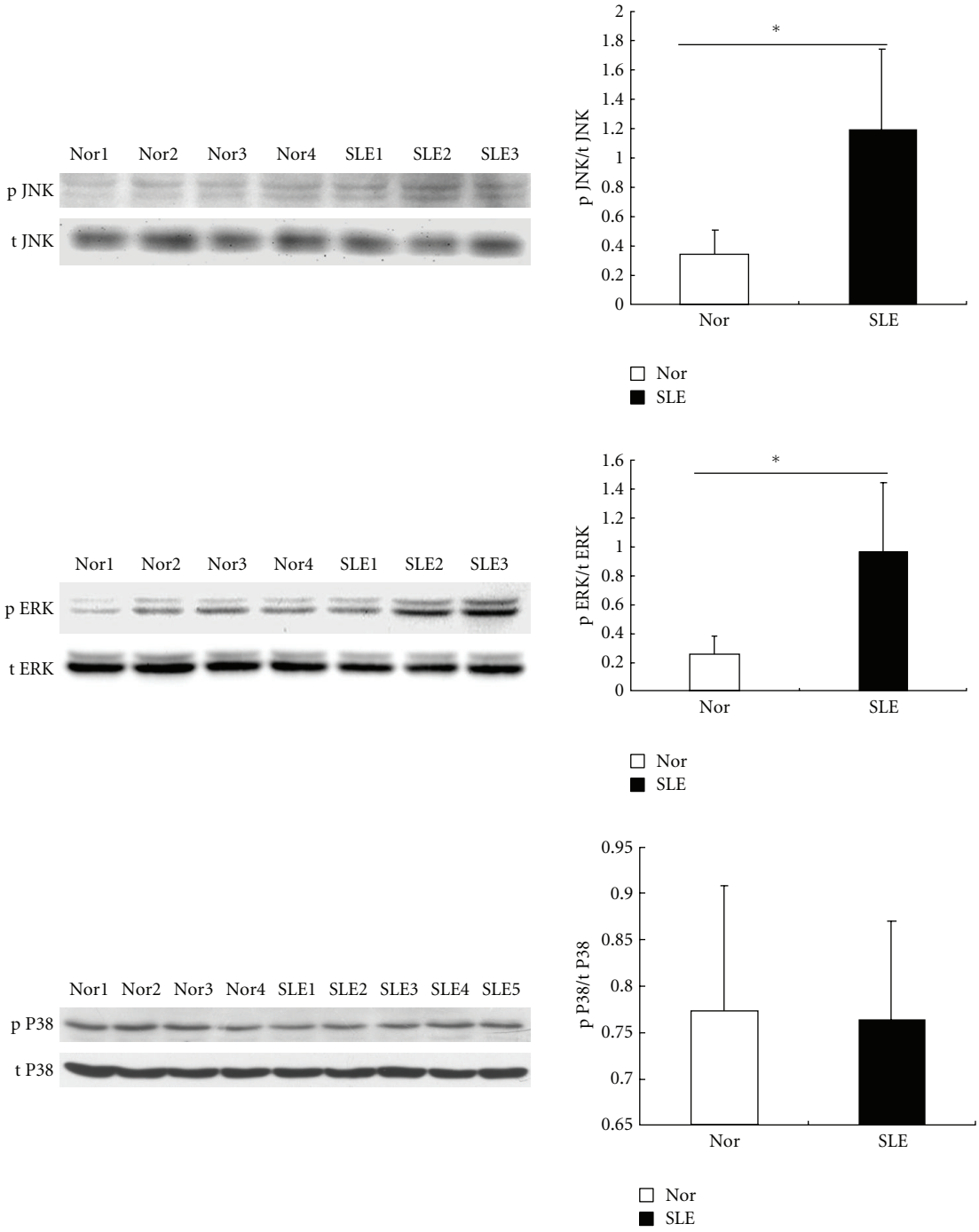

Figure 6: MAPK pathway in BMMSCs from SLE patients. Immunoblotting showed the phosphorylation of JNK and ERK1/2 was higher in BMMSCs from SLE patients. Results were shown as mean ± SEM. $^*P < 0.05$ (SLE versus normal controls by Student's t-test). SLE: systemic lupus erythematosus, Nor: normal controls.

Rux2, Msx2, and osterix [40]. According to the results of the microarray, most of genes in the BMP pathway were decreased including Smad-1, Smad-5, BMPR1A and the target gene Id-1. As the phosphorylation process controls the activity of Smad-1, Smad-5, and BMPR1A, we only confirmed the mRNA level of some of the target genes and the protein level of BMP-5.

Id genes are thought to be the most targeted genes by BMP-Smad signaling. Four Id proteins (Id-1 to -4) have been identified in mammals, which are critical in controlling the differentiation and proliferation of myeloid lineages [41]. Previous studies indicated that Id-3 was overexpressed in SLE peripheral blood cells [42] and Id-1 transcription was upregulated by IL-6 stimulation in the B6.Sle1.Yaa mice

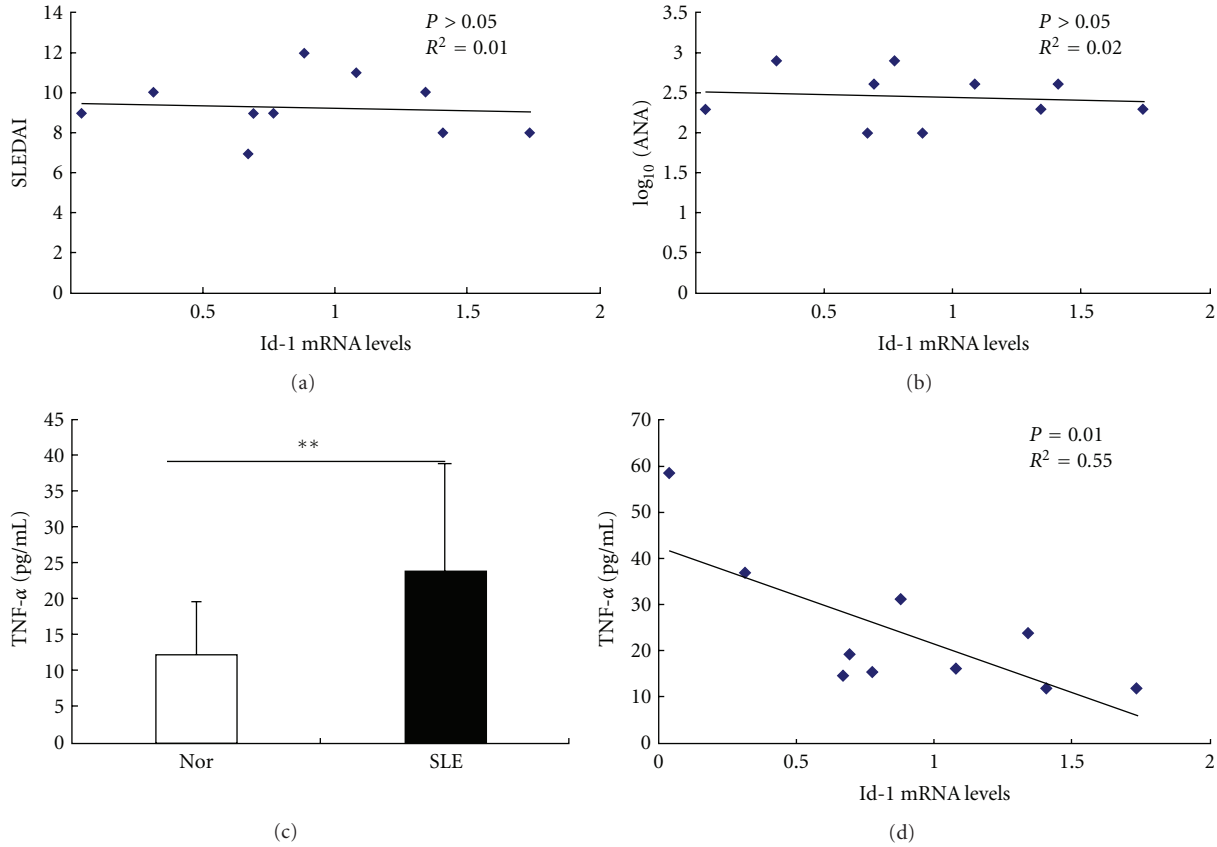

Figure 7: The correlation of Id-1 mRNA expression with serum TNF-α levels in SLE patients. (a, b) Id-1 mRNA level was not correlated with antinuclear antibodies (ANA) levels and SLE disease activity index (SLEDAI) score of SLE patients. (c) The serum level of TNF-α in SLE patients ($n = 10$) were higher than that of normal controls ($n = 20$). $^{**}P < 0.01$ versus normal controls by Student's t-test. (d) Id-1 mRNA expression was reversely correlated with serum TNF-α level of SLE patients ($P = 0.01$, $r^2 = 0.55$). SLE: systemic lupus erythematosus, Nor: normal controls.

model [43]. When compared with normal controls, we found that the Id-1 mRNA level was lower in BMMSCs derived from SLE patients, however, its level was not correlated with ANA level or SLADAI score of SLE patients. Similar to the results described by others [44, 45], TNF-α level was also found higher in the serum of SLE patients in our study, which was reversely correlated with Id-1 mRNA level. This result further confirmed those of microarray and suggested there might be a dysregulated BMP pathway in BMMSCs from SLE patients, which possibly contribute to the osteogenesis impairment and osteoblastic niche deficiency in MRL/lpr mice and SLE patients [15].

MAPK cascade is an important pathway that transmits extracellular signals into cytoplasma to initiate cellular processes such as proliferation, differentiation, and development. The three well-characterized subfamilies of MAPKs include the extracellular signal-regulated kinases (ERK1/2), c-Jun NH2-terminal kinases (JNK-1/2/3), p38. Many growth factors can trigger MAPK pathway including epidermal growth factor (EGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF). In addition, chemokines such as SDF-1 could stimulate human MSCs migration through increased phosphorylation of ERK [46], and Wnt3a could induce a rapid and transient activation of MAPKs p38 and ERK1/2 leading to increased alkaline phosphatase activity and nodule mineralization in murine C3H10T1/2 mesenchymal cells [47]. Moreover, many inflammatory cytokines, such as IL-1 and TNF-α, could inhibit the osteoblastic differentiation via phosphorylation of ERK1/2 and SAPK/JNK [48, 49]. We found in this study an increased phosphorylation of ERK1/2 and SAPK/JNK in BMMSCs from SLE patients and postulate that this activation might result from the inflammatory environment in the bone marrow of SLE patients.

5. Conclusion

In conclusion, our present study revealed absolutely different gene profile pattern of BMMSCs from SLE patients and showed disordered actin cytoskeleton in BMMSCs from SLE patients. Furthermore, we found abnormalities in cell cycling regulation, BMP/TGF-β and MAPK pathways. Our findings suggest BMMSCs, as a component of bone marrow, may play an important role in the etiopathogenesis of SLE.

Conflict of Interests

The authors indicate no potential conflict of interests.

Acknowledgments

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Clinical Study

Double Allogenic Mesenchymal Stem Cells Transplantations Could Not Enhance Therapeutic Effect Compared with Single Transplantation in Systemic Lupus Erythematosus

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The clinical trial of allogenic mesenchymal stem cells (MSCs) transplantation for refractory SLE patients has shown significant safety and efficacy profiles. However, the optimum frequency of the MSCs transplantation (MSCT) is unknown. This study was undertaken to observe whether double transplantations of MSCs is superior to single transplantation. Fifty-eight refractory SLE patients were enrolled in this study, in which 30 were randomly given single MSCT, and the other 28 were given double MSCT. Patients were followed up for rates of survival, disease remission, and relapse, as well as transplantation-related adverse events. SLE disease activity index (SLEDAI) and serologic features were evaluated. Our results showed that no remarkable differences between single and double allogenic MSCT were found in terms of disease remission and relapse, amelioration of disease activity, and serum indexes in an SLE clinical trial with more than one year followup. This study demonstrated that single MSCs transplantation at the dose of one million MSCs per kilogram of body weight was sufficient to induce disease remission for refractory SLE patients.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by multiorgan involvement and loss of tolerance against self-antigens followed by antibody production. Current treatments of severe SLE flares consist of toxic immunosuppressive drugs, most commonly cyclophosphamide (CYC), mycophenolate mofetil, and leflunomide [1]. However, the therapeutic options in cases of SLE refractory to standard treatments are indeed limited, and the disease remains potentially fatal in some patients [2].

Mesenchymal stem cells (MSCs) have potent immunosuppressive capacity, which is demonstrated by the inhibition of T lymphocytes proliferation and proinflammatory cytokines production *in vivo* and *in vitro*. MSCs, furthermore, suppress antibody production of B cells and the generation and function of antigen presenting cells [3, 4]. The immunomodulation of MSCs is for a large extent mediated by soluble factors and induced under inflammatory conditions [5]. Previous studies showed that MSCs/osteoblast linage played a critical role in maintaining the hematopoietic stem cell (HSC) niche [6, 7]. Recently, it has been demonstrated that MSCs themselves constitute an essential HSC niche component, and they are spatially associated with HSC niche *in vivo* bone marrow [8].

As the first example of efficacy, clinical trials for prevention and treatment of graft-versus-host disease (GVHD) after HSC transplantation show that MSCs can

modulate allogenic immune responses and effectively treat human disease. Now these multipotential cells have been applied in various physical and immune injuries including liver cirrhosis, multiple sclerosis, and Crohn's disease [9–11]. Our previous studies also showed that allogenic bone marrow or umbilical-cord-derived MSCs transplantation is safe and effective in treating drug-resistant SLE patients [12–14]. In these pilot clinical studies, all patients received once intravenously MSCs infusion. Additionally, we found that some patients were also well responsive to another dose of MSCs after disease relapse. On the other hand, animal studies indicated that multiple MSCs transplantations could enhance clinical efficacy in lupus mice [15]. However, it is unknown whether multiple MSCs infusions are superior to single transplantation in patients, and the optimal doseage and frequency for MSCs therapy is still obscure. So in this study, we compare the efficacy between single and double transplantations of allogenic MSCs in SLE patients. The conclusion of this study can provide further potentiality of allogenic MSCs transplantation in clinical application for SLE.

2. Materials and Methods

2.1. Patients. From March 2007 through February 2010, 58 patients with SLE refractory to standard therapies were enrolled in allogenic MSCs transplantation (MSCT) trial at the Affiliated Drum Tower Hospital of Nanjing University Medical School after signing informed consent. The study was approved by the Ethics Committee at The Drum Tower Hospital of Nanjing University Medical School and registered at ClinicalTrials.gov (Identifier: NCT00698191). All enrolled patients had at least 4 of 11 American College of Rheumatology criteria for SLE [16]. The inclusion and exclusion criteria have been shown as previously [12]. The trial was conducted in compliance with current Good Clinical Practice standards and in accordance with the principles set forth under the Declaration of Helsinki (1989).

2.2. MSCs Purification and Identification. Bone-marrow-derived MSCs (BMMSCs) were obtained from healthy family donors after signing informed consents. Bone marrow mononuclear cells were separated by density gradient centrifugation as previously described [13, 14]. Those without appropriate bone marrow donors were infused with umbilical-cord-derived MSCs (UCMSCs). UCMSCs were prepared by the Stem Cell Center of Jiangsu Province. Fresh umbilical cords were obtained from informed and healthy mothers in local maternity hospitals after normal deliveries. The purification procedure was described as previously [12].

Criteria for release of MSCs for clinical use included presence of visible clumps, spindle-shape morphology, and absence of contamination by pathogens (as documented by aerobic and anaerobic cultures before release), as well as by virus for hepatitis B surface antigen, hepatitis B core antibody, hepatitis C virus antibody, human immunodeficiency virus antibodies I and II, cytomegalovirus IgM, and syphilis antibody (as determined by enzyme-linked immunosorbent assay [ELISA] before release), cell viability greater than 92% (as determined by trypan blue testing), and immune phenotyping proving expression of CD73, CD105, CD90, CD29 (>90%), and absence of CD45, CD34, CD14, CD79, and HLA-DR (<2%).

2.3. MSCs Transplantation Procedures. Randomization was conducted between once and double MSCT groups. The enrolled 58 refractory SLE patients were randomly assigned into once or double MSCT groups. Of all the patients, 30 were randomly given a single MSCs transplantation, and the other 28 patients received double allogenic MSCs transplantations, with an interval for 1 week. Before MSCT, all patients were administered CYC (10 mg per kilogram per day) intravenously on days 4, 3, and 2 to inhibit active lymphocytes. Patients received allogenic MSCs intravenously at the density of one million cells per kilogram of body weight in each transplantation.

2.4. Follow-Up and Outcome Characteristics. After MSCT, all patients returned for scheduled followup at 1, 3, 6, and 12 months and then yearly thereafter. Medical history, physical examination, and serologic testing were performed. Complete remission was defined as SLEDAI score < 3 and steroid requirement ≤ 10 mg/day of prednisone or its equivalent, combined with British Isles Lupus Assessment (BILAG) D scores or better in all organs but not hematological system [17, 18]. Complete remission for hematological system was defined as white blood cell count > 4,000/μL, hemoglobin > 11 g/dL, platelet count > 100×10^9/L, and steroid maintenance ≤ 10 mg/day of prednisone or its equivalent. Disease relapse was defined as an increase in SLEDAI score ≥ 3 from the previous visit, or experience 1 new domain with a BILAG A score or 2 new domains with a BILAG B score after a previous response [17, 18]. Transplantation-related mortality included all deaths associated with transplantation of MSCs, except those related to recurrence of underlying disease. The investigators assessed and recorded adverse events and their severity throughout the study.

After UC-MSCT, the dose of prednisone and immunosuppressive drugs was tapered when clinical efficacy was achieved for each patient. The withdrawal of prednisone and immunosuppressant was permitted if patient's condition continued to improve. No other immunosuppressant was used unless disease relapsed. If the patient underwent disease relapse, he or she will withdraw from the study and will be given higher dose of prednisone or other immunosuppressants according to disease conditions. The patient's clinical data after relapse and change of clinical regimens will not be included for analysis.

2.5. Statistical Analysis. Descriptive statistics was used to summarize patient characteristics. Differences in patient demographics prior transplantation were analyzed by unpaired *t*-test, Chi-Square test, or Fisher's exact test. All tests were 2 sides. Rates of overall survival, disease complete remission, relapse, and adverse event at different visit times

at two groups were analyzed by a Kaplan-Meier survival curve and were statistically tested with the log-rank test. We calculated the hazard ratio (HR) and their 95% CIs using the univariate Cox proportional hazards model. Serial data were compared within and between groups by repeated measures ANOVA. All P value of less than 0.05 was considered as a significant difference.

3. Results

3.1. Patient Demographics and Disease Manifestations before MSCT. Fifty-eight patients with refractory SLE enrolled in this trial, and all patients underwent allogenic MSCT and were followedup for more than 12 months. The mean followup was 27 months (range from 12 to 48 months) in single transplantation group and 26 months (range from 12 to 40 months) in double transplantation group. Patients' demographics pretransplantation have been shown in Table 1. The two cohorts were balanced in gender, race, MSCs source, clinical manifestations, and disease activity prior transplantation. Medium disease duration was shorter in single than in double transplantation group (mean ± SD 60.2 ± 50.0 months versus 92.1 ± 64.3 months, $P = 0.039$).

3.2. Overall Survival, Disease-Free Survival, and Relapse. With medium followup of over 24 months in both cohorts, one death was observed in double transplantation group. The survival rate was 100% for single and 96.4% for double transplantation group, respectively (log-rank = 1.071, $P = 0.301$). Rate of complete remission was 53.3% (16/30) in single transplantation group and 28.6% (8/27) in multiple transplantation group during 4-year followup by Kaplan-Meier survival curves (log-rank = 3.374, $P = 0.066$, Figure 1(a)). In a multivariable Cox regression model for complete remission, there was no difference between single and double transplantation group (HR 0.38, 95%CI 0.14–1.02; $P = 0.060$). Disease duration (HR 1.00, 95%CI 0.99–1.01, $P = 0.290$) and MSC source (HR 0.69, 95%CI 0.28–1.69, $P = 0.420$) were also not associated with complete remission. Rate of disease relapse was not statistically different in single transplantation group (8/30, 26.7%) compared to that of double transplantation group (6/27, 22.2%, log-rank = 0.075, $P = 0.784$, Figure 1(b)). The average time to relapse was 21 months (mean value, from 6 to 45 months) and 12 months (mean value, from 3 to 24 months) in single and double transplantation group, respectively. Cox regression showed that no difference in disease relapse was found between the two groups (HR 1.16, 95%CI 0.39–3.49, $P = 0.790$).

We calculated the overall percentage of disease relapse that occurred in two groups (8/30, 26.7% in single MSCT group; 6/27, 22.2% in double MSCT group). Additionally, the exact rate of disease relapse was calculated by Kaplan-Meier survival curve (Figure 1(b)), and the variable was correlated with time point when relapse occurred. In the present data, for those who had disease relapse, most relapse events occurred after 24 months followup. For example, in once MSCT group, 7 patients completed 30 months followup and had achieved a previous clinical response, in which 4 had relapse at 40, 36, 48, and 30 months, respectively (Figure 1(b), Supplementary Material available online at doi:10.1155/2012/273291).

Table 1: Patient demographics pretransplantation.

Variable	No. of patients	
	Single MSCT ($n = 30$)	Double MSCT ($n = 28$)
Age in years	30 (12–47)	33 (16–54)
Gender, n (F/M)	25/5	26/2
Race, n (Asian/others)	30/0	28/0
Disease duration (m)	62 (7–232)	92 (12–264)
MSCs source, n		
Bone marrow (BM)	12	9
Umbilical cord (UC)	18	19
Medium followup for survivors (m)	27 (12–48)	26 (12–40)

MSCT, mesenchymal stem cells transplantation.

3.3. Disease Activity and Serum Indexes. Disease activity shown by SLEDAI scores decreased significantly in both groups after allogenic MSCs transplantation by repeated measures ANOVA ($F = 59.36$, $P < 0.001$, Figure 2(a)). There was no correlation between SLEDAI decline and MSCs transplantation frequency ($F = 3.31$, $P = 0.074$). Serum albumin also significantly improved after MSCT at each group ($F = 50.89$, $P = 0.000$), and there was no difference between the two groups ($F = 0.018$, $P = 0.896$, Figure 2(b)). Serum complement 3 (C3) and anti-double-strand DNA (dsDNA) antibody similarly improved in both groups by the same analyzed methods (Figures 2(c) and 2(d)).

3.4. Amelioration of Renal Function and Hematologic Indexes by Allogenic MSCT. Twenty-six patients (26/30, 86.7%) in single MSCT group and 24 patients (24/27, 88.9%) in double MSCT group underwent renal involvement at baseline, shown by the presence of proteinuria, or hematuria, or renal disfunction. The 24 hour proteinuria significantly declined after allogenic MSCT within each group by repeated measures ANOVA ($F = 19.29$, $P = 0.001$). However, once MSCT group demonstrated much lower levels of proteinuria compared to double MSCT group at the first 12-month visits ($F = 5.31$, $P = 0.026$, Figure 3(a)). For those who had renal disfunction at baseline, serum creatinine significantly ameliorated after MSCT within each group ($F = 6.30$, $P = 0.003$), and there was no difference between the two groups ($F = 0.401$, $P = 0.534$, Figure 3(b)). Twelve patients (12/30, 40.0%) in single MSCT group and 13 patients (13/28, 46.4%) in double MSCT group suffered hematologic involvement at baseline; platelet counts and hemoglobin levels significant improved after allogenic MSCT in each group ($F = 10.001$, $P = 0.000$ for platelet counts, $F = 9.237$, $P = 0.000$ for hemoglobin levels); no difference was found between the two groups ($F = 0.098$, $P = 0.760$ for platelet counts, $F = 0.015$, $P = 0.905$ for hemoglobin levels, Figures 3(c) and 3(d)).

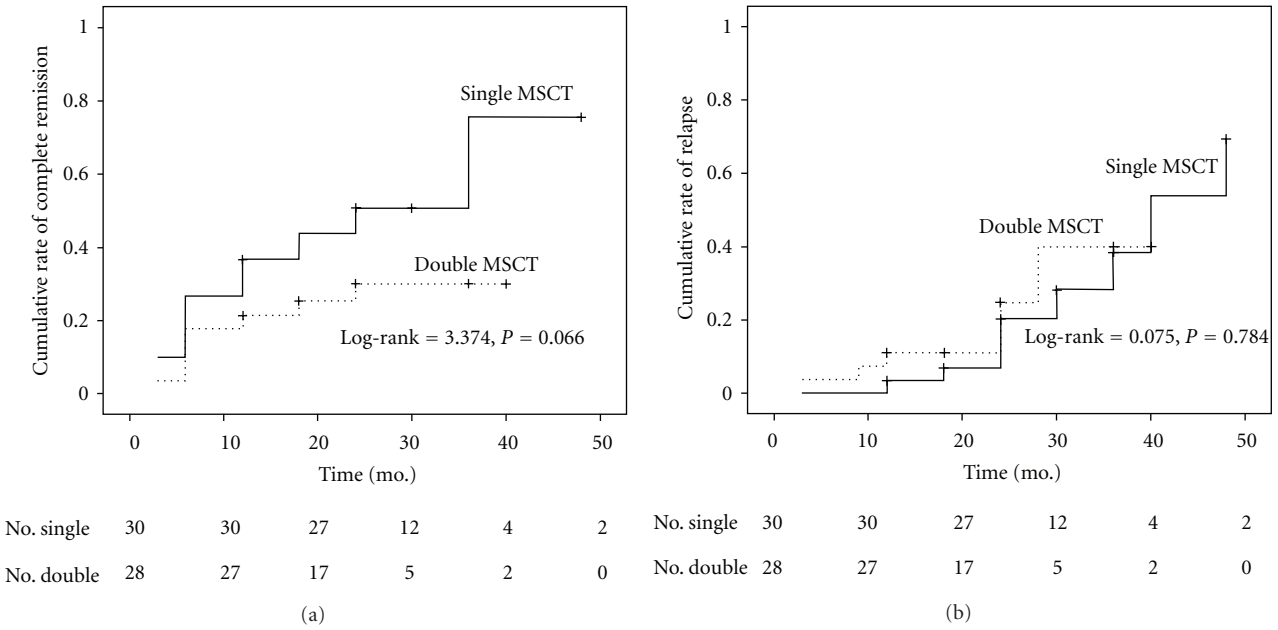

Figure 1: Rate of complete remission (a) and disease relapse (b) for patients with single and double MSCs transplantations, by Kaplan-Meier survival curve analysis.

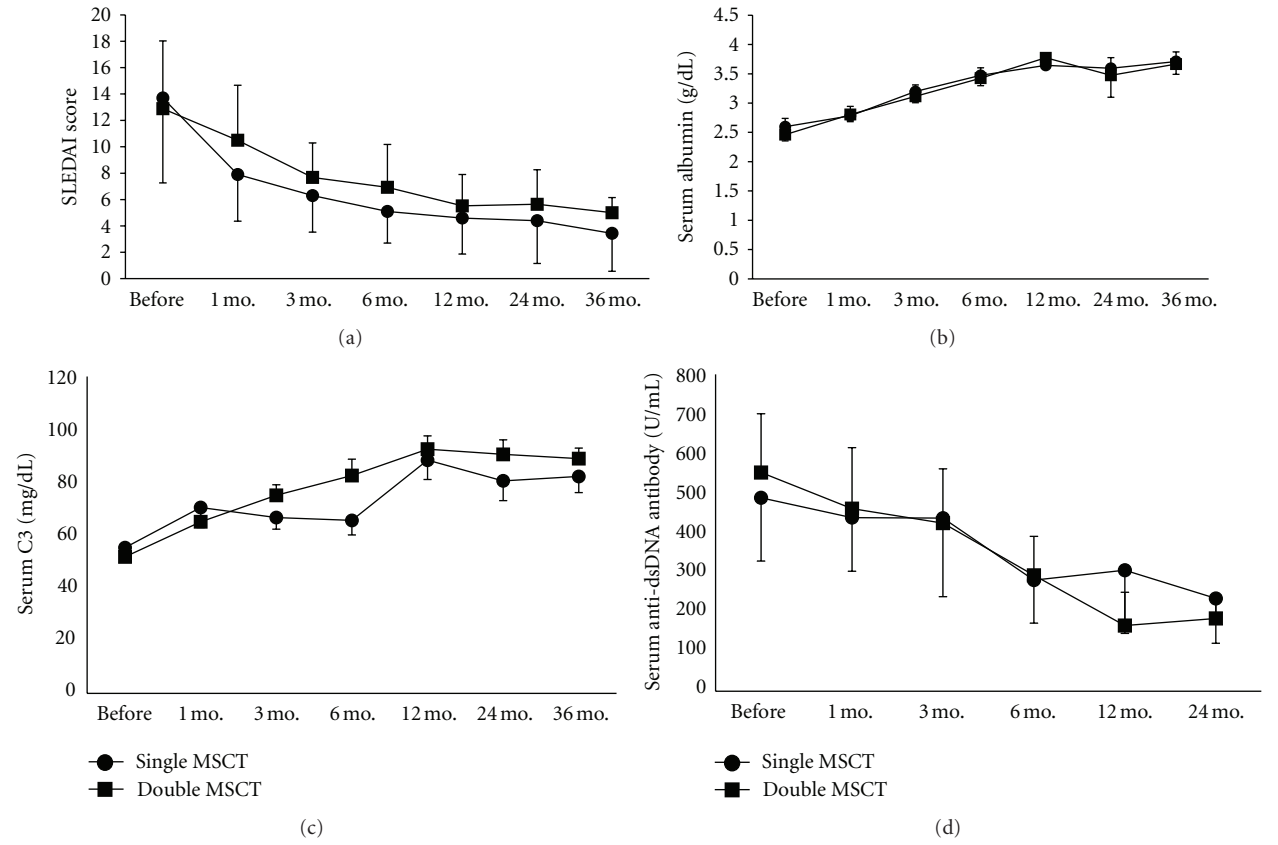

Figure 2: Comparisons of SLEDAI score (a), serum albumin (b), Complement 3 (C3, (c)), and anti-double-strand DNA antibody (dsDNA, (d)) for patients with single and double MSCs transplantations, by repeated measures ANOVA. Values are the mean ± SEM.

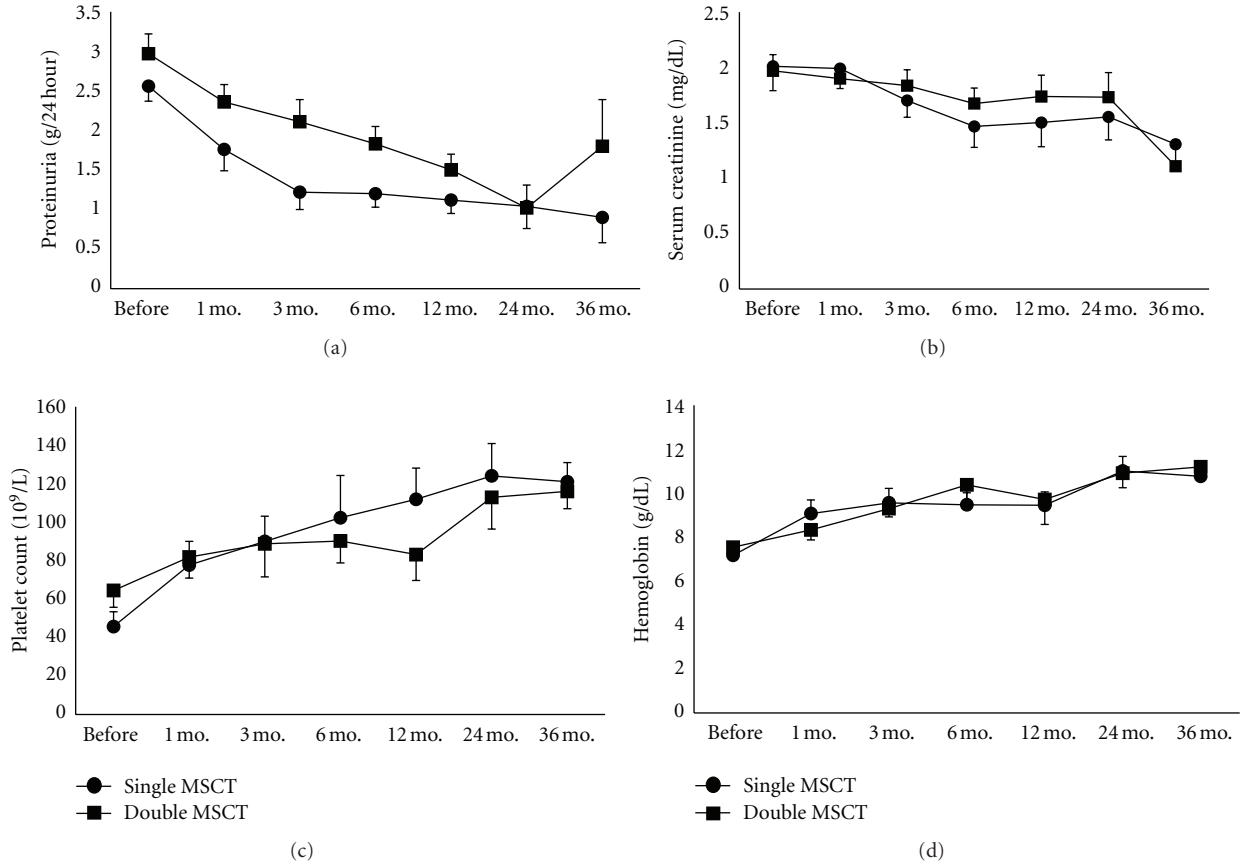

Figure 3: Comparisons of proteinuria (a), serum creatinine (b), platelet count (c), and hemoglobin level (d) between patients given single or double MSCs transplantations, by repeated measures of ANOVA. Values are the mean ± SEM.

3.5. Adverse Events. One patient in double transplantation group underwent uncontrolled disease recurrence 6 months after MSCT due to upper respiratory tract infection. She was not responsive to conventional treatments and finally died of acute heart failure. During 4 years followup, 7 patients in single transplantation group (23.3%) and 9 patients in double transplantation group (32.1%) suffered infection events, and no statistical difference was found between the two groups. Of 7 patients in single transplantation group, 3 had upper respiratory tract infection, 3 had intestinal infection, and one had oral fungi infection. Of 9 patients in double transplantation group, 4 had upper respiratory tract infection, 2 had intestinal infection, one had herpes zoster infection, one had pneumonia, and one had pulmonary tuberculosis. All the adverse events were not considered transplantation related.

3.6. Maintenance Therapy. Two patients in both single (2/30; 6.7%) and double transplantation groups (2/28; 7.1%) had discontinued immunosuppressive drugs in the last followup. Dose of prednisone was tapered to 5–10 mg/day for 24 patients (24/30, 80.0%) in single MSCT group and 22 patients (22/28, 78.6%) in double MSCT group, respectively. Maintenance therapy regimen was defined as the dose of prednisone was not more than 10 mg/day, combined with the dose of immunosuppressive drug was not more than 0.4–0.6 gm/3 months of CYC, 10 mg/day of leflunomide, or 0.5 gm/day of mycophenolate mofetil. Eleven and 7 patients in single (11/30; 36.7%) and double (7/28; 25.0%) transplantation groups achieved above-mentioned maintenance therapy in the last followup. Time to reach maintenance therapy was not different between single (11.8 months, 3–24 months) and double (10.0 months, 4–15 months) transplantation groups.

4. Discussion

Systemic infusions of mesenchymal stem cells have been widely used in clinical applications. However, the appropriate dose of cells for each patient is still unknown. The dose of MSCs in current studies relied to a large extent on clinical experience and lack of rigorous standards. In a phase II clinical trial for MSCs transplantation in GVHD and followed up for 5 years [19], the therapeutic dose of MSCs ranged from 0.8 million to 9.0 million per kilogram for responders and from 0.6 million to 1.9 million per kilogram for nonresponders. However, no significant correlation has been made between the dose of MSCs received and clinical outcomes. Furthermore, single, double, and repeated doses of MSCs have been administered, but with no obvious pattern to the observed outcomes. A small clinical study showed that repeated intermittent MSCs infusions, ranged

from 4 to 8 times, with a 3- to 14-day interval, 10 to 20 million MSCs each time, could successfully improve signs and symptoms, as well as Th1/Th2 rebalance for 4 patients with sclerodermatous chronic GVHD [20]. Recently, Lim et al. [21] applied different dose of third-party-derived-bone marrow MSCs for two patients with GVHD (ranged from 0.5 to 2 million cells per kilogram each infusion), and the outcomes showed that a dose of one million per kilogram was as effective as that of 2 million per kilogram of recipient. Nevertheless, these case studies were insufficient to provide evidence for clinicians and larger-scale clinical trials are needed to determine the optimal cells dose for a better clinical application.

This study for the first time represents a large single-institution series of refractory SLE patients receiving single or double MSCs transplantations. We found a considerable improvement in disease remission for patients transplanted single and double allogenic MSCs. In previous studies, we have proposed that single allogenic MSCs transplantation ameliorated disease phenotype in SLE mice and humans [13]. Additionally, multiple infusions of allogenic UC MSCs, at 18, 19, and 20 weeks, seemed to significantly ameliorate lupus nephritis in MRL/*lpr* mice, compared to single transplantation [15], our animal and clinical data suggest that there may exist disparity between lupus mice and humans.

Although the routes of administration are different between diseases, such as intraportal injection for liver cirrhosis [22] and intrathecal injection for multiple sclerosis or amyotrophic lateral sclerosis [23], intravenous infusion is intensively recommended and applied for most type of diseases [24, 25]. In the present study, we focused on comparing the difference between single and multiple transplantations of allogenic MSCs intravenously, with each dose of one million cells per kilogram of body weight. The dose of infused MSCs for each transplantation was based on the previous successful treatment with the same dose in refractory SLE patients and lupus models [12–14]. Additionally, the current consensus report of the International MSCT Study Group has preferred a dose of 1-2 million MSCs per kilogram for a single intravenous infusion [26]. Based on our previous studies and current reports [15, 27], we chose one-week interval between two times of MSCs transplantation for patients. The current data revealed an optimal dose of infused allogenic MSCs for SLE patients. However, whether this is the case in other disorders still needs further investigations.

Most enrolled patients were unresponsive to CYC treatment before MSCT (for at least 6 months), the low dose of CYC given 4 days before MSCs infusion to each patient was used to inhibit active lymphocytes responses but not to treat disease. So we do not think the same pretreatment regimens before MSCT in both groups would influence the clinical response between once and double MSCT. Furthermore, the dose of CYC in the present study is much lower than that used in hematopoietic stem cells transplantation (total 30 mg/kg versus 200 mg/kg), and our previous animal studies had demonstrated that the addition of CYC before MSCT could not enhance clinical efficacy in MRL/*lpr* lupus mice [28]. Moreover, allogenic MSCT could act more effective than CYC in treating MRL/*lpr* lupus mice [13]. Recently, we have compared the clinical efficacy between patients given and not given CYC for pretreatment at baseline, and the results showed no difference between the two groups (unpublished data). So patients' clinical response was not resulted from CYC pretreatment.

The role of transplanted MSCs *in vivo* is mainly dependent on their multiple differentiation and tissue repairing, as well as extensive immune modulation [7, 29]. Although most of *in vitro* experiments showed that the immunoregulatory effect of MSCs on T cells or B cells is in a dose-dependent manner [30, 31], the reason that repeated transplantations of allogenic MSCs *in vivo* failed to enhance therapeutic efficacy in SLE patients is unclear. It is undoubted that the dose of MSCs in patients is not the more the better, and the appropriate dose of MSCs is most important for clinical treatment. There is no necessity of double transplantations for SLE patients for each therapy. More studies are needed to investigate the role of multiple infused MSCs *in vivo*.

5. Conclusion

This study provides evidence that single transplantation at the dose of one million MSCs per kilogram of body weight is sufficient to induce disease remission in the treatment for refractory SLE patients, and double MSCT had no enhanced effect.

Authors' Contribution

D. Wang and K. Akiyama contributed equally to this work.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Epstein-Barr Virus and Systemic Lupus Erythematosus

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The etiology of SLE is not fully established. SLE is a disease with periods of waning disease activity and intermittent flares. This fits well in theory to a latent virus infection, which occasionally switches to lytic cycle, and EBV infection has for long been suspected to be involved. This paper reviews EBV immunobiology and how this is related to SLE pathogenesis by illustrating uncontrolled reactivation of EBV as a disease mechanism for SLE. Studies on EBV in SLE patients show enlarged viral load, abnormal expression of viral lytic genes, impaired EBV-specific T-cell response, and increased levels of EBV-directed antibodies. These results suggest a role for reactivation of EBV infection in SLE. The increased level of EBV antibodies especially comprises an elevated titre of IgA antibodies, and the total number of EBV-reacting antibody isotypes is also enlarged. As EBV is known to be controlled by cell-mediated immunity, the reduced EBV-specific T-cell response in SLE patients may result in defective control of EBV causing frequent reactivation and expression of lytic cycle antigens. This gives rise to enhanced apoptosis and amplified cellular waste load resulting in activation of an immune response and development of EBV-directed antibodies and autoantibodies to cellular antigens.

1. Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a rare autoimmune disease with an incidence of 6–35 new cases per 100.000 per year and typically presents in women (90% of cases) in the reproductive age [1–3]. The American College of Rheumatology (ACR) updated the clinical criteria for the classification of SLE in 1997, stating that 4 out of 11 criteria should be present consecutively or simultaneously during a period of observation in order to classify SLE (Table 1) [4]. The criteria involve dermatologic symptoms including a butterfly rash on the malar region of the face, discoid rash, photosensitivity, and oral or nasopharyngeal ulcers. Additional criteria comprise arthritis, serositis, renal disorders, and neurologic disorders (including seizures or psychosis). Different hematologic disorders are also included: anemia, leucopenia, lymphocytopenia, and thrombocytopenia. The last two criteria are immunologic disorders including: the presence of antinuclear antibodies (ANAs), which are observed in 80–90% of SLE patients. Most common are autoantibodies directed against double-stranded DNA (dsDNA) (58–70% of SLE patients [2, 5]), but also antibodies to other nuclear components such as histones, Ro52, Ro60, La, and Sm are frequently found [3–6]. The clinical presentation of SLE is influenced by a variety of factors including ethnicity, gender, age, socioeconomic factors, and age of onset [1]. The typical course of the disease is illustrated by periods of disease flares alternating with waning disease activity, and the typical treatment of SLE consists of immunosuppressive medication, which clinically improves the condition of the patients [7].

The etiology of SLE is believed to be multifactorial with genetic and environmental factors, both contributing to the development of this very complex disease. SLE is concordant in 24% of monozygotic twins and approximately 2% of dizygotic twins [8], indicating a genetic contribution. Certain major histocompatibility complex (MHC) II alleles, including HLA-DR2 and HLA-DR3, have been indicated to serve as risk factors in the development of SLE [5], and various HLA-DQ and HLA-DR alleles have been shown to be associated with the production of specific autoantibodies and other clinical manifestations of SLE [5]. Numerous other genes have been shown to be associated with the SLE

Table 1: Symptoms and clinical manifestations of SLE* [3, 4, 6] and IM [29].

SLE	IM
Malar rash	Skin rash
Discoid rash	Palatal exanthema
Photosensitivity	
Oral/nasopharyngeal ulcers	Pharyngitis
Arthritis	Arthralgias
Serositis	
Renal disorders	Renal disorders
Hematologic disorders	
Anemia	Anemia
Leucopenia	Granulocytopenia
Lymphocytopenia	
Thrombocytopenia	Thrombocytopenia
Immunological disorders	Lymphoadenopathy
ANAs	ANAs
Anti-dsDNA	Anti-DNA
Anti-Sm	
Anti-histone	Anti-histone
Anti-ribonucleoprotein	Anti-ribonucleoprotein
Rheumafactor	Rheumafactor
Neurologic disorders (seizures/psychosis)	Neurological disorders (encephalitis/meningitis)
	Headaches
Fatigue	Fatigue
Muscle aches	Muscle aches
Low-grade fever	Fever
Loss of appetite	Loss of appetite
	Malaise
	Hepatosplenomegaly

* ACR criteria highlighted in bold.

pathogenesis especially components of interferon pathways (e.g., *IRF5*, *STAT4,* and *SPP1*), which probably reflects general intrinsic immune deficiencies in SLE patients [9, 10].

Impaired T-cell proliferation, and abnormal cytokine production has also been demonstrated to play a role in SLE pathogenesis [11]. A T helper 1/T helper 2 cytokine imbalance is observed in SLE patients. An enhanced T helper 17 cell response has also been detected and correlated with disease activity in SLE patients, which suggests a role for interleukin-17 (IL-17) in the pathogenesis of SLE [12, 13].

Another risk factor for developing SLE is deficiencies in the classical complement pathway, especially C1q (93%) and C4 (75%) deficiency. C1q deficiency may be inherited or acquired as a result of the production of C1q autoantibodies, which can be detected in some SLE patients. This results in decreased clearance of apoptotic materials leading to accumulation of apoptotic blebs [14–17]. Nuclear autoantigens are clustered at the surface of these blebs. As they are recognized by the immune system as "nonself," they may initiate autoimmune responses [18]. This gives rise to the production of autoantibodies directed against conserved cellular components. The production of autoantibodies results in the formation of circulating immune complexes. When the concentration and size of these complexes reach a critical level, they may deposit in the subendothelium inciting inflammation and tissue damage [16, 19].

Environmental risk factors for SLE development are ultraviolet radiation and certain drugs and chemicals [21–23], and infections are known to be major environmental factors. Especially Epstein-Barr virus (EBV) infection has been shown to be highly associated with the development of SLE, as presented in the following sections.

2. Epstein-Barr Virus Infection

EBV, also known as human herpesvirus 4 (HHV4), is comprised of a 172 kb linear dsDNA genome inside an envelope-enclosed icosahedral capsid (Figure 1). EBV is a ubiquitous infectious agent, latently infecting approximately 95% of the world's population [24]. It is transmitted via saliva and replicates initially at mucosal surfaces in oropharyngeal and nasopharyngeal epithelial cells, especially in the tonsillar area. Next, the virus enters the underlying tissues and infects resting B cells via binding of its viral envelope glycoprotein 350 (gp350) to the B-cell type 2 complement receptor (CD21) [25, 26].

Central to the understanding of EBV's disease biology is the ability of the virus to shift between an active lytic cycle and a latent state, from which the virus occasionally reactivates.

Primary EBV infection during childhood is asymptomatic, but infection in adolescence causes infectious mononucleosis (IM) in 30–70% of cases, where the virus infects up to 20% of the B cells in the body [27, 28]. The reason for the age-related difference in disease is unknown. The most common symptoms and clinical manifestations of IM are skin rash, palatal exanthema, arthralgias, renal disorders, anemia, granulocytopenia, thrombocytopenia, pharyngitis, lymphoadenopathy, hepatosplenomegaly, fatigue, muscle aches, fever, loss of appetite, headaches, and malaise. Furthermore, the central nervous system can be involved, including development of encephalitis or meningitis (Table 1) [29].

Many studies link EBV infection with various autoimmune diseases (e.g., SLE and multiple sclerosis [30–35]) and some cancers, including lymphoid malignancies (e.g., Burkitt's lymphoma [36]) and epithelial cell malignancies (e.g., nasopharyngeal carcinoma [37]). Several cutaneous manifestations have also been associated with EBV infection including hydroa vacciniforme, a photosensitivity dermatosis of childhood mediated by infiltrations of EBV-specific $CD8^+$ cytotoxic T cells in the skin [38].

2.1. EBV Lytic Cycle. During the primary EBV infection, the virus is in its lytic cycle of existence. The early lytic genes, *BZLF-1* and *BRLF-1*, encoding two transcription factors, are essential for the induction of the lytic replication cycle of EBV and also in the reduction of promoter activity in the

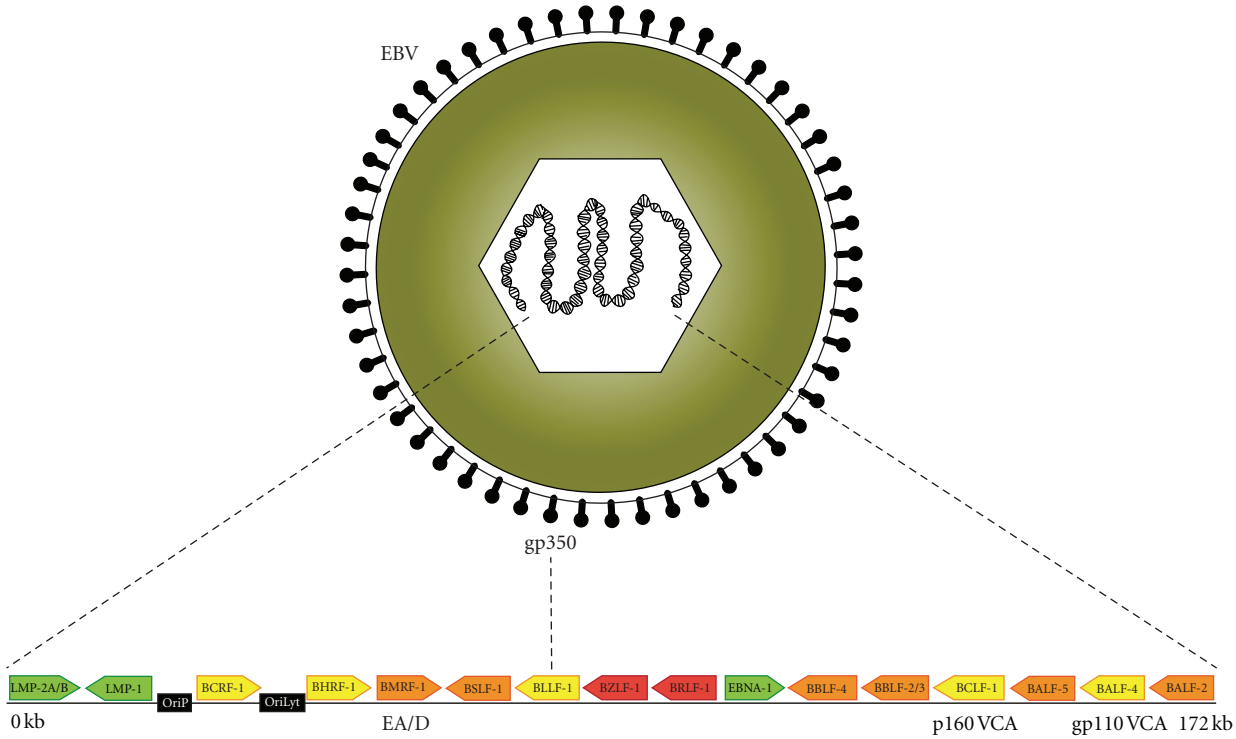

Figure 1: EBV structure and partial map of the genome. The EBV virion comprises a 172 kb linear dsDNA genome inside an icosahedral capsid enclosed by an envelope with viral glycoproteins (gp350) utilized for infection of B cells. The positions of the origins of latent and lytic replication of the viral genome, OriP and OriLyt, respectively, are illustrated in black boxes in the EBV genome map. Selected genes and their relative placement are shown as arrows pointed in the direction of translation [20]. In the latent state, only a few antigens are expressed including EBNA-1, LMP-1, and -2A/B (shown in green). Lytic replication begins with induction of the two early transcription factors (shown in red), which activate early viral promoters generating the initiation complex at OriLyt consisting of 6 viral gene products (illustrated in orange). During lytic cycle, various lytic antigens are expressed (shown in yellow) [20]. Gene products of *BMRF*-1, *BCLF*-1, and *BALF*-4 are depicted as EA/D, p160 VCA, and gp110 VCA, respectively.

latent state of infection [39, 40]. They activate the early viral promoters required for generation of the initiation complex at the lytic origin of viral replication, *oriLyt*, consisting of 6 viral gene products. The 6 viral genes are *BALF-5*, encoding the viral DNA polymerase, *BMRF-1*, encoding Epstein-Barr virus diffuse early antigen (EBV-EA/D) [41–45], which is the viral DNA polymerase accessory protein, *BALF-2*, encoding a single-stranded DNA-binding protein, *BSLF-1* and *BBLF-4*, encoding the primase and the helicase, respectively, and *BBLF2/3*, which encodes the helicase-primase-associated protein [46–51]. The gathering of the initiation complex and the binding of the gene product of *BZLF-1* to *oriLyt* result in lytic replication of the virus.

During lytic cycle, the viral DNA is replicated by a mechanism, where the majority of the 90–100 viral genes are expressed [48]. Multiple rounds of replication are initiated within *oriLyt*, resulting in viral gene expression and viral genome replication with a 100- to 1000-fold amplification [26, 50]. This gives rise to the shedding of infectious virus into saliva that can infect other B cells and epithelial cells and also be transmitted to a new host [52].

Several lytic cycle antigens expressed during the lytic cycle of infection are involved in immune evasion. These include the *BCRF-1* gene encoding a viral IL-10 homologue and *BHRF-1* encoding restricted early antigen (EA/R), a viral Bcl-2 homologue. Like human IL-10, viral IL-10 inhibits the synthesis of interferon-γ (IFN-γ) and suppresses $CD8^+$ cytotoxic T-cell responses and upregulation of MHC I expression [53]. EA/R protects both infected B cells and epithelial cells from apoptosis [54].

Another EBV lytic cycle antigen, EBV-EA/D, is localized both in the cytoplasm and in the nucleus of infected cells, where it colocalizes with the viral DNA polymerase. EBV-EA/D binds dsDNA without sequence specificity and is a part of the EBV DNA-binding complex together with the viral DNA polymerase. It is essential for the polymerase to replicate the viral genome, and EBV-EA/D is therefore termed the EBV DNA polymerase accessory protein [55–58]. EBV-EA/D is also demonstrated to be widely distributed on the newly synthesized EBV genome during lytic replication and is therefore suggested to stabilize the newly synthesized viral DNA [59].

In addition, EBV-EA/D has been shown to have transcription factor activity, inducing activation of several promoters downstream of the *oriLyt* component, which is required for viral lytic replication [45, 55]. Studies have proposed that EBV-EA/D somehow functions as a coactivator for the *BZLF-1* gene product, improving its transactivation

of both the *BALF-2* gene promoter [60] and the *BHLF-1* gene promoter [44]. Different sites of the EBV-EA/D protein have been associated with its different functions. Amino acids 378–404 are required for its transactivator functions [61], and amino acids 194–238 are necessary for stimulation of the viral DNA polymerase [62].

Later in the lytic cycle of infection, the late lytic viral proteins are synthesized: EBV viral capsid antigen (EBV-VCA) and membrane antigen (MA). EBV-VCA is a protein composed of a 110 kDa glycoprotein (gp110) and a 160 kDa protein (p160) encoded by *BALF-4* and *BCLF-1*, respectively. gp110 is involved in virus maturation and improves the efficiency of the virus to infect B cells and epithelial cells [63], whereas p160 is essential for the assembly of the viral capsid [64].

2.2. Latent State. After primary infection, EBV usually enters the latent state as a consequence of the host's immune response. The result of primary EBV infection is numerous EBV-infected B cells, which have induced continuous proliferation and prevented apoptosis resulting in differentiation into immortalized resting memory B cells. These can exit the tonsils and enter the peripheral circulation, and they can persist for life in the host [52]. The EBV genomic DNA will undergo circularization and thus consists of a closed circular plasmid that behaves as the host's chromosomal DNA, which results in severely restricted expression of viral genes. Based on these and other immune evasion mechanisms, the virus becomes undetectable by the immune system [25, 26].

In the latent state of infection, nearly all of the approximately 80 viral promoters are silenced, and a maximum of 9 genes are expressed. These include the nuclear antigens (EBNA-1, -2, -3A, -3B, and -3C), the leader protein (LP), and the latent membrane proteins (LMP-1, -2A, and -2B) [52, 66]. LMP-1 and LMP-2A both act as survival signals of the infected B cell. LMP-1 serves as the signal that normally comes from the CD40 signal transduction pathway initiated by $CD4^+$ T-cell help, and LMP-2A provides the signal normally generated by antigen binding of the B-cell receptor. Thus, these two latent EBV antigens rescue the infected B cells from apoptosis [66, 67].

EBNA-2 is known to be the most important transcription factor and controls the expression of all other latent viral genes. It blocks lytic replication in the majority of EBV-infected cells, ensuring the presence of latently infected B cells and thereby obstructing EBV elimination by the immune response of the host [52]. EBNA-1 is the only viral antigen required for maintenance of the viral genome as it acts as a replication factor during latent infection, where the EBV genomic DNA only is replicated once every cell cycle [26, 52]. When resting memory B cells are latently infected for a longer period of time, EBV only expresses EBNA-1. The EBNA-1 protein contains a glycine and alanine repeat domain, which ensures that the protein is not degraded by the proteasome of the host. Therefore, no EBNA-1 peptides are presented at the surface of the infected B cells, and the virus is thus hidden from the immune system [25, 68].

2.3. Reactivation and Switch to the Lytic Cycle. Occasionally, EBV can reactivate and switch back to the lytic cycle. The triggers for EBV reactivation are unknown. However, differentiation of infected B cells into plasma cells might trigger the activation of the promoter for early lytic genes, which eventually will result in replication and switch to lytic cycle [25, 52, 69]. Yet, the signals and timing involved in this process are unknown and must be a dynamic correlation between the host's immune response towards EBV and the infection state. It is established that activation of the lytic program happens in latently infected memory B cells passing through the lymphoid tissue associated with the pharynx mucosa [26]. Because of this ability of the virus to reactivate, it serves as a constant antigenic challenge to the host.

2.4. Response from the Immune System to EBV. Both latent and lytic EBV antigens are potent immunogens, and a vigorous immune response is initiated during EBV infection. This response involves all parts of the immune system and will control, but not eliminate, the infection. The expansion of EBV-infected B cells during lytic cycle is especially controlled by $CD8^+$ cytotoxic T cells, which kill infected B cells and also induce the latent state in remaining EBV-infected B cells [70]. Cell-mediated immunity is also crucial in preventing the latent infection from entering lytic replication [25]. IFN-γ is suggested to be an important mediator of the immune response against EBV, as the level of IFN-γ is highly increased in patients with IM [71]. The clinical symptoms do not disappear until the amounts of both infected B cells in lytic cycle and of activated T cells are reduced, which occurs after approximately 4 weeks for normal immunocompetent individuals [25]. The $CD8^+$ cytotoxic T-cell response toward EBV accounts for the cutaneous symptoms associated with EBV infection (Table 1) [72].

A humoral immune response is also initiated during EBV infection, and EBV-infected individuals have distinct serologic profiles during the latent and acute phases. In early stages of the primary infection, antibodies toward EBV-VCA and EBV-EA/D are generated, whereas EBNA-1 antibodies develop later. EBV-VCA IgM antibodies are diagnostic for recent active infection [73]. Antibodies of the IgG isotype to EBV-VCA and EBNA-1 will persist throughout life [74]. EBV-EA/D-directed antibodies are known as a strong indication of lytic replication of the virus [74]. Serum IgA antibodies toward the *BZLF-1* gene product and EBV-EA/D have been shown to be produced during active disease and are suggested to be stimulated by EBV replication in mucosal sites [75]. The antibodies produced against EBV antigens counteract the viral infection mainly by antibody-dependent cell-mediated cytotoxicity [72].

3. Association between EBV Infection and SLE

Many studies have revealed a connection between SLE and EBV infection. Essentially all adult SLE patients are infected with EBV (99.5%) [24]. However, the statistical significance of this finding is reduced by the large proportion of healthy adults infected as well (95%). In young people below the

Table 2: EBV-EA/D antibodies in SLE patients and healthy controls [65].

No. of antibody isotypes (IgG, IgA, IgM)	% SLE patients	% Healthy controls
0	12%	65%
1	23%	25%
2	28%	10%
3	37%	0%

age of 20 years, the difference between SLE patients and healthy controls is more evident, as the prevalence of EBV infection in the control population is lower, with only 70% being infected, while essentially all pediatric SLE patients are infected with EBV (99.6%) [76, 77].

As demonstrated in Table 1, SLE and EBV-induced IM are known to have similar symptoms and clinical manifestations, indicating an association. Most interesting, presence of rheumafactor and autoantibodies against cellular components like DNA, histones, and ribonucleoproteins is found in IM patients as well as in SLE patients [78, 79]. EBV infection may somehow result in both diseases according to the genetic predisposition and the immune response against EBV in the individual.

SLE patients have been shown to have at least a 10-fold increased frequency of EBV-infected peripheral B cells compared to healthy controls. This increase is associated with increased disease activity in SLE patients and is independent of intake of immunosuppressive medication [31]. In addition, an abnormally high viral load in the peripheral blood mononuclear cells (PBMCs) has been demonstrated in SLE patients compared to healthy controls in several studies [33–35, 80]. Kang et al. found, by the use of real-time quantitative PCR, a 40-fold increase of EBV load when comparing SLE patients to healthy controls [33], and Moon et al. found at least a 15-fold increase of EBV load in SLE patients [34]. Furthermore, Lu et al. found a significantly elevated level of EBV DNA in serum from 42% of the examined SLE patients compared to only 3% of the healthy controls [80]. These findings suggest EBV active lytic cycle with profound viral replication in SLE patients. Thus, it may be indicated that reactivation of EBV is associated with the development of SLE.

Studies on normal immunocompetent carriers of EBV demonstrate that they usually show little or no mRNA expression by EBV. Gross et al. have demonstrated that SLE patients have abnormal expression of 4 viral mRNAs: *BZLF-1, LMP-1, LMP-2*, and *EBNA-1* in their PBMCs [31]. The measured expression levels of mRNAs were often higher than in individuals with IM indicating very active virus. *BZLF-1* is one of the early lytic genes, facilitating the initiation of the lytic replication of the virus, and expression of this mRNA in SLE patients clearly indicates reactivation of the virus. In addition, an abnormal latency state is indicated in the SLE patients by the increased expression of the three latent state mRNAs. The enhanced expression of *LMP* mRNAs might result in improved survival of EBV-infected B cells, as the encoded antigens serve as survival signals that normally comes from the CD40 signal pathway and by antigen binding to the B-cell receptor [66, 67].

Also, Poole et al. measured the levels of EBV mRNA in PBMCs from SLE patients and healthy controls infected with EBV [81]. They found a 3.2-fold increase in the *BLLF-1* mRNA encoding gp350, which is essential for the binding and infection of new B cells. Also, the *BCRF-1, EBNA-1*, and *LMP-2* mRNAs were increased 1.7-fold in SLE patients compared to healthy controls. These results suggest that the EBV infection is active and harder to control in the SLE patients.

Serologic evidence of a connection between EBV infection and SLE development has been illustrated several times by examining the presence of antibodies to EBNA-1, EBV-VCA, and EBV-EA in sera from SLE patients. Studies on antibodies to EBNA-1 and EBV-VCA are contradictive. Most studies show no difference in the prevalence of IgG and IgM antibodies to either EBNA-1 or EBV-VCA between SLE patients and healthy controls [82–85]. However, studies on pediatric SLE patients and one study on adults show that only two-thirds of healthy controls compared to all SLE patients are seropositive for these antibodies [24, 76, 77]. Furthermore, an elevated amount of both EBNA-1 and EBV-VCA IgA antibodies has been detected in SLE patients [80, 83, 86, 87].

In addition, elevated titers of IgG antibodies to early lytic antigens including EBV-EA/D, EBV-EA/R, and the *BALF-2* gene product have been found in approximately half of SLE patients compared to only 8–17% of healthy controls by several research groups [82, 84, 85, 88, 89]. Most interesting, elevated levels of IgA antibodies to these antigens have also been found in SLE patients, characteristic of epithelial infection. Lau et al. demonstrated that 15% of SLE patients compared to none of the healthy controls were positive for EBV-EA IgA antibodies by immunofluorescence [90], and Draborg et al. found a positive rate of IgA EBV-EA/D antibodies of 58% regarding SLE patients, whereas none of the serum samples from healthy controls showed IgA antibody binding to EBV-EA/D [65]. Furthermore, when compiling the positivity for EBV-EA/D-reacting antibody isotypes (IgG, IgM, and IgA) for each individual, 65% of the SLE patients were positive for two or three isotypes. None of the healthy controls were positive for three isotypes, and only 10% were positive for two isotypes, whereas the majority (65%) had no antibodies against EBV-EA/D (Table 2) [65]. These results could not be explained by intake of immunosuppressive medication, indicating that the antibodies do not occur upon reactivation of EBV due to an iatrogenically suppressed

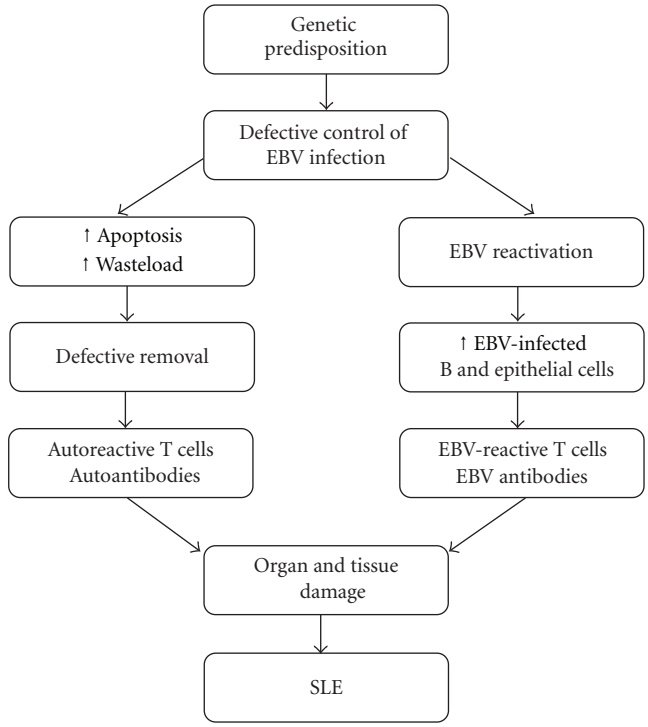

Figure 2: Hypothesis of development of SLE from EBV infection. Genetic insufficiencies may result in poor control and thereby more frequent reactivation of the latent EBV infection. The increased number of EBV-infected cells will, upon apoptosis, initiate an innate and adaptive immune response against the released cellular antigens and EBV antigens due to defective removal of waste products. This will result in production of autoantibodies and EBV antibodies as an attempt to control the virus-induced inflammation. Furthermore, activation of both autoreactive and EBV-reactive T cells will occur. The response from the immune system cause organ and tissue damage leading to development of SLE.

immune system. Presumably, the results of high prevalence of IgA antibodies against EBV reflect the host's attempt to control reactivation or reinfection of EBV in epithelial cells. Additionally, the presence of multiple EBV-EA/D antibody isotypes indicates a more disseminated EBV infection in SLE patients than in healthy controls.

The constant attempts of the host's immune system to control the virus apparently lead to attack on cells expressing EBV-EA/D, resulting in killing of infected cells before assembly of mature EBV particles. This results in release of EBV-EA/D (presumably bound to dsDNA) and intracellular antigens (including those involved in EBV replication and protein synthesis). Since this occurs both in B cells and epithelial cells, the antibody response involves both IgG and IgA to EBV-EA/D and autoantigens (e.g., dsDNA and ribonucleoproteins), depending on the individuals infection distribution and immune system.

Actually, IgA deficiency has been shown to be a risk factor for development of SLE as it results in frequent infections, and approximately 6% of SLE patients have been shown to suffer from IgA deficiency [91, 92]. It could be speculated that defects in controlling EBV infection result in SLE development in different ways. Presumably, some individuals develop SLE due to lack of production of IgA antibodies to counteract an epithelial EBV reactivation. Other individuals are not able to control EBV infection as a result of other immune defects and therefore attempt to control EBV with production of IgA antibodies against EBV lytic cycle antigens (especially EBV-EA/D).

An additional mechanism by which EBV can contribute to development of SLE is molecular mimicry. EBNA-1 has been shown to cross-react with SLE-associated autoantigens resulting in cross-reactive antibodies followed by epitope spreading, which eventually can result in development of SLE [93–95].

The reduced EBV-specific T-cell reactivity observed in SLE patients is possibly a consequence of defective EBV-specific T cells, which indicate poor control of EBV infection. Actually, the defective control of the virus has been demonstrated to involve an impaired EBV-specific T-cell response in SLE patients [33, 96]. Studies conducted by Berner et al. on EBV-specific T cells in SLE patients showed a tendency of an increased frequency of $CD8^+$ T cells toward a specific epitope of the lytic cycle BMLF1 protein. These results were obtained by analysis of PBMCs using MHC I tetramers. Using an ELISPOT assay, the EBV-reactive $CD8^+$ T cells were found to be incapable of producing IFN-γ upon stimulation [96]. This indicates that the EBV-specific $CD8^+$ T cells of SLE patients may have a defect in their ability to become activated upon stimulation and will thereby produce poor effector responses. In addition, Kang et al. found a tendency of SLE patients to have a decreased amount of EBV-specific

$CD8^+$ cytotoxic T cells producing IFN-γ, when samples of whole blood were stimulated with EBV [33]. Simultaneously, they showed a significantly increased frequency of EBV-specific $CD4^+$ T cells producing IFN-γ in SLE patients when stimulated [33]. These results suggest that the impaired EBV-specific T-cell response in SLE patients comprises a defect in EBV-specific $CD8^+$ T-cell cytotoxicity and a compensatory increased frequency of EBV-specific $CD4^+$ T cells.

The above-mentioned associations between active and uncontrolled EBV infection and SLE indicate that EBV and possibly also other viruses have a pathogenic role in the development of SLE. Other viruses besides EBV have been suggested to be associated with SLE including cytomegalovirus [97], parvovirus B19 [98], hepatitis B [98], and human endogenous retroviruses [99]. Overall, infections are presumably involved in SLE induction, and SLE patients have an increased susceptibility to many kinds of infections [100]. These findings are related to the intrinsic immune defects found in SLE patients.

4. Conclusion

The much investigated association between EBV infection and the development of SLE indicates genetic and/or acquired difficulties with suppressing the infection and keeping EBV in its latent state. This is demonstrated by defective EBV-specific T cells, an abnormally high viral load, expression of viral genes, and high levels of EBV IgA antibodies in SLE patients.

Presently, the major genetic predisposing factors are deficiencies in components of the classical complement pathway [17], certain MHC alleles [5], components of IFN pathways, and other immune-regulatory pathways [9, 10, 91]. Acquired antibodies to C1q may also contribute to disease development. These factors may contribute in different ways. Genetically determined immune deficiencies and the presence of particular MHC alleles may first of all limit the ability to control EBV infection and reactivation, and complement deficiencies impair the removal of necrotic and apoptotic cell debris [14–16, 18]. This theory covers many aspects of SLE, but does not explain the female preponderance. Presumably, the solution to this problem shall be found in genetically determined immune system differences or in pregnancy/maternity-related influences on the immune system.

As demonstrated in Figure 2, it is hypothesized that lack of control of EBV infection could result in more widespread latent infection and more frequent reactivation. This entails increased numbers of EBV-infected B cells and epithelial cells and may lead to enhanced apoptosis of cells and amplified cellular waste load. An immune response is therefore initiated with development of autoantibodies against cell components. Lack of control of EBV infection may thus be a contributing factor to development of SLE. EBV reactivation may also give rise to release of EBV lytic cycle antigens resulting in the demonstrated production of EBV-directed antibodies reflecting the hosts attempt to control the reactivation.

Clinically, the constant interplay between EBV reactivation, reinfection, and the host's immune response results in individual disease patterns and clinical presentations, spanning from initial mild symptoms to ultimate classification as SLE as more and more ACR criteria are fulfilled.

In conclusion, the demonstrated associations between EBV and SLE suggest that infection with and reactivation of EBV has a pathogenic role as an environmental trigger inducing or promoting the development of SLE in genetically predisposed individuals.

Abbreviations

ACR: American College of Rheumatology
ANA: Antinuclear antibody
dsDNA: Double-stranded DNA
EA/D: Diffuse early antigen
EA/R: Restricted early antigen
EBNA: Epstein-Barr nuclear antigen
EBV: Epstein-Barr virus
gp: Glycoprotein
HHV4: Human herpesvirus 4
IFN: Interferon
IL: Interleukin
IM: Infectious mononucleosis
LMP: Latent membrane protein
LP: Leader protein
MA: Membrane antigen
MHC: Major histocompatibility complex
PBMC: Peripheral blood mononuclear cell
SLE: Systemic lupus erythematosus
VCA: Viral capsid antigen.

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Clinical Study

Association of Intrarenal B-Cell Infiltrates with Clinical Outcome in Lupus Nephritis: A Study of 192 Cases

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Background. Lupus nephritis (LN) remains a major cause of morbidity and end-stage renal disease. Dysfunction of B lymphocytes is thought to be important in the pathogenesis of SLE/LN. Intrarenal B cells have been found in several forms of inflammatory kidney diseases although their role in LN renal is not well defined. *Methods*. Intrarenal B cells were analyzed in 192 renal biopsies from patients diagnosed with lupus nephritis. Immunohistochemical staining of serial sections was performed for each LN patient using CD20, CD3, and CD21 antibodies. *Results*. Intrarenal B cells were more likely to be associated with class IV LN and were mainly distributed in the renal interstitium, with very few in the glomerulus. The systemic lupus erythematosus disease activity index (SLEDAI), blood urea nitrogen, and serum creatinine levels were all significantly greater in the LN-B cell groups (all $P < 0.05$). LN renal activity and chronicity indices correlated with B-cells infiltrates (all $P < 0.0001$). Renal biopsies were classified into four distinct categories according to the organizational grade of inflammatory cell infiltrates. Germinal center- (GC-) like structures were not identified in any LN biopsies. *Conclusion*. It is hypothesized that intrarenal B cells enhance immunological responses and exaggerate the local immune response to persisting autoimmune damage in the tubulointerstitium.

1. Background

Lupus nephritis (LN) is the main cause of morbidity and mortality in systemic lupus erythematosus (SLE) [1]. LN develops in up to 60% of SLE patients during the course of the disease and its treatment remains a challenge [2]. LN is characterized by immune complex deposition and inflammation in glomeruli and the tubulointerstitium. Many studies have indicated that systemic loss of B-cell tolerance results in the local deposition of immune complexes [3, 4].

Intrarenal mononuclear cells have long been believed to be composed mainly of monocytes and T cells. Classically, B cells have been considered to exert long-range effects mostly via activation in secondary lymphoid organs such as lymph nodes and spleen, with subsequent proliferation and differentiation into antibody-producing plasma cells. Consequently, few researchers have focused on the role of B cells as part of the renal infiltrate. However, a high prevalence of intrarenal B cells has been noted in immune-mediated diseases, such as renal transplant rejection and glomerulonephritis [5–7] thus indicating that local B-cell infiltrates play a role in tissue injury such as tissue fibrosis, neolymphangiogenesis, and ectopic lymphogenesis [8]. Investigations in the MRL/*lpr* mouse model of lupus nephritis have indicated that B cells exert a pathogenic role in the absence of soluble autoantibody production [9]. Moreover, Steinmetz et al. observed that the majority of B cells in lupus nephritis patients displayed a mature non-antibody-producing phenotype with antigen-presenting ability [10]. Recently, the contribution of B cells to the formation of lymphoid-like structures in renal tissue has been proposed [10, 11]. B cells within these lymphoid structures secrete autoantibodies [12] and are required locally to maintain activated T cells [11]. These observations provide evidence

of the functional importance of this intrarenal lymphoid tissue, although the clinical impact remains to be elucidated. It is hypothesized that intrarenal B cells form part of a local system with pivotal involvement in the pathogenesis of lupus nephritis. No detailed data are currently available. In this study, renal B-cell infiltrates were analyzed in a large number of human lupus nephritis patients to reveal the relationship between B-cell infiltration and clinical parameters in order to further elucidate the mechanism in LN.

2. Methods

2.1. Patients. A prospective study of 192 patients who attended the Department of Rheumatology of Renji Hospital at the Shanghai Jiaotong University School of Medicine was carried out. All patients fulfilled the American College of Rheumatology classification criteria for the diagnosis of SLE [13]. Clinical evidence of LN was obtained in all cases and LN diagnosis was confirmed by examination of renal biopsy specimens. Plasma samples were collected on the day of renal biopsy. The following demographic, clinical, and serologic data were collected at the time of renal biopsy: sex, age, duration of SLE and LN, systemic lupus erythematosus disease activity index (SLEDAI), 24 h proteinuria, levels of blood urea nitrogen, serum creatinine, serum C3, C4. The presence or absence of antinuclear antibodies (ANA), antiSm, anti-ribonucleoprotein (anti-RNP), anti-double-stranded DNA (anti-dsDNA), and antinucleosome antibodies was determined. SLEDAI was used to estimate global disease activity.

Informed patient consent was obtained prior to participation in the study, and the study protocol was approved by the institutional review board of Shanghai Jiaotong University.

2.2. Histology of Renal Biopsy Samples . All patients underwent ultrasound-guided renal needle biopsy. Renal tissues obtained by biopsy were fixed in 10% neutral-buffered formalin, dehydrated gradually, and embedded in paraffin. Paraffin-embedded tissue sections were stained with hematoxylin and eosin, periodic acid-Schiff, Masson's trichrome, and periodic acid-silver methenamine. Small portions of fresh renal tissue were snap-frozen, and 4 μm cryostat-cut sections were incubated with fluorescein isothiocyanate (FITC-) conjugated rabbit antisera against human IgG, IgA, IgM, C1q, and C3 (Dako, Denmark), and the direct immunofluorescence of these sections was examined. The biopsy specimens were classified using the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification of LN [14].

2.3. Activity and Chronicity Indices of Renal Tissue Injury. Renal tissue injury was evaluated on the basis of activity and chronicity indices according to methods reported by Austin et al. [15]. Activity index was calculated as the sum of the scores (on a scale of 1–3) of endocapillary proliferation, karyorrhexis, fibrinoid necrosis (the score was multiplied by 2), cellular crescents (the score was multiplied by 2), hyaline deposits, leukocyte exudation, and interstitial inflammation. The score of the chronicity index was the sum of the scores (on a scale of 1–3) for glomerular sclerosis, fibrous crescents, tubular atrophy, and interstitial fibrosis.

2.4. Immunohistochemical Staining of Renal Biopsy Samples. Immunohistochemical staining of serial sections was performed for each LN patient using the following antibodies: CD20 (L26, Dako; Glostrup, Denmark), CD3 (Dako; Glostrup, Denmark), and CD21 (1F8; Dako; Glostrup, Denmark).

Paraffin-embedded tissue sections were placed on positively charged slides and incubated in a stove at 60°C for 1 h. Sections were deparaffinized and rehydrated through a series of washes with xylene and graded alcohols. Endogenous peroxidase was blocked by treatment with 3% H_2O_2 for 30 min. Antigen retrieval was performed by flooding the slides with 10 mM citrate buffer (pH 6.0) and heating in a microwave at 1,000 W for 10 min. Primary mouse monoclonal anti-human CD20, CD3, and CD21 antibodies were applied to the slides at a dilution of 1 : 50 in 1% bovine serum albumin/phosphate buffered saline (BSA/PBS) and subsequently incubated overnight at room temperature. Slides were then incubated with a secondary goat anti-mouse IgG antibody (H+L; Dako) for 30 min. Sections were washed with PBS (pH 7.4) between each step (3 times for 5 min). Finally, sections were counterstained with Mayer's hematoxylin, air-dried, cleared in xylene, and coverslipped.

2.5. Quantification of Immunofluorescence and Immunohistochemical Staining Scores . The intensity of glomerular staining of IgG, IgA, IgM, C3, and C1q was semi-quantitatively assigned a score of 0, 1, or 2.

Results of 192 LN patient renal biopsies were classified according to the organizational stage of inflammatory cell infiltrates as described by Steinmetz et al. [10]. A scattered pattern of intrarenal CD20-positive B cells were graded as 1. Nodular aggregates consisting of CD3-positive T cells and CD20-positive B cells without microanatomical compartmentalization were graded as 2. Distinct T-cell and B-cell zones without a central dendritic cell network of aggregates were graded as 3. Aggregates with the highest level of microanatomical organization, consisting of distinct T- and B-cell compartments with a central network of CD21-positive follicular dendritic cells (fDCs) were graded as 4. Typical examples of these grades of infiltrates are shown in Figure 1. All biopsy specimens were scored by a renal pathologist with no prior knowledge of the clinical and laboratory analysis details of patients.

2.6. Statistical Analysis. All statistical analyses were performed using SPSS version 11.0 software (SPSS Inc., Chicago, USA). Categorical variables were compared using Fisher's exact test or chi-square test. Differences between the median values of defined patient groups were compared using the nonparametric Mann-Whitney U test. A Spearman's rank correlation was used to detect correlations among different study parameters. $P < 0.05$ was considered statistically significant.

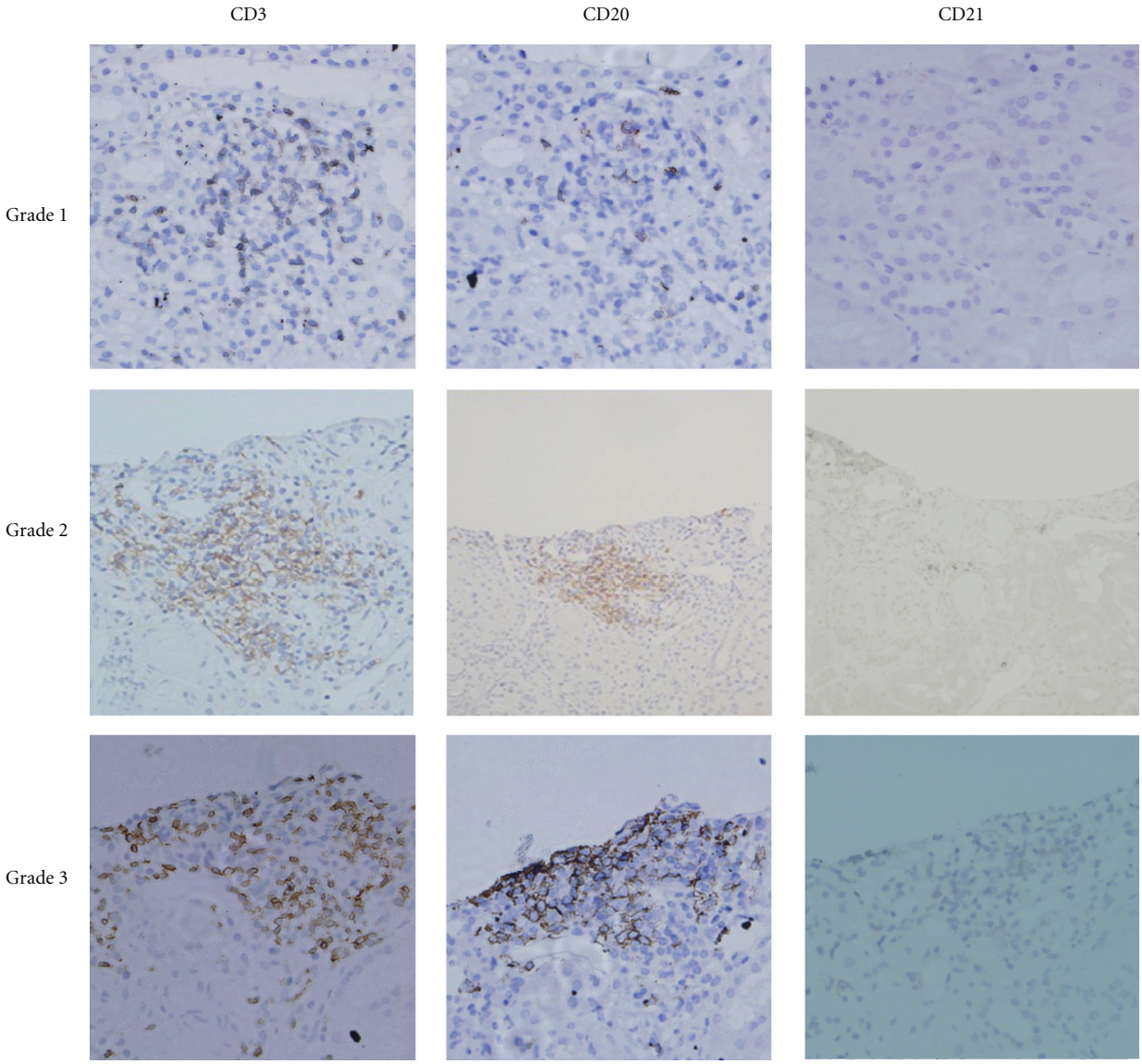

Figure 1: Microanatomical organization of inflammatory infiltrates. Serial staining for CD3, CD20, and CD21 allowed classification into four different grades. Grade 0 aggregates consist of T cell infiltrates alone (no B cells) (not shown). Grade 1 aggregates consist of scattered T- and B-cell infiltrates (original magnification: ×400). Grade 2 aggregates show a cluster-like structure. No T- and B-cell zones are evident. Grade 3 aggregates show clearly distinguishable T- and B-cell areas (original magnification: ×200).

3. Results

3.1. Demographic, Clinical Characteristics, and Laboratory Results of LN Patients. Firstly, in this prospective study, 192 LN patients (167 women and 25 men; mean age ± SD: 33 ± 13 years) were separated into 2 groups: LN with intrarenal B cells (LN-B group) and LN without intrarenal B cells (LN-non-B group). The LN-B group comprised 118 (61.5%) patients (101 women and 17 men, 34 ± 14 years) and the LN-non-B group comprised 74 (38.5%) patients (66 women and 8 men, 33 ± 14 years). No significant difference was detected between the two groups in terms of age or gender ($P > 0.05$). SLEDAI, blood urea nitrogen, and serum creatinine levels were all significantly greater in the LN-B-cell group than in the LN-non-B-cell group (all $P < 0.05$). However, the duration of SLE or LN, the level of C3/C4, and the proteinuria (g/24 h), were not statistically different between the two groups. Furthermore, no association between intrarenal B-cell infiltration and anti-dsDNA antibodies was identified (all $P > 0.05$; Table 1). No association between intrarenal B-cell infiltration and the level of ANA, anti-Sm, or antinucleosome antibodies between the two groups was identified (all $P > 0.05$; data not shown).

3.2. Microanatomical Organization of Inflammatory Infiltrates. All biopsy samples from LN patients were stained for CD20 as a pan-B-cell marker, in addition to CD3 and CD21 as T cell and fDC markers, respectively. Serial sections

Table 1: Demographics, clinical characteristics, and laboratory analysis of LN patients.

	LN-B-cell $n = 118$	LN-non-B-cell $n = 74$	P value
Sex (male/female)	17/101	8/66	0.513
Age (years)	34 ± 13	33 ± 13	0.526
SLE duration (months)	57 ± 60	43 ± 63	0.294
LN duration (months)	25 ± 37	26 ± 49	0.852
SLEDAI	14 ± 6	13 ± 5	0.042
Proteinuria (g/24 h)	3.13 ± 2.97	2.69 ± 2.56	0.311
Blood urea nitrogen (mmol/L)	10.43 ± 7.67	6.24 ± 3.40	0.000
Serum creatinine (μmol/L)	89.91 ± 66.89	59.75 ± 22.05	0.000
Serum C3 (g/L)	0.51 ± 0.21	0.54 ± 0.26	0.427
Serum C4 (g/L)	0.082 ± 0.049	0.084 ± 0.076	0.827
Anti-dsDNA (positive/negative)†	72/11	44/9	0.506

†Anti-dsDNA antibodies were not detected in 35 patients in the LN-B-cell group and 21 patients in LN-non-B-cell group.
SLEDAI: systemic lupus erythematosus disease activity index; SLE: systemic lupus erythematosus; LN: lupus nephritis; anti-dsDNA: anti-double-stranded DNA antibody.

were prepared and examined for each patient as previously described [10]. Infiltrates consisting of T cells alone (no B cells, the same to non-B-cell group) were graded as 0. Scattered B- and T-cells were graded as 1. Nodular aggregates without distinct T- and B-cell zones were graded as 2. Leukocyte clusters in which distinct T- and B-cell regions had formed were graded as 3. The highest organizational level clearly showed separate B- and T-cell compartments with a central fDC network that is a classical feature of germinal centers of lymph follicles in secondary lymphoid tissue. These aggregates were graded as 4. B cells were mainly distributed in renal tubules, renal interstitial vessels and interstitial distribution, with very few in the glomeruli (Figure 1). No grade 4 infiltration was observed in biopsies from lupus nephritis patients in this study.

3.3. Inflammatory Infiltrates and Renal Histology. The distribution of the ISN/RPS classification of the 192 patients was as follows: seven were class I, five were class II, 13 were class III, 71 were class IV, 43 were class V, 28 were class (III + V), and 24 were class (IV + V). The distribution of the LN-B-cell group was as follows:, one of the biopsies was class I, one was class II, five were class III, 56 were class IV, 20 were class V, 15 were class III + V, and 19 were class IV + V. When compared with LN-non-B-cell group, the LN-B-cell group was more likely to be associated with class IV LN ($P < 0.05$). The activity and chronicity indices were also significantly higher in the LN-B-cell group than in the LN-non-B-cell group (all $P < 0.001$), with median (25–75th percentile) activity index values of 5 (3–7) and 2 (1–4.5), respectively, and median (25–75th percentile) chronicity index values of 3 (1–4) and 1 (0–2.5), respectively (Table 2).

Table 2: Comparison of histologic parameters of LN-B-cell and LN-non-B-cell groups.

	LN-B-cell $n = 118$	LN-non-B-cell $n = 74$	P value
ISN/RPS classification			<0.001*
I	1 (0.8)	6 (8.1)	
II	1 (0.8)	4 (5.4)	
III	5 (4.2)	8 (10.8)	
IV	56 (47.5)	15 (20.3)	
V	20 (16.9)	23 (31.1)	
III + V	15 (12.7)	13 (17.6)	
IV + V	19 (16.1)	5 (6.7)	
Activity index	5 (3–7)	2 (1–4.5)	0.000
Chronicity index	3 (1–4)	1 (0–2.5)	0.000

*P value for the difference in the ISN/RPS classification distribution between the two groups.

3.4. Clinical Features of Different Intrarenal Cell Grades. Examination of renal biopsy samples from 192 patients with LN revealed. There were 74 (38.5%) patients in grade 0, 86 (44.8%) patients in grade 1, 17 (8.9%) patients in grade 2, and 15 (7.8%) patients in grade 3. No patients were identified in grade 4. The clinical parameters for blood urea nitrogen levels for patients in grade 0 was lower than those in other grades (all $P < 0.05$). There were no statistical differences between grades 1, 2 and 3 (all $P > 0.05$) (Figure 2(a)). An identical pattern was observed in serum creatinine levels (Figure 2(b)). In the LN-B-cell grade 1 group, the SLEDAI level was higher than the grade 0 group ($P < 0.05$) although there were no statistical differences between the other groups (all $P > 0.05$) (Figure 2(c)). No statistical differences were identified between any groups in the 24-hour urinary protein levels (all $P > 0.05$) (Figure 2(d)) or in other clinical parameters, such as the levels of C3/C4 and anti-dsDNA (data not shown).

In the four different groups of LN patients, the activity and chronicity indices of grade 0 were significantly lower than the other grades (all $P < 0.05$) although there were no significant differences between grades 1, 2, and 3 (all $P > 0.05$) (Figure 3). No significant differences in immune complex deposition were identified in the four different groups of LN patients (all $P > 0.05$, data not shown).

4. Discussion

Lupus nephritis is one of the most frequent and serious complications in SLE patients. Although the pathogenesis of lupus nephritis is not clear, production of pathogenic antibodies is traditionally viewed as the principle contribution of B cells to the pathogenesis of immune-mediated glomerulonephritis [9, 16]. However, it is increasingly apparent that B cells have a much broader role in such diseases, functioning as antigen-presenting cells, regulators of T cells, macrophages, and dendritic cells and involved in the formation of local lymphocytic expansion [17].

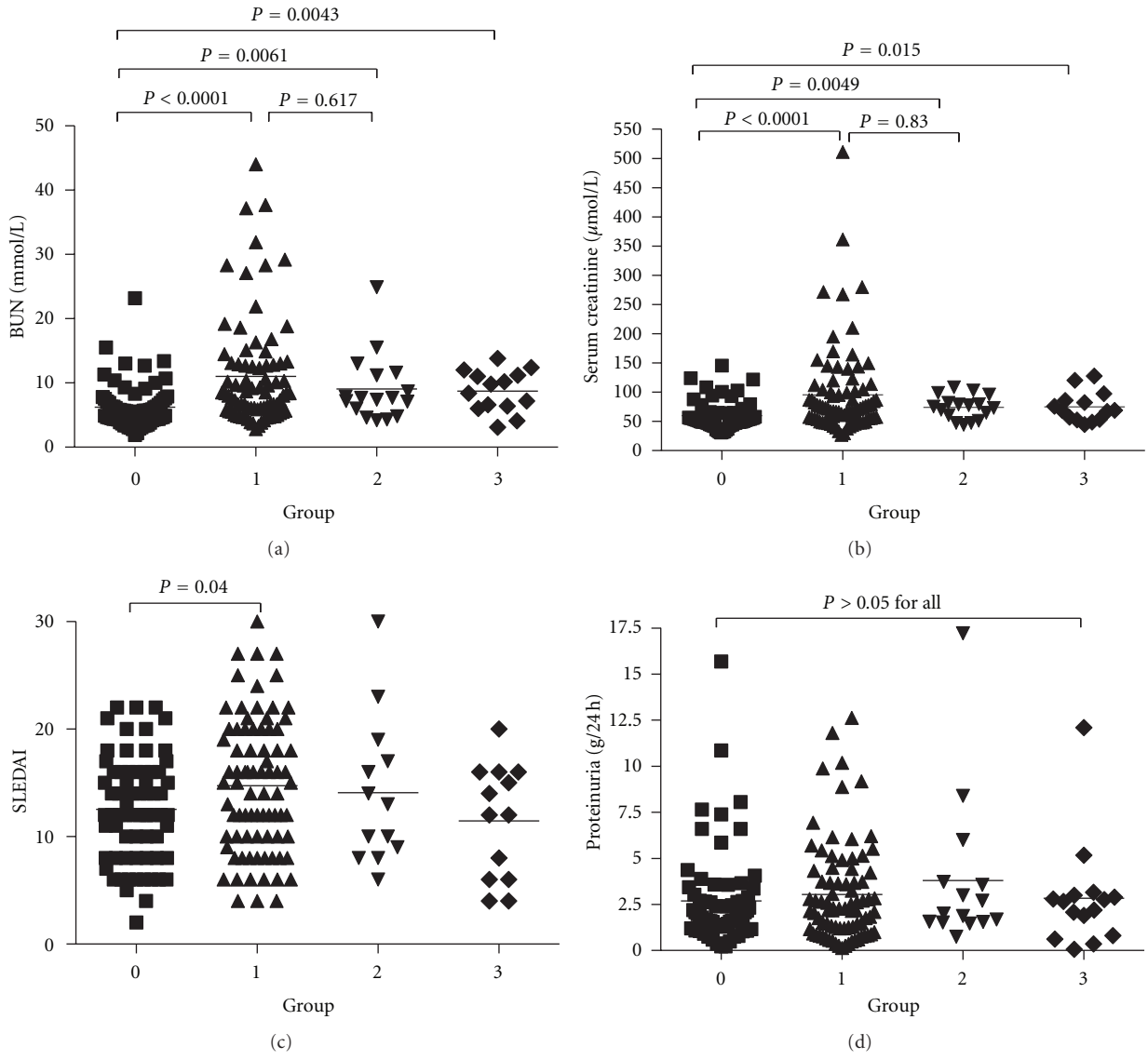

Figure 2: Serum blood urea nitrogen and creatinine levels, SLEDAI, and 24-hour urinary protein level in four different groups of LN patients.

The human B lymphocyte-specific marker, CD20, is a cell surface molecule which is widely expressed in B-cell differentiation subsets, including mature B cells and all subgroups of pre-B cells [18]. Steinmetz et al. observed that most locally infiltrating B cells in nephritis displayed a mature non-antibody-producing phenotype with antigen presenting capacity [10]. However, the report lacked a detailed description of the biopsy materials investigated. In this study, infiltrates were characterized by immunostaining using the B-cell marker CD20, the T-cell marker CD3, and CD21 as a marker of fDCs. Renal biopsies from 192 LN patients were analyzed and classified into four different groups according to their microanatomical structures. It was observed that B cells are predominantly detected in the tubulointerstitial compartment, with very few in the glomerular tuft. In previous studies of the characterization of the leukocyte subsets in human glomerulonephritis, such as IgA nephropathy and renal allograft rejection [19], B cells were rarely seen within glomeruli which is consistent with the results of this study. Although the number of B cells was found to be relatively low compared with T cells, there was a significant correlation between B cells and the degree of renal function. In this study, it was observed that SLEDAI, blood urea nitrogen, and serum creatinine levels were all significantly greater in the LN-B-cell group compared with the LN-non-B-cell group. Furthermore, intrarenal B cells were more likely to be associated with class IV LN and the activity and chronicity indices were also significantly higher in the LN-B-cell group compared with the LN-non-B-cell group. Therefore, it can be speculated that the formation of interstitial B-cell aggregates is a common response in LN and plays a pivotal role in renal injury.

B cells are recruited to most chronically inflamed tissues and areas resembling secondary lymphoid tissue have been

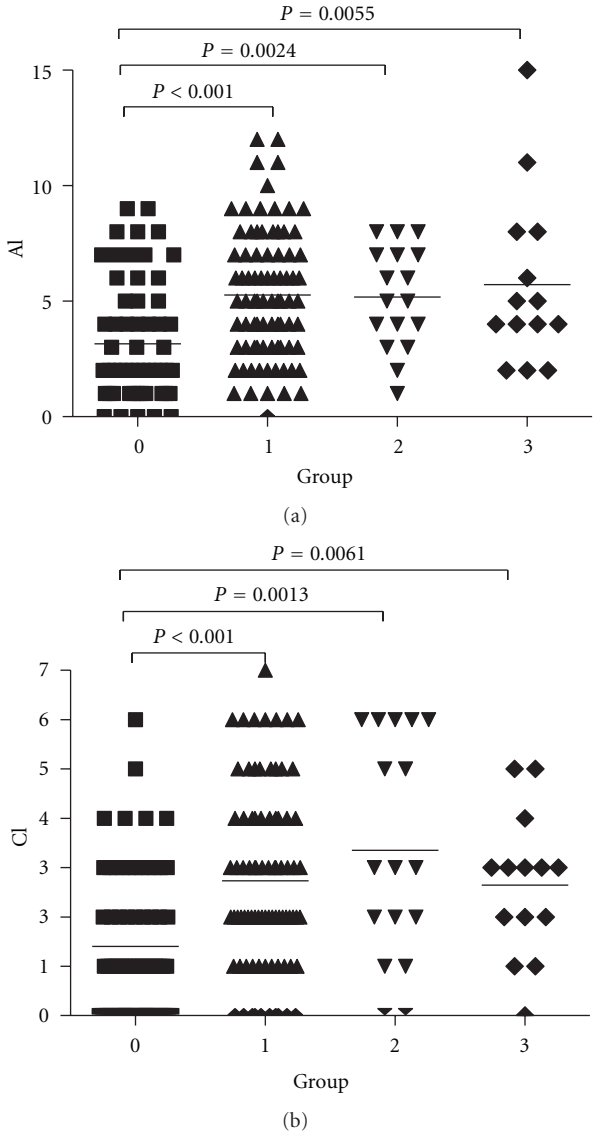

Figure 3: Lupus nephritis activity index (AI) and chronicity index (CI) in the four different B-cell groups of LN patients.

found in several forms of inflammatory kidney disease, including renal transplant rejection and glomerulonephritis [20]. However, the pathophysiological significance of these B cells remains to be clearly defined. Recent studies on lupus nephritis patients revealed that intrarenal B cells form local lymphoid tissue, described as tertiary lymphoid neogenesis, displaying a mature non-antibody-producing phenotype with antigen presenting capacity [10]. It is hypothesized that local proliferation in these germinal centers leads to the expansion and perpetuation of inflammatory cell aggregates resulting in persistence and chronicity of the renal inflammation. It has recently been reported that some lupus renal biopsies contain apparently functional germinal center- (GC-) like structures associated with *in situ* B-cell clonal expansion and somatic hypermutation [21]. However, in this study, intrarenal ectopic lymphoid tissue was not identified in any LN patients. These anomalies may result from differences in study design and ethnicity of the patient population. In accordance with previous studies, detailed classification of intrarenal lymphoid clusters was applied. However, no correlation was identified between clinical parameters and the presence of the three different classes of intrarenal B lymphoid aggregates.

In renal allografts, the accumulation of CD20-positive B cells in the tubulointerstitium has been associated with steroid-resistant acute allograft rejection and with graft loss [18]. Rituximab is a CD20-specific chimeric antibody that depletes B cells, thereby inhibiting differentiation into antibody-producing plasma cells [22]. Depletion of local B cells inside the kidney could also prevent formation of intrarenal ectopic lymphoid structures. Improvement in disease activity in response to rituximab has been shown in some patients, although responses in many patients are only partial or completely fail [23–25]. These differences may reflect heterogeneity in LN pathogenesis and also emphasize the need for further investigation of the role of intrarenal B cells in LN.

In order to further elucidate the mechanisms responsible for intrarenal B-cell formation, studies evaluating the role of chemokines such as BCA-1/CXCL13 and its specific receptor CXCR5, in the induction and maintenance of the spatial organization of B-cell infiltrates in LN biopsies have been performed [21, 26]. Local expression patterns of B-cell survival factors such as APRIL and BLyS are important factors for intrarenal B cells in lupus nephritis patients [26]. Studies indicate that antagonists of these chemokines, such as anti-BAFF/BCA-1 antibodies or corresponding receptors, may have therapeutic efficacy in patients with autoimmune disorders of this type [27]. It is hoped that an understanding of the expression patterns of renal B-cell and effectors would be beneficial to physicians in the choice of appropriate targeting reagents for different patients populations. It is speculated that intrarenal B cells form part of a local system that enhances the immunological response by functioning as antigen presenting cells, and as a source of cytokines promoting T cell proliferation and lymphatic neoangiogenesis. In this way, intrarenal B cells could enhance the local immune response to persisting autoantigens in the tubulointerstitium, resulting in persistence and chronicity of renal inflammation.

5. Conclusions

This study revealed a correlation between clinical parameters and the presence of the four different intrarenal lymphoid aggregates in a large number of LN patients. It is hypothesized that intrarenal B cells enhance immunological responses and exaggerate the local immune response to persisting autoimmune damage in the tubulointerstitium. Investigation of follow-up biopsies from these patients is required to clarify this association and the function of intrarenal B cells in LN.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Y. Shen and C.-Y. Sun performed most of the experiments and prepared the paper. F.-X. Wu and Y. Chen worked on the clinical data presentation and participated in the statistical analysis. M. Dai and Y.-C. Yan worked on the renal biopsy and biopsy specimens scores. C.-D. Yang was responsible for the main experimental design, data interpretation, and finalizing the paper. All authors read and approved the final paper.

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Review Article

Role of MHC-Linked Susceptibility Genes in the Pathogenesis of Human and Murine Lupus

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Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by the production of autoantibodies against nuclear antigens and a systemic inflammation that can damage a broad spectrum of organs. SLE patients suffer from a wide variety of symptoms, which can affect virtually almost any tissue. As lupus is difficult to diagnose, the worldwide prevalence of SLE can only be roughly estimated to range from 10 and 200 cases per 100,000 individuals with dramatic differences depending on gender, ethnicity, and location. Although the treatment of this disease has been significantly ameliorated by new therapies, improved conventional drug therapy options, and a trained expert eye, the underlying pathogenesis of lupus still remain widely unknown. The complex etiology reflects the complex genetic background of the disease, which is also not well understood yet. However, in the past few years advances in lupus genetics have been made, notably with the publication of genome-wide association studies (GWAS) in humans and the identification of susceptibility genes and loci in mice. This paper reviews the role of MHC-linked susceptibility genes in the pathogenesis of systemic lupus erythematosus.

1. Introduction

Chronic autoimmune diseases have complex pathogeneses and the course of events leading to these diseases is not well understood. They arise from a dysfunction of the immune system, recognizing self-antigens as foreign, which can lead to inflammation and severe damage of tissues and organs. One of these complex inflammatory diseases is called systemic lupus erythematosus (SLE). The etiology of lupus is multifactorial with environmental, hormonal, ethnic, and genetic factors [1].

In the 70s and 80s of the last century mouse models of spontaneous lupus, like (NZB × NZW) F1 hybrids, BXSB mice (which carry the disease-accelerating *Yaa* gene on the Y chromosome [2–4]), MRL/*lpr* mice (MRL mice homozygous for a *fas* mutation [5, 6]) or MRL/*gld* mice (MRL mice homozygous for a *fasL* mutation [7, 8]) were established [9–12]. Research based upon these mice revealed that a number of genes, loci, and pathways are directly associated with lupus in both mouse and human species (reviewed in [13–17]). In addition, by means of these models signaling pathways were identified that are dysregulated in both human and murine lupus. Hence, mouse models will continue to serve as invaluable instruments for studying the genetic basis of lupus susceptibility, because they depict the genetic facets of the human systemic lupus erythematosus (SLE).

Recent findings suggest that aberrant epigenetic mechanisms may be involved in the pathogenesis of lupus [18], and a number of genes have been claimed to be targets of these alterations [19]. However, the mechanisms underlying epigenetic changes are poorly understood. Deciphering the contribution of epigenetic alterations to the pathogenesis of lupus will provide promising insights in this complex autoimmune disease and epigenetic pharmaceuticals will offer new therapeutic options to treat SLE.

One of the genetic risk factors for the development of lupus (or other immune-mediated diseases) are genes linked to the major histocompatibility complex (MHC) [20]. In humans, HLA antigens have long been associated with SLE and, therefore, these susceptibility genes are extensively studied [21]. Certain HLA class II genes or haplotypes

seem to be particularly involved on lupus pathogenesis [14, 22–24]. HLA class III genes, such as those encoding the complement components C2 and C4, may also be considered as risk factors for the development of a lupus-like disease in different ethnicities [25]. In mice, it could also be shown that the MHC class II locus directly participates in lupus disease susceptibility similar to that observed in humans [26]. The effect of MHC-linked complement factors on disease expression is strongly dependent on the background genes, reflecting the genetic unification of inbred mice in comparison to wildtype mice.

However, the role of certain MHC haplotypes, genes, or alleles in lupus pathogenesis is still controversially discussed. For this reason and to update the most recent scientific research on this topic, this paper reviews the role of MHC genes and alleles in the pathogenesis of both human and murine lupus.

2. The Major Histocompatibility Complex (MHC)

2.1. Historical Overview. More than a century ago, it was observed that tissue transplants (now called allografts) of one animal were rejected when transferred to a different laboratory mouse. At the Jackson Laboratory Gorer showed in 1937 that so-called H or "histocompatibility antigens" on the surface of mouse cells account for this [27, 28]. Seven years later, it was Medawar who showed that allograft rejection is a host versus graft reaction [29, 30]. At the same time, Snell developed congenic mice strains that were genetically identical except at the H-2 locus. With the aid of these mice he could show that the H-2 antigens were "controlled" by genes at the H-2 complex on chromosome 17 and called this multigene locus "major histocompatibility complex" (MHC) [31–33]. In 1958, the first human alloantigen present on leucocytes was detected by Dausset, which was later called HLA-A2 [34, 35]. A few years later Payne and coworkers depicted the first human multiallelic system, now known as the HLA class I loci HLA-A and HLA-B [36]. However, it was clear from the beginning that allograft rejection or acceptance is not the physiological function of MHC molecules. In the early sixties, experiments of Benacerraf et al. with guinea pigs and synthetic amino acid polymers showed that there is a single genetic locus which controls the immune system's ability to respond to foreign antigens and called the (autosomal dominant) genes of this locus "immune response genes" (or Ir genes) [37–39]. In the late 1960's, McDevitt found that the Ir genes were linked to the MHC [40, 41]. The concept of immune response genes was refined by Zinkernagel and Doherty (in 1974), who made the breakthrough discovery that the ability of virus-specific T lymphocytes to combat a virus infection is dependent upon the simultaneous recognition of both "foreign" molecules of the virus and self molecules (i.e., major histocompatibility proteins) [42]. This limitation or narrowing of antigen recognition by T cells was called "MHC-restricted antigen recognition" or in short, "MHC restriction" and was subsequently confirmed in many other systems. One year before Zinkernagel and Doherty made their pioneering discovery, the first disease-associated MHC allele, namely, HLA-B27, was reported. HLA-B27 is strongly associated with ankylosing spondylitis [43, 44].

2.2. Genetics of HLA and H-2. The major histocompatibility complex is located on the short arm of chromosome 6 in humans and on the telocentric chromosome 17 in mice [45, 46]. The genes coding for the classical transplantation antigens as well as the so-called "class III" polypeptides are located within this multigene region [47–49]. About 40% of the expressed MHC genes encode proteins related to immune defense [48]. Whereas the classical class I and class II transplantation antigens are expressed on cells and tissues (with the exception of proteins involved in antigen processing and presentation of antigens to the immune system, such as LMPs, TAPs, and Tapasin), the class III antigens are secreted proteins which do not play a role in tissue acceptance or graft rejection. Class III antigens comprise proteins with immune functions such as components of the complement cascade (C2, C4, and factor B), cytokines (TNF-α, LTA, LTB), steroid metabolism (Cyp21B), heat shock proteins (hsp70), and many other genes not directly associated with immune responses [50]. For historical reasons, human MHC polypeptides are called "human leukocyte antigens" (HLA) and mouse MHC proteins "histocompatibility 2" (H-2) antigens.

In humans, the MHC is the most gene-dense region of the genome, and the MHC genes themselves are the most polymorphic genes known so far. Among the ~3 billion base pairs of the human or murine genome, arranged on 23 and 20 chromosomes, respectively, there are 20,000–30,000 protein-coding genes [51–53]. That means that an average of one gene was found for every 100,000 to 150,000 base pairs. The human MHC, however, contains more than 120 functional genes and additional nonfunctional pseudogenes in both mice and humans distributed over 3.6 Mbp [54–57]. The outcome of this is an average of approximately one gene for every 30,000 base pairs.

MHC molecules are codominant expressed and clustered in so-called "haplotypes". The term was introduced by Ceppellini et al. (in 1967), who used familial genotype data, to explain the coinheritance of alleles at two closely linked loci [58]. This organization is thought to facilitate recombination events that generate new alleles and therefore, contribute to the high polymorphism of MHC proteins. Polymorphism derives from the creek word "$\pi o \lambda \upsilon \mu o \rho \phi \acute{\iota} \alpha$" (polymorphia) and means "many or complex shapes". The polymorphism found in the MHC class II genes is generally limited to exon 2, which encodes the peptide-binding groove [59]. Due to the high frequency of MHC alleles, most individuals will be heterozygous for each different MHC gene locus. Each MHC molecule in the population has a different spectrum of peptide binding. This insures that no one pathogen can destroy the whole population by developing protein sequences that are incapable of binding to an MHC molecule, and thus evading the immune system (Figure 3).

In contrast to humans the number of MHC (H-2) alleles is strongly reduced in inbred mice because of the homozygosity at their MHC loci. As many peptides are not recognized by the remaining alleles/haplotypes, these mice often

have an impaired immune response against pathogens. In fact, the MHC genes of mice were first called "immune response (Ir) genes because of strain-dependent defects in responses to certain antigens [38].

2.3. Evolution of MHC Diversity. In the sixties and seventies, two different models have been developed to explain the high heterogeneity of the MHC genes: Negative frequency dependence (rare allele advantage) and heterozygote advantage (overdominance model) [60–62]. The negative frequency dependence postulates that rare MHC alleles (of recent origin) may have a selective advantage, as no pathogen may be adapted to it [63]. The overdominance model states that polymorphism will be advantageous because heterozygous individuals are able to recognize a wider range of pathogens and parasites [60]. A main difference between these two types of (balancing) selection is that overdominance is based upon a stable polymorphism, whereas a polymorphism maintained by frequency dependence will be dynamic [64]. However, there is still a controversy, if the heterozygote advantage on its own is sufficient to explain the high degree of MHC polymorphism [65]. For instance, it has recently been shown that balancing selection can also result from MHC-dependent choice of mates [66].

Evolution of MHC genes and alleles is driven by the need to maximize peptide binding diversity in order to recognize a maximum of potential pathogens. Polymorphism and polygeny are two (independent) genetic mechanisms for increasing variety of MHC class I and class II proteins. Polygeny acts on the individual level, whereas polymorphism is (primarily) a population-relevant criterion. Thus, a maximum number of class I and II genes would ensure the greatest conceivable protection of a single individual against pathogens. However, polygeny is limited by a mechanism called "MHC restriction": T cells recognize fragmented antigens (self and foreign) only in conjunction with MHC proteins [42, 67]. To avoid autoimmune reactions, T cells that strongly react with MHC molecules presenting self-peptides are deleted. In consequence of these opposed requirements, the immune surveillance is a delicate balance between self and foreign as well as between (self-)tolerance and immune response. Furthermore, these two opposing demands create a dilemma: On the one hand, many MHC genes would present a maximum of different peptides but on the other hand, the presentation of many different self-antigens would strongly reduce the T cell diversity. Thus, MHC restriction limits T cell antigen recognition and response. As a consequence of this, the diversity of MHC class I and II proteins of a single individual is limited (and optimized) to six different molecules (3 genes $\times$ 2 alleles). The optimal number is called "immunogenetic optimum" [68]. Due to the limited number of MHC genes, some agents may evolve polypeptides that evade the immune system of single individuals, but the enormous polymorphism within a population diminishes the possibility that a pathogen can exterminate a whole species (individual C). However, there is a major drawback of this kind of defense strategy: if the size of a population decreases strongly, some MHC haplotypes will disappear, leading to a reduction of MHC diversity, which in turn will negatively affect survival of the population [69]. In summary, the number of different MHC genes is a delicate balance between the key requirement of an entire population/species and the core requests of its individuals.

3. How Is Lupus Erythematosus Influenced by the MHC?

Variations within the MHC locus seem to be associated with a great variety of autoimmune diseases. Consequently, the contribution of HLA genes to lupus pathology has recently been extensively studied [21, 70–72]. However, due to the extensive linkage disequilibrium among alleles throughout this locus, the causal relationship between these MHC variations and autoimmune pathogenesis have remained elusive for the great majority of these diseases, including lupus [73].

Although the pathogenesis of the disease is still poorly understood and a number of environmental factors have been postulated, genetic predisposition is clearly a major risk parameter for SLE [74, 75]. There is strong evidence for a genetic component based upon a high concordance rate of SLE in monozygotic twins as well as the occurrence of SLE in 5–12% of the relatives of affected patients [76–79]. The complex nature of SLE reflects a polygenic inheritance of the disease rather than a monogenic mode. Several genes are known to contribute to SLE susceptibility [80, 81], because they affect key pathways, implicating immune complexes, host immune signal transduction, and interferon pathways (reviewed in [82]). Only in a small proportion of patients (<5%), a single gene seems to be responsible for the disease onset. Many of these genes relate to the early complement components from which the C2 and C4 genes are linked to the MHC (Figure 1 and [83–85]).

The mechanisms underlying antigen recognition are of great importance to human autoimmune diseases. A number of genes have been claimed to be associated with susceptibility to anti-self responses. Because of their considerable heterogeneity, the immunoglobulin genes, the T cell receptor genes, and the major histocompatibility complex (MHC) genes have soon been suspected of playing a distinct role in the pathology of lupus and other autoimmune diseases. Particularly, the MHC class II allotypes HLA-DR2 and -DR3 seem to be related to (and/or positively correlated with) lupus disease [86–88]. Genes, like angiotensin-converting enzyme (ACE) or angiotensinogen (AGT), that specifically increase kidney susceptibility to lupus pathogenesis have also been described [89].

Advances in high throughput technology have enabled the genotyping of hundreds of thousands of single nucleotide polymorphisms (SNPs) in a single individual and genome-wide association studies (GWAS) in lupus patients [90]. GWAS in European- or East Asian-ancestry populations [91–94] and high-density screenings [20, 95] have identified several independent SNPs in the MHC region associated with SLE. Some of these SNPs could be confirmed in a recent targeted association study [96]. GWAS may also been used to decipher complex ethnic disparities in SLE prevalence rates. For unknown reasons, the prevalence of lupus in African and Hispanic Americans is two to fivefold higher compared to

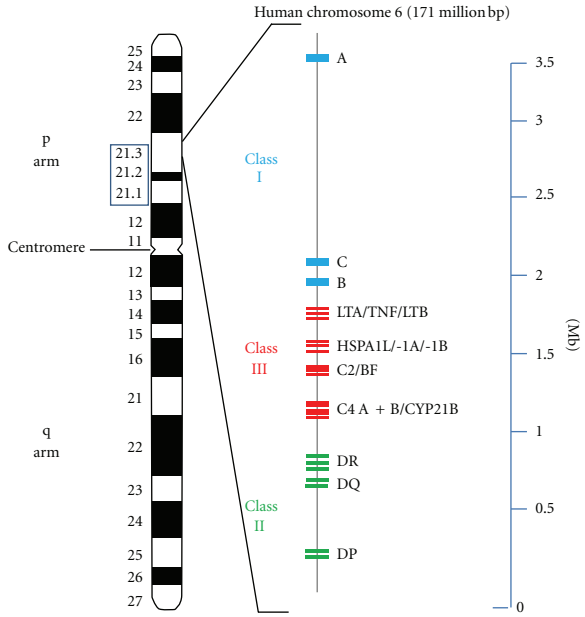

Figure 1: HLA gene cluster and lupus susceptibility genes on human chromosome 6. Ideogram of chromosome 6 (left) and schematic diagram of the MHC-complex-associated genes ranging from 6p21.1 to 6p21.3 (middle). The class I gene complex contains three major loci (A, C, and B), as well as additional (unmentioned) loci. The resulting class I polypeptides associate with the invariable beta-2 microglobulin, encoded by a gene on chromosome 15. The HLA-B locus is known as the most polymorphic gene within the human genome. Class II MHC molecules are composed of two glycosylated polypeptide subunits (called α and β chain) of approximately equal length. Whereas HLA-DP and -DQ code for one alpha- and one beta-chain polypeptide, respectively, the genetics of HLA-DR is more complex: It consists of one locus coding for the alpha subunit and 4 loci coding for beta subunits. Unlike the other DR loci, DRA is not polymorphic. Even though the DR β-chain is encoded by 4 loci, no more than two are present on a single chromosome. DRB1 is the most polymorphic gene of the class II locus. Class I and class II antigens are membrane proteins whereas almost all class III polypeptides are serum proteins (including the complement components C2, C4A, C4B, and factor B) or can be detected in other body fluids. Therefore, the term "class III" is misleading, as this locus does not contain a distinct class of genes. The coding regions of the genes are shown as small blue (class I), green (class II), and red (class II) rectangles, respectively. Abbreviations: LTA: lymphotoxin A, LTB: lymphotoxin B, TNF: tumor necrosis factor alpha, HSPA1L: heat shock 70 kDa protein 1-like, HSPA1A: heat shock 70 kDa protein 1A, HSPA1B: heat shock 70 kDa protein 1B, BF: complement factor B, CYP21B: cytochrome P450 21-hydroxylase and Mb: mega base pairs.

Americans of European ancestry [97]. A recent SNP screening of the MHC region revealed for independent SNP signals for African American women [98]. The strongest signal of this study (the SNP rs9271366), was also associated with SLE in a previous Chinese GWA study of Han and coworkers [91]. It has also been shown by GWAS that several established non-MHC lupus loci are not related to other autoimmune diseases, which suggests a limited genetic overlap between these diseases and SLE [99]. In summary, it can be stated that genome-wide and -targeted association studies, despite of their methodological and application-related limitations, are useful tools to localize lupus-associated genes.

In the past few years, progress has been made in identifying lupus susceptibility genes in mice [100, 101]. Meanwhile, a large number of lupus susceptibility loci have been detected in mouse models, and some of the corresponding susceptibility genes have been identified by now (reviewed in [10, 102–106]) including those linked to MHC [14, 107, 108]. An important milestone in murine lupus genetics was the identification of the SLE loci 1–3 by Mohan et al. and Morel et al. in NZM2410 mice [109–111], a lupus-prone strain derived from a cross between NZB and NZW mice [112]. The identification of these loci provided the starting basis for a rapidly growing number of publications that dissected the role of single loci or genes in lupus development [113–119]. Several B6-based lupus congenic strains has been characterized, that carry the NZM2410-derived SLE-susceptibility loci *Sle1*, *Sle2*, and *Sle3* (reviewed in [17]). It has been shown that these three loci act in an additive way and that the coexpression of them is necessary to develop the full severity of the disease [107, 120]. Subsequently, it has been demonstrated by congenic dissection and polygenic analyses that both protective suppressor and harmful susceptibility loci form the genetic basis for murine lupus and that they act in a highly complex manner that involves several genes [121, 122]. Meanwhile, for a subset of these murine genes, involvement in human SLE has been established [17].

Based upon these models, there is considerable evidence that single MHC genes contribute to the development of systemic lupus erythematosus [26, 123–125]. However, in both mice and humans, lupus susceptibility results from accumulating effects of a large number of individual gene variants [126] of which the MHC-linked loci are reviewed below.

3.1. MHC Class I Genes. The association between MHC loci and susceptibility to lupus has been known since 1971, when HLA-B8 was shown to be associated with this disease [21]. In particular, the ancestral haplotype A1-B8-DR3 has been linked to lupus susceptibility [127–130]. Nevertheless, early studies have focused upon MHC class II genes in lupus pathogenesis, since class II-restricted $CD4^+$ T cells have been associated with the generation of autoantibodies [131]. Although the dysregulation of class I levels is predicted to result in autoimmunity [132], the relevance of MHC class I proteins to lupus, however, is less clear. Recent studies have implicated a distinct role for MHC (H-2) class I molecules in mouse lupus pathogenesis: McPhee et al. could demonstrate that β2-microglobulin-deficient (β2m) BXSB-Yaa and -SJL mice (i.e., mice deficient in class I antigen presentation) developed much more aggressive and lethal forms of a lupus-like disease that characterizes these strains [133]. These results are in line with previous findings in the (NZB × NZW) F1 mouse model of lupus disease [125]. A more sophisticated role for class I proteins could be demonstrated for β2m-deficient MRL/*lpr* mice: While inhibiting nephritis, β2m deficiency accelerates spontaneous lupus skin disease [134]. In another report, Mozes et al. could show that MHC class I-deficient mice are resistant to experimental SLE,

although these mice were not generally poor responders to antigen [135]. Furthermore, MHC class I-deficient MRL/*lpr* mice demonstrate a substantial reduction in CD4/CD8 double-negative (DN) T cells and symptoms of the lupus-like disease [136]. In summary, these results indicate that class I-dependent T cells are key players for the murine lupus-like syndrome.

3.2. MHC Class II Genes. SLE is associated with class II genes of the MHC, but it is not yet clear which haplotypes, genes, or alleles are primarily responsible for disease association. Initial reports looking at the involvement of HLA in SLE assumed a direct involvement of haplotypes containing DR2 and/or DR3 to disease pathogenesis [22, 137–140], but later reports indicated for both humans and mouse models that HLA DR molecules may have an increased association with the production and specificity of autoantibodies rather than with the disease itself [75, 141–143]. Meanwhile, an immense number of studies based on different ethnicities have identified HLA class II associations with SLE.

The presence of antinuclear antibodies (ANA) is a serological hallmark of lupus erythematosus (found in the serum of most patients) [144], and the role of HLA genes in autoantibody expression has been intensely researched, because it indicates the activation of autoaggressive B cells and the breakdown of tolerance to self-antigens. A subspecies of antinuclear autoantibodies, called Ro/SSA (a ribonuclear protein) is present in 25–50% of SLE cases [145, 146] and the level and occurrence of theses autoantibodies correlate with the presence of HLA-DR2/DR3 and HLA-DQw1/DQw2 heterozygotes [147]. In mouse models, heterozygosity at the MHC (H-2) locus has also been associated with lupus susceptibility and enhanced autoantibody production [148, 149]. For (NZB × NZW) F1 hybrid mice, it has been hypothesized that H-2A or H-2E MHC class II genes are two likely candidates [81]. DQA1*0102 and DQA1*0301 alleles were observed to be strongly associated with the presence anti-Ro/La and anti-dsDNA antibodies in Chinese but not in a Malaysian control group [150]. However, a German lupus study showed that all HLA-DR and -DQ (homozygous and heterozygous) combinations appear with frequencies expected from the observed gene frequencies, suggesting that gene complementation at MHC class II loci seems not to contribute to lupus susceptibility [151].

Other autoantibodies are detected in patients with SLE but the HLA associations with these are less clear. Antiphospholipid antibodies are frequently observed in patients with SLE [152–154] and a significant association of DR7-positive patients (in linkage disequilibrium with the HLA-DR gene B4) that carry anticardiolipin antibodies could be observed by Savi et al. [155]. Azizah et al. found a significant association of the DQB1*0601 allele with anti-Sm/RNP, DR2 with anti-Ro/La, and DR2, DRB1*0501, and DRB1*0601 with anti-dsDNA antibody expression [156].

It has been shown that the HLA haplotype DR3-DQ2-C4AQ0 is strongly associated with SLE in Caucasians [157, 158]. A strong association with lupus was also determined by DNA typing for DQA1* 0501 in Scandinavian patients [159]. However, this allele was in linkage disequilibrium with DR3 and DR5. A strong association to SLE is found with DRB1*03 and DOB1*0201 alleles of central European patients [160]. A genetic predisposition of HLA DR2- and/or HLA DR3-containing haplotypes for SLE has also been described for German, Kuwaiti, and Chinese lupus patients [161–163].

Strong associations of class II genes with lupus susceptibility have also been shown by GWA studies. Studies based on sequence length polymorphisms in European populations identified a potential association of the class II HLA-DRB1 alleles HLA-DRB1*08:01, -*03:01, and -*15:01 with SLE [73, 164]. Two of these alleles (HLA-DRB1*03:01 and -*15:01) have also been identified in a recent study of the IMAGEN consortium using high-density SNP typing across the MHC [20]. In a study of Ruiz-Narvaez et al. the strongest SLE-associated SNP was the rs9271366 near the HLA-DRB1 gene [98]. This SNP was also associated with higher risk of SLE in a previous GWAS [91]. Although there are hardly any GWAS results concerning class III genes, the SNP rs419788 in intron 6 of the class III gene SKIV2L was found to be independently associated with SLE [165]. However, in a recent report this SNP was not found to be independent from the rs3135391 (HLA-DRB1*15:01) signal [96].

In summary, these results indicate that both DR2 and DR3 and their associated DQ alleles seem to play a role in SLE [146, 166]. However, most of the results concerning the contribution of individual MHC class II polymorphisms to SLE have been obtained from population-based case-control studies and need to be confirmed in family-based studies [146].

MRL/*lpr* mice spontaneously develop aggressive autoimmune kidney disease characterized by an immune complex glomerulonephritis, which is associated with increased (or de novo) renal expression of major histocompatibility complex (MHC) class II molecules and a massive systemic expansion of CD4-CD-double negative (DN) T cells [167–169]. However, these mice are homozygous for the H-2^k haplotype, which is shared by several other strains, that do not develop lupus-like symptoms. In addition, it has been shown that genes encoded within or closely linked to the MHC region regulate autoantigen selection and isotype switching to IgG3 but have minimal effect on end-organ damage or survival in MRL/*lpr* mice [170]. On the other hand, MHC (H-2) class II expression appears to be required for the development of autoaggressive $CD4^+$ T cells involved in autoimmune nephritis, because MHC class II-deficient MRL/*lpr* mice do neither produce serum anti-DNA antibodies nor develop proliferative renal disease in contrast to their wild-type counterparts [168].

In contrast to New Zealand black (NZB) and New Zealand white (NZW) mice, F1 hybrids of these strains (with a H-$2^{d/z}$ haplotype) spontaneously develop a severe lupus-like immune complex glomerulonephritis associated with the production of antinuclear autoantibodies [171]. Morel et al. have focused on the genetic dissection of lupus-prone NZM2410 mice, which are derived from this cross [110, 112] and identified four epistatic modifiers (*Sles1–4*) by linkage analysis. The cumulative effect of these suppressive loci accounts for the benign autoimmunity in NZW mice [122].

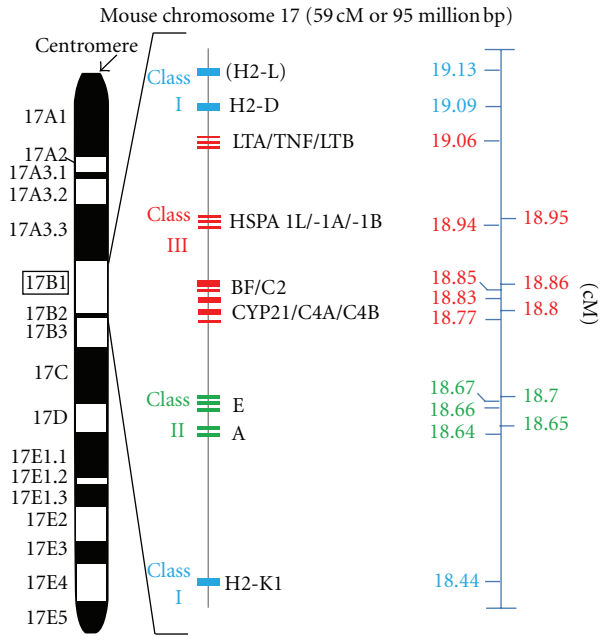

Figure 2: H-2 gene cluster and lupus susceptibility genes on mouse chromosome 17. Ideogram of chromosome 17 (middle) and schematic diagram of the MHC-complex-associated genes at 17 B1 (boxed left). The coding region of the genes is shown as small blue (class I), green (class II), and red (class II) rectangles, respectively. There are 11 mouse MHC subclasses, ranging from 18.4 to 20.3 centimorgan (cM). However, the organization of the "classical" MHC classes is similar in human and mouse. The MHC class I consists of two major loci, K and D, which are (unlike the human MHC class I counterpart) separated by the class II and class III genes. The class II gene complex is known as the I region (from "immune response") and the class II genes are also termed "Ir genes". Class III lupus susceptibility genes are tumor necrosis factor alpha (TNF), cytochrome P450 21-hydroxylase a1 (CYP21) as well as the complement factors C2, C4A, and C4B, respectively. Note, that cM is a nonlinear genetic distance unit of recombinant frequency, which is influenced by several factors. Further abbreviations: LTA: lymphotoxin A, LTB: lymphotoxin B, HSPA1L: heat shock 70 kDa protein 1-like, HSPA1A: heat shock 70 kDa protein 1A, HSPA1B: heat shock 70 kDa protein 1B, and BF: complement factor B. Source of mapping data: IMGT information system (http://www.imgt.org/IMGTrepertoireMHC/LocusGenes/): Artzt et al. (1991), Mammalian Genome 1: 280ff. [232], and Endo et al. [233], Gene 205: 19ff. [233].

The strongest one, *Sles1*, being encoded by an MHC (H-2^z) class II locus, was sufficient to completely prevent autoimmunity initiated by Sle1 in (NZW × B6.NZMc1)F1 mice.

MHC H-$2^{d/z}$ heterozygosity (H-2^d of NZB and H-2^z of NZW mice) promotes lupus disease, as congenic H-$2^{d/d}$and H-$2^{z/z}$ homozygous crosses do not develop severe disease [172, 173]. On the other hand, Zhang and coworkers found that H-2A$^{d/d}$ homozygous (NZB × NZW)F1 mice lacking H-2E molecules developed severe SLE similar to that seen in wild-type F1 mice, whereby the effect of H-2E is greatly influenced by the haplotype of H-2A molecules [174]. The authors propose two different mechanisms to explain their results: First, compared with H-$2^{d/d}$ F1 mice, the self-antigen presenting capacity of DCs in H-$2^{d/z}$ F1 is much higher, so that effects of E molecules may be insufficient for disease suppression and, alternatively, generation of H-$2^{d/z}$ F1 unique self-reactive T cells restricted to haplotype mismatched H-2Aα/β heterodimers in the thymus may play a role in an H-2E molecule-independent manner. However, one should keep in mind that H-$2^{d/z}$ heterozygosity is a necessary but not sufficient condition for the development of autoimmunity in NZB/W F1 mice [175]. Kotzin and coworkers wanted to dissect the role of Ea^z, Eb^z, Aa^z, and Ab^z MHC class II molecules to lupus susceptibility, but they could not observe an increased contribution of these polypeptides to the seriousness of the disease in transgenic approaches [26, 123].

BXSB mice spontaneously develop a male-biased lupus-like syndrome that is accelerated by the *Yaa* (Y-linked autoimmune accelerator) gene [9, 176]. The BXSB MHC locus (H-2b haplotype) plays a crucial role in disease expression since congenic BSXB.H-2d mice have a less severe syndrome [2]. As B6·Yaa (H-2b/b) mice do not develop lupus symptoms, there are also non-MHC-linked genes in the BSXB genome that contribute to disease development [104]. It has been shown that lupus was initiated by a translocation of 17 genes, including *TLR7*, from the X to the Y chromosome [3, 4]. TLR7 overexpressing transgenic mice have demonstrated that duplication of the *TLR7* gene is the sole requirement for this accelerated autoimmunity, as reduction of TLR7 gene dosage abolishes the Yaa phenotype [177]. Furthermore, TLR7 and additional nucleic acid-binding TLRs, consisting of the toll-like receptors 3 and 9, exacerbate lupus-like disease in other autoimmune-prone strains [178]. Although a TLR7 gene copy-number variation could be detected in the human genome, it was not significantly increased among SLE patients as compared with the healthy control group, and no significant concordance between the number of gene copies and the SLE phenotype was found [179]. However, other reports describe SNPs in the human *TLR7* gene that associate with lupus [180, 181]. García-Ortiz and coworkers reported an association between increased *TLR7* gene copy numbers and childhood-onset SLE in the Mexican children [182].

However, even after more than 30 years of research, the precise contribution of HLA class II genes to lupus pathogenesis remains ambiguous and is still a matter of discussion.

3.3. MHC Class III Genes. Class III genes of the MHC encode proteins that are not involved in antigen presentation (Figures 1 and 2). C2, C4A, C4B, and factor B are complement components that constitute both the C3 convertases of the classical and alternative pathway [183, 184]. Tumor necrosis factor alpha (TNF-α) and its related proteins lymphotoxin-α and -β are immune modulating cytokines of the TNF superfamily [185, 186], and the heat shock protein 70 (Hsp70) orthologues are a triplet of genes, which are important components of the chaperone machinery [187, 188].

3.3.1. Complement Components. The complement system plays an important role in innate and adaptive immunity [189]. Its main biological function is to recognize foreign particles, macromolecules, and apoptotic cells, and to

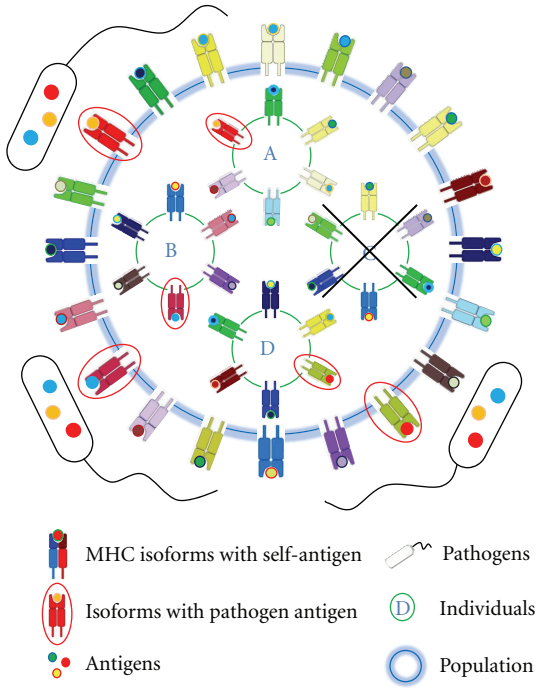

Figure 3: Protective effect of MHC polymorphism on populations (simplified scheme). Evolution of MHC genes and alleles is driven by the need to maximize peptide binding diversity in order to recognize a maximum of potential pathogens. Thus, the extreme polymorphism of MHC molecules of vertebrates is thought to reflect a pathogen-driven selection. This insures that no germ can exterminate the whole population by developing peptides that cannot be bound by any MHC molecule. However, compared to the enormous diversity of MHC molecules within a population (outer circle), their heterogeneity within a single individual is restricted to a few different MHC polypeptides (individuals A–D). This can be attributed to a mechanism called "MHC restriction" (see MHC chapter) that limits polygeny of the MHC genes (in general to 3 genes per MHC class I and class II). As a consequence some agents may evolve polypeptides that evade the immune system of single individuals (individual C) and harm or even kill them. In consequence of these opposed requirements, the immune surveillance is a delicate balance between (self-)tolerance and immune response that ensures survival of a population and/or a species at the expense of single individuals.

support their elimination either by opsonisation or lysis [190]. Although rare, inappropriate complement activation as well as complement deficiencies are involved in the pathophysiology of systemic lupus erythematosus [25, 191, 192].

Lupus is casually associated with the homozygous deficiency of the most early components of the complement activation pathway (C1q, C1r, C1s) [189, 193]. However, MHC-linked C2 and C4 deficiencies are also associated with SLE [194], and approximately 40% of C2 deficient individuals develop SLE-like symptoms [195]. In fact, homozygous C2 deficiency is thought to be the most common inherited complement defect associated with lupus [196, 197]. In addition, Fielder et al. found a high frequency of null alleles at the C2, C4A, and C4B loci in families of SLE patients [198]. In humans and mice, C4 is encoded by two tandemly arranged genes (C4A and B) within the MHC ([199] and Figures 1 and 2). About 40 protein variants for C4 have been documented [200]. It has been shown that low copy numbers of the C4 gene are a risk factor for SLE in European Americans [201] and a large C4A-CYP21A gene deletion (particularly associated with HLA-B44, -DR2, and -DR3 alleles) in black Americans [202]. On the other hand, C3 deficiency is only rarely associated with lupus development, because homozygous hereditary C3 deficiency is a seldom genetic disease [203]. It is thought that absence of complement proteins results in a defective immune complex clearance and, in consequence, to a deposition of the complexes in various organs [204, 205]. An alternative hypothesis postulates that self-reactive B cells, which are specific for lupus autoantigens, are not effectively silenced (or eliminated) without complement [206]. In fact, recent findings suggest, that enhanced B cell function is the defining pathogenic event of lupus pathogenesis, leading to autoimmunity and organ damage [207].

Aberrant splicing of the C4 mRNA (caused by an intronic insertion of the B2 sequence in the C4 gene) is the basis for low C4 expression in H-2^k mice, such as lupus-prone MRL mice [208, 209]. An association between complement deficiency and SLE has also been shown for complement-deficient mouse models [210]. C1q- and C4-deficient mice develop a lupus-like disease and exhibit impaired clearance of apoptotic cells [211]. Indeed, apoptotic cells are thought to be a major source of the autoantigens of SLE [212]. This has led to the hypothesis that the delayed clearance of apoptotic material leads to a persistence of proinflammatory activities which may then initiate autoimmunity.

3.3.2. Heat Shock Protein (HSP) Genes. Heat shock proteins (hsp) are highly conserved proteins that regulate protein folding. They are induced by a variety of stresses like heat, growth factors, inflammation, and infection [213]. The expression of hsp90 is found to be increased in the mononuclear cells of about one-fourth of SLE patients and antibodies to this protein are detected in patients with SLE [146]. Levels of hsp90 protein in SLE patients seem to correlate with IL-6 and hsp90 autoantibody levels, supporting the following scenario: Elevated levels of IL6 in SLE patients induce higher levels of hsp90 protein which in turn results in the production of hsp90 autoantibodies [214].

Another heat shock protein that play a role in SLE pathogenesis is HSPA1B, a member of the hsp70 gene family. The HSPA1A, HSPA1B, and HSPA1L are MHC class III genes in murines and humans, which code for highly homologous polypeptides [215]. HSPA1B encodes a polypeptide that is thought to be involved in disease susceptibility [216]. Association of a polymorphism (A to G transition) in the coding region of the HSPA1B gene with SLE in African Americans has been reported in a case-control study [217].

3.3.3. Tumour Necrosis Factor (TNF) Gene. Tumour necrosis factor alpha (TNF-α) is an inducible member of the TNF/TNFR superfamily with a broad range of immunological effects [218]. Macrophages are the major source of TNF-α, although it can be produced by many other cell types

as well [219]. It is generally known as a proinflammatory cytokine, stimulating the acute phase response and increasing MHC class I and II expression as well as antigen-driven lymphocyte proliferation [220–222]. Dysregulation of TNF-α production has been implicated in a variety of human diseases, including lupus. A rare polymorphism (G to A transition) in the promoter region has been found to be increased in patients with SLE in a case-control study [223, 224], which is probably due to linkage disequilibrium with DR3 [225]. However, other reports based on Caucasian SLE patients describe an independent contribution of TNF polymorphisms and HLA-DR3 to SLE susceptibility [226, 227].

As in humans, the murine TNF-α gene is located within the MHC [228]. The NZW mouse strain carries a unique TNF allele, that expresses only limited amounts of TNF-α [229]. It has been proposed that this polymorphism ameliorates murine lupus symptoms [228, 230] and, indeed, it has been shown by Kontoyiannis and Kollias, that autoimmunity and lupus nephritis is accelerated in NZB mice with an engineered heterozygous deficiency in tumor necrosis factor [231].

4. Concluding Remark

The MHC genes including TNFα, HSP70, and class II genes have been associated with systemic lupus erythematosus. However, in most cases, genetic susceptibility to lupus is not caused by a single gene or allelic variation. Defects in complement genes are well-documented exceptions, which may predispose to lupus because of the persistence of antibody complexes or activation of self-reactive B cells. The role of TNFα, HSP70, or MHC class II gene loci in lupus pathology is more difficult to evaluate. This is due, among others, to the linkage disequilibrium of the MHC, which makes it difficult to prove a direct contribution of single genes or alleles to lupus susceptibility. Furthermore, the identification of susceptibility or suppressor genes is complicated by the plain fact that SLE is a highly heterogeneous disease that appears when susceptibility and suppressor loci are unbalanced. In addition, environmental, epigenetic, hormonal, and infectious factors may alter the epigenetic status quo and may trigger lupus in genetically-susceptible individuals. On the other hand, analysing the influence of environmental factors on the epigenetic status of well-defined MHC haplotypes or MHC gene polymorphisms may open promising perspectives for future studies.

For these reasons, deciphering the contribution of MHC locus and its gene products to the pathogenesis of human and murine lupus will add the next important piece of the puzzle that will further clarify the etiology of this complex autoimmune disease.

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Research Article

Systemic-Lupus-Erythematosus-Related Acute Pancreatitis: A Cohort from South China

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Acute pancreatitis (AP) is a rare but life-threatening complication of SLE. The current study evaluated the clinical characteristics and risk factors for the mortality of patients with SLE-related AP in a cohort of South China. *Methods*. Inpatient medical records of SLE-related AP were retrospectively reviewed. *Results*. 27 out of 4053 SLE patients were diagnosed as SLE-related AP, with an overall prevalence of 0.67%, annual incidence of 0.56‰ and mortality of 37.04%. SLE patients with AP presented with higher SLEDAI score (21.70 ± 10.32 versus 16.17 ± 7.51, $P = 0.03$), more organ systems involvement (5.70 ± 1.56 versus 3.96 ± 1.15, $P = 0.001$), and higher mortality (37.04% versus 0, $P = 0.001$), compared to patients without AP. Severe AP (SAP) patients had a significant higher mortality rate compared to mild AP (MAP) (75% versus 21.05%, $P = 0.014$). 16 SLE-related AP patients received intensive GC treatment, 75% of them exhibited favorable prognosis. *Conclusion*. SLE-related AP is rare but concomitant with high mortality in South Chinese people, especially in those SAP patients. Activity of SLE, multiple-organ systems involvement may attribute to the severity and mortality of AP. Appropriate glucocorticosteroid (GC) treatment leads to better prognosis in majority of SLE patients with AP.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic, autoimmune, inflammatory disease characterized by the presence of a plethora of autoantibodies, immune complex formation, and multiple organ system involvement. Gastrointestinal (GI) manifestations are common in SLE patients, but acute pancreatitis is rare [1–6]. It was reported that 19.2%–50% of SLE patients presented with gastrointestinal symptoms [7–11], whereas pancreatitis occurred in about 0.7%–8.2% of patients with SLE [7, 8, 11, 12] and the annual incidence was approximately 0.4–1.1‰ [3–5]. Our knowledge about SLE-related acute pancreatitis (AP) is mostly based on individual case reports or small case series. Despite its rarity, AP can be a life-threatening complication of SLE if not treated appropriately. Prevalence of SLE is relatively high in Chinese people, which is 0.7∼1/1000 in comparison to 0.51/1000 in United States [13]. But so far very few case reports on SLE-related AP in Chinese population have been published. The current study aims to clarify the clinical characteristics, severity, mortality, and outcome of SLE-related acute pancreatitis in south China.

2. Materials and Methods

A retrospective review of inpatient medical records between January 2000 and January 2012 was performed at the First Affiliated Hospital of Sun Yat-Sen University in South China. 4053 patients were classified as SLE during the past 12 years who fulfilled at least four of the American College of Rheumatology (ACR) revised classification criteria for SLE (1997) [14]. A diagnosis of acute pancreatitis (AP) was established by the presence of typical clinical symptoms (including abdominal pain, nausea, and vomiting) and confirmed by more than a three-fold elevation of serum amylase or lipase or evidence of imaging findings-computer tomography [CT] scan or ultrasonography (USG) [15]. Among these SLE patients, 27 were with dual simultaneous

diagnosis of AP, and another 23 age- and gender-matched SLE patients without AP were randomly selected. Review of the clinical files of these 50 SLE patients was performed and data was extracted.

The SLE Disease Activity Index (SLEDAI) [16] was used to evaluate SLE activity during AP, and patients were defined as active SLE if the SLEDAI score was equal to or greater than 6. The Systemic Lupus International Collaborating Clinics/ACR (SLICC/ACR) damage index [17] was used to ascertain organ damage in SLE. The Atlanta criteria [18] were used to classify the severity of acute pancreatitis. Severe acute pancreatitis (SAP) was defined as the presence of at least three of Ranson's criteria and eight or more Acute Physiology and Chronic Health Evaluation II (APACHE II) score, or with the evidence of organ failure (systolic blood pressure < 90 mmHg, $PaO_2 \leq 60$ mmHg on room air, creatinine > 2 mg/dL, gastrointestinal bleeding > 500 mL/24 h, DIC or severe hypocalcemia ≤ 7.5 mg/dL) or local complications (i.e., pancreatic necrosis, abscess, or pseudocyst). The positivity of CT scan was defined as diffuse or segmental enlargement of the pancreas, illegibility of peripancreas fat, low/high density area in contrast, and peripancreas effusion [19]. The positivity of USG was defined as pancreatic enlargement, decreased echodensity, and possible fluid collections [20].

Demographic information including gender, age at SLE onset, duration between the onset of SLE and AP, history of alcohol consumption, gallstone, metabolic abnormalities (hypertriglyceridemia and hypercalcemia), clinical symptoms, laboratory findings, medications (especially corticosteroid, and immunosuppressive agents (ISA)) and outcome were documented. Acute pancreatitis related to mechanical obstruction (choledocholithiasis), toxic-metabolic etiologies (alcohol intake, drugs, hypercalcemia, or hypertriglyceridemia), infection, or trauma were ruled out in every case [21].

2.1. Statistical Analysis. Statistical analysis was done using the SPSS program 13.0 and Prism software version 5.0. The Mann-Whitney U test was used for continuous variables and the chi-square or Fisher's exact test for categorical variables. Survival rates were estimated using the Kaplan-Meier method. A P value <0.05 was considered statistically significant in all comparisons.

3. Results

3.1. Demographic and Clinical Characteristics of SLE-Related Acute Pancreatitis. 27 out of 4053 SLE patients were diagnosed as SLE-related AP during the past 12 years, with an overall prevalence of 0.67% and annual incidence of 0.56‰. One patient developed 2 episodes of pancreatitis and the other 26 patients had only one episode at the time of hospitalization. The demographic and clinical features of each SLE-related AP patient were shown in Table 1.

3.2. Comparison of Demographic and Clinical Features in SLE Patients with and without SLE-Related AP. The majority of patients (92.59%, 25/27) were females and the mean age at SLE onset was 26.96 ± 13.30 years (ranged from 14 to 57 years). Time interval between the onset of SLE and AP ranged from 1 week to 20 years, and more than half of the patients (51.85%, 14/27) developed AP within the first year of the onset of SLE. All these 27 patients were classified as active SLE with average SLEDAI score of 21.70 ± 10.32 at the onset of AP. The clinical features related to acute pancreatitis in these 27 SLE patients were nonspecific. Abdominal pain (92.59%), fever (77.78%) and nausea/vomiting (74.07%), were the most frequent manifestations and other symptoms included diarrhea (44.44%), loss of appetite (44.44%) and GI tract hemorrhage (14.81%).

Other organ system involvement was found in all SLE-related AP patients with an average number of 5.70 ± 1.56 (ranged from 3 to 8 organs), including hematological system, kidney, liver, serositis, mucocutaneous involvement, respiratory system, arthritis, and central nervous system.

Clinical features and laboratory findings were compared between these two groups and the results were shown in Table 2. SLE patients with AP presented with higher SLEDAI score (21.70 ± 10.32 versus 16.17 ± 7.51, $P = 0.03$), more organ system involvement (5.70 ± 1.56 versus 3.96 ± 1.15, $P = 0.001$), higher frequence of fever (77.78% versus 39.13%, $P = 0.006$), hepatological and hematological disorders (82.61% versus 34.78%, $P = 0.01$; 100% versus 60.87%, $P = 0.001$), serositis (62.96% versus 26.09%, $P = 0.01$), elevated CRP (81.82% versus 47.62%, $P = 0.02$), positive anti-La antibody (33.33% versus 0, $P = 0.003$), and higher mortality (37.04% versus 0, $P = 0.001$) compared to SLE patients without AP.

3.3. Comparison of Clinical Features between SAP and MAP Patients. According to Atlanta criteria, 27 SLE-related AP patients were divided into SAP group (severe acute pancreatitis, $n = 8$, 29.63%) and MAP group (mild acute pancreatitis, $n = 19$, 70.37%). The comparison of the demographic and clinical data between SAP and MAP patients as shown in Table 3. The results indicated that the age of onset of AP in SAP patients as significantly younger than MAP (19.63 ± 10.88 versus 30.05 ± 13.25, $P = 0.016$). SAP patients presented with significantly higher mortality (75% versus 21.05%, $P = 0.014$) and more abnormal hematologic findings (thrombocytopenia and leucopenia, 100% versus 52.63%, $P = 0.026$; 87.5% versus 31.58%, $P = 0.013$, resp.) compared to MAP. The Kaplan-Meier survival curves showed death rate within 30 days after onset of acute pancreatitis in SAP and MAP groups (Figure 1).

3.4. Comparison of Clinical Features between Pediatric- and Adult-Onset SLE-Related AP. SLE-related AP patients were divided into pediatric-onset group (under 18 years of age, $n = 10$) and adult-onset group ($n = 17$). Demographic and clinical characteristics were compared between these two groups. Pediatric-onset SLE-related AP had higher rate of severe AP (60% versus 11.76%, $P = 0.014$), higher serum amylase level (17.55 ± 16.09 versus 6.53 ± 5.42, $P = 0.007$), lower percentage of positive anti-Ro antibody (25% versus 84.62%, $P = 0.01$), and lower rate of anti-La antibody

Table 1: The demographic and clinical characteristics of each SLE patient with AP.

Case	Age at SLE onset (y)	Duration between onset of SLE and AP (m)	SLEDAI score at onset of AP	Number of involved organs concomitant with AP	GC treatment after onset of AP	Outcome
1	23	0.5	9	5	Increased dose	In remission
2	16	48	12	4	Stop	In remission
3	18	12	14	6	Increased dose	Died
4	22	72	14	7	Increased dose	In remission
5	16	36	16	7	Increased dose	In remission
6	57	24	17	4	Increased dose	In remission
7	36	0.5	17	6	Initial treatment	Died
8	48	180	18	7	maintaining	Died
9	14	36	21	4	maintaining	Died
10	14	12	23	7	Increased dose	Died
11	19	2	23	6	Increased dose	In remission
12	14	2	27	7	Increased dose	Died
13	46	1	25	4	Increased dose	In remission
14	22	0.25	18	3	Decreased dose	In remission
15	51	240	18	6	Decreased dose	In remission
16	42	4	19	7	Increased dose	In remission
17	20	84	18	5	Decreased dose	Died
18	39	24	13	5	Increased dose	In remission
19	15	2	33	6	Decreased dose	Died
20	15	36	41	7	Increased dose	In remission
21	26	3	41	8	Increased dose	Died
22	39	1	27	6	Increased dose	In remission
23	20	72	10	3	maintaining	In remission
24	36	12	38	6	maintaining	Died
25	16	2	8	3	Increased dose	In remission
26	30	48	47	8	Increased dose	In remission
27	14	72	19	6	maintaining	In remission

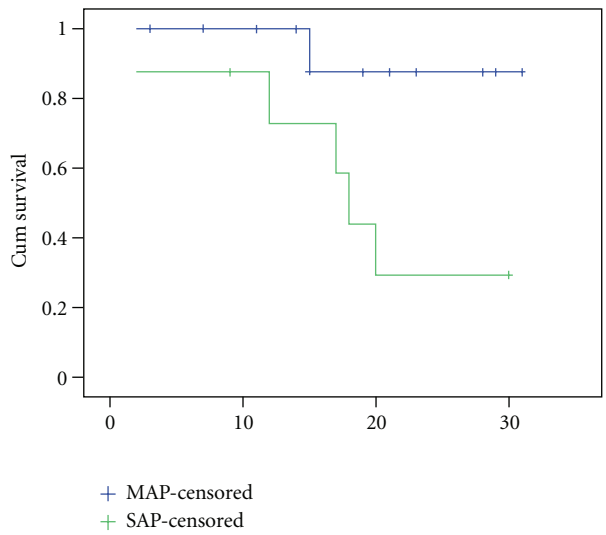

Figure 1: Kaplan-Meier survival curves for the time (days) from onset of SLE-related acute pancreatitis to death.

(0 versus 53.85%, $P = 0.02$) compared to adult-onset SLE-related AP. However, the difference in mortality was not statistically significant between pediatric and adult patients (50% versus 29.41%, $P = 0.26$).

3.5. Comparison of Clinical Features between Mortality and Nonmortality SLE Patients with AP. The risk factors for mortality were further analyzed. 27 SLE-related AP patients were divided into mortality group ($n = 10$) and nonmortality group ($n = 17$). The clinical manifestations were compared between these two groups and shown in Table 4. The mortality group had higher percentage of hypoalbuminemia (90% versus 47.06%, $P = 0.031$), hyperbilirubinemia (40% versus 5.88%, $P = 0.047$), hematuria (100% versus 41.18%, $P = 0.002$), and granular casts (70% versus 23.53%, $P = 0.024$) compared to nonmortality group. Severity of acute pancreatitis was the most powerful risk factor for mortality in SLE-related AP (OR 11.25, 95% CI (1.611, 78.57) and $P = 0.014$).

Table 2: Comparison of demographic and clinical features in SLE patients with and without AP.

	SLE with AP ($n = 27$)	SLE without AP ($n = 23$)	P
Female (%)	25 (92.59%)	20 (86.96%)	0.42
Age on SLE diagnosis (y)	26.96 ± 13.30	28.39 ± 9.98	0.26
GCs dose (mg)	61.19 ± 37.63	50.96 ± 28.82	0.18
SLEDAI score	21.70 ± 10.32	16.17 ± 7.51	**0.03**
SLICC/ACR damage index	1.19 ± 0.92	0.96 ± 1.19	0.11
Mortality	10 (37.04%)	0	**0.001**
Fever (%)	21 (77.78%)	9 (39.13%)	**0.006**
Neuropsychiatric (%)	7 (25.93%)	1 (4.35%)	**0.042**
Pulmonary (%)	11 (40.74%)	5 (21.74%)	0.13
Articular (%)	16 (59.26%)	16 (69.57%)	0.32
Mucocutaneous involvement (%)	18 (66.67%)	16 (69.57%)	0.54
Renal (%)	24 (88.89%)	20 (86.96%)	0.59
Hepatological (%)	19 (82.61%)	8 (34.78%)	**0.01**
Hematological (%)	27 (100.00%)	14 (60.87%)	**0.001**
Serositis (%)	17 (62.96%)	6 (26.09%)	**0.01**
Number of organs involved	5.70 ± 1.56	3.96 ± 1.15	**0.001**
Positive anti-dsDNA (%)	24 (88.89%)	19 (82.61%)	0.41
Positive anti-Sm (%)	6/21 (28.57%)	10 (43.48%)	0.24
Positive anti-Ro (%)	13/21 (61.90%)	14 (60.87%)	0.60
Positive anti-La (%)	7/21 (33.33%)	0	**0.003**
Positive ACL-IgG (%)	4/21 (19.05%)	1/22 (4.55%)	0.168
Positive ACL-IgM (%)	4/21 (19.05%)	1/22 (4.55%)	0.16
Positive anti-β_2 GPI (%)	3/21 (14.29%)	2/22 (9.09%)	0.48
Low C3 (%)	26/26 (100%)	22 (95.65%)	0.47
Low C4 (%)	21/26 (80.77%)	20 (86.96%)	0.42
Elevated CRP (%)	18/22 (81.82%)	10/21 (47.62%)	**0.02**

4. Treatment and Outcome

Among these 27 SLE-associated AP patients, 26 were on steroid treatment before the onset of AP and the average dosage of GCs was 61.19 ± 37.63 mg/day (ranged from 10 mg/day to 120 mg/day). AP was considered as the initial presentation of SLE in one patient (patient 7 in Table 1), and standard GC treatment started after diagnosis. Additional immunosuppressive agents (ISA) were also administrated in 22 patients before the onset of AP, including 18 on hydroxychloroquine, 2 azathioprine, 8 methotrexate, 5 cyclophosphamide, and 1 FK506. After the episodes of AP, oral medicines were stopped because of fasting. Methotrexate or cyclophosphamide were continuously prescribed in 5 patients but switched to I.V. injection. 1 patient developed recurrent episode of AP when increasing the dosage of GC for the relapse of SLE, and GC treatment was stopped (patient 2) after onset of AP. 25 patients were continuously treated by GCs and/or ISA during their episode of AP. 16 patients were given aggressive treatment of GCs and/or ISA (12 patients obtained clinical and laboratory improvement (75%) and 4 died), 5 patients were treated with the maintenance dose of GCs and/or ISA (2 patients in remission (40%) and 3 died), and 4 patients were treated with decreased dose of GCs because of fever and concerning of potential infections (2 patients in remission (50%) and 2 died) (The results showed in Figure 2). Totally, 10 patients died and the overall mortality rate was 37.04% (10/27).

5. Discussion

SLE-related AP is relatively rare compared to other organ injury involved in lupus. The incidence of clinical AP associated with SLE varies from 0.7 to 4% [5, 8, 12, 22], with the annual incidence of 0.4–1.1‰ [3, 4]. Most previous studies on this issue were individual case reports or small case series. So far, the Hopkins lupus cohort [12] reported the largest case series with 63 SLE-attribute pancreatitis out of 1740 SLE patients (3.5%), and a Taiwan series reported 40 out of 2976 SLE patients (1.34%). This study was the first report of the SLE-related AP in south China. In current cohort, 27 out of 4053 SLE patients were diagnosed as SLE-related AP, with the prevalence of 0.67%, and annual incidence of 0.56‰, which is comparable with the findings of previous literatures [3–5, 8, 12, 22].

The pathogenic mechanism of SLE-related AP is very complex and multifactors. Vascular damage (including vasculitis, intimal thickening, immune complex deposition, occlusion of arteries, and arterioles), autoantibody production, abnormal cellular immune response, and drug toxicity may be responsible for the development of pancreatitis [8].

Table 3: Comparison of demographic and clinical characteristics between SLE-related severe acute pancreatitis (SAP) and mild acute pancreatitis (MAP).

	SAP ($n = 8$)	MAP ($n = 19$)	P value
Demographic characteristics			
Female	7 (87.50%)	18 (94.74%)	0.513
Age of onset AP (y)	19.63 ± 10.88	30.05 ± 13.25	**0.016**
Interval between onset of SLE and AP (m)	23.38 ± 28.25	44.36 ± 64.73	0.822
Early AP (⩽1 year)	5 (62.50%)	9 (47.37%)	0.678
SLEDAI score at onset of AP	22.13 ± 6.24	21.53 ± 11.77	0.44
SLICC/ACR damage index	1.25 ± 0.89	1.16 ± 0.96	0.854
Number of organs involved	5.75 ± 1.28	5.68 ± 1.70	0.893
Intensive therapy of GC/ISA	5 (62.50%)	11 (57.89%)	1
Mortality	6 (75%)	4 (21.05%)	**0.014**
Clinical characteristics			
Fever	8 (100.00%)	13 (68.42%)	0.136
Mucocutaneous involvement	6 (75.00%)	12 (63.16%)	0.676
Articular involvement	3 (37.50%)	10 (52.63%)	0.678
Serositis	5 (62.50%)	10 (52.63%)	0.696
Neuropsychiatric involvement	2 (25.00%)	5 (26.32%)	1
Renal involvement	7 (87.50%)	15 (78.95%)	1.0
Laboratory findings			
Serum amylase*	18.09 ± 18.15	7.46 ± 5.88	0.077
Serum lipase*	8.53 ± 3.14	7.63 ± 5.45	0.616
Elevated serum transaminase	7 (87.5%)	11 (57.89%)	0.201
Thrombocytopenia	8 (100%)	10 (52.63%)	**0.026**
Leucopenia	7 (87.50%)	6 (31.58%)	**0.013**
Positive anti-dsDNA	8 (100.00%)	16 (84.21%)	0.532
Positive anti-Sm	1/7 (14.29%)	5/14 (35.71%)	0.613
Low C3	8/8 (100.00%)	18/18 (100.00%)	1
Low C4	7/8 (87.50%)	14/18 (77.78%)	1
Anti-Ro	2/7 (28.57%)	11/14 (78.57%)	0.056
Anti-La	1/7 (14.29%)	6/14 (42.86%)	0.337

*Times in excess of the upper limit of normal (ULN).

In the current cohort, more than half patients (51.85%) developed acute pancreatitis within 1 year of the onset of SLE, and all 27 patients were active SLE with dramatically elevated SLEDAI scores and other simultaneous SLE manifestations, especially the hematologic and renal involvement. SLE patients with AP presented with higher SLEDAI scores compared to patients without AP. Previous studies [3, 4, 22, 23] also demonstrated that episodes of SLE-related pancreatitis significantly increased in the active SLE group. AP was considered as one of the clinical features of active SLE and was associated with the activity of the disease itself. These results indicated that SLE itself can be the primary etiologic factor or cofactor predisposing to AP.

SLICC/ACR damage index score represents disease burden in SLE patients. It was significantly higher in SLE patients with pancreatitis compared to SLE patients without pancreatitis in Hopkins cohort [12]. Although SLE-related AP had more organ system involvement in current study, the damage index score was low, and there was no significant difference between SLE patients with and without AP (1.19 ± 0.92 versus 0.96 ± 1.19, $P = 0.11$). The reason of the low-damage index score might lie in the relatively younger onset age, shorter duration of disease, and less-chronic organ damage.

Our study found that pediatric-onset AP tended to be more severe compared to adult-onset AP. SAP group had significant higher prevalence of thrombocytopenia and leucopenia than MAP group. Mortality patients has higher rate of hypoalbuminemia, hematuria, granular casts, and hyperbilirubinemia than nonmortality group, which indicated that multiple organ systems involvement, especially hematological, renal, and liver injury in SLE patients might be the major causes due to the severity and mortality of AP. In general population, the mortality rate of AP is about 3.8% ~ 10% [24–27]. Approximately 15~20% of all AP cases were SAP which accounted for a mortality rate of 16.3% ~ 30% [27–29]. SLE-related AP patients had much higher mortality. Wang et al. [23] reported that the mortality rate was 27.5% in all SLE-related AP and 78.57% in SAP. Richer et al. [30] reported that 57% of childhood-onset lupus with pancreatitis developed SAP with the mortality of 45%. In our cohort, the overall mortality rate of SLE-related AP was

Table 4: Comparison of demographic and clinical characteristics between mortality and non-mortality group.

	Mortality ($n = 10$)	Non-mortality ($n = 17$)	P value
Demographic characteristics			
Female	10 (100%)	15 (88.24%)	0.387
Age of onset AP (y)	24.10 ± 12.02	28.65 ± 14.07	0.123
SLEDAI score at onset of AP	25.00 ± 9.40	19.76 ± 10.61	0.065
Number of organs involved	6.20 ± 1.14	5.41 ± 1.73	0.167
Intensive therapy of GC/ISA	4 (40%)	12 (70.59%)	0.124
Clinical characteristics			
Fever	10 (100%)	11 (64.71%)	**0.042**
Mucocutaneous involvement	6 (60%)	12 (70.59%)	0.439
Articular involvement	6 (60%)	7 (41.18%)	0.293
Serositis	7 (70%)	8 (47.06%)	0.226
Neuropsychiatric involvement	4 (40 %)	3 (17.65%)	0.204
Laboratory findings			
Serum amylase*	14.79 ± 17.34	8.15 ± 6.03	0.241
Serum lipase*	6.46 ± 3.51	8.20 ± 5.58	0.368
Elevated serum transaminase	8 (80%)	10 (58.82%)	0.244
Hypoalbuminemia	9 (90%)	8 (47.06%)	**0.031**
Proteinuria	10 (100%)	12 (70.59%)	0.077
Hematuria	10 (100%)	7 (41.18%)	**0.002**
Granular casts	7 (70%)	4 (23.53%)	**0.024**
Hyperbilirubinemia	4 (40%)	1 (5.88%)	**0.047**
Positive anti-dsDNA	10 (100%)	14 (82.35%)	0.232
Positive anti-Sm	2/8 (25%)	4/13 (30.77%)	0.59
Low C3	9/9 (100%)	17/17 (100%)	1.0
Low C4	7/9 (77.78%)	14/17 (82.35%)	0.58
Anti-Ro	3/8 (37.5%)	10/13 (76.92%)	0.09
Anti-La	1/8 (12.5%)	6/13 (46.15%)	0.133

* Times in excess of the upper limit of normal (ULN).

37.04% compared to 0 in SLE patients without AP ($P = 0.001$), and mortality rate in SAP was 75%. The severity of AP might be the most important risk factor for the mortality of SLE-related AP patients (OR 11.25, 95% CI (1.611, 78.57), and $P = 0.014$).

In accordance with other literatures, the manifestations of SLE-related AP in this cohort were nonspecific and similar to non-SLE acute pancreatitis. Abdominal pain (92.59%), fever (77.78%), and nausea/vomiting (74.07%) were the most common symptoms. These symptoms could also be attributed to other gastrointestinal diseases or adverse reactions of medication and may lead to misdiagnosis in general practice. It was reported that the rate of misdiagnosis of AP in SLE was up to 88.6% [31]. Delayed diagnosis and improper treatment may contribute to unfavorable prognosis, even lifethreatening [32]. Likewise, the mortality rate of the Hopkins Lupus Cohort (3%) was considerably lower than average of other reported studies due to close monitoring, early diagnosis, and treatment [12]. So, AP should be paid more attention in any SLE patient with abdominal pain when mechanical obstruction or toxic-metabolic etiologies, infection, or trauma were ruled out.

Some immunosuppressants, such as corticosteroids, azathioprine, and cyclosporine have been implicated to cause pancreatitis in several case reports. Only 2 patients in our study took azathioprine but the medication was discontinued after the onset of AP. The current study couldn't verify the relationship between azathioprine and acute pancreatitis in SLE patients. There is still a controversy over steroid treatment in SLE-related AP. Increasingly accumulated evidence showed that steroids do not trigger acute pancreatitis or cause increased mortality on AP [22, 33, 34], but instead, they have a possible therapeutic effect on SLE-related pancreatitis [5, 35–37]. In Hopkins cohort, appropriate treatment with corticosteroids added a survival benefit in SLE-related AP. In current study, 16 SLE-related AP patients received intensive GC and/or ISA treatment, and 75% of them exhibited favorable prognosis.

In summary, SLE-related acute pancreatitis is rare but with high-mortality rate, which is even higher in those severe acute pancreatitis with multiple organ system involvement. Activity of SLE, hematological system, renal, and liver injury in SLE patients may attribute to the mortality of AP. Early diagnosis of acute pancreatitis in SLE patients, especially

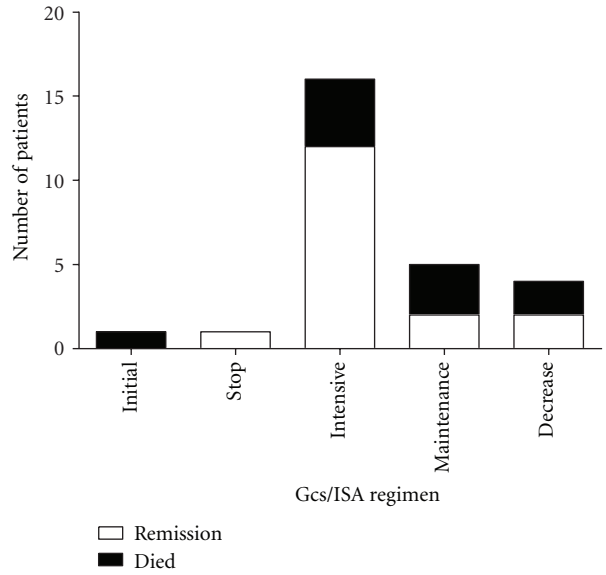

Figure 2: Treatment regimen and outcome of the SLE-related AP.

those with abdominal pain, and appropriate glucocorticosteroid treatment is beneficial for a better therapeutic outcome in the majority of patients.

Abbreviations

AP: Acute pancreatitis
SAP: Severe acute pancreatitis
MAP: Mild acute pancreatitis
GC: Glucocorticosteroid
ISA: Immunosuppressive agents.

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Review Article

Balance between Regulatory T and Th17 Cells in Systemic Lupus Erythematosus: The Old and the New

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Pathogenic mechanisms underlying the development of systemic lupus erythematosus (SLE) are very complex and not yet entirely clarified. However, the pivotal role of T lymphocytes in the induction and perpetuation of aberrant immune response is well established. Among T cells, IL-17 producing T helper (Th17) cells and regulatory T (Treg) cells represent an intriguing issue to be addressed in SLE pathogenesis, since an imbalance between the two subsets has been observed in the course of the disease. Treg cells appear to be impaired and therefore unable to counteract autoreactive T lymphocytes. Conversely, Th17 cells accumulate in target organs contributing to local IL-17 production and eventually tissue damage. In this setting, targeting Treg/Th17 balance for therapeutic purposes may represent an intriguing and useful tool for SLE treatment in the next future. In this paper, the current knowledge about Treg and Th17 cells interplay in SLE will be discussed.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disorder affecting almost all organs and tissues [1, 2]. In genetically predisposed subjects, environmental factors, such as viral infections and smoking, induce the breakdown of self-tolerance eventually triggering autoimmune response [1, 2]. The clinical heterogeneity of the disease often represents a challenge for clinicians and reflects the complexity of underlying pathogenic mechanisms. The aberrant crosstalk between different immune cells such as B and T lymphocytes represents a milestone in the natural history of SLE and, in general terms, of all autoimmune conditions. Self-antigen presentation by antigen presenting cells may be identified as the *primum movens* that leads to recruitment, activation, and expansion of autoreactive lymphocytes. This cascade culminates with disease-specific autoantibody production by B cells and eventually with target tissue injury [1–3]. B lymphocytes are well-recognized actors in SLE pathogenesis, and this is further confirmed by the effectiveness of B-cell depleting therapies in these patients [4]. Moreover, an altered T-cell homeostasis which plays a pivotal role in the development of the disease and the longstanding paradigm of T helper (Th) 1/Th2 cell immune response was recently challenged by the recognition of Th17 cells and regulatory T cells (Treg) [5, 6]. This intriguing evidence pointed out the need to call into question previous discoveries. The aim of this paper is to discuss the current knowledge about the interplay between Treg and Th17 cells in the pathogenesis of SLE and potential therapeutic intervention in this setting.

2. Th17 Cell Subsets in SLE

Th17 cells were identified according to their capability to produce IL-17, and initially they were thought to be just a variant of Th1 cells and to origin from a common precursor [5, 7]. Nonetheless, further investigation ruled out this possibility and found that Th17 commitment of naïve T cells, by the expression of STAT-3 and retinoic acid orphan receptor-(ROR-) γt, was attributable to the presence of both transforming growth factor-(TGF) β and IL-6 in the surrounding microenvironment [8–12].

First cloned in 1995, IL-17 family cytokines display a broad spectrum of action including the capacity to induce the production of inflammatory and tissue-damaging molecules.

In particular, IL-17A is able to stimulate the production of chemokines and cytokines from multiple cells like epithelial cells and fibroblasts [13] and to promote the proliferation, maturation, and recruitment of neutrophils, macrophages, and lymphocytes through the induction of colony-stimulating factors and chemokines [13].

Concerning IL-17 in SLE, recent data from humans and mice clearly support the role of this cytokine and Th17 cells in lupus pathogenesis. To note, it has been observed by several groups that SLE patients, including those with new-onset disease, had increased serum or plasma levels of IL-17, expansion of IL-17-producing T cells in the peripheral blood, and infiltration of Th17 cells in target organs like the kidneys [14–20]. Some studies showed that circulating IL-17 levels correlate with disease activity [14–17] and are associated with kidney involvement [20]. In addition, increased expression of IL-17 and RORγt mRNA has been found in urine sediments from lupus patients [21]. Taken together, these results point out the pivotal role played by IL-17 in mediating target organ damage in both early and long-standing stages of the disease [22].

Notably, the involvement of IL-17 in the perpetuation of lupus nephritis was recently underlined by the elegant study performed by Crispín et al., in which a novel pathogenic Th17 cell subset was identified [23]. This small T-cell population, named double negative (DN) according to the lack of both CD4 and CD8 molecules expression, appeared to be responsible for most of IL-17 production in sera and kidney of SLE patients. This may be explained by the hypothesis that all DN T cells are already committed *in vivo* towards a Th17 phenotype, whereas $CD4^+$ cells require additional stimuli to differentiate into IL-17 producing T cells. Interesting, IL-17 can also promote humoral immunity that plays a major role in lupus pathogenesis. IL-17, alone or in combination with B-cell activating factor (BAFF), increases the survival and proliferation of human B cells as well as the differentiation of B cells into antibody-producing cells [12, 24]. In conclusion, IL-17 could promote inflammation in lupus by affecting both cellular and humoral immune response. In this setting, additional studies are needed to find out the mechanisms for increased Th17 cell response and the therapeutic implication of targeting IL-17 in SLE, as discussed in detail below.

3. Treg-Cell Subsets in SLE

The other face of the coin in the pathogenesis of SLE is represented by Treg cells [25]. Since their first identification in late 90s, Treg cells became a hot topic in immunology because of the recognition of a link between impairment of this cell population and development of autoimmunity [6, 26–29]. Indeed, Treg cells display suppressive activity towards autoreactive lymphocytes thus preventing the onset of aberrant self-immune response [30]. Initially, Treg-cells were isolated in humans and mice according to high surface levels of CD25 (IL-2Rα) and intracellular expression of the forkhead winged helix (Fox) P3 transcription factor. FoxP3 expression is required for commitment of Treg cells and maintenance of their functional activity [31]. To date, several Treg cell subsets have been identified. They differ from each other for either phenotypic features or origin. Natural Treg cells (nTreg) are produced in the thymus in the very early phases of life following an appropriate T-cell receptor (TCR) stimulation and in the presence of a peculiar cytokine microenvironment. Conversely, inducible Treg cells (iTreg) result from the differentiation of naïve T cells in secondary lymphoid organs during the entire life [32, 33]. However, this classification appears slightly restrictive nowadays, since deeper understanding of Treg cell physiology has been achieved. Our group and other investigators observed that T cells lacking CD25 and expressing FoxP3 displayed suppressive activity towards effector T cells from both healthy subjects and pathological conditions [34–37] Interestingly, we provided the clue that the surface expression of glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) on $CD25^-$ T cells was able to confer them a regulatory phenotype and function [34]. In addition, as will be discussed in detail below, iTreg cells may originate from activated T cells when appropriate stimuli are present in the surrounding microenvironment [38]. Abnormalities of this fine tuning may result in the development of autoimmunity.

In recent years, Treg-cell assessment in SLE has been performed by several groups [39]. Unfortunately, although much effort has been spent to shed some light on Treg imbalance in SLE, conclusive data are still lacking [40]. The majority of these studies reported either reduced number or impaired function of circulating Treg cells in SLE [41–44]. On the opposite, others failed to observe any abnormalities in this T-cell subset and found resistance of effector T cells to regulatory activity of Treg cells [45, 46]. These discrepancies may arise from fair differences between isolation protocols and flow-cytometry technicalities resulting in strong difficulties in comparing results from different studies. Moreover, data regarding Treg cells in SLE target organs, such as kidney, are poor, and, hence, regulatory mechanisms controlling Treg homeostasis within affected tissues are still a matter of debate [47, 48]. According to the aforementioned data regarding putative Treg cells lacking CD25, Zhang et al. recently evaluated circulating $CD25^-$ T cells in SLE patients and surprisingly found an increase of the $CD25^-FoxP3^+$ fraction [49]. Further analysis, however, allowed to conclude that this cell subpopulation was actually divergent from conventional Treg cells, as they failed to exert suppressive activity *in vitro* towards $CD25^-FoxP3^-$ effector T cells [50]. In this setting, albeit FoxP3 is universally accepted as specific marker of Treg cells, it must be taken into account that in some cases its expression may be misinterpreted. Noteworthy, it has been recently suggested that, besides the expression itself, FoxP3 intensity is the true discriminator between effector and regulatory T cells. Indeed, $FoxP3^{low}$ T cells often produced IL-2 independently on CD25 surface levels, whereas $FoxP3^{high}$ T cells did not [51]. Furthermore, we demonstrated that among the $CD25^-$

cell subset, only those coexpressing GITR on the surface display *in vitro* suppressive activity that can be reverted by antibody-mediated GITR blockade [34]. In conclusion, the definite role of Treg-cells in SLE is still uncertain and further studies are required to shed some light on this controversial issue. At the same time, the identification of more specific phenotypic Treg cell markers in an attempt to minimize variability between different studies are surely needed.

4. The Interplay between Th17 and Treg Cells in SLE: Who's Who?

Besides the above-mentioned difficulty to clarify the effective role played by Th17/Treg cells in SLE pathogenesis, recent studies made this matter even more complex providing the clue of a plasticity between the two T-cell subsets [38]. As suggested by Lee et al., it appeared that TGF-β plays a dual role on naïve T cells depending on the presence or absence of IL-6. The combination of TGF-β and IL-6 allows the differentiation toward a Th17 phenotype, whereas if TGF-β is present alone, iTreg cells will be generated [10, 11, 52]. Furthermore, transition between Th17 and Th1 cells may also be possible. Indeed, several groups have generated Th17 cells *in vitro* and adoptively transferred to induce autoimmune disease in mice. After transfer *in vivo*, Th17 cells quickly acquire the ability to produce IFN-γ, as Th1 cells do, and lose their capacity to release IL-17 [53, 54]. A similar behavior was observed *in vitro* following several culture rounds. It has been proposed that the shift from Th17 to Th1 cell may be due, at least in part, to the fact that Th17 cells express IL-12 receptors and readily produce IFNγ in response to IL-12 exposure [55]. In addition, also an epigenetic mechanism may underlie the plasticity of $CD4^+$ T helper cell differentiation, as recently suggested [56].

These findings draw our attention to new intriguing scenarios in which the cytokine milieu is the key player that globally drives immune response towards either health or disease [57]. However, it has been already described that SLE flares may occur as a consequence of cytokine imbalance and eventually of the Th17/Treg ratio in lupus-prone mice [8]. More recently, it has been demonstrated that such imbalance is not limited to SLE flares but is hallmark of the disease, since also patients with quiescent disease display a Th17/Treg ratio favoring Th17 cells [58, 59]. Taken together, these evidences prompt therapeutic approaches aimed to restore an adequate cytokine network and Th17/Treg balance in SLE. Indeed, although Th17 cells play a key role in the pathogenesis of the disease, Th1 and other effector cells are also involved in the perpetuation of autoimmune response [14, 60–63]. Therefore, selective Th17 targeting may not be sufficient to counteract chronic inflammation in SLE patients. On the other hand, restoring the immune balance between Th17 and Treg cells may help to achieve a better clinical response. These observations are strengthened by the evidence that selective Th17 blockade led to disease worsening in a murine model of colitis and exacerbated acute graft-versus-host disease, reasonably for a rebound increase of Th1 cells [64, 65]. In this context, it is noteworthy that previous studies have confirmed the ability of some agents, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), rapamycin, and the vitamin A metabolite all-trans-retinoic acid, to promote the conversion of Th17 to Treg cells in experimental autoimmune encephalomyelitis [66–68]. It is to note, however, that TCDD is toxic in animals and humans, while all-trans-retinoic acid and rapamycin have not been yet tested in humans. Moreover, the nucleosomal histone peptide epitope H471-94 appears to be able to induce generation of Treg cells and suppression of inflammatory Th17 cells in lupus-prone mice, through the induction of tolerogenic dendritic cells rather than via a direct effect on Treg/Th17 cells [69].

In conclusion, the aforementioned data suggest that targeting Th17 and Treg cells for therapeutic purposes in SLE may be possible. However, further investigations aimed to identify well-tolerated and powerful compounds that induce the diversion of T-cell differentiation from Th17 cells to Treg cells are needed [70, 71].

5. Conclusions

Aberrant T-cell homeostasis is a crucial event in SLE pathogenesis, and Th17/Treg imbalance appears to represent an important key pathogenic player. However, many aspects of such a deregulation in the course of the disease are still uncertain, and conclusive data about specific underlying mechanisms are lacking. Promising results concerning therapeutic targeting Th17/Treg cell balance may open new lines of investigation for SLE treatment in the near future.

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Review Article

Autoantibodies and Resident Renal Cells in the Pathogenesis of Lupus Nephritis: Getting to Know the Unknown

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Systemic lupus erythematosus is characterized by a breakdown of self-tolerance and production of autoantibodies. Kidney involvement (i.e., lupus nephritis) is both common and severe and can result in permanent damage within the glomerular, vascular, and tubulo-interstitial compartments of the kidney, leading to acute or chronic renal failure. Accumulating evidence shows that anti-dsDNA antibodies play a critical role in the pathogenesis of lupus nephritis through their binding to cell surface proteins of resident kidney cells, thereby triggering the downstream activation of signaling pathways and the release of mediators of inflammation and fibrosis. This paper describes the mechanisms through which autoantibodies interact with resident renal cells and how this interaction plays a part in disease pathogenesis that ultimately leads to structural and functional alterations in lupus nephritis.

1. Introduction

Lupus nephritis is a severe organ manifestation of systemic lupus erythematosus (SLE) that can affect up to 70% of the SLE population [1]. Depending on the severity of disease, 10–30% of these patients will progress to end-stage renal failure. Lupus nephritis is characterized by the production of anti-double-stranded (ds) DNA antibodies and immune-mediated injury in the glomerular, vascular, and tubulo-interstitial compartments of the kidney [2–9]. If left untreated, destruction of the normal renal parenchyma and their replacement with fibrous tissue ensues [7]. Lupus nephritis follows a relapsing-remitting pattern in which the frequency of flares differs between individual patients. Clinical manifestations of active lupus nephritis include proteinuria, active urinary sediments, and progressive renal dysfunction [10, 11].

Anti-dsDNA antibodies have been shown to contribute to the pathogenesis of lupus nephritis. Many features of lupus nephritis can be replicated in nonautoimmune mice after either intraperitoneal administration of human or murine anti-dsDNA antibodies or inoculation with the transgene that encodes the secreted form of an IgG anti-DNA antibody [12, 13]. It has remained intriguing how these antibodies deposit in the kidneys and trigger intrarenal pathogenic mechanisms. Various mechanisms of antibody binding have been proposed, some of which remain controversial. The origin of anti-dsDNA antibodies, their pathogenic role, and the characteristics associated with nephritogenic property have been extensively studied in experimental and *in vitro* systems [2, 3, 6, 13–15]. The data to date shows that polyreactivity and the ability to interact with various cell surface, intracellular, or extracellular molecules could be a pivotal property that allows the antibodies to elicit injury in the kidney [16–19]. This paper will discuss the contributing roles of resident renal cells in the pathogenesis of lupus nephritis through their interaction with anti-dsDNA antibodies, thereby inducing inflammatory and fibrotic processes in the kidney. Mechanisms through which lymphocytes and macrophages contribute to the pathogenesis of lupus nephritis have been discussed in recent papers [20–22].

1.1. Anti-dsDNA Antibodies and Lupus Nephritis. Production of autoantibodies is a cardinal feature of SLE [23]. The production of antibodies towards chromatin material, in particular to dsDNA, is strongly associated clinically with

Table 1: Autoantibodies with pathogenic potential in patients with lupus nephritis.

Autoantibodies	Prevalence (%)	Binding to kidney structure/resident renal cells	References
Anti-dsDNA	70–96	GBM	[29–31]
		Mesangial cells	
		Glomerular epithelial cells	
		Glomerular endothelial cells	
		Proximal tubular epithelial cells	
Anti-nucleosome	60–90	GBM	[29, 31, 32]
		Mesangial cells	
		Glomerular epithelial cells	
		Glomerular endothelial cells	
Anti-Ro	25–44	GBM	[29, 33]
Anti-Smith	10–60	GBM	[29, 30, 33]
Anti-C1q	40–97	GBM	[29, 31, 34, 35]
		Glomerulus	
		Tubular basement membrane	
Anti-α-actinin	20	Glomerulus	[16, 17, 29, 36]
		Mesangial cells	
		Podocytes	
Anti-annexin II	32–65	Glomerulus	[18]
		Mesangial cells	
Anti-ribosomal P protein	75	Glomerulus	[37, 38]
		Mesangial cells	

lupus nephritis [4–6, 23–28]. Other autoantibodies have also been described in patients with lupus nephritis [18, 29–38] and these are listed in Table 1.

Anti-DNA antibodies constitute a subgroup of anti-nuclear antibodies that bind to either single-stranded or double-standard DNA [2]. These antibodies form part of the normal spectrum of natural antibodies in healthy individuals which are predominantly of the IgM class and react weakly with self-antigens. In lupus patients, these "natural" antibodies undergo an isotype switch to IgG that increases their pathogenic potential [2]. Somatic mutations in the encoding immunoglobulin genes can also result in the secretion of high-affinity IgG anti-dsDNA antibodies [2, 39]. It is this subset of anti-dsDNA antibodies that have been implicated in pathogenesis of SLE and glomerulonephritis. Anti-dsDNA antibodies of the IgG subclass, in particular those of the IgG_1 and IgG_3 subclass which can fix complement, are important in pathogenesis and also as a disease biomarker [2, 40, 41]. Anti-dsDNA antibodies have been detected in the sera of SLE patients before clinical onset of disease [42], and the prevalence of anti-dsDNA antibodies in patients with lupus nephritis is 70–96% compared to 0.5% in patients with nonlupus autoimmune disease or in healthy subjects [29, 31, 43]. Other factors that determine the nephritogenicity of anti-DNA antibodies include avidity of antigen binding, charge, and amino acid sequence in the complementarity determining region, as reviewed by Foster et al. [8] and Isenberg et al. [27].

Circulating levels of anti-dsDNA antibodies correlate with disease activity in many patients [4, 24, 27, 44]. Winfield et al. demonstrated that the affinity of circulating anti-dsDNA antibodies to dsDNA correlated with the activity of nephritis [14]. They also noted that the anti-dsDNA activity in IgG fractions eluted from nephritic glomeruli was higher than that in corresponding serum samples [14].

2. Mechanisms through Which Lupus Autoantibodies Mediate Kidney Injury

Onset of lupus nephritis is initiated by the deposition of anti-dsDNA antibodies in the renal parenchyma. The exact mechanism through which anti-dsDNA antibodies are deposited in the kidney to mediate kidney injury remains to be fully elucidated. Three mechanisms have been proposed, and they include (1) the deposition of preformed circulating DNA/anti-dsDNA immune complexes in the kidney, (2) binding of antibodies to antigens deposited within the kidney—the "planted antigen" theory, and (3) direct binding to cross-reactive antigens present either on the surface of resident renal cells or in their extracellular environment.

2.1. Entrapment of Circulating Preformed DNA/Anti-dsDNA Immune Complexes. It had been postulated that renal injury in lupus patients was due to the passive entrapment of circulating preformed DNA/anti-dsDNA immune complexes

in the glomerulus. This theory has now been disproved since preformed immune complexes are difficult to detect in the blood, and studies have demonstrated that they are only transiently localized to the glomeruli before they are rapidly removed by the liver [45]. Following administration to nonautoimmune mice, preformed DNA/anti-dsDNA immune complexes have no affinity for components of the glomerular basement membrane (GBM) [46]. Furthermore, after administration of these immune complexes to lupus-prone mice, the level of anti-dsDNA antibodies and disease activity decreased [47].

2.2. "Planted Antigen" Theory. In the "planted antigen" theory, chromatin materials released into the circulation from apoptotic or necrotic cells are entrapped within the GBM where they serve as "planted antigens" to mediate binding of anti-dsDNA antibodies. Studies have suggested that the positively charged histone component of nucleosomes may initially bind to heparan sulfate proteoglycans in the GBM through charge-charge interactions, which exposes the DNA component of the nucleosome to act as a "planted antigen" or intermediate bridge for anti-dsDNA antibody binding [48, 49]. Subsequent studies have corroborated that anti-dsDNA antibodies can bind to the glomerulus through nucleosomes [26, 48–50]. Kramers et al. demonstrated that the perfusion of anti-dsDNA antibodies complexed to nucleosomal material into Wistar rats resulted in their deposition in the glomerular capillaries [51]. Subcutaneous administration of heparin to NZB/W F1 mice resulted in reduced nucleosome-containing immune complexes in the GBM and delayed development of disease manifestations suggesting that heparin may compete with extracellular heparan sulfate proteoglycans for nucleosome binding, thereby reducing immune complex formation in the kidney parenchyma [52]. Ultrastructural studies by Rekvig's group have demonstrated that anti-dsDNA antibodies colocalize with chromatin material in electron-dense deposits in the diseased kidney [26, 50]. The role of nucleosomes in the pathogenesis of lupus nephritis has been reviewed by Mortensen and Rekvig [53]. Anti-nucleosome antibodies have also been detected in SLE patients particularly in patients with renal flare [54]. Some have proposed that anti-nucleosome antibodies may be a disease biomarker for lupus nephritis [54, 55].

2.3. Cross-Reactivity with Non-DNA Antigens. Autoreactivity to native DNA *per se* does not appear to be a property of anti-dsDNA antibodies that are responsible for inducing renal injury. Immunization of non-autoimmune mice with mammalian DNA failed to induce the production of pathogenic anti-dsDNA antibodies or clinical manifestations of disease. Rather, there is emerging evidence that polyreactivity of anti-dsDNA antibodies, independent of chromatin material acting as a bridge for binding, confers pathogenic potential. Polyreactivity of anti-dsDNA antibodies may be related to structural or conformational similarity, or molecular mimicry [56]. Cross-reactivity of anti-dsDNA antibodies was first observed by Raz et al., who demonstrated that human and murine anti-DNA antibodies could bind directly to renal antigens in isolated rat kidneys, and this resulted in the induction of proteinuria [57]. Krishnan et al. demonstrated that anti-dsDNA antibodies from lupus-prone mice when injected intravenously into BALB/c mice could bind to the GBM and mesangial matrix and induce disease manifestations, and that these processes were independent of the binding of the antibodies to chromatin material [58]. Waters et al. observed that NZM congenic mice developed chronic glomerulonephritis in the absence of anti-dsDNA antibodies [59]. Christensen et al. also noted that nephritis developed in Toll-like receptor-9- (TLR-9) deficient lupus-prone mice despite the absence of anti-dsDNA antibodies [60]. It is thus possible that the reactivity of antibodies towards DNA or chromatin material *per se* may not be critical for the development of lupus nephritis, but rather the ability of autoantibodies to bind to various antigens in the renal parenchyma. α-actinin, heparan sulfate proteoglycan, laminin, fibronectin and collagen have been reported as putative antigens that are recognized by anti-dsDNA antibodies [16–18, 61–63]. However, some of the data were derived from experiments with murine monoclonal anti-DNA antibodies with uncertain clinical relevance in human lupus. Also, some studies employed solid-phase binding assays, which could introduce binding artifacts and conformational changes to the surface-bound antigens [64], and therefore, *in vitro* and experimental studies should be undertaken to confirm such findings. More recently, our group showed that human anti-dsDNA antibodies could bind to annexin II on the surface of human mesangial cells and induce changes in cell function [18].

3. Anti-dsDNA Antibody Binding to Kidney Cells and Renal Injury

Renal injury in lupus nephritis is initiated by the deposition of autoantibodies and/or immune complexes in the renal compartments. Downstream pathogenic effector mechanisms include activation of the complement and coagulation cascades, infiltration of acute and chronic inflammatory cells, and induction of mediators of inflammation or fibrosis from resident kidney cells and infiltrating cells. Polyclonal B-cell activation and autoantigen-driven expansion of autoreactive B cells result in the increased production of polyclonal anti-dsDNA antibodies in lupus patients and their deposition in sites of injury [65, 66]. Morphologic changes in the kidney are variable as reflected by the spectrum of pathological changes in lupus nephritis [67]. Previous studies have demonstrated heterogeneity in the molecular pathogenesis between patients with lupus nephritis [68]. Depending on the type, duration, and severity of lupus nephritis, immune deposits can be found in the mesangium, subendothelial, subepithelial, and tubulointerstitial regions [67]. Deposition of cationic immune deposits in the mesangial or subendothelial compartments can initiate the recruitment of inflammatory cells and the activation of resident mesangial and endothelial cells [56]. Immune deposition in the subepithelial area is associated

Table 2: Binding of anti-dsDNA antibodies to resident renal cells and the effect on cellular functions.

	Mesangial cells	Endothelial cells	Proximal renal tubular epithelial cells
Mechanism of binding	Indirect binding through DNA, histones, and nucleosomes Cross-reactive binding to heparan sulfate ribosomal P protein laminin α-actinin annexin II	Indirect binding through DNA, histones, and nucleosomes Cross-reactive binding to hevin unidentified proteins with M.W. of 30–35, 44, 68, 110, and 180 kDa	Indirect binding through DNA Cross-reactive binding to A and D snRNP proteins
Internalization of anti-dsDNA antibodies into resident renal cells	Occurs after binding to annexin II	Occurs after binding to fibronectin	Occurs after binding to unidentified protein(s)
Effect on cell proliferation	Increase	Increase	Increase
Induction of apoptosis	Yes	Yes	Yes
Effect on cell viability	Decrease	Decrease	Decrease
Effect on inflammation	Increased synthesis of: IL-1β, IL-6, and TNF-α hyaluronan	Increased synthesis of: IL-1β, IL-6, and IL-8 adhesion molecules von Willebrand factor	Increased synthesis of: IL-1β IL-6 TNF-α
Effect on fibrosis	Activation of PKC-α, -βI, and βII signaling pathways and increased synthesis of TGF-β1 and fibronectin	Increased gene expression of TGF-β1	Induced epithelial-to mesenchymal transdifferentiation

with podocyte injury and proteinuria, while the GBM acts as a barrier for leukocyte infiltration [56]. Irrespective of the site of initial or predominant injury, downstream events such as deposition of extracellular matrix and renal scarring constitute a final common pathway.

Data from *in vitro* studies have demonstrated that anti-dsDNA antibodies can bind to mesangial cells, glomerular epithelial cells (podocytes), endothelial cells, and proximal tubular epithelial cells [4, 5, 69–72], and that such binding led to functional changes in these cells [5, 18, 72–77]. The following discussion will focus on the results to date on the interaction between anti-dsDNA antibodies and resident renal cells in the context of the pathogenesis of lupus nephritis (Table 2).

3.1. Anti-DNA Antibodies and Mesangial Cells. Mesangial cells constitute up to 40% of the total cells in the glomerulus and are situated centrally within the glomerulus [78]. They are contractile and have morphological and functional properties similar to smooth muscle cells. Mesangial cells are able to synthesize a plethora of cytokines, growth factors, and matrix proteins which, together with their contractile property, provide structural support to the capillary loops and contribute to kidney homeostasis. Mesangial cells contribute to the synthesis and remodeling of extracellular matrix, which together with the cells constitute the mesangium. Qualitative and quantitative changes to the mesangial matrix can have a profound effect on mesangial cell function and behavior [78, 79]. As a corollary, these properties also explain the pathophysiology that follows mesangial cell injury.

Deposition of immunoglobulins and activation of complement within the mesangium is a cardinal feature in lupus nephritis, while complement activation plays an important role in the pathogenesis of different types of glomerular diseases [80]. Fenton et al. demonstrated that deposition of immune complexes in the mesangium of NZB/W F1 mice during the early phase of disease was accompanied by the appearance of anti-dsDNA antibodies, which preceded the downregulation of DNase 1 mRNA and activity [81]. In this regard, reduced renal expression of DNase 1 in lupus-prone mice is thought to be a mechanism for reduced fragmentation and clearance of chromatin material [53]. There is evidence that mesangial cells can synthesize C3, which is increased when the cells are incubated with immune complexes [82]. Mesangial cells thus have the potential to contribute to complement activation in the kidney and to complement-mediated injury in the mesangium.

The administration of anti-dsDNA antibodies to either predisease NZB/W F1 or BALB/c mice results in their deposition in the glomerulus, including the mesangium, through indirect chromatin-mediated or direct cross-reactive binding [13, 18, 83]. We and others have demonstrated that anti-dsDNA antibodies can bind to mesangial cells through chromatin material [69] or through the direct interaction with cross-reactive antigens such as α-actinin, annexin II,

or ribosomal P protein, and that such binding may or may not be dependent on the Fc portion [16–18, 38, 84]. The functional consequences of this interaction include increased mesangial proliferation, apoptosis, activation of the PKC and MAPK signaling pathways, and increased synthesis of proinflammatory cytokines and profibrotic mediators such as hyaluronan, IL-1β, IL-6, TNF-α, TGF-β1, and fibronectin [18, 28, 72, 75, 76, 84–86].

The data that α-actinin can mediate the binding of anti-dsDNA antibodies to mesangial cells is intriguing, since α-actinin is an intracellular constituent of the mesangial cytoskeleton. α-actinin is present in multiple subcellular regions such as cell-cell and cell-matrix contact sites, in addition to cellular protrusions and lamellipodia [87, 88]. It is thus possible that part of the α-actinin molecule may extrude through the plasma membrane of mesangial cells to permit its binding with anti-dsDNA antibodies, although this needs to be confirmed by further studies. We have previously demonstrated that α-actinin expression is increased within the mesangium of patients with proliferative renal diseases [88]. Consistent with our finding, Zhao et al. also observed increased α-actinin expression in mesangial cells isolated from MRL/lpr mice [89], thereby suggesting increased availability of α-actinin for anti-dsDNA antibody binding. The pathogenic role of α-actinin as a cross-reactive antigen has recently been questioned by Mjelle et al. who demonstrated that anti-dsDNA antibodies did not colocalize with α-actinin in kidneys obtained from NZB/W F1 mice, but instead bound to glomerular structures containing extracellular nucleosomes [90].

Annexin II is a calcium-dependent, phospholipid binding protein that is expressed in various organs including the kidney and can exist either as a monomer, heterodimer, or heterotetramer [91]. Annexin II is present within the cytoplasm and on the plasma membrane of various cells [91], and its translocation from the cytoplasm to the plasma membrane is increased following its phosphorylation that can be induced by various cytokines and growth factors such as IFGF and EGF or heat stress [92, 93]. It functions as a plasminogen receptor, thus regulating fibrin homeostasis and angiogenesis and also membrane trafficking [94]. Autoantibodies to annexin II are detected in patients with antiphospholipid syndrome, and annexin II has been shown to activate endothelial cells following their exposure to antiphospholipid antibodies [95]. We have recently demonstrated that annexin II is a cross-reactive antigen on the surface of human mesangial cells that mediates the binding of human polyclonal anti-dsDNA antibodies [18]. We further demonstrated that following the binding of anti-dsDNA antibodies to annexin II, the antibodies were internalized and translocated to the cytoplasm and nucleus in a time- and temperature-dependent manner [18]. The ability of anti-dsDNA antibodies to penetrate live cells was first observed by Alarcon-Segovia and Llorente in human mononuclear cells [96], and subsequently by Yanase et al. in rat H35 hepatoma cells [97, 98]. It is noteworthy that entry of antibodies into cells is not unique to anti-dsDNA antibodies, since this phenomenon has also been observed with autoantibodies against nuclear ribonucleoprotein, ribosomal P protein, La, and Ro in human and animal cells [99–102].

Data from our laboratory showed that binding of anti-dsDNA antibodies to annexin II on mesangial cell surface induced annexin II synthesis in the cells, and the latter was mediated through the activation of p38 MAPK, JNK, and AKT/PI3K signaling pathways. There was also a concomitant increase in cell proliferation, induction of IL-1β, TNF-α, IL-6, and hyaluronan secretion, activation of the PKC signaling pathway, and upregulation of TGF-β1 and fibronectin synthesis [18, 75, 76]. These findings thus propose a new paradigm by which anti-dsDNA antibodies contribute to progressive inflammatory and fibrotic processes in the pathogenesis of lupus nephritis. The effect on IL-6 is worth highlighting, since this cytokine has been shown to increase mesangial cell proliferation and exacerbate glomerulonephritis [103]. Our results from animal experiments and human renal biopsies also showed that annexin II expression was increased in the mesangium and GBM of NZB/W F1 mice and patients with active lupus nephritis, and it colocalized with IgG and C3 deposition [18]. Intercepting the interaction between anti-dsDNA antibodies and annexin II on mesangial cells may therefore be a potential novel approach for the treatment of lupus nephritis.

The mechanism(s) through which autoantibodies are internalized, and the functional consequence of this process, have yet to be fully elucidated, but it has been suggested that some of these autoantibodies may be internalized through an Fc receptor-dependent mechanism, which was associated with cellular changes such as cytotoxicity and apoptosis [104]. Internalization and nuclear localization of anti-dsDNA antibodies may also be dependent on their polyreactivity and the presence of nuclear localizing motifs in the CDR3 region of the heavy chain [105]. It is possible that these autoantibodies are transported intracellularly via clathrin-associated vesicles or are accompanied by chaperones [106]. The administration of murine anti-dsDNA antibodies to non-autoimmune mice resulted in their localization in the cell nuclei of many organs including the kidney, and this was associated with glomerular hypercellularity, increased collagen expression in the mesangial matrix, and proteinuria [107]. However, cellular entry and localization of these anti-dsDNA antibodies were shown to be dependent on the antigen-binding region of the molecule but not mediated through the Fc-receptor, although the role of Fc-mediated inflammation in the mesangium through other pathways cannot be excluded [107, 108].

3.2. Anti-DNA Antibodies and Endothelial Cells. The glomerular capillary endothelium differs from other endothelial cells in that they are flattened and highly fenestrated [109]. Endothelial cell activation and injury is a common occurrence in various immune-mediated glomerular diseases where there is complement activation in the subendothelial space, as observed in severe proliferative lupus nephritis [67, 110]. Activation of glomerular endothelial cells results in the upregulation of adhesion molecules [111], which serves

to stabilize the adhesion of infiltrating leukocytes to the subendothelial and mesangial regions during immune-mediated renal injury.

Anti-endothelial cell antibodies have been detected in the serum of a high proportion of lupus patients, especially during active disease [112–118]. Serum levels of anti-endothelial cell antibodies correlate with the severity of lupus nephritis and serological evidence of endothelial dysfunction [116, 119]. Anti-endothelial cell antibodies have also been shown to induce glomerulonephritis in normal rabbits [120]. Fujii et al. observed that intraperitoneal injection of 17H8a, a hybridoma clone derived from MRL/lpr mice, into SCID mice resulted in the deposition of 17H8a antibodies in the subendothelium and the formation of glomerular lesions similar to those in lupus-prone mice [121]. Furthermore, the 17H8a antibodies could be internalized by human umbilical vein endothelial cells (HUVECs) and glomerular endothelial cells through a mechanism that was mediated by fibronectin and actin polymerization [121]. It was not reported whether 17H8a antibodies were reactive towards dsDNA.

Subsets of murine monoclonal anti-dsDNA antibodies can bind to HUVEC indirectly through chromatin material, indicating that anti-dsDNA antibodies contribute to the repertoire of anti-endothelial cell antibodies [69, 70, 117]. We have previously reported that, in the presence of DNA, some murine anti-dsDNA antibodies are able to bind to an HUVEC plasma membrane protein with an M.W. of 46 kDa, and the ability of DNA to bind to the surface of HUVEC was increased in the presence of IL-1α or TNF-α [70]. Histones could also facilitate the binding of murine monoclonal anti-dsDNA antibodies and DNA to HUVEC, and the degree of binding was influenced by the relative concentrations of antibody, DNA, and histones [69]. In the human setting, polyclonal anti-dsDNA antibodies isolated from different patients with lupus nephritis could bind to HUVEC through two distinct mechanisms. Human polyclonal anti-dsDNA antibodies that required DNA to bind to HUVEC also bound to a protein of M.W. 46 kDa, whereas direct cross-reactive binding was mediated through membrane proteins with M.W. of 30–35, 44, 68, 110, and 180 kDa [122]. Incubation of HUVEC with human polyclonal anti-dsDNA antibodies induced the expression of VCAM-1, ICAM-1, and von Willebrand factor, which was associated with increased expression of IL-1, IL-6, and IL-8, which had been shown to play an important role in the inflammatory processes in lupus nephritis [73, 74, 123, 124].

Accumulating evidence suggests that type I interferons (IFN) such as IFN-α and IFN-γ play an important role in the pathogenesis of lupus nephritis [125, 126]. These proinflammatory peptides are synthesized by both infiltrating and resident renal cells including glomerular endothelial cells [127]. Recent studies have demonstrated that type I IFN synthesized by resident renal cells promoted end-organ disease in an experimental model of autoantibody-mediated glomerulonephritis [128]. It is possible that anti-dsDNA antibodies may also induce synthesis of IFN-α and IFN-γ in endothelial cells and other intrinsic renal cells to mediate downstream inflammatory processes although further studies are warranted to confirm this.

3.3. Anti-DNA Antibodies and Proximal Renal Tubular Epithelial Cells. Approximately 70% of lupus nephritis patients have demonstrable immune aggregates along the renal tubular basement membrane. The tubulo-interstitium occupies up to 90% of the kidney volume. Variable degrees of tubulo-interstitial inflammation and fibrosis are found in practically all forms of chronic progressive renal diseases regardless of the inciting injury, including those which start off as a predominantly glomerular disease, and the severity of tubulo-interstitial changes inversely correlates with renal prognosis [129, 130]. Proximal renal tubular epithelial cells constitute the predominant cell type in the tubulo-interstitium. These cells are responsible for solute transport and reabsorption. They have the ability to synthesize growth factors and matrix proteins and have a high proliferative potential and thus are important in the regeneration of the tubular epithelium in response to acute tubular injury. Upon stimulation by proinflammatory or profibrotic mediators, proximal renal tubular epithelial cells exhibit phenotypic alterations and undergo epithelial-to-mesenchymal transdifferentiation (EMT) [131]. EMT is characterized by the loss of epithelial cell adhesion, cell activation with actin reorganization and *de novo* synthesis of α-smooth muscle actin, disruption of the underlying basement membrane, and increased cell migration and invasion [132–134]. There is compelling evidence that the presence of myofibroblasts predicts progressive fibrosis in animal and human renal diseases [135–140].

We have demonstrated that the deposition of immune complexes in the tubulo-interstitium correlated with circulating anti-dsDNA antibody levels, tubulo-interstitial expression of IL-6, and tubulo-interstitial abnormalities that included tubular atrophy, inflammatory cell infiltration, and interstitial fibrosis [5]. The level of tubulo-interstitial IL-6 expression, predominantly contributed by the proximal tubular epithelial cells, correlated with the infiltration of immune cells into the tubulo-interstitium.

HK-2 cells are normal proximal renal tubular epithelial cells that have been immortalized by transduction with the human papilloma virus [141]. We have demonstrated that anti-dsDNA antibodies from patients with lupus nephritis induced phenotypic changes in HK-2 cells that were analogous to epithelial cells undergoing EMT [5]. We have also demonstrated that anti-dsDNA antibodies, especially those derived from patients during active disease, could induce IL-6 secretion in HK-2 cells [5]. Depending on the disease status, the induction of IL-6 secretion in HK-2 cells by anti-dsDNA antibodies can be through distinct mechanisms. During remission, anti-dsDNA antibodies induced IL-6 secretion either directly or were mediated through IL-1β. In contrast, anti-dsDNA antibodies isolated from patients with active disease induced IL-6 secretion through the prior induction of both IL-1β and TNF-α [5]. The heterogeneity may be related to distinct properties of different clones of anti-dsDNA antibodies. Mediators secreted by mesangial cells and HK-2 cells upon stimulation with anti-dsDNA antibodies can induce IL-6 secretion in the other cell types, indicating that there could be bidirectional communication

or crosstalk between the glomerulus and tubulo-interstitium [5]. Consistent with our findings, Ronda et al. also observed that immunoglobulins isolated from the sera of patients with SLE could induce IL-6 secretion in proximal renal tubular epithelial cells, and this was accompanied by the activation of the ERK signaling pathway [142].

Koren et al. have reported that murine and human anti-dsDNA antibodies cross-reacted with A and D snRNP proteins in porcine proximal tubular epithelial cells (PK15 cells). Of the two murine monoclonal antibodies tested that were derived from NZB/W F1 mice, one anti-dsDNA antibody was internalized and localized in the cytoplasm and nuclei of cells, while the second murine monoclonal antibody remained at the cell surface and was not internalized. The concomitant addition of complement and either murine or human anti-dsDNA antibodies to PK15 cells resulted in cell lysis, which was more prominent with the subset of anti-dsDNA antibodies that were not internalized [143]. Zack et al. demonstrated that the murine anti-dsDNA antibody mAb 3E10 could bind to renal tubules in normal human renal tissue, and intraperitoneal injection of mAb 3E10 into normal BALB/c mice primed with pristane resulted in antibody binding to the plasma membrane of proximal renal tubular epithelial cells and their subsequent internalization and translocation into the nucleus [71]. This binding was dependent on DNA and the Fab portion of the antibody [71]. These studies have thus begun to shed light on the pathogenic pathways through which anti-dsDNA antibodies can induce tubulo-interstitial injury through their interaction with proximal renal tubular epithelial cells.

3.4. Anti-DNA Antibodies and Podocytes. Podocytes are highly differentiated epithelial cells that are found on the outer surface of the glomerular capillary tuft and serve as the final barrier to urinary protein loss by the formation of foot processes and interposed slit diaphragms [144]. We and others have demonstrated that anti-dsDNA antibodies can bind to podocytes *in vitro* and *in vivo* [4, 90, 145]. The administration of a human monoclonal anti-dsDNA/anti-α-actinin antibody to SCID mice resulted in their subepithelial and subendothelial deposition, which was associated with widespread, segmental effacement of podocyte foot processes and proteinuria [145]. Although it is tempting to speculate that anti-dsDNA antibodies may bind to podocytes through α-actinin, studies by Mjelle et al. suggest otherwise [90]. These researchers proposed that nucleosomes mediated the binding of anti-dsDNA antibodies to glomerular structures *in vivo* [90]. Subepithelial immune deposits along the peripheral capillary loops is a cardinal feature in patients with class V lupus nephritis, which results in podocyte hypertrophy, their increased synthesis of matrix proteins, and subsequent thickening of the GBM [146].

4. The Role of Toll-Like Receptors in the Pathogenesis of Lupus Nephritis

TLRs are a class of innate immune receptors that regulate inflammatory and immune responses. They are essential for the induction of adaptive immune responses against microbial infection [147]. Although they are predominantly expressed on leukocytes, resident renal cells also express distinct members of the TLR family depending on the cell type [148]. Accumulating evidence suggests that TLRs contribute to the pathogenesis of lupus nephritis, where nonmicrobial, host-derived nucleic acids activate TLRs and exacerbate disease manifestations [149–151]. TLR-9 recognizes hypomethylated CpG-containing DNA motifs including those of mammalian origin released from injured or stress-induced cells [149, 152]. Activation of TLR-9 by DNA-containing immune complexes is mediated through high-mobility group box proteins and receptor for advanced glycation end-products [149], and once activated, it augments cytokine production in dendritic cells [149, 153]. TLR-9 activation also regulates the production of anti-dsDNA antibodies in lupus-prone mice [60, 154]. The expression of TLR-9 in resident renal cells is arguable since some researchers have shown TLR-9 to localize solely on infiltrating cells [155], whilst other researchers have observed increased TLR-9 expression in tubular epithelial cells and glomerular cells during active lupus nephritis [156, 157]. Increased tubular expression of TLR-9 correlates with proteinuria and tubulo-interstitial injury in lupus patients, whereas increased glomerular TLR-9 expression is associated with a higher activity index [156, 157]. Inhibition of TLR-9 signaling in lupus-prone mice attenuates the development of glomerulonephritis [158] although its pathogenic role in the development of lupus nephritis has recently been questioned [159]. Mesangial and tubular epithelial cells also express TLR1-4 and TLR-6 [148, 155], but their role in the pathogenesis of lupus nephritis remains to be defined.

5. Conclusion

Renal involvement is a major cause of morbidity and mortality in SLE. Pathological manifestations in lupus nephritis are diverse, initiated by the deposition of immunoglobulins and formation of immune complexes in the glomerular and tubulo-interstitial compartments of the kidney. There is emerging evidence that the interaction between anti-dsDNA antibodies and resident kidney cells, notably mesangial cells, proximal renal tubular epithelial cells, glomerular endothelial cells, and possibly podocytes, plays a significant role in disease pathogenesis. Cell surface binding followed by translocation of antibodies to the cytoplasm and/or nucleus precedes the induction of proinflammatory and profibrotic pathways. Distinct mechanisms may apply to different subsets of antibodies, and at different phases of disease. Not only does the elucidation of these processes provide researchers with a better understanding of the role of anti-dsDNA antibodies in pathogenesis, but also it offers potential novel approaches for disease intervention.

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Review Article

Modulatory Function of Invariant Natural Killer T Cells in Systemic Lupus Erythematosus

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Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease with complex immunological and clinical manifestations. Multiple organ failure in SLE can be caused by immune dysfunction and deposition of autoantibodies. Studies of SLE-susceptible loci and the cellular and humoral immune responses reveal variable aberrations associated with this systemic disease. Invariant natural killer T (iNKT) cells are a unique subset of lymphocytes that control peripheral tolerance. Mounting evidence showing reductions in the proportion and activity of iNKT cells in SLE patients suggests the suppressive role of iNKT cells. Studies using murine lupus models demonstrate that iNKT cells participate in SLE progression by sensing apoptotic cells, regulating immunoglobulin production, and altering the cytokine profile upon activation. However, the dichotomy of iNKT cell actions in murine models implies complicated interactions within the body's milieu. Therefore, application of potential therapy for SLE using glycolipids to regulate iNKT cells should be undertaken cautiously.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease with complex immunological and clinical manifestations. Reduced immune tolerance and abnormal activation of T and B cells lead to autoantibody production mainly against protein-nucleic acid complexes, such as chromatin, and small ribonucleoprotein particles. These autoantibodies complexed with their cognate self-antigens deposit within capillaries of various organs and subsequently mediate systemic disorders. The commonly affected organs include the skin, heart, kidneys, lungs, joints, and central nervous system. This disease usually begins in the 20–45-year age range, although it can occur at nearly any age. SLE is more common in women than in men (>8 : 1). Studies using animal models suggest a role of estrogens in the disease development. The induction of SLE depends on hereditary factors and environmental agents, and inherited genes, infections, ultraviolet light, and some medications are all involved. In general, triggers causing cell death, inefficient clearance of apoptotic cells, and improper exposure of intranuclear antigens to an uncontrolled immune system are potential causes of SLE [1].

Reduced immune tolerance leading to an overt immune response normally precludes various autoimmune disorders. Regulatory T-cells play important roles in mediating peripheral tolerance and immune cell homeostasis. Among them, the natural killer T (NKT) cells are a unique subset of T lymphocytes. NKT cells, which express both NK1.1 and the T cell receptor (TCR) in humans and most murine models, are heterogenous containing both CD1d-restricted and CD1d-nonrestricted populations. CD1d-restricted NKT

cells might recognize glycolipids presented by CD1d for development and activation. Type I NKT cells within the CD1d-restricted population express an invariant TCR in the mouse (Vα14Jα18) and human (Vα24Jα18) combined with a limited but not invariant TCRβ chain repertoire (preferentially Vβ8.2, Vβ7, or Vβ2 in the mouse and Vβ11 in human) [2]. These cells are thus classified as invariant NKT (iNKT) cells that account for more than 80% of CD1d restricted NKT cells in mice. Type II NKT cells are also CD1d-restricted; however, they express variable TCR$\alpha\beta$ chain combination and are difficult to identify. The most potent agonist of CD1d-restricted NKT cells, α-galactosylceramide (α-GalCer), a synthetic glycolipid similar to that from an extract of marine sponges, is used widely to define the number and function of type I NKT cells [3]. In this paper, we use the term "iNKT cells" to describe CD1d-restricted NKT cells; however, methods used to identify these cells are described in the text when relevant to avoid confusion.

iNKT cells are innate-like lymphocytes. Immediately upon activation through TCR engagement, iNKT cells secrete a wide array of cytokines and chemokines. These cells also exert cytolytic activity through granzyme B and FasL-induced apoptosis. iNKT cells can upregulate CD80, CD86, and CD40 on antigen-presenting cells (APCs) to mediate downstream immune responses. Therefore, iNKT cells are considered effector cells that bridge the innate and adaptive immune response [4]. iNKT cells are associated with various autoimmune diseases, including type I diabetes experimental autoimmune encephalomyelitis, and arthritis [5]. Studies also indicate that the number and function of circulating iNKT cells decrease in SLE patients although the immunophysiological role of iNKT cells in SLE is unclear.

Various murine lupus models have been used to investigate the effects of the aberrant number and function of iNKT cells on disease activity. MRL/*lpr* mice, which have a defective point mutation in Fas, spontaneously develop inflammatory lesions affecting the skin and kidneys with marked lymphoproliferation and autoantibody production. CD1d-deficient MRL/*lpr* mice show exacerbated skin lesions [6]. The other widely used murine model, NZB/W F1 (BWF1) mice show an increase in activated iNKT cells with age; however, CD1d deficiency accelerates the onset and progression of nephritis [7]. A chemical-induced lupus model showed that exposure to hydrocarbon oils, such as pristane, facilitates SLE progression through an unknown mechanism. CD1d deficiency exacerbated lupus nephritis in this model, suggesting a regulatory role of iNKT cells [8].

In this paper, we discuss recent studies using different murine models to identify the possible roles of iNKT cells in SLE.

2. Numerical Deficiency of iNKT Cells in Human SLE

Changes in the number of iNKT cells are associated with many autoimmune disorders in humans, such as SLE, psoriasis, rheumatoid arthritis, and myasthenia gravis. In human SLE, iNKT cell number is measured using various methods.

Measurement of the expression of TCR Vα24Jα18 mRNA level indicates that the numbers of invariant TCR Vα24Jα18$^+$ CD4 CD8 double negative (DN) T cells are reduced in peripheral blood lymphocytes and in the rheumatoid synovium of patients with SLE [9, 10]. Flow cytometry shows that the number of DN NKT cells expressing TCR Vα24/Vβ11 is lower in the blood of SLE patients than in healthy controls [11]. Because SLE patients develop progressive lymphopenia, the absolute cell number is affected by the reduction in total lymphocyte number. The proportion of iNKT cells can be calculated to determine the level. The frequency of NKT cells (percentages of CD56$^+$CD3$^+$ T cells among all lymphocytes) is lower in patients with SLE than in controls [12]. Studies using 6B11 monoclonal antibody, which binds specifically to the conserved CDR3 region of the Vα24Jα18 TCR [13, 14], have shown that both the percentage and absolute number of iNKT cells are lower in SLE patients than in healthy controls [15]. Another subpopulation of human Vα24$^+$CD8$^+$ iNKT cells express mainly CD161 (NK1.1) and recognize CD1d molecule [16], and the cell number of this population is lower in patients with SLE than in healthy controls [17].

iNKT cell deficiency correlates with Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) [15, 18], suggesting that iNKT cells are involved in the control of disease activity. Although immunosuppressive drugs correlated significantly with log-transformed absolute iNKT cell numbers ($P = 0.036$) in one study [15], the direct effect of medication on iNKT cell numbers was excluded because SLE patients without drug exposure had consistently lower iNKT cell numbers than did healthy controls. Another study found no correlation between drug therapy and the proportion of NKT cells [19]. Thus, the reduction in NKT cells in SLE patients does not appear to be a secondary response to drug therapy.

3. Functional Deficiency in iNKT Cells in Human SLE

In addition to the reduction in iNKT cells in human SLE, the poor response of iNKT cells to α-GalCer has also been demonstrated in SLE patients [11], whose proliferative response of PBMCs was measured in cells cocultured with α-GalCer. The magnitude of the responses varied between subjects, and both good and poor responders were prevalent among both patients and healthy controls. However, the proliferation indices were significantly lower in patients than in healthy controls (median 7.5 versus 28.7, $P < 0.001$) [20]. α-GalCer potently activated iNKT cells to produce IFN-γ and IL-4. The levels of both mRNA and cytokines in the supernatant of α-GalCer-induced PBMCs were lower in SLE patients than in healthy controls.

The lower response of iNKT cells results mainly from their impaired function rather than a defect in the presentation ability of CD1d-bearing cells. In one study, the percentages of CD1d$^+$ PBMCs and monocytes were similar in SLE patients and healthy controls, and the expression level of CD1d on PBMCs and monocytes was also indistinguishable

between SLE patients and healthy controls [20]. To define further the defective function of iNKT cells, sorted antigen-presenting cells (APCs) from patients or controls were cocultured with patients' iNKT cells. $CD3^+6B11^+$ iNKT cells from an SLE patient failed to proliferate upon α-GalCer activation in the presence of monocytes from a healthy control, but iNKT cells from a healthy control were expanded successfully in the presence of monocytes from a healthy control [20]. Another study confirmed that $V\alpha24^+$ DN iNKT cells from nonresponders fail to proliferate in the presence of APCs from responders, whereas APCs from nonresponders could expand iNKT cells from responders [11]. Another study observed an increase in apoptosis of iNKT cells from patients after 7 days of incubation with α-GalCer [15], suggesting that the poor response of iNKT cells might partly result from the susceptibility to activation signaling-induced cell death.

Although the CD1d expression level on B cells and $CD1d^+$ B cells is significantly lower in patients than in controls, in vitro coculture experiments indicate that monocytes, but not B cells, are effective APCs for iNKT cells [15].

These data show that iNKT cells in SLE patients are dysfunctional and suggest that activating this population may have therapeutic potential.

4. Function of iNKT Cells Associated with SLE Disease

Various murine models have shown the importance of iNKT cells in SLE progression and systemic disorders. These models have been analyzed and described in detail [21, 22]. In this paper, we focus on recent studies that clarify the functions of iNKT cells and their associations with SLE.

4.1. Detection of Apoptotic Cells and Triggering of the Immune Response. SLE can cause severe multiple organs failure resulting from autoantibodies induction. These autoantibodies target nuclear antigens that are theoretically inaccessible. It is hypothesized that the inefficient clearance of apoptotic cells is the source of the antigen pool and that secondary necrotic bodies fuel the inflammation [23–25]. Several genetic studies have identified SLE-susceptible loci, such as CRP [26], and C1q [27], which is involved in clearance of dead cells, and these data support the concept that impaired apoptotic cell clearance is involved in SLE. Recent data suggest that cleavage of autoantigens by granzyme B during cytotoxic-T-lymphocytes- (CTL)-induced apoptosis is involved in human systemic autoimmune diseases [28]. Because CTL-induced targets are often pathogen-infected cells, the molecular mimicry between microbial antigens and autoantigens is not the only explanation for the initiation of autoimmunity after infection.

In one study of C57BL/6 mice, injection with irradiated apoptotic cells induced autoantibody production [29]. In this mouse model, deficiency in iNKT cells exacerbated the effects of the disease by increasing the production of autoantibodies and glomerular deposition of IgG immune complex [30]. Injection of apoptotic cells rapidly upregulated the expression of CD69 in splenic iNKT cells; the number of IFN-γ-producing iNKT cells decreased and the number of IL-10-producing iNKT cells increased in the injected mice. Syngenic apoptotic cell transfer into $CD19^{-/-}$ mice induced iNKT cells to limit the activation of wild-type B but not $CD1d^{-/-}$ B cells that were adoptive-transferred, respectively, into $CD19^{-/-}$ recipient. The production of both IgM and IgG3 anti-DNA antibodies was reduced. These data suggest that autoreactive B cells can be regulated by iNKT cells triggered by apoptotic cells in a CD1d-dependent manner.

Increased levels of lysophosphatidylcholine and other oxidized lipids are exposed on the outer leaflet of apoptotic cells [31]. Immunization with these apoptotic cells induces the production of IgM that recognizes oxidized lipids. NKT cells may survey the lipid derivatives on apoptotic cells presented by APCs and then mediate immune tolerance. It was shown recently that apoptotic cells with phosphatidylserine exposed on the outer membrane leaflet can rapidly activate iNKT cells through recognition by T-cell Ig-like mucin-like-1 (Tim-1) on iNKT cells [32]. However, airway hyperactivity was observed rather than improved outcome in this model.

4.2. Modulation of Antibody Production. The fact that SLE progression can be caused by various abnormal stimuli of lymphocyte activation suggests the presence of high immunoglobulin levels in the plasma of SLE patients. However, as expected for a heritable trait, such as SLE, analysis of the blood from relatives of SLE patients with subclinical phenotypes should more precisely reflect the pathogenic mechanism and the relationships with genetic and cellular aberrations.

High plasma IgG levels have been noted in both patients with SLE and their relatives [12, 19]. The levels of total IgG and anti-dsDNA IgG in patients with SLE and their relatives are associated with a low frequency of $V\alpha24^+$ iNKT cells. This result suggests that iNKT cells play an important role in the regulation of IgG production.

Although an inverse relationship between iNKT cells and IgG production has been observed in humans, murine models reveal a dichotomy in the regulation of IgG production by iNKT cells. One study showed that CD1d-reactive iNKT cells contribute to the development of lupus in BWF1 mice by promoting autoantibody production by B cells [7]. Another study showed that purified iNKT cells but not conventional T cells augment the in vitro secretion of IgM, IgG, and anti-dsDNA antibodies by BWF1 B cells [33] and that CD1d and CD40 are indispensable for this interaction. In addition, adoptive transfer into irradiated *nu*/*nu* BALB/c mice of T cells from the spleen of transgenic BALB/c mice expressing the TCR Vα4.4Jα24 and Vβ9 chain recognizing CD1d on syngenic B cells induced lupus and severe immune complex glomerulonephritis, including the production of anti-dsDNA antibodies, in the host mice [34].

Another view suggests that iNKT cells have a suppressive role in the regulation of IgG production. In a model using heterozygous $J\alpha18^{+/-}$ mice, which show similar pathophysiology to human SLE by having a reduced rather than complete absence of iNKT cells, the mice had a significantly higher anti-dsDNA IgG level and increased activation of

autoreactive B cells [30]. Pristane-injected BALB/c mice showed increased autoantibody production and exacerbated nephritis [35, 36]. Further studies of mice with chemically induced diseases examine that the deficiencies in CD1d-restricted cells contribute to the disease.

In lipopolysaccharide-activated mouse models, reconstitution of active Vα14$^+$ iNKT cells in Jα18$^{-/-}$ BALB/c mice downregulated anti-dsDNA antibody and rheumatoid factors production but did not change total IgG levels [37]. iNKT cells increased total IgG production and the appearance of activation markers on B cells through soluble mediators and helper T cells, whereas autoreactive B cells were impaired in a contact- and CD1d-dependent manner. This highlights the ability of iNKT cells to distinguish autoreactive from nonautoreactive B cells. Differences in CD1d expression on autoreactive and nonautoreactive B cells suggest differences in regulation between these cells because CD1d expression is higher on dsDNA-responsive autoreactive B cells.

The potent agonist of iNKT cells, α-GalCer, is used widely to study the effect of iNKT cells in various disease models. With the administration of C8-α-GalCer (with an 8-carbon acyl chain), which skews the serum cytokine secretion toward a Th2 pattern, 50% of BWF1 mice developed lupus nephritis by 30 weeks. And 50% of control BWF1 mice developed proteinuria by about 36 weeks [38]. In contrast to α-GalCer, injection of β-galactosylceramide, a 12-carbon acyl chain containing glycolipid which rapidly reduced the ratio of iNKT cells in the liver and spleen [39], ameliorated lupus and reduced anti-dsDNA IgG2a production. This implies a complicated role of iNKT cells during the progression of autoimmunity and that alternative agonists of iNKT cells produce different outcomes in murine SLE models.

4.3. Modulation of the Cytokine Profile. Abnormal cytokine profiles have been implicated in the loss of immune tolerance and in a variety of autoimmune diseases. Type I NKT cells produce variety of proinflammatory cytokines, including Th1-, Th2-, and Th17-related cytokines. However, the pathophysiology of human SLE is contradictory to be related to the cytokine alteration by NKT cells in patients. Although early reports demonstrated defective Th1 and excessive Th2 responses in lupus [40], recent data suggest that the levels of both Th1 (IFN-γ, IL-12, and IL-18) and Th2 (IL-4, and IL-10) cytokines are increased in the sera of lupus patients [41, 42]. Intracellular cytokine staining reveals comparable IL-4- and IFN-γ-expressing lymphocytes in PBMCs from SLE patients without nephritis and healthy donors [43, 44]. However, in a subgroup of patients with severe lupus nephritis, the intracellular cytokine ratio shifts to a Th1 phenotype [44, 45]. In disease-alleviated SLE patients, decreased IFN-γ-producing cells and increased IL-4-producing CD4$^+$ T cells were observed after corticoid treatment [43] and low-dose UV phototherapy [46], respectively. Although Th1/2-related cytokines might contribute to SLE progression and severity, the cytokine profiles of activated iNKT cells from SLE patients are yet to be determined.

In addition to Th1- and Th2-related cytokines, iNKT cells can also express IL-17 and IL-21 [47, 48]. IL-17 has recently been implicated in the pathogenesis of SLE [49]. Evidence indicates that production of IL-17 is abnormally high in sera of SLE patients [42] and is correlated with SLE disease severity [49, 50]. When activated by IL-17, the PBMC of patients with lupus nephritis produced higher level of total IgG, anti-dsDNA IgG, and IL-6 [51]. IL-17 production is also high in murine models affected by lupus nephritis [52–55]. It shows spontaneously developed germinal centers in the spleen where IL-17$^+$ T cells colocalize with IL-17R$^+$ B cells [55] providing the suggestion that IL-17$^+$ T cells impact B cells in lupus disease. The main source of IL-17 in SLE patients derives from double negative (DN) TCR$\alpha\beta^+$CD4$^-$CD8$^-$T cells [56]. DN T cells are scarce in healthy individuals, but they expand in peripheral blood of SLE patients and infiltrate into kidney with lupus nephritis where they produce proinflammatory cytokines, including IL-17, IL-1β, and IFN-γ [56–58]. Also in lupus murine models, DN T cells are important IL-17 producer [52]. It also demonstrates elevated plasma levels of IL-21 as well as percentages of IL-21 expressing T cells in SLE patients compared with healthy controls [59, 60]; nevertheless, there is no correlation between IL-21 and disease severity or anti-ds DNA titers [59].

The study of CD1-lipid reactive T cells is much more complicated in humans than in mice. In addition to CD1d, CD1a-, b-, and c-restricted T cells in humans are relatively diverse with CD4$^+$, CD8$^+$, or CD4$^-$CD8$^-$ double negative (DN) populations. Although Vα24 DN NKT cells are numerically decreased in SLE patients, the influence of the subsets of other CD1-lipid reactive T cells on SLE pathogenesis in humans should be further investigated.

In murine models, treatment of adult BWF1 mice (age 8–12 weeks) with α-GalCer exacerbated the disease activity, whereas treatment of young BWF1 mice (age 4 weeks) ameliorated SLE symptoms [61]. Moreover, transfer of NK1.1$^+$ T cells from aged SLE mice to young BWF1 mice (before the onset of renal failure) induced proteinuria and swelling of the glomeruli. It has been indicated that iNKT cells expand in aged BWF1 mice and the authors reported that α-GalCer administration induced predominant IFN-γ production in old mice [7]. Use of a blocking anti-CD1d monoclonal antibody to treat BWF1 mice augmented the Th2 responses and ameliorated lupus [61]. These results suggest that the impact of α-GalCer treatment on disease in BWF1 mice varies with age and imply that the cytokine profile of iNKT cells influences the progression of SLE.

In pristane-induced nephritis models, the effect of α-GalCer differs between mouse strains. In BALB/c mice, Th2 responses are induced by treatment with α-GalCer, which protects mice against nephritis. Conversely, in SJL/J mice, treatment with α-GalCer increases the Th1 responses and exacerbates disease [62]. The differences in the effect of α-GalCer seem to correlate with the cytokine profile produced by activated iNKT cells. It is the common regulatory mechanism in several autoimmune diseases, such as experimental autoimmune encephalomyelitis, and type 1 diabetes.

iNKT cells mediate various immune responses, including maintenance of self-tolerance, tumor surveillance, and the response to microbial pathogens. Given the limited TCR

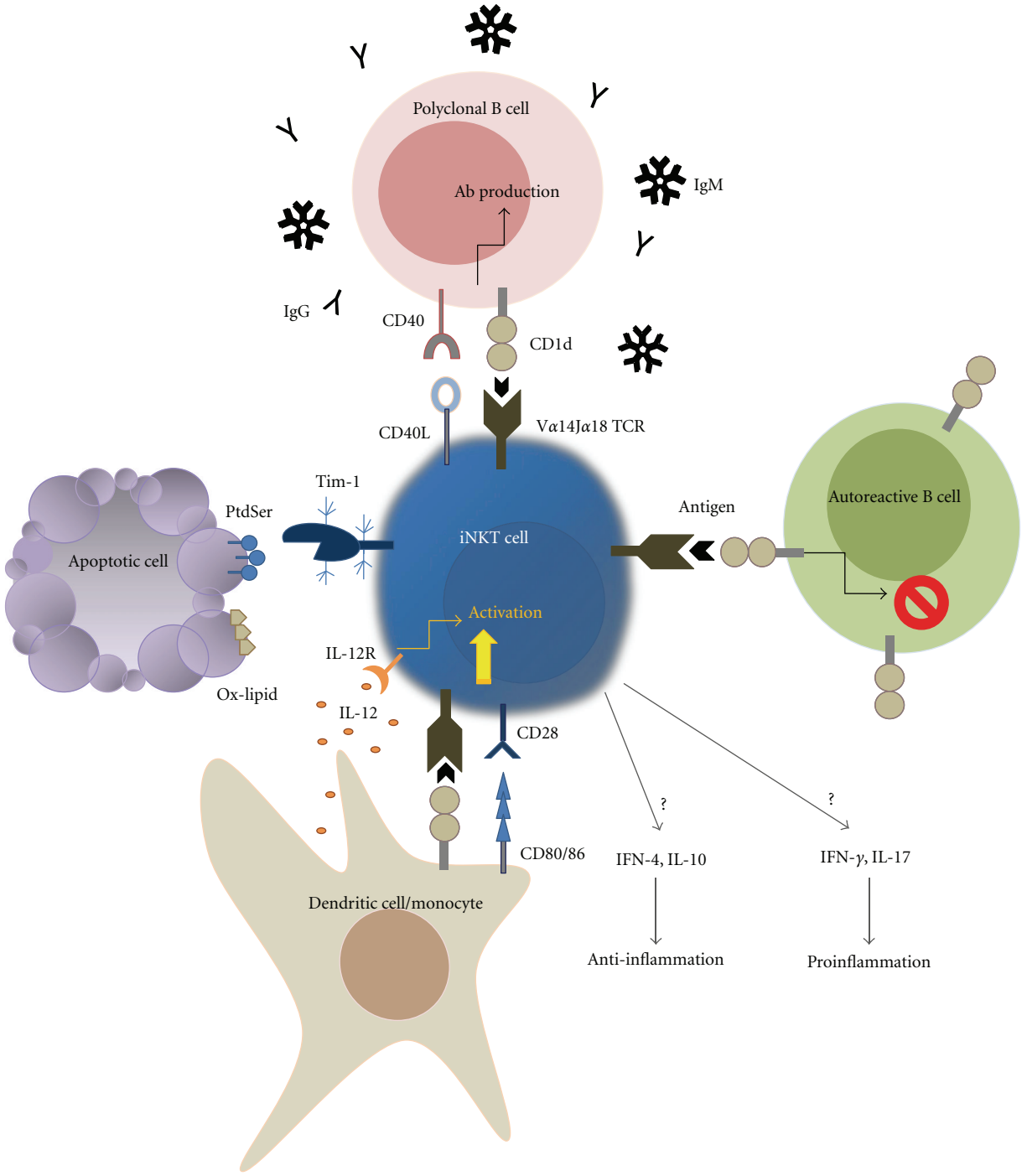

Figure 1: The function of iNKT cells in murine lupus models. iNKT cells in the mouse that express invariant TCR, Vα14Jα18, are CD1d-restricted T lymphocytes. The antigens presented by CD1d can be microbial components, endogenous antigen, iGb3, or oxidized lipid (Ox-lipid) derivatives from apoptotic cells. DCs and monocytes are potent APCs that activate iNKT cells both directly through TCR engagement and indirectly through IL-12. Immediately upon activation, iNKT cells release Th1-, Th2-, and T17-related cytokines, depending on the antigen presented and/or the characteristics of the APCs. The proinflammatory cytokines, IFN-γ and IL-17, lead predominantly to SLE exacerbation. iNKT cells can sense apoptotic blebs through Tim-1, which recognizes phosphatidylserine (PtdSer) exposed on the outer leaflet membrane, and can mediate immune suppression (see text). By contrast, iNKT cells activate B cells and thus upregulate total IgG and IgM levels in a CD1d-dependent manner, but iNKT cells can also inhibit the activation of autoreactive B cells. CD1d expression levels suggest that iNKT cells are capable of discriminating self- from nonself-reactive B cells.

diversity, attention has focused on the mechanisms underlying the activation of iNKT cells [63]. In addition to the microbial glycolipid antigens engaging directly with the invariant TCR on iNKT cells [3], indirect activation of iNKT cells by cytokines or endogenous antigen presentation through microbial-stimulated dendritic cells (DCs) is also possible [64–66]. This may explain the ability of various stimuli to activate iNKT cells in the body and implies that iNKT cells might mediate both beneficial and detrimental outcomes depending on the milieu produced by the activated DCs.

The beneficial roles of iNKT cells are involved in immune tolerance and can ameliorate or prevent tissue inflammation [67, 68]. The suppressive effect is mediated globally through tolerogenic DCs, B cells, or regulatory T cells or directly by skewed cytokine production and induction of apoptosis through Fas-FasL engagement of autoreactive lymphocytes [69]. SLE patients have reduced proportions and functions of iNKT cells, which imply that the suppressive effect is mediated by this population. However, a reduced population of iNKT cells cannot be a diagnostic clinical marker of SLE because the frequency of iNKT cells varies markedly between healthy people. Although the suppressive effect was identified recently in a murine lupus model, the function of iNKT cells in humans needs to be clarified.

Long-term anergy of iNKT cells by reactivation can be induced in mice [70]. The unresponsiveness to αGalCer includes reduced proliferative activity and failure of IFN-γ production. This suggests that the aberrant proportion and function of iNKT cells in SLE patients may reflect only the outcome after repeated exposure to cognate self-antigens. By contrast, Green et al. did not exclude the possibility that the reduced level of iNKT cells results from attack by upregulated antibody in SLE patients [12]. Therefore, iNKT cells may be a potential therapeutic target in the treatment of SLE patients, although the complicated interactions between iNKT cells and other immune cells and the exact function of iNKT cells require further consideration.

5. Conclusion

In this paper, we have discussed the association between iNKT cells and SLE in clinical and murine models. In human SLE patients, the reduced proportion and function of iNKT cells correlate with disease activity and iNKT cells correlate inversely with IgG levels. Recent studies indicate that iNKT cells can sense apoptotic cells and mediate immune tolerance and suggest that iNKT cells can distinguish autoreactive B cells from nonautoreactive B cells to suppress autoreactive antibody production in a CD1d-dependent manner. However, other studies have reported that iNKT cells upregulate total IgG and IgM levels (Figure 1). These findings suggest that iNKT cells are involved in suppressive regulation in SLE.

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Clinical Study

Diminished Expression of Complement Regulatory Proteins on Peripheral Blood Cells from Systemic Lupus Erythematosus Patients

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CD55, CD59, CD46, and CD35 are proteins with complement regulatory (Creg) properties that ensure cell and tissue integrity when this system is activated. The aim of this study was to evaluate the Creg expression on peripheral blood cells from SLE patients and its association with cytopenia and disease activity. Flow cytometric analyses were performed on blood cells from 100 SLE patients and 61 healthy controls. Compared with healthy controls, we observed in SLE patients with lymphopenia and neutropenia decreased expression of CD55, CD59, and CD46 ($P < 0.05$). In SLE patients with anemia, CD59 and CD35 were decreased on red blood cells. Furthermore, there was a negative correlation between CD55 and CD59 on neutrophils and the disease activity. The results suggest there is an altered pattern of Creg expression on the peripheral blood cells of SLE patients, and the expression is correlated with disease activity and/or with activation of the complement system.

1. Introduction

The complement system (CS) represents the first defense line of innate immunity; it acts facilitating the phagocytosis of immune complexes, pathogens, and apoptotic cells and forming the membrane attack complex (MAC), resulting in cell lysis. This powerful defense system is composed of multiple components (>60 different proteins and activation products) that trigger in a cascade-type system [1].

The complement as a central defense system is immediately activated within seconds upon entry of a pathogen into the human host through three pathways: the classical (triggered by antibody-antigen complexes), the lectin (triggered by carbohydrates on the surface of bacteria), and the alternative pathways (spontaneous and continuous process which is initiated by the conformational change of C3). These three pathways use different proteins to produce C3 and C5 convertases, which involve cleavage of C2 and C4 (classical and lectin pathway) or the cleavage of factor B by factor D (alternative pathway). All result in the formation of the lytic MAC (membrane-attack complex: C5b-9) [2, 3]. Activation of the complement system is a powerful drive to initiate inflammation but can, if unregulated, lead to severe tissue damage and disease. Based on their potent damaging capacity, the generation and targeting of complement effector compounds are tightly regulated [4].

Normal cell membranes express complement regulatory (Creg) proteins that regulate activation of the complement system and provide essential protection against self-damage [5]. There are four major human cell surface Creg

proteins: CD59 (membrane inhibitor of reactive lysis—MIRL), which is a complement membrane inhibitor that blocks assembly of the MAC by binding to C8 and C9 [6], CD55 (decay accelerating factor—DAF), which accelerates the disassembly of preformed C3 and C5 convertases [7], CD46 (membrane cofactor protein), which acts as a cofactor for the factor-I-mediated cleavage of the activated complement components C3b/C4b [8], and CD35 (complement receptor type I, CR1), which is also involved in the regulation of C3 fragment deposition and serves as a cofactor for the degradation of C3b by factor I [4]. These Creg proteins are present on the cell surface of whole blood cells, except the CD46, which is not expressed on RBCs. It has been reported that the production and the expression of some of these complement regulatory proteins are altered in autoimmune diseases and that inherited deficiencies of the complement system components are associated with a high prevalence of systemic lupus erythematosus (SLE), glomerulonephritis, and vasculitis [9–11].

The complement system is integrally involved in the pathogenesis of tissue injury in SLE. Tissue deposition of immunoglobulin is a characteristic feature of SLE and can cause continued complement activation by the classical pathway [10]. Therefore, potential differences on the expression of the Creg proteins could implicate different susceptibilities to complement-mediated damage and be clinically significant. Particularly, altered expression on blood cells could be related to cytopenic changes common in this disease. Earlier studies have shown that expression of CD35 [12–16], CD55, and CD59 [17, 18] on erythrocytes and CD55 and CD59 [19–21] on lymphocytes are decreased in patients with SLE, but some of these findings were controversial. The current study aimed to evaluate the expression of CD55, CD59, CD46, and CD35 expression on peripheral blood cells from SLE and healthy controls using flow cytometry and its correlation with cytopenias on these patients.

2. Material and Methods

2.1. Subjects. One hundred patients that fulfilled the American College of Rheumatology classification criteria [22] for SLE and 61 healthy controls with no history of autoimmune diseases were included in the present study. SLEDAI (SLE disease activity index) [23] and SLICC (systemic lupus international collaborating clinics) damage index [24] were applied to each patient as a measurement of disease activity and cumulative damage, respectively.

SLE patients were followed up at the Rheumatology Clinic of Hospital de Clínicas de Porto Alegre, Brazil. The exclusion criterion was concomitant presence of overlap with another autoimmune disease. Peripheral blood samples were collected in Na-EDTA Vacutainer tubes. All SLE patients were receiving an immunosuppressive drug at the time of blood collection (mycophenolate mofetil, cyclophosphamide, azathioprine, methotrexate, cyclosporine, and/or rituximab).

This study was performed with approval of the ethics committee of the Hospital de Clínicas de Porto Alegre, and all subjects were informed about the objectives and procedures of the study and gave their written informed consent.

2.2. Flow Cytometric Analysis of CD55, CD59, CD35, and CD46 on the Cell Membrane. For red blood cell (RBC) staining, 100 uL of diluted blood (with an optimal dilution with phosphate-buffered saline (PBS) to achieve 10000 RBC/uL) as placed into polystyrene tubes (Becton Dickinson (BD) Biosciences, San Diego, CA, USA) and as subjected to two-colour staining with 8 uL/test of fluorochrome-conjugated monoclonal antibodies (MoAbs) against CD55PE, CD59FITC, CD35PE, and CD46FITC (BD Biosciences, San Diego, CA, USA). After 20 min incubation at room temperature, samples were resuspended in 0.5 mL of PBS and cells were analysed on the flow cytometer.

For leukocyte staining, 100 uL of whole blood (with an optimal dilution to achieve 5000 cells/uL) as placed into polystyrene tubes and as subjected to two-colour staining with 8 uL of each antibody of fluorochrome-conjugated MoAbs against CD55PE, CD59FITC, CD35PE and CD46FITC (BD Biosciences, San Diego, CA, USA). After 15 min incubation at room temperature, 1.0 mL of FACSlyse (BD Biosciences, San Diego, CA, USA) was added and lysis was allowed for 10 min at room temperature. Samples were washed once and resuspended in 0.5 mL of PBS.

50000 events were acquired and analysed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, San Diego, CA, USA). Membrane intensity of CD55, CD59, CD46, and CD35, which is proportional to the number of CD55, CD59, CD46 and CD35 epitopes on the membrane, was estimated in the gated subpopulations by one-parameter histograms, and the relative mean fluorescence intensity (MFI) was recorded. The definition of positive and negative cells was set when staining with isotype control was performed, in order to set the gates and distinguish positive staining from autofluorescence and nonspecific antibody binding.

2.3. Serological Studies. Measurement of complement 3 (C3) and complement 4 (C4) is used to determine whether primary deficiencies or activation-related consumption of the complement components is present in SLE patients. C3 and C4 measurements were performed using the ADVIA 1800 chemical analyzer system (Siemens) on patient's sera.

2.4. Complete Blood Cell Count (CBC). A CBC was performed using the Sysmex XE-2100 (Sysmex Corporation, Japan). Slides revised were prepared with SP-100 SYSMEX using a staining program was as follows: May-Grünwald (Bio Lyon, France) pure time: 2.5 min, MG dilute time: 3 min, Giemsa (Bio Lyon, France) time: 7 min, rinse 0 min, and drying time 5 min, as instructed by the supplier.

2.5. Statistics. Data were compared using the Mann-Whitney U test, Student's t-test, and Spearman's correlation coefficient when appropriate. The level of statistical significance was established at $P < 0.05$. Statistical analysis was conducted using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

Table 1: Demographic, clinical, and laboratory features of SLE patients.

Patients' features	SLE ($n = 100$)	Healthy controls ($n = 61$)
Females (%)	93	67.2
Age (year) median (interquartile range)	42 (31–53)	45 (30–61)
SLEDAI[a] median (interquartile range)	2 (0–5)	—
SLICC-DI[b] median (interquartile range)	1 (0–2)	—
Malar rash (%)	58	—
Nephritis (%)	45	—
Arthritis (%)	67	—
AIHA[c] (%)	28	—
RBC ($\times 10^{12}$ cells/uL)	4.15 (0.55)[d]	4.4 (0.36)
Hemoglobin (g/dL)	12.0 (1.6)[d]	13.5 (1.2)
Platelets ($\times 10^3$ cells/uL)	208 (65)[d]	228 (45)
Leucocytes ($\times 10^3$ cells/uL)	5.43 (4.07–7.91)[e]	6.96 (6–8.59)
Lymphocytes ($\times 10^3$ cells/uL)	1.32 (0.85–1.79)[e]	2.25 (1.75–2.85)
Neutrophils ($\times 10^3$ cells/uL)	3.58 (2.22–5.29)[e]	3.77 (3.08–4.74)
Monocytes ($\times 10^3$ cells/uL)	0.48 (0.37–0.68)[e]	0.58 (0.6–0.75)
Thrombocytopenia* (%)	16	0
Leukopenia* (%)	17	0
Lymphopenia* (%)	38	0
Neutropenia* (%)	13	0
Anemia* (%)	21	0
C4 level	25.4 (16.8)	—
C3 level	108.4 (28.1)	—

[a]SLEDAI: systemic lupus erythematosus disease activity index.
[b]SLICC-DI: systemic lupus international collaborating clinics damage index.
[c]AIHA: autoimmune hemolytic anemia (positive Coombs' test).
[d]Mean ± SD.
[e]Median (interquartile range).
*Lymphopenia: <1200 lymphocytes/uL, neutropenia: <1500 neutrophils/uL, anemia: hemoglobin < 11 g/dL, and thrombocytopenia: platelets < 150.000 cells/uL.

3. Results

The description of the 100 patients and 61 healthy controls is summarized in Table 1. Of the SLE patients, 38% had lymphopenia (lymphocytes: <1200/uL), 13% had anemia (hemoglobin < 11 g/dL), 21% had neutropenia (neutrophils < 1500/uL), and 16% had thrombocytopenia (platelets < 150.000/uL). These disease manifestations and cell counts were at the time the blood sample was taken, and the patients were not subdivided by the number of cytopenic manifestations. None of these cytopenias were observed in the healthy control group.

3.1. Neutrophil Analyses. In SLE patients, the MFIs of all Cregs on neutrophils (granulocytes) were significantly lower than those of healthy controls (Table 2). When comparing neutropenic (13/100) with non-neutropenic SLE patients, all Cregs, with the exception of CD46, were significantly decreased (Figure 1).

There was a negative correlation between CD55 ($r = -0.278, P = 0.019$) and CD59 ($r = -0.23, P = 0.048$) expression on neutrophils and the SLEDAI; beside that, there was a positive correlation between CD55 ($r = 0.237, P = 0.021$) and CD35 ($r = 0.334, P = 0.030$) expression on neutrophils and C3 serum levels in SLE patients, and CD55 ($r = 0.334, P = 0.001$) with C4 level.

When analyzing only neutropenic SLE patients, a positive correlation was shown between CD59 on neutrophils and C4 serum levels ($r = 0.828, P = 0.006$).

3.2. Lymphocyte Analyses. In SLE patients, the MFIs of CD55, CD59, and CD46 on lymphocytes were significantly lower than those of healthy controls (Table 2). When comparing lymphopenic (38/100) with non-lymphopenic SLE patients, only CD55 and CD59 were significantly decreased (Figure 2).

There was a positive correlation between CD55 ($r = 0.231, P = 0.026$) expression on lymphocytes and C3 serum levels in SLE patients, and no association with SLEDAI or SLICC.

3.3. Monocyte Analyses. In SLE patients, only the MFI of CD55 on monocytes was significantly lower than that of healthy controls (Table 2). There was no correlation between Creg expression on monocytes and C3 and C4 level or SLEDAI and SLICC in SLE patients.

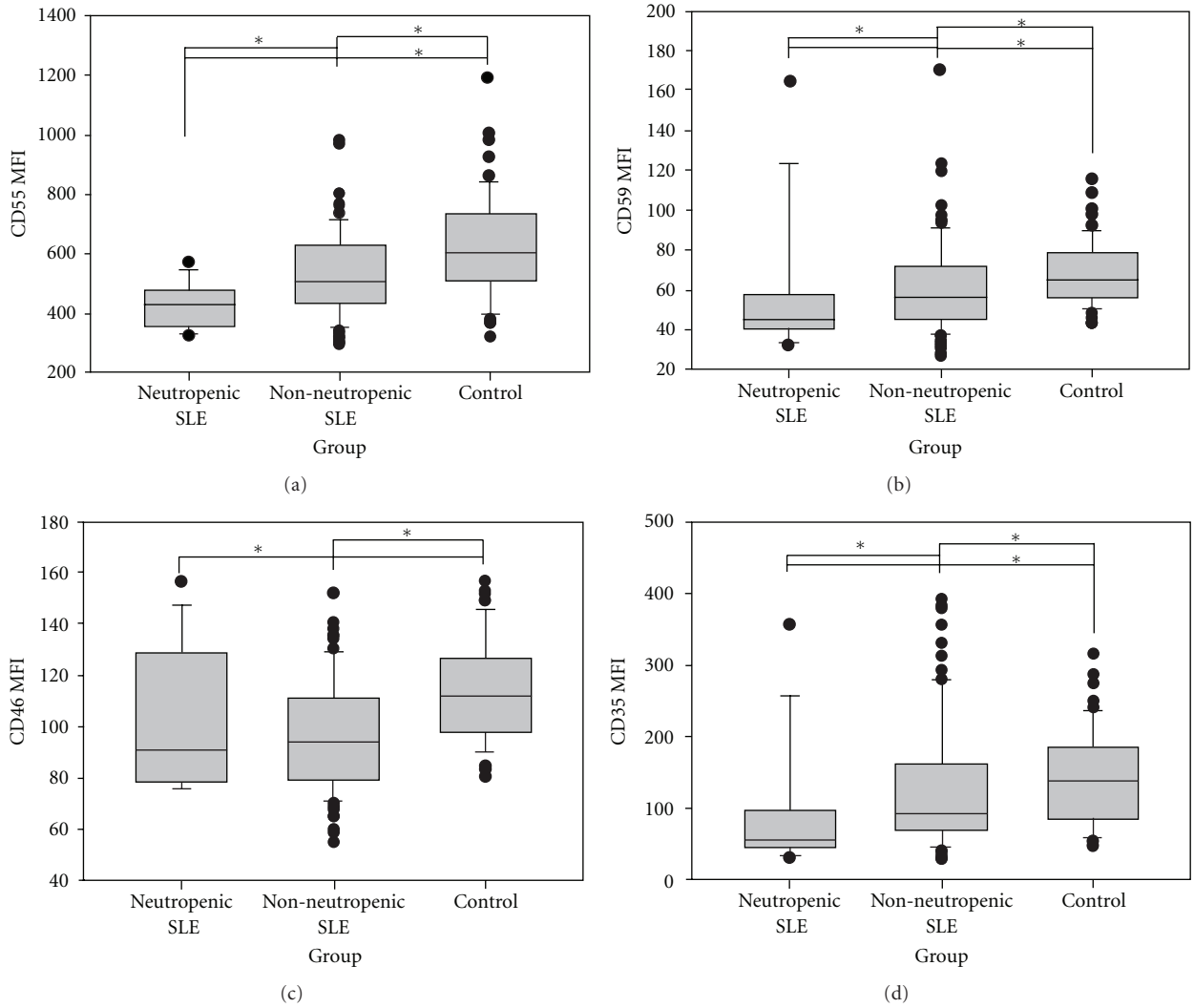

Figure 1: Creg surface expression of neutrophil cell. The figure displays mean fluorescence intensity (MFI) of CD55, CD59, CD46, and CD35 on gated neutrophil from SLE patients with neutropenia, without neutropenia and controls. Median and interquartile range from all subjects studied in each group were shown. *Significant statistical difference ($P < 0.05$).

3.4. Red Blood Cell Analyses. In SLE patients, the MFIs of CD59 and CD35 on RBC were significantly lower than those of healthy controls (Table 2). When comparing anemic (21/100) with nonanemic SLE patients, there were no MFI CD59 and CD35 statistic difference (Figure 3).

There was a positive correlation between CD35 ($r = 0.218, P = 0.049$) expression on RBC and C4 serum levels in SLE patients and no association with SLEDAI or SLICC. When analyzed only anemic patients, this latter correlation was stronger ($r = 0.526, P = 0.021$). CD46 was not analyzed because it is not expressed on RBCs.

4. Discussion

Our study revealed significantly lower Creg expression on several blood cells from SLE patients when compared with healthy controls, more marked in cytopenic patients, and in many cases associated with higher disease activity and lower serum C3 and C4 levels. Although there are a few publications evaluating some of the Creg proteins in specific blood cells in SLE patients, our study is the first to encompass all the membrane-bound Cregs and all blood cells in a large sample of SLE patients. This allows a clear view of the expression profile of these proteins and their relations with decreased blood cell numbers and with disease activity.

We have previously reported a decreased expression of CD55 (but not of CD59) on neutrophils from SLE patients [21], and decreased CD35 expression on neutrophils has also been shown [16, 25]. In this study, beside confirming the decreased expression of CD55 and CD59, it was demonstrated that the higher the disease activity, the lower their expression on neutrophils. Furthermore, there might be a direct correlation between the lower CD55 and CD35 expression and activation of the classical complement pathway, as indicated by the lower C3 and C4 serum levels. These findings suggest that the decreased expression of Cregs

Table 2: The mean of membrane fluorescence intensity (MFI) of CD55, CD59, CD46, and CD35 on the blood cells of SLE patients and controls.

Cell	Creg	SLE patient MFI[a]	Control MFI[a]	P[b]
Neutrophils	CD55	515 ± 132	611 ± 168	0.001*
	CD59	61 ± 24	68 ± 15	0.034*
	CD35	88 (67–154)	138 (86–185)	0.007*
	CD46	97 ± 21	113 ±19	<0.001**
Lymphocytes	CD55	302 ±147	350 ± 121	0.041*
	CD59	24 (13–31)	30 (25–38)	0.012*
	CD35	23 (21–28)	28 (21–59)	0.053
	CD46	62 (49–77)	79 (65–97)	<0.001****
Monocytes	CD55	953 ± 313	1057 ± 241	0.021*
	CD59	23 (18–33)	22 (15.5–33)	0.422*
	CD46	74 ± 21	78 ± 16	0.217
	CD35	122 (66.2–202)	138 (85–198)	0.296**
RBC	CD55	188 ± 44	201 ± 43	0.153
	CD59	73 (53–110)	112 (102.5–148)	<0.001**
	CD35	9.1 ± 2.5	15 ± 5.0	<0.001**

* Significant statistical difference ($P < 0.05$).
** Significant statistical difference ($P < 0.001$).
[a]Media ± SD or median (25–75 interquartile range).
[b]Mann-Whitney U test or Student's t-test.

may be due to their consumption trying to protect the cell against complement-mediated lysis, perhaps triggered by specific autoantibodies.

On lymphocytes, the CD55, CD59, and CD46 MFI showed significant differences between SLE and controls. Lymphopenic patients presented the lower expression of these Cregs. Similarly to our results, Garcia-Valladares et al. [19] investigated the MFI of CD55 and CD59 in T and B lymphocytes from SLE patients with lymphopenia. Both T and B cells from lymphopenic patients showed decreased membrane expression of CD55 and CD59 when compared to controls. Tsunoda et al. [20] found that the proportion of CD59 on activated T CD8+ lymphocytes in SLE patients was significantly reduced compared to controls and that it could be correlated with disease activity and to be involved in the induced apoptosis of these cells. Our data showed that the decreased expression was unrelated to disease activity and accumulated damage using SLEDAI and SLICC, as has been reported [19, 21], but demonstrated that the lower the C3 level and consequently the greater complement activation, the lower the expression of CD55 on lymphocytes in these patients.

The MFIs of CD59 and CD35 on RBCs from SLE patients were significantly reduced when compared to healthy controls, but this deficiency does not seem to be associated with anemia or autoimmune hemolytic anemia (AIHA), since the nonanemic and patients with no secondary AIHA also demonstrated reduced CD59 and CD35 MFI on their red cells. Our data about the decreased CD35 expression on RBC from SLE patients corroborate the findings of the literature [12–16]. Furthermore, we found that the low expression of CD35 in SLE patients was correlated with low C4 levels.

The diminished expression of CD59 on RBCs from SLE patients with secondary AIHA was previously reported by Richaud-Patin et al. [17]. However, in contrast with our results, SLE patients with no AIHA exhibited a normal expression of these molecules. It is important to mention that the number of patients evaluated in our study with and without AIHA was 28 and 72, respectively, which is much greater than that of the study of Richaud-Patin et al.

We also observed a decreased CD35 and CD59 expression on RBCs from SLE patients with nephritis ($n = 45$) ($P < 0.05$, data not shown). This finding corroborates in part the findings of Arora et al. [18], who have demonstrated that, in 15 lupus nephritis patients, the expression of CD35 was significantly reduced compared to the expression on erythrocytes from normal individuals. On the other hand, these authors observed that CD55 and CD59 levels were highly elevated in RBCs, in contrast with our results.

The cause of this generally decreased expression of Creg proteins in SLE blood cells is still unclear. Richaud-Patin et al. [17] have hypothesized that the diminished expression of CD55 and CD59 proteins on red cells might be due either to the impaired synthesis of the GPI (glycosylphosphatidylinositol) anchor or to the abnormal coupling of the protein to the membrane on red blood cell precursors. However, our findings do not support these hypotheses, since in that case the expression of Cregs would be uniformly reduced on all blood cells, while different patterns of diminished expression depending on each cell type were observed in our study.

A decline in CD35 expression at both mRNA transcript and protein level in SLE has been described, and it has been suggested to be acquired [26]. However, nothing is known about the factors involved in this downregulation

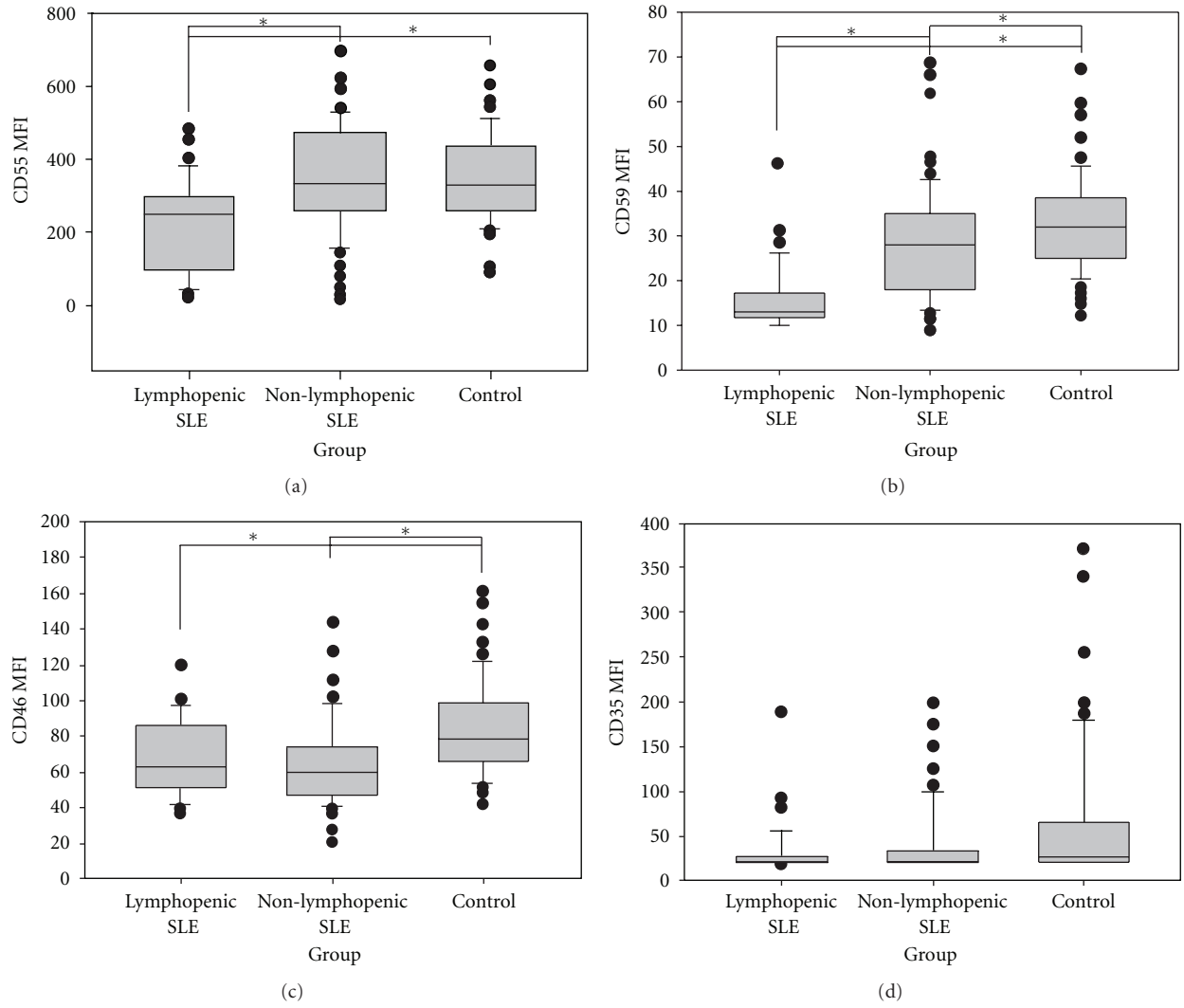

Figure 2: Creg surface expression of lymphocytes cell. The figure displays mean fluorescence intensity (MFI) of CD55, CD59, CD46, and CD35 on gated neutrophil from SLE patients with lymphopenia, without lymphopenia, and controls. Median and interquartile range from all subjects studied in each group were shown. *Significant statistical difference ($P < 0.05$).

of CD35 gene expression [27]. Lach-Trifilieff et al. [28] demonstrated that there is no lack of CD35 expression on young RBC (reticulocytes), in which CD35 is known to be low, and in most cases the low CD35 on RBC is due to an accelerated loss occurring in the circulation. Holme et al. showed that erythrocyte CD35 numbers are reduced during periods of increased disease activity and tend to return to normal during remission [29].

The fact that there was an association of decreased Creg expression with disease activity, low complement levels, and decreased peripheral blood cell numbers in our study indicates that the mechanism is related to the disease itself. The production of autoantibodies against specific cell self-antigens, Creg consumption, and complement-mediated lysis may be the most plausible explanation, as has also been partially suggested by other studies [5, 21, 30]. On the other hand, the use of immunosuppressive drugs may have influenced our results, being a limiting factor in our study and because of the nonhomogenous treatments and multiples therapies was limited to determine the clear association of a specific drugs with Creg decrease and/or cytopenia. We believe that the random inclusion of patients can reduce this influence if it really exists.

The decreased expression of the Cregs may also involve other functions of these proteins. For instance, CD59 has been implicated in the process of signal transduction and T-cell activation [31], and it has been reported that CD59 cross-linking induces internalization of this molecule and endocytosis of the lymphocyte membrane [32]. By another suggestion, it seems that the epitopes against which the monoclonal antibodies are directed somehow express themselves in a differential manner, depending on the cells' activation state [33].

In conclusion, it was evident that there are differences in the patterns of expression of Creg proteins on the peripheral blood cells from SLE patients, since the diminished MFI

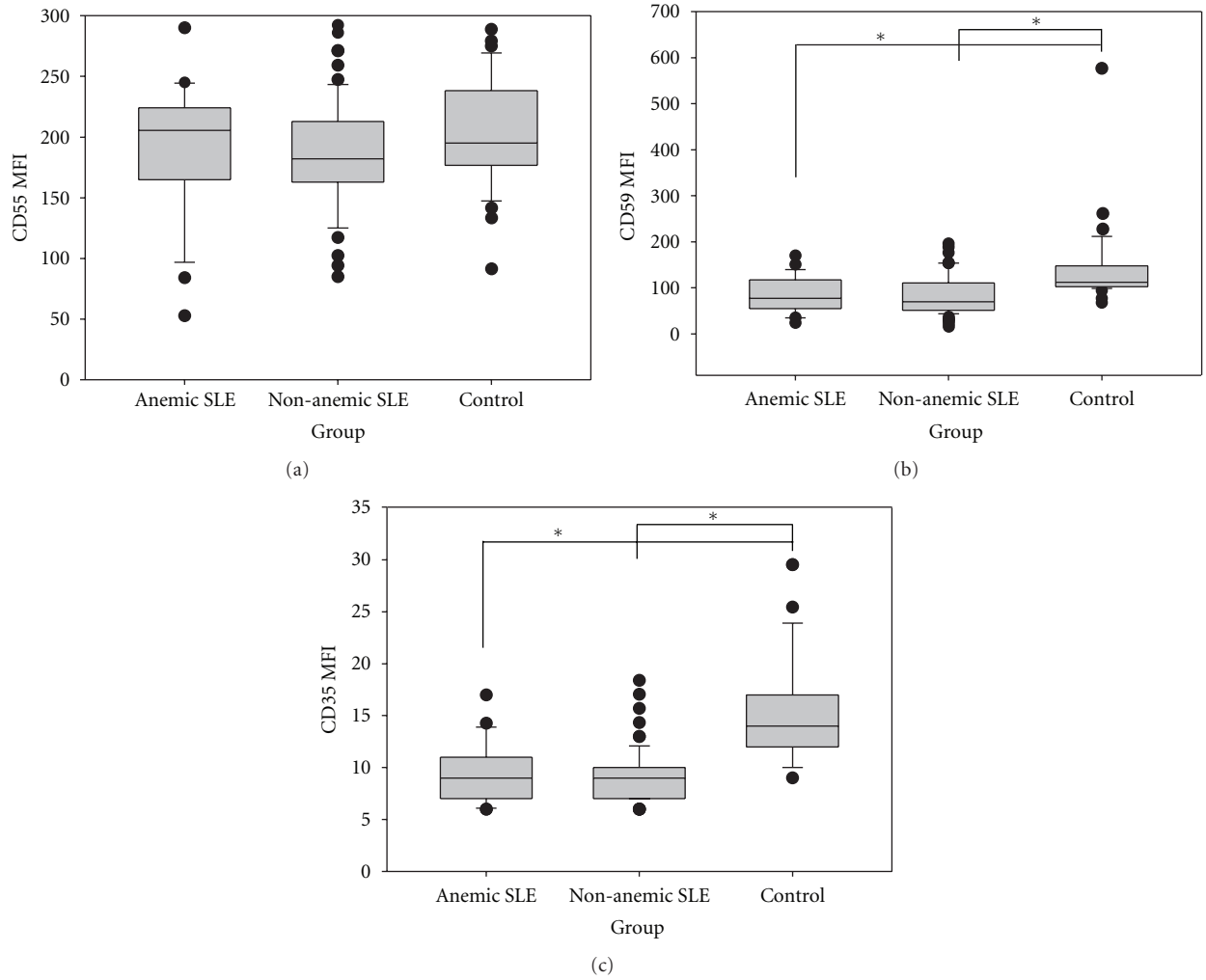

Figure 3: Creg surface expression of RBC. The figure displays mean fluorescence intensity (MFI) of CD55, CD59, CD46, and CD35 on gated RBC from SLE patients with anemia, without anemia, and controls. Median and interquartile range from all subjects studied in each group were shown. *Significant statistical difference ($P < 0.05$).

expressions of all Cregs proteins were found on neutrophils cells; CD55, CD59, and CD46 on lymphocytes; CD55 on monocytes; CD59 and CD35 on RBC. Moreover, these differences, even for the lower most part, seem to correlate with disease activity, complement activation, and blood cell cytopenias. The cause of the decreased expression on cell surface from SLE patients is not yet established, and the mechanisms by which cells are destroyed or sequestered remain rather obscure. We believe this is an adaptive phenomenon that happens due to a consumption of the Creg proteins when trying to prevent complement-mediated cell lysis. Moreover, the fact that each of these four hemopoietic lineages might show underexpression of Cregs independently from the others suggests the participation of different physiopathologic processes. Deeper understanding of these processes, and the role of Cregs, could be important for the development of novel therapies for the blood cell involvement in SLE and other autoimmune-mediated diseases.

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Research Article

Beneficial Effect of *Bupleurum* Polysaccharides on Autoimmune-Prone MRL-lpr Mice

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Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease leading to inflammatory tissue damage in multiple organs. The crude polysaccharides (BPs) isolated from the roots of *Bupleurum smithii* var. *parvifolium* have anticomplementary activity and immunomodulatory functions on macrophages. To study its potential benefit on SLE, we examined effects of BPs on MRL-lpr mice, which have similar disease features to human SLE. MRL-lpr mice were treated orally with BPs 15, 30, or 60 mg kg^{-1} day^{-1} for 12 weeks and their SLE characteristics were evaluated. The results revealed that BPs elongated life span, improved kidney function, delayed lymphadenopathy, and reduced autoantibodies. It seemed to be mediated by inhibition of complement and macrophages activation and suppression of interferon-γ (IFN-γ) and interleukin-6 (IL-6) gene expression in the kidney. These results implicate that BPs may be an immunomodulator for the treatment of autoimmune diseases like SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease involving inappropriate inflammatory responses, resulting in multiorgans dysfunctions like lymphadenopathy and glomerulonephritis. It is characterized by a polyclonal expansion of autoreactive lymphocytes and production of multiple autoantibodies, mostly in young females [1]. The main effectors of disease pathology are the diverse autoantibodies, immune complexes, complement activation, and autoreactive cells [2].

As an important link between the innate and adaptive immune system, monocytes/macrophages have been found to play an essential role in the pathogenesis of SLE [3]. Altered functions of these cells may play a dynamic role not only in the initiation of autoimmunity through abnormalities in phagocytosis but also in the perpetuation of the disease through abnormal signals such as increased costimulation of autoreactive T and B cells and in tissue damage [4].

MRL-$Fas^{lpr/lpr}$ (MRL-lpr) mice with Fas mutation spontaneously develop an autoimmune disease similar to human SLE. Deposition of immune complex in the kidney triggers the production of proinflammatory mediators, resulting in macrophage and lymphocyte infiltration, and ultimately to glomerulosclerosis with renal failure in these mice [5]. Aberrant macrophage activities have been shown in numerous studies in MRL-lpr mice [6–8]. Indeed, MRL-lpr mice provide a more attractive model because their syndrome is rapid, spontaneous, and predictable. Because of this, we used MRL-lpr mice to attempt to evaluate therapies.

Radix Bupleuri (dried roots of *Bupleurum chinense* or *Bupleurum scorzonerifolium*), known as Chai-Hu, is one of the most frequently prescribed crude herbs in the prescriptions of traditional Chinese medicine for the treatment of inflammatory diseases [9] and autoimmune diseases [10]. Our previous experiments confirmed that crude polysaccharide isolated from *Bupleurum smithii* var. *parvifolium* (BPs) showed inhibitory properties toward complement activation [11] and had potent immunomodulatory activity on macrophages [12]. It had also been proved that BPs have beneficial effect on autoimmune disease induced by *Campylobacter jejuni* in BALB/c mice via inhibiting humoral

immune hyperfunction and alleviating the activation of complement [11]. The present work is to study the effect of BPs on MRL-lpr mice and to learn its possible mechanism.

2. Materials and Methods

2.1. Isolation and Characterization of Bupleurum Polysaccharides (BPs). The roots of *Bupleurum smithii* var. *parvifolium* were purchased from Shanghai Hua-Yu Chinese Materia Medica Co. Ltd and its identity was verified by Professor Shenli Pan at Fudan University. A voucher specimen (DFC-CH-H2003121602) of the plant material has been deposited in the Herbarium of Materia Medica, Department of Pharmacognosy, School of Pharmacy, Fudan University, Shanghai, China. The isolation and chromatographic studies of the crude polysaccharides from *Bupleurum smithii* var. *parvifolium* were completed as previously described [11].

2.2. Mice and Experimental Protocol. Eight-week-old female MRL-lpr and BALB/c mice were obtained from Slaccas-Shanghai Lab Animal Ltd. (SPF II Certificate; number SCXK2007–2005) and kept under specific pathogen free and normal housing conditions in a 12-hour light and dark cycle. All experimental protocols described in this study were approved by the Animal Ethical Committee of School of Pharmacy, Fudan University.

BPs and prednisone were ground and suspended in normal saline for administration, respectively. In the longitudinal study, twelve-week-old BALB/c mice were orally received normal saline as the control, and twelve-week-old MRL-lpr mice were orally received normal saline, BPs 60, 30, and 15 $mg \cdot kg^{-1} \cdot day^{-1}$, or prednisone 5 $mg \cdot kg^{-1} \cdot day^{-1}$ for 12 weeks.

During the physical exam, each mouse was palpated to determine the extent of lymph node enlargement that was present. Lymph node enlargement was scored as follows: 0, none; 1, mild enlargement (palpable, but not easily visible); 2, moderate enlargement (easily visible, but not interfering with mobility); and 3, severe enlargement (easily visible and interfering with mobility regardless of extent of interference) [13].

Urine was collected over 24 h in metabolic cages and stored at −80°C at week 24. Mice were sacrificed at the end of week 24 of age and serum was stored −80°C until measurement of antinuclear antibodies. Lymph node, spleen, thymus, and kidneys were removed promptly and one kidney from each mouse was stored into 10% formaldehyde before further analysis. Remaining kidneys were snap frozen in liquid nitrogen prior to storage at −80°C. The index of lymph node, spleen, or thymus was expressed as the ratio of lymph node, spleen, and thymus wet weight (g) versus body weight (g) (100×).

2.3. Immunoassay of Antibodies. Enzyme-linked immunosorbent assay (ELISA) was carried out for the detection of specific antibodies in sera of MRL-lpr and BALB/c mice (control group). For the detection of anti-dsDNA antibodies and anti-ssDNA antibodies, 96-well plates (Costar, Corning, NY) were coated with calf thymus DNA (Sigma) or denatured calf thymus at 50 μg/mL. 10 μg/mL histone from calf thymus (Sigma) was used for detection of antihistone antibodies. Murine serum was diluted at 1 : 200 in phosphate-buffered saline and horseradish-peroxide (HRP-) conjugated goat anti-mouse IgG antibodies were diluted at 1 : 1000 (Sino-American Biotechnology Company, Shanghai, China). Optical density (OD) was monitored at 492 nm using a well scanner ELISA reader (Multiskan FC, Thermo scientific). Results were indicated in Enzyme Index (EI). $EI = 100 \times OD_{tested} / (\text{Mean } OD_{\text{control group}} + 3SD)$ [14].

For the detection of total IgG, 96-well plates were coated with goat anti-mouse IgG (Wuhan Boster Biological Technology, Ltd.) 10 μg/mL, 100 μL/well. Sera were diluted at 1 : 10,000, 100 μL/well. HRP-conjugated goat anti-mouse IgG antibodies were added at a 1 : 5,000 dilutions. The mice IgG standard (Sino-American Biotechnology Company, Shanghai, China) was used for standard curve fitting and immunoglobulin concentration calculating.

2.4. Assessment of Creatinine and Urinary Protein. Mouse creatinine was estimated in serum samples using a Creatinine Jaffe method kit (FengHui Medical Technology Company, Shanghai, China). Proteinuria was measured by Coomassie brilliant blue test [11]. Albumin (bovine serum) was used to make standard curves. Murine urine was centrifuged at 1000 × g for 10 min. The supernatant was diluted at 1 : 3 in normal saline. The optical density was measured at 540 nm after addition of Coomassie brilliant blue solution.

2.5. Hematoxylin and Eosin (H and E) Staining and Immunohistochemistry. The kidney was fixed with 10% formaldehyde and embedded in paraffin. Sections of 5 μm thick were cut and stained with hematoxylin and eosin (H and E). Glomerular injury was blindly semiquantified by a renal pathologist. Sections were graded as follows: 0, normal; 1, a small increase of cells in the glomerular mesangium; 2, a larger number of cells in the mesangium; 3, complex endocapillary hypercellularity sometimes with mild sclerosis or necrosis; and 4, glomerular crescent formation, sclerosis, tubular atrophy and casts [15]. Usually, there were different grade lesions observed in a kidney; the most severe alteration was referred to as the grade of each mouse kidney and was taken into analysis.

For the detection of IgG deposits, the 5 μm sections were deparaffinized, rehydrated, and incubated with peroxidase-conjugated goat anti-mouse IgG (Sino-American Biotechnology Company, Shanghai, China). Staining was visualized using chromogenic substrate solution 3-3′ diaminobenzidine (DAB).

2.6. Real-Time PCR. Total RNA was extracted from snap-frozen kidneys using Trizol reagent (Invitrogen, USA), and cDNA was synthesized from 2 ug total RNA by using random hexamers and SuperScript II Reverse Transcriptase (Invitrogen). SYBR Green I Dye detection system was used for quantitative PCR (qPCR) on Step One Plus Real-Time PCR System (Applied Biosystems, USA). 2 uL cDNA was

amplified in a 20 uL PCR reaction system using recombinant Taq DNA polymerase (TAKARA, Japan). All reactions were performed in triplicate, and negative controls contained no template DNA. We used GAPDH RNA as an endogenous control for normalization. cDNA was subjected to 2-step PCR method: 95°C for 2 min; 40 PCR cycles (94°C for 10 s, 59°C for 10 s, and 72°C for 40 s) to detect MCP-1, IFN-γ, and IL-6 or subjected to 40 PCR cycles (94°C for 10 s, 57°C for 10 s, and 72°C for 40 s) to detect MHC-II, and subjected to 40 PCR cycles (94°C for 10 s, 57°C for 10 s, and 72°C for 40 s) to detect GAPDH. To verify that the primer pair produced only a single product, a dissociation protocol was added after thermocycling, determining dissociation of the PCR products from 60 to 95°C. Data were analyzed using the comparative threshold cycle ($\Delta\Delta$Ct) method.

2.7. Western Blotting. Kidney tissues of mice (100 mg) were separated by 12% SDS-PAGE under reducing conditions and transferred to PVDF membranes (Milipoler). Membranes were blocked with Superblock overnight at room temperature. Blots were probed with 1 : 200 dilution of primary antibody for C3, F4/80, IFN-γ, IL-6, MCP-1, MHC-II, and GAPDH (Santa Cruz, USA) for 2 hours in 5% milk/TBST. Membranes were next incubated with 1 : 2,000 dilution peroxidase-labeled second antibody (Santa Cruz, USA) for 2 hours. All membranes were visualized using DAB buffer. Densitometric analysis of the film was performed using a Model GS-2008 imaging densitometer (TANON) and analyzed using TANON analysis software.

2.8. Statistical Analysis. Quantitative variables were expressed as means ± SD. One-way analysis of variance (ANOVA) was used. If any significant change was found, post hoc comparisons were performed using Fisher's PLSD. Nonparametric data were analyzed by the Mann-Whitney U-test. P value < 0.05 was considered significant.

3. Results

3.1. BPs Decreased Mortality Percentage and Lymphadenopathy of MRL-lpr Mice. The effect of BPs on survival was estimated by comparing the BP-treated group with vehicle-treated model group. After treatment for 12 weeks, the survival percentage of MRL-lpr mice (24-week-old) increased in BP- 30 mg·kg^{-1}·day^{-1}- (100%), BP- 60 mg·kg^{-1}·day^{-1}- (100%) and Prednisone- (100%) treated groups (Figure 1).

The lymphadenopathy of MRL-lpr mice was graded as lymph node enlargement. There was no significant difference in lymphadenopathy among five groups at 16 weeks of age. BPs 60 mg·kg^{-1}·day^{-1} significantly delayed the lymphadenopathy after 8 weeks of treatment and prednisone after 9 weeks of treatment ($P < 0.05$) (Table 1).

3.2. BPs Decreased Organ Index of MRL-lpr Mice. The index of lymph node, thymus, and spleen increased significantly in vehicle-treated model group when compared with control group. BPs inhibited lymph node swelling ($P < 0.01$), administration of 30 and 60 mg·kg^{-1}·day^{-1} BPs inhibited thymus swelling ($P < 0.05$), while prednisone treatment significantly decreased the index of lymph node, thymus, and spleen ($P < 0.05$) (Figure 2).

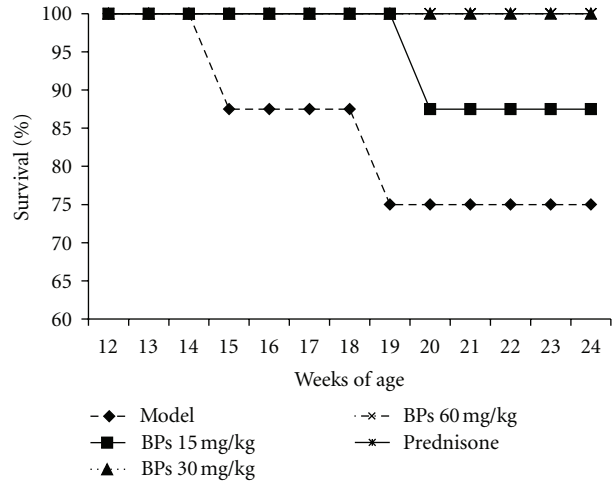


Figure 1: Effect of BPs on survival rate of MRL-lpr mice. MRL-lpr mice were grouped randomly and treated with BPs 15, 30, and 60 mg·kg^{-1}·day^{-1}, prednisone 5 mg·kg^{-1}·day^{-1}, or model from week 12 to week 24 ($n = 8$ per group); were data expressed as means ± SD.

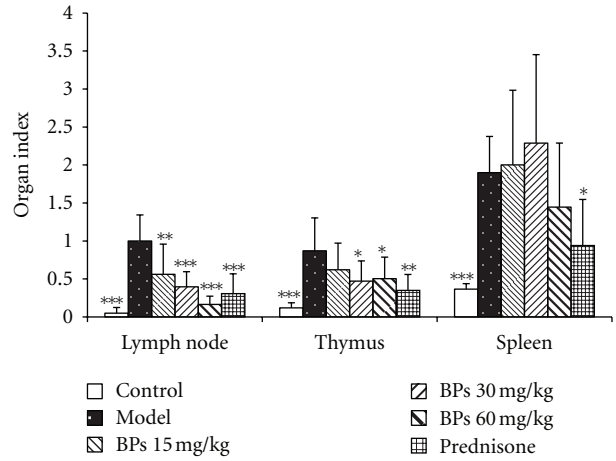


Figure 2: Effect of BPs on the index of lymph node, thymus, and spleen in BALB/c and MRL-lpr mice. MRL-lpr mice were grouped randomly and treated with BPs 15, 30, and 60 mg·kg^{-1}·day^{-1}, prednisone 5 mg·kg^{-1}·day^{-1}, or model from week 12 to week 24; data were expressed as means ± SD ($n = 6$–8); $^{*}P < 0.05$, $^{**}P < 0.01$; $^{***}P < 0.001$ compared with vehicle treated-model group, tested by ANOVA and Fisher's PLSD.

3.3. BPs Reduced Autoantibody and Total IgG Levels in MRL-lpr Mice. The sera were collected from MRL-lpr and BALB/c mice to assay autoantibody concentration by ELISA. Anti-dsDNA, anti-ssDNA, and anti-histone antibody levels were significantly elevated in the vehicle-treated MRL-lpr mice (model group) compared with BALB/c mice (control group) ($P < 0.001$) (Figure 3) and were significantly reduced

Table 1: Effect of BPs on lymphadenopathy of MRL-lpr mice.

Weeksof age	Model	lymphadenopathy score BPs			Prednisone
		15 mg·kg^{-1}	30 mg·kg^{-1}	60 mg·kg^{-1}	5 mg·kg^{-1}
16	0 (0~1)	0.5 (0~1)	1 (0~1)	0 (0~1)	0 (0~0)
18	1 (0~2)	1.5 (0~2)	1 (0~2)	0 (0~2)	0 (0~1)
20	2 (0~3)	2 (0~3)	1.5 (0~3)	0 (0~3)[a]	1 (0~2)
21	2 (1~3)	2 (0~3)	2 (0~3)	0 (0~3)[a]	1 (0~2)[a]
22	3 (2~3)	2 (0~3)	2 (0~3)	0 (0~3)[b]	1 (0~2)[b]
23	3 (2~3)	3 (0~3)	2.5 (0~3)	0 (0~3)[b]	1 (0~3)[a]
24	3 (2~3)	3 (0~3)	3 (0~3)	0 (0~3)[b]	1.5 (0~3)[a]

note: Data were expressed as median (minimum~maximum) (n = 6–8); [a]$P < 0.05$, [b]$P < 0.01$ compared with vehicle treated model group, tested by Mann-Whitney U test.

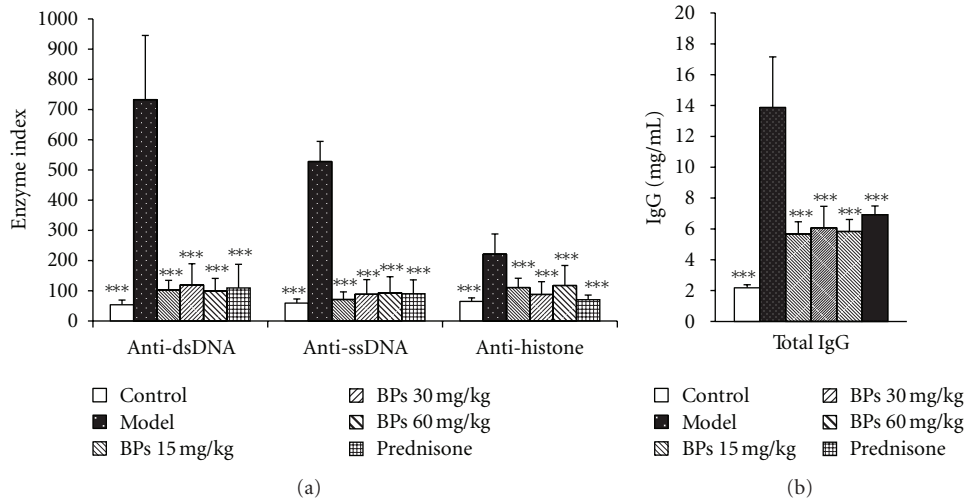


Figure 3: Effect of BPs on antinuclear antibodies and total IgG production in BALB/c and MRL-lpr mice. MRL-lpr mice were grouped randomly and treated with BPs 15, 30, and 60 mg·kg^{-1}·day^{-1}, prednisone 5 mg·kg^{-1}·day^{-1}, or model from week 12 to week 24; data were expressed as means ± SD (n = 6–8); ***$P < 0.001$ compared with vehicle-treated model group, tested by ANOVA and Fisher's PLSD. Enzyme Index = 100 × OD_{tested} /(Mean $OD_{control\ group}$ + 3SD).

by BPs 15, 30, 60, or prednisone 5 mg·kg^{-1}·day^{-1} ($P < 0.001$). It was indicated that BPs blocked the production of autoantibodies in MRL-lpr mice, which are closely related to SLE.

Furthermore, total IgG levels were assayed. Similarly, total IgG levels were significantly elevated in the vehicle treated MRL-lpr mice compared with control group ($P < 0.001$) (Figure 3) and were reduced by BPs 15, 30, 60, or prednisone 5 mg·kg^{-1}·day^{-1} significantly ($P < 0.001$).

3.4. BPs Impaired Proteinuria and Reduced Serum Creatinine of MRL-lpr Mice. To evaluate whether BPs had effects on kidney function, MRL-lpr mice were treated by BPs for 12 weeks and urine samples were collected for protein determination. In comparison with control group, model group exhibited significant increase in the level of urinary protein ($P < 0.001$), indicating a certain degree of kidney dysfunction. However, BPs treatment resulted in a reduction in the levels of urinary protein (Figure 4). BPs 60 mg·kg^{-1}·day^{-1} and prednisone 5 mg·kg^{-1}·day^{-1} significantly reduced urinary protein level ($P < 0.01$).

At 24 weeks of age, MRL-lpr mice showed increased serum creatinine levels ($P = 0.057$), BPs treatment mildly reduced serum creatinine levels, but only 30 mg·kg^{-1}·day^{-1} group showed significance (Figure 4).

3.5. BPs Suppressed Lupus Nephritis and Reduced Renal IgG Deposition in MRL-lpr Mice. We compared the extent of nephritis between vehicle-treated and BPs-treated MRL-lpr mice at 24 weeks. Vehicle-treated MRL-lpr mice developed diffuse proliferative glomerulonephritis presented as diffuse mesangial matrix expansion, profound mesangial cell proliferation, and focal segmental glomerulosclerosis. Cellular crescents and global glomerulosclerosis were often seen, and the number of both resident cells and infiltrating leukocytes was increased in glomeruli. Most animals revealed profound tubulo interstitial inflammation as characterized by periglomerular and diffuse interstitial leukocyte infiltrates,

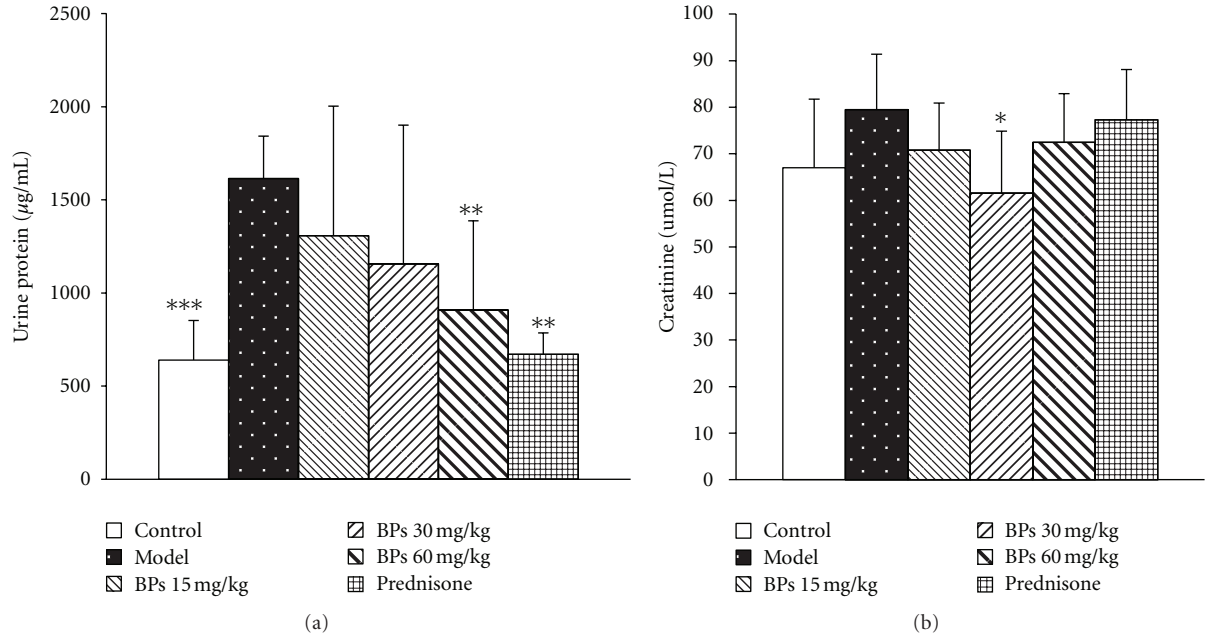


Figure 4: Effect of BPs on urinary protein and serum creatinine levels of MRL-lpr and BALB/c mice. MRL-lpr mice were grouped randomly and treated with BPs 15, 30, and 60 mg·kg^{-1}·day^{-1}, prednisone 5 mg·kg^{-1}·day^{-1}, or model from week 12 to week 24; mice were sacrificed and the supernatant of urine was diluted 1 : 3 in normal saline; the serum creatinine was measured with Jaffe method; data were expressed as means ± S.D. (n = 6–8); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with vehicle-treated model group, tested by ANOVA and Fisher's PLSD.

Table 2: Effect of BPs on glomerulonephritis scores of BALB/c and MRL-lpr mice.

Glomerulonephritis score					
Control	Model	BPs			Prednisone
		15 mg·kg^{-1}	30 mg·kg^{-1}	60 mg·kg^{-1}	5 mg·kg^{-1}
0(0∼1)[c]	3.5 (3∼4)	2 (1∼3)[c]	2 (1∼2)[c]	2 (1∼2)[c]	1.5 (1∼2)[c]

note: Data were expressed as median (minimum∼maximum) (n = 6–8); [b]$P < 0.001$ compared with vehicle-treated model group, tested by Mann-Whitney U test.

tubular atrophy, and intraluminal cast formation. Treatment with BPs suppressed lupus nephritis as documented by a significant reduction of the glomerulonephritis scores that encompasses glomerular cell proliferation, endocapillary hypercellularity, crescent formation, sclerosis, tubular atrophy and casts (Table 2, Figure 5).

To determine whether BPs might affect renal disease by reducing IgG deposition in the kidneys, tissue sections from mice were stained for the presence of IgG. Vehicle-treated MRL-lpr mice showed a patchy dense immunoperoxidase indicative of mesangial and tubulointerstitial IgG deposition. In contrast, BPs treatment significantly decreased IgG deposition (Figure 5).

3.6. BPs Suppressed Inflammatory Mediators and Markers Expression in Kidney. Using RT-PCR and Western blotting, we examined the expression of IFN-γ, IL-6, MCP-1, MHC-II, and F4/80 in the kidney of mice at 24 weeks. The expression of IFN-γ, IL-6, MCP-1 mRNA, and protein was greatly elevated in vehicle-treated MRL-lpr mice and was reduced in varying degree from BP-treated mice compared with the vehicle-treated mice. BPs treatment reduced the mRNA and protein expression of the surface marker MHC-II and F4/80 (Figures 6 and 7).

C3 levels were also reduced in the kidney of BPs treatment group, as was shown in Western blotting results (Figure 7).

4. Discussion

Systemic lupus erythematosus is a chronic autoimmune inflammatory disease that affects various organs, including skin, joint, kidney, and blood. Even without clear pathogenesis mechanism, it has been suggested that the main effectors of disease pathology are the diverse autoantibodies, immune complexes, complements, and autoreactive cells. Altered biology of immune cells and possibly other cell types invariably contributes to the progression of the diseases [2].

Macrophages have been considered to play important roles in the pathogenesis of SLE in numerous studies [4]. Owing to the intrinsic defects of macrophages, SLE is one of the widely acknowledged examples in which apoptotic

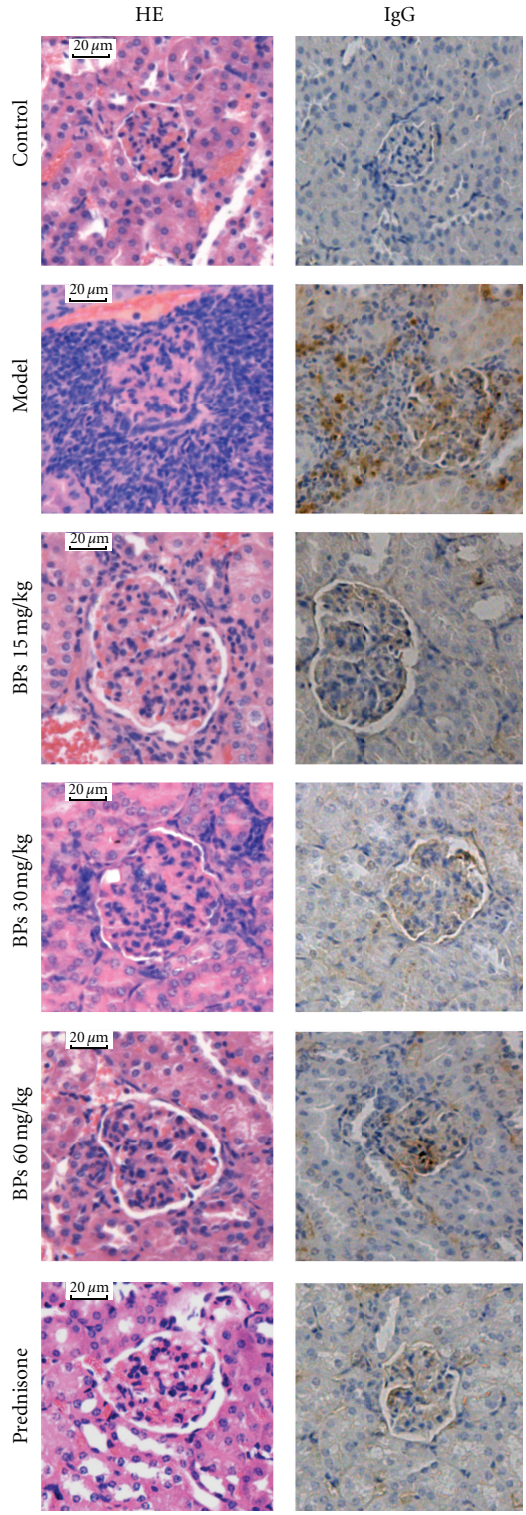


Figure 5: Haematoxylin and eosin-stained and immunohistochemistry of kidney sections. Light microscopy was 200x. Section of a BALB/c group mouse: normal kidney sections (HE, IgG). Section of a vehicle-treated model mouse: glomerular sclerosis, tubular atrophy, and increased infiltrating leukocytes (HE), a patchy dense immunoperoxidase indicative of IgG deposition (IgG). Section from 15, 30, 60 $mg \cdot kg^{-1} \cdot day^{-1}$ BP- and prednisone-treated mouse: mild mesangial cell proliferation (HE), less IgG deposition (IgG).

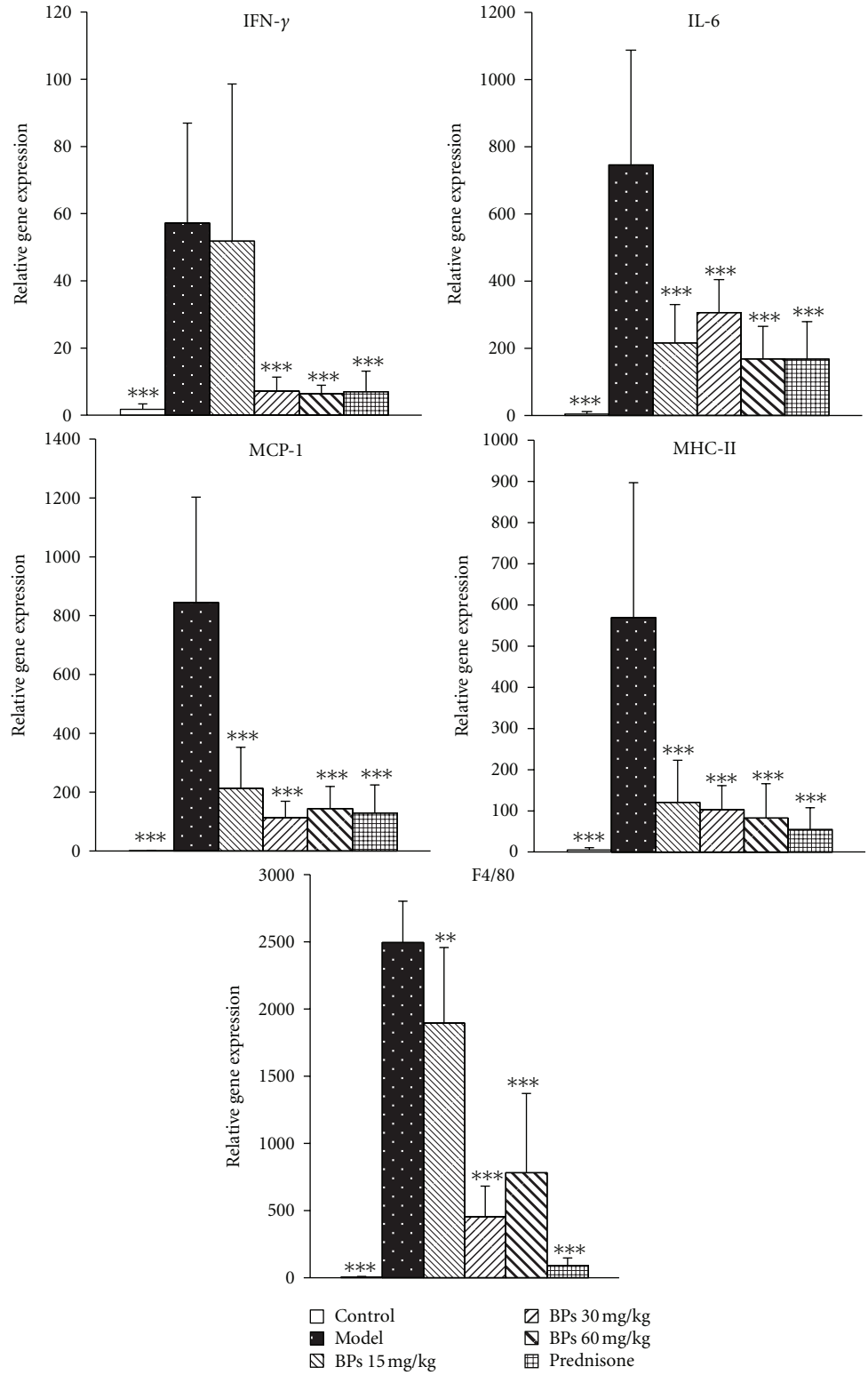
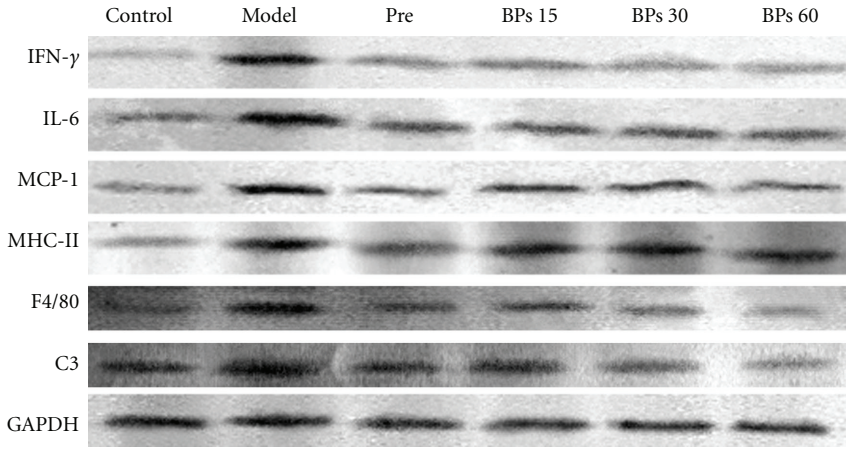
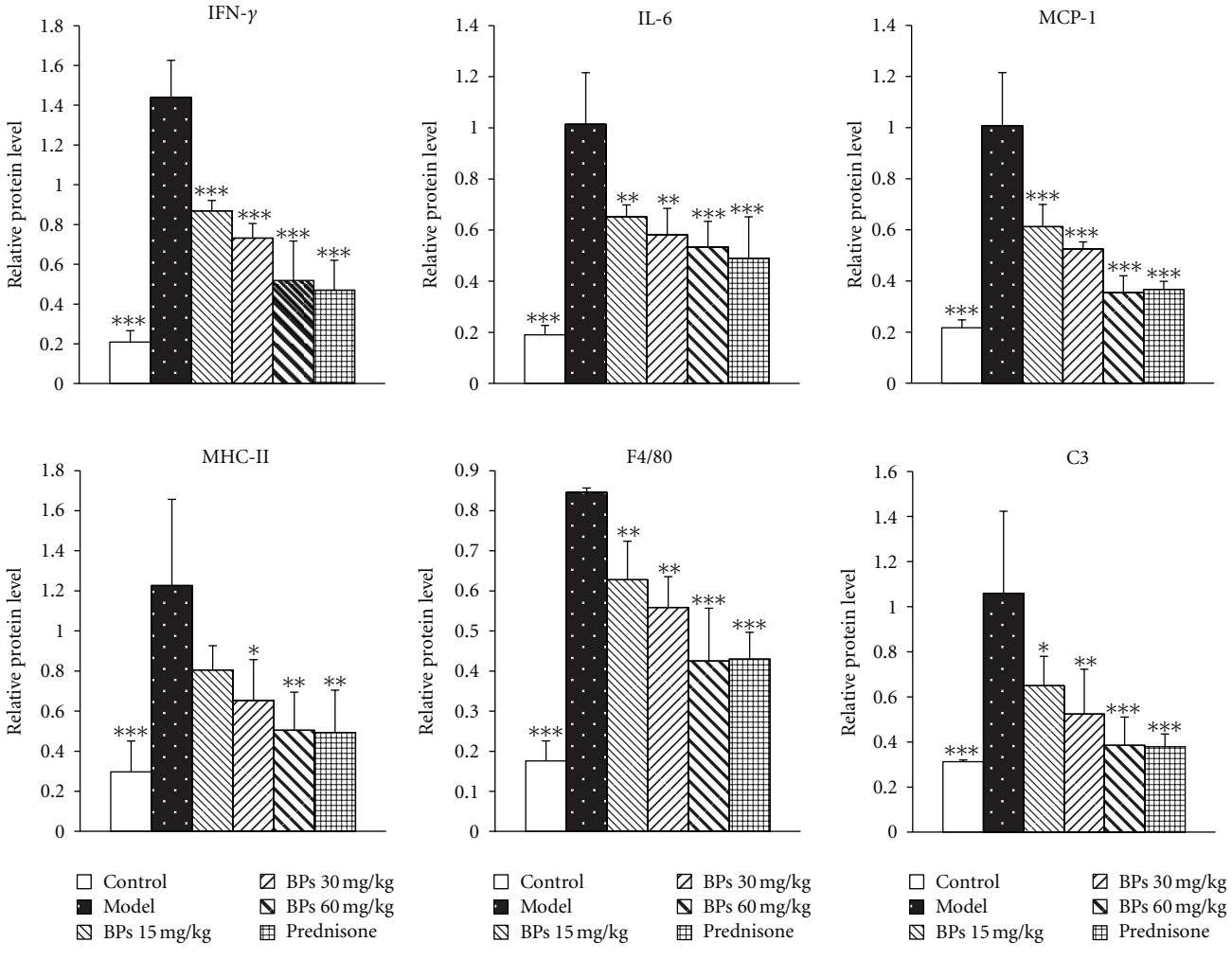


Figure 6: IFN-γ, IL-6, MCP-1, MHC-II, and F4/80 mRNA expression in the kidney from MRL-lpr mice. The mRNA expression of IFN-γ, IL-6, MCP-1, and MHC-II in the kidney was prepared from the BALB/c mice (control) and MRL-lpr mice administered vehicle solution (model), 15, 30, and 60 mg·kg^{-1}·day^{-1} BPs, or 5 mg·kg^{-1}·day^{-1} prednisone. The levels of mRNA were analyzed by real-time PCR; GADPH was shown as the loading control. Each data point represented the mean of individual mouse in three independent experiments. Values were presented as means ± S.D. ratio of IFN-γ, IL-6, MCP-1, MHC-II, and F4/80 mRNA to GADPH mRNA ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with vehicle-treated model group, tested by ANOVA and Fisher's PLSD.



(a)



(b)

Figure 7: Protein expression of IFN-γ, IL-6, MCP-1, MHC-II, F4/80, and C3 in the kidneys of MRL/lpr mice. (a) Renal expressions of IFN-γ, IL-6, MCP-1, MHC-II, F4/80, and C3 were examined by Western blot analysis. The presented blot was a representative of those obtained from three mices. GAPDH was used as loading control. (b) Results of quantitative analysis were expressed as means ± S.D. ratio of IFN-γ, IL-6, MCP-1, MHC-II, F4/80, and C3 to GADPH ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with vehicle-treated model group, tested by ANOVA and Fisher's PLSD.

cell clearance is disturbed in both mice and patient [3]. Recent studies also confirmed that aberrant function of lupus macrophages appeared to play a dynamic role in the initiation and perpetuation of the systemic autoimmune response and organ damage [4]. Moreover, aberrant monocyte surface marker expression and numerous abnormalities of the cytokine network have been described in patients suffering from SLE [3].

Our laboratory has done a series of experiments concerning the effect of crude *Bupleurum* polysaccharide (BPs) on the immune system. It was suggested that BPs have potent immunomodulatory activity on macrophages *ex vivo* by enhancing phagocytic activities and inhibiting LPS-induced production of proinflammatory mediators [12]. It had been demonstrated in our previous study that BPs were a major component that contributed to the anticomplementary activity and had a beneficial effect on systemic lupus erythematosus-like syndrome induced by CJ-S_{131} in BALB/c mice [11]. BPs also inhibited LPS-induced phosphorylation of NF-κB, TNF-α, IL-1β, IL-6, IL-12, and IFN-β production in peritoneal macrophages (in press).

Here we find that BPs treatment for 12 weeks has several beneficial effects on MRL-lpr mice including protection from lethality, amelioration of glomerulonephritis, improvement of kidney function, and prevention of lymphadenopathy and thymus enlargement. The improved pathology was associated with reduced production of autoantibodies and inhibited expression of inflammatory cytokines and chemotactic factors in the kidneys. We suggest that these benefitial effects might be related to BPs anticomplement activity and immunomodulatory functions on macrophages. Since analyses on blood, urine, and kidney were performed at the end of the treatment period and the sickest mice in model group had died, the final analyses are likely not an accurate measure of the effects of these agents. More experiments will be needed and the analyses of desease progression should be carried out in our further experiment.

In healthy individuals, the immune system defends the body against microbes by distinguishing self from foreign antigens. For reasons not completely understood, immune tolerance against self-antigens fails in SLE and the immune system actively responds to a wide array of autoantigens [16]. Antinuclear antibodies such as anti-dsDNA antibodies are unique to patient with SLE [17]. One of the most important features of anti-dsDNA antibody is its association with glomerulonephritis [18]. In the present study, the serum from vehicle-treated MRL-lpr mice had higher levels of total IgG, which may contain a large amount of antinuclear antibodies (ANA) mainly. The kidney sections also had higher IgG deposition and expressed hypercellular glomeruli. High levels of protein in urine and creatine in sera also indicated kidney dysfunction in MRL-lpr mice. According to the previous results, it is indicated that the kidney dysfunction in MRL-lpr mice might be related to their aberrant immune responses. We demonstrated that BPs decreased the total IgG, anti-dsDNA, anti-ssDNA, and anti-histone antibodies, as well as renal IgG deposition, reduced glomerular hypercellularity, and suppressed lupus nephritis in MRL-lpr mice. Therefore, it is suggested that BPs improves glomerulonephritis of MRL-lpr mice through modulation of these pathological phenomena.

Lupus nephritis is triggered by glomerular immune complex deposits that activate the components of the classical complement pathway, which finally leads to the assembly of membrane attack complex [19]. In the present study, the favorable effects of BPs treatment on markers of active lupus nephritis were associated with a significant reduction of complement C3 in kidney, a marker of intraglomerular complement activation. This was in line with our previous findings that BPs had anticomplementary activity.

Leukocyte infiltration is a hallmark of severe lupus nephritis, and macrophages play an important role in amplification of the inflammatory process in the kidney [20]. MHC-II is an important molecule expressed on antigen presenting cells, such as macrophages, dendritic cells, and B cells. Enhanced MHC class II antigen expression is a common feature of autoimmunity and may play a key role in the initiation and progression of lupus nephritis [21]. F4/80 is a cell surface glycoprotein predominantly expressed on murine macrophages [22]. In this study, renal RT-PCR and Western blotting revealed that MHC-II and F4/80 were significantly upregulated in the kidney of MRL-lpr mice. This is likely the reflection of aberrant activation and increased infiltration of macrophages in this model [4]. We found that the expressions of MHC-II and F4/80 were significantly inhibited by BPs, which might reflect the immune inhibitory effect of BPs on overactivated macrophages.

MCP-1 can induce transendothelial migration of monocytes, and infiltration of monocytes/macrophages can in turn facilitate tissue destruction [23]. MCP-1 deficient MRL-lpr mice were protected from progressive renal injury by reduced leukocyte recruitment [24]. In this study, BPs treatment resulted in a decrease in renal expression of MCP-1, indicating that the effect of BPs on kidney inflammation might be mediated by reduced MCP-1 expression and macrophage recruitment.

Infiltrating mononuclear cells are the major source of IL-6 in diseased kidneys affected by lupus nephritis [25], IL-6 can in return promote macrophage activation, it is elevated in the serum and urine of some lupus patients, and murine lupus models support a role for IL-6 in nephritis [26, 27]. In our previous study, we found that BPs decreased LPS-induced excessive production of NO and proinflammatory cytokines, including IL-1β, IL-6, and TNF-α, but had mild effects on these cytokines when they were in physiologic levels [12]. We found that the renal expression of IL-6 was significantly elevated in the kidney of MRL-lpr mice and was inhibited by BPs treatment, indicating the suppression effect of BPs on macrophage secretions.

Tissue macrophages can be recruited and activated as a consequence of the actions of a range of inflammatory mediators such as cytokines. IFN-γ is increased in the serum of some SLE patients and its level has been shown to correlate with disease activity [28]. Furthermore, IFN-γ is a potent cytokine in inducing MHC-II antigen expression in infiltrating monocytes [29]. We found that the expression of IFN-γ was significantly elevated in the kidney of MRL-lpr mice and was inhibited by BPs treatment, indicating that

the anti-inflammatory effects of BPs might also be associated with decreased renal expression of proinflammatory cytokines.

In addition to the local suppression of macrophages, the therapeutic effects of BPs might also be related to a systemic blunting of autoimmunity, as reflected by decreased serum levels of autoantibodies and renal immune complex deposition. Since macrophage-derived cytokines are required for the differentiation of B cells into antibody-secreting plasma cells [30] and for the survival and proliferation of B cells [31], one attractive hypothesis could be that the inhibitory effect of BPs on autoantibody production and deposition might be mediated by macrophages. Another plausible hypothesis could be that the effect of BPs treatment is due to less autoantibody deposition in the kidneys with all the other effects being secondary. Although our previous work showed that BPs has an effect on macrophages, and in this study we found a decrease in surface markers and cytokines associated with macrophages (either recruiting them, activating them, or produced by them), further studies are needed to identify whether macrophages are the direct function target of BPs treatment.

In summary, this study demonstrated that BPs improves lupus nephritis mainly by suppressing abnormal autoimmunity of SLE. Our analysis proves the therapeutic efficacy of BPs in the treatment of SLE in MRL-lpr mice. Taken with the current data, BPs could be a new agent for the treatment of autoimmune disease.

Acknowledgments

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Review Article

Gender Differences in the Pathogenesis and Outcome of Lupus and of Lupus Nephritis

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Systemic Lupus Erythematosus (SLE) typically affects females at far greater rates than males; however male SLE patients often have more severe disease than females. The gender disparities have been reported in clinical manifestations and in serological and hematological indices as well. In particular, SLE complicated with nephritis is more frequent in men than women, and several groups identified male gender as a risk factor for progression to renal failure. The specific differences in pathogenesis amongst genders have yet to be conclusively defined, though genetic, hormonal, and immune responses have been analyzed thus far. Further research is warranted to further elucidate these differences and permit the development of gender-tailored treatment regimens.

1. Introduction

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease that can potentially cause inflammation and damage to any organ system. There are multiple genetic, hormonal, and environmental factors that are known to influence the development and nature of the disease. SLE is characterized by periods of high activity alternating with periods of remission and typically presents in females of childbearing age. During these reproductive years, the ratio of females to males is nine to one, with a lower ratio seen before puberty and a decline later in life. The increased rate of SLE in females implicates hormones as essential in disease manifestations, and this influence of sex hormones is also seen in animal models of the disease. Typically, in most mouse models, females have worse outcomes, and administration of estrogens exacerbates while androgens ameliorate disease [1].

2. Gender Differences in SLE Disease Manifestations

Despite the noted relationship of estrogens and increased autoimmune diseases in females, there is a growing body of the literature reflecting both different disease manifestations and a difference in severity of SLE in males versus females. Approximately 4% to 22% of SLE patients in reported series are male, and this number increases to 30% in studies regarding familial aggregation [2]. As a minority, males with SLE have been frequently subjected to treatments studied mostly in females, and become grouped along with females regarding most health-related issues. As gender differences may affect drug action and availability, tailored treatments for males and females might improve outcomes and overall prognosis for both genders [2]. Several groups have studied the sex disparities in this disease and have suggested gender, along with ethnicity, age of disease onset, or autoantibody profiles as a means to identify SLE subgroups [3]. More severe skin lesions, serositis, renal disease, thrombotic events, and seizures have been reported in males by several authors [4], though conflicting results have been presented regarding these gender differences among SLE patients and the precise role of gender in damage accrual has not yet been defined [4].

The LUpus in MInorities, NAture versus nurture (LUMINA) cohort is a well-known multiethnic US Cohort consisting of Hispanic, African American, and Caucasian patients. To further understand the impact of gender on

manifestations and outcome of SLE, researchers in the LUMINA Study Group compared disease activity in males versus females by the SLAM (Systemic Lupus Activity Measure) and damage accrual by the Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index (SLICC/SDI) at baseline and at the last visit. In addition to the above disease indices, socioeconomic/demographic, clinical, and serological features were compared for this study [4].

Of the 618 LUMINA patients enrolled at the time of the study, 555 were female, 63 male. Caucasians were overrepresented amongst males. Poverty was less frequent, while smoking and alcohol use more common in males. Males in larger numbers experienced more difficulty in access to health care, but they showed more adequate illness-related behaviors at both points in time. These "illness-related behaviors" were measured using the Illness Behavior Questionnaire (IBQ), which assesses maladaptive responses to illness, including hypochondriacal responses, denial, and changes in affect. The IBQ was designed to indicate the extent to which these behaviors explain exaggerated response to health issues [5]. There was a tendency toward more frequent renal involvement (63.5% versus 52.1%, $P = 0.085$) and positive lupus anticoagulant antibody (LAC) in males (21.4% versus 9.1%, $P = 0.004$), but other antibodies occurred at comparable rates. Shorter disease duration at baseline, higher SDI at any time, and LAC at any time were factors independently associated with SLE in men (Table 1). Musculoskeletal involvement was less frequent in males in this cohort. Damage was shown to accrue faster in males with SLE; male sex was a stronger predictor of damage at baseline (55.6% versus 39.5%, $P = 0.014$) and positively associated with damage over the course of disease as measured at the last visit (71.4% versus 61.3%, $P = 0.115$). This study identified male gender as a risk factor for accelerated damage in SLE, in addition to reflecting differences in severity and manifestations of SLE by gender [4].

3. Gender Differences in Disease Manifestations: Focus on Renal Disease

Renal disease in SLE is a source of major morbidity and mortality; it develops in approximately 60% of patients with SLE, with a reported 5–22% of these patients progressing to end-stage renal disease requiring dialysis or transplant [6]. Studies have shown that lupus nephritis (LN) is more frequent in men than in women [7]. Multiple factors including male sex, black race, presence of antiphospholipid antibodies, increased creatinine at the time of diagnosis, anemia, frequent nephritic flares, hypertension, and excessive prolonged proteinuria are all considered risk factors for increased progression to end-stage renal disease (ESRD) [6]. However, the influence of gender on long-term renal outcome is controversial [7], and the differences in pathology of nephritis in males verses females have yet to be formally elucidated or studied. Higher prevalence, poorer renal outcome, and poorer overall survival rate have been demonstrated in several studies [8], as well as higher prevalence of Class IV, diffuse proliferative nephritis (DPGN), and active glomerular disease amongst males [2].

Lu et al. reviewed the existing literature published from 1975–January 2009 using the PubMed database to identify potential clinical characteristics in male lupus. Renal involvement was frequently more common in the studies reviewed, in both adult and pediatric SLE populations. In some, DPGN, the class with typically the poorest prognosis, was seen as the predominant finding on biopsy in males. Increased risk of renal failure and ESRD were also observed amongst males in greater numbers in various papers reviewed [2].

Of the patients in our own institution participating in the Einstein Lupus Cohort, approximately 300 SLE patients have been entered into the registry, of which 35 (11.7%) are males. Of the male patients, 16 (47%) have renal disease, whereas around 33% of the female patients have renal disease. Forty-one (46%) females have proliferative nephritis (class IV or mixed IV and V), 26 have class III or mixed III and V. Biopsy data on the remaining female patients is unavailable. Eight male patients (50%) have proliferative nephritis; seven have class IV, one with mixed class III and IV. Three males have mixed class III and V, two male patients class III. Two patients have pure membranous disease (class V) and one did not have a biopsy [9]. Our numbers are consistent with the other cohorts where males have renal disease in greater numbers and specifically more proliferative nephritis than the female counterparts.

4. Gender Differences by Geographic Region

4.1. Asia. Several groups have analyzed the clinical expression and outcomes of SLE in males in different ethnic populations and geographic regions. To evaluate whether male patients in their local Chinese population had differences in clinical features at diagnosis, course of disease and features, rate and severity of disease relapses, organ damage, and cumulative damage scores, Mok et al. performed a retrospective review of 51 male patients and 201 female patients at the Rheumatology and Nephrology Clinics of the Queen Mary Hospital in Pokfulam, Hong Kong [1]. Disease activity was measured by the SLEDAI and organ damage by the SLICC/ACR Damage Index. At the time of diagnosis, there was a trend, but not a statistically significant difference, in the following: males had less arthritis, alopecia, anti-Ro antibody, less Raynaud's, and more discoid lesions and thrombocytopenia. Regarding renal disease, 11 males had renal biopsy at presentation; six male (55%) patients had DPGN, while 30 females had biopsies at presentation and 20 (67%) had DPGN, which was not a significant difference. There was also no difference in the presence of anti-double-stranded DNA antibodies on presentation. In this population, there was no difference in subsequent rate of development of DPGN; however, a significantly higher proportion of males had impaired renal function, with glomerular filtration rate (GFR) <50% normal, and a trend toward higher cardiovascular damage. The overall percentage of males requiring dialysis was not different from females.

Table 1: Nonrenal manifestations of SLE more prominent in males by cohort.

Cohort	Laboratory abnormalities found increased in males	Clinical manifestations increased in males	Clinical manifestations decreased in males
LUMINA [4]	Lupus anticoagulant	Organ damage accrual	Musculoskeletal (MSK) disease
Hopkins [16]	Lymphopenia	Neuropsychiatric	Malar rash
	anti-Sm	Renal	Photosensitivity
	direct Coombs	Cardiovascular disease	Oral ulcers
	Lupus anti-coagulant	Peripheral vascular disease	Alopecia
	low C3		Raynaud's phenomenon
	anti-dsDNA		Arthralgia
Mok [1]	Thrombocytopenia	Discoid lesions	MSK disease
		Cardiovascular damage	Alopecia
			Raynaud's disease
GLADEL [12]	Leukopenia	Constitutional symptoms	Arthralgia
	Lymphopenia	Neurologic manifestations	MSK disease
	Hemolytic anemia	at onset	Skin disease
	Thrombocytopenia	Cardiovascular disease	
	IgG ACL Ab	Arterial HTN	
	Low C3		
Molina [8]	dsDNA ab	Arthritis	Raynaud's disease
		Vascular thrombosis	
		CNS manifestations	
		Cardiopulmonary disease	
Stefanidou [13]	Thrombocytopenia	Stroke	Alopecia
		APLS	Arthralgia
		GI symptoms	Photosensitivity
		Vascular thrombosis	Raynaud's disease

Total number of overall relapses was significantly less in males and severe flares also lower [1].

The above observation that disease course of SLE and male nephritis, aside from reduced GFR, was not different from males to females, differs from various other reports including another study of Acute Kidney Injury (AKI) in Chinese patients with lupus nephritis from a single center at the Peking University. AKI was defined as the presence of any one of the following items: an abrupt (within 48 hours) reduction in kidney function currently defined as an absolute change in serum creatinine of more than or equal to 0.3 mg/dL; a percentage increase in serum creatinine of ≥50% (1.5-fold from baseline); or a reduction in urine output (documented oliguria of less than 0.5 mL/kg per hour for more than 6 hours). AKI was previously identified as a risk factor for progression to ESRD. However, little was known about the patient characteristics of these patients or that gender may be a risk factor for AKI. The clinical, laboratory, renal histopathology, treatment, and outcome data were retrospectively collected and compared between lupus nephritis patients with and without AKI. The impact of AKI on renal outcome was evaluated [10].

Among 322 patients with renal-biopsy-proven lupus nephritis, 66 (20.5%) were identified as AKI. Male predominance was observed in patients with AKI ($P < 0.001$). The mean value of serum creatinine was 3.82 ± 2.59 mg/dL upon diagnosis. Most patients had hematuria (90.9%) and leukocyturia (71.2%). More than half of the patients presented with nephrotic syndrome (68.2%), with the amount of urine protein between 0.76 and 21.04 g/24 h (mean 6.57 ± 4.36). Regarding the pathological classification of lupus nephritis, the proportion of class IV was significantly higher ($P < 0.001$), and the proportion of classes III and V was significantly lower in the AKI group ($P < 0.001$ for both). In the AKI group, there was a significantly higher score of the total activity indices, endocapillary hypercellularity, cellular crescents, karyorrhexis/fibrinoid necrosis, subendothelial hyaline deposits, interstitial inflammation, leukocyte infiltration, the total chronicity indices, tubular atrophy, and interstitial fibrosis. In comparison with the non-AKI group, patients with AKI had significantly higher proportions of serositis ($P < 0.001$), neurologic disorder ($P = 0.026$), anemia ($P < 0.001$), thrombocytopenia ($P = 0.013$), and nephrotic syndrome ($P = 0.011$), but significant

Table 2: Comparison of gender differences in four separate cohorts of SLE patients.

Cohort		Male	Female	P value
LUMINA [4]	Patient no. (total)	63	555	
	Renal involvement	63.5%	52.1%	$P = 0.085$
GLADEL [12]	Patient no. (total)	123	1091	
	Renal involvement	58.5%	44.6%	$P = 0.004$
Molina [8]	Patient no. (total)	107	1209	
	Renal involvement	58%	44%	$P = 0.004$
Stefanidou [13]	Patient no. (total)	59	535	
	Renal involvement	27.1%	16.1%	$P = 0.002$

lower serum C3 ($P < 0.001$). The SLEDAI scores, renal pathological activity indices and, chronicity indices were significantly higher in the AKI group ($P < 0.001$ in all cases).

Regarding outcome, the AKI group had a significantly poorer renal outcome compared with non-AKI group ($P < 0.001$). In the AKI group, patients with crescentic glomerulonephritis and thrombotic microangiopathy had the worst renal outcome. AKI was an independent risk factor for renal outcome along with male gender, age, activity index score, presence of crescents, chronicity score, interstitial inflammation, glomerular sclerosis, fibrous crescents, tubular atrophy, and interstitial fibrosis. The findings were consistent with the previous studies where mostly male lupus patients presented with more severe renal involvement and poorer outcome than female lupus patients, in clinical manifestations, laboratory characteristics, pathological features, and outcome of patients with AKI [10].

4.2. South America. Moving to a different geographic region and ethnic group, a case-control study assessing renal outcome of lupus nephritis in male patients at the Sao Paulo University Medical School in Brazil, by Resende, et al. was published in 2011 [11]. The primary endpoint was doubling of serum creatinine and/or end-stage renal disease. The secondary endpoint was defined as a variation of GFR per year, calculated as the difference between final and initial estimated GFR (eGFR) adjusted by follow-up time for each patient. At baseline, male and female patients were not statistically different regarding WHO LN class, eGFR, follow-up time, and 24-hour proteinuria, as well as age, albumin, C3, antinuclear antibody, anti-DNA antibody, and hematuria. There was no difference in the primary outcome, but male gender was significantly associated with a worse renal function progression, as measured by GFR per year calculated as the difference between final and initial estimated GFR (eGFR) adjusted by follow-up time for each patient. The multivariate linear regression model showed that male gender remained statistically associated with a worse renal outcome even after adjustment for eGFR, proteinuria, albumin, and C3 complement at baseline.

The Grupo Latinoamericano de Estudio del Lupus (GLADEL) started in 1997 as a multinational inception prospective cohort in Latin American centers having expertise in the diagnosis and management of SLE. The data from the first 1214 patients was incorporated in a computer database available to all groups and interconnected among them. M. A. Garcia et al. used the data to analyze the influence of gender in the disease pattern and prognosis in a prospective cohort of SLE patients from 34 centers in nine Latin American countries: Argentina, Brazil, Colombia, Cuba, Chile, Guatemala, México, Peru, and Venezuela [12]. Of the 1214 SLE patients included in the GLADEL cohort, 123 were male. Demographic characteristics as well as clinical manifestations, laboratory profile, activity, and damage scores were evaluated at onset and during the course of the disease and compared with female patients. The median age at onset of the male patients was 27 and that at diagnosis 29.2 years. Delay to diagnosis was shorter in males (134 versus 185 days, $P = 0.01$). At onset, men more frequently showed constitutional symptoms and a higher prevalence of neurologic manifestations (4.5 versus 0.8%, $P \leq 0.053$). During disease course, renal disease, characterized by persistent proteinuria and/or cellular casts, was significantly higher in males (58.5% versus 44.6%, $P = 0.004$), as was hemolytic anemia. Males more frequently had leukopenia, lymphopenia, thrombocytopenia, hemolytic anemia, IgG anticardiolipin antibodies, and low C3, along with any form of cardiovascular manifestation (56.1 versus 41.4%, $P = 0.002$), particularly arterial hypertension. Although not statistically significant, mortality was also higher in men [12]. Arthralgia and/or arthritis were more frequent among women (Tables 1 and 2).

Molina et al. had found similar results in an earlier cross-sectional study of 107 Latin American male patients compared to a group of 1,209 Latin American female patients with SLE [8]. In this population at hospitals in Columbia and Mexico, the three most common findings in males were arthritis, skin, involvement, and renal disease. Renal involvement (58% versus 44%, $P = 0.004$) and vascular thrombosis occurred at significantly higher rates in males. The rate of nephrotic syndrome (31% versus 22%, $P = 0.04$) and the presence of dsDNA antibodies were significantly higher in males, as was the use of moderate-to-high doses of corticosteroids. Diffuse proliferative nephritis was the most common biopsy finding in both groups. Although there was no difference in mortality from all causes, SLE-related mortality was higher in the male group. The use of cytotoxic agents, dialysis, and renal transplantation was higher in the male group,

though not statistically significant. Extrarenal manifestations more prevalent amongst males included CNS involvement, osteonecrosis, and severe cardiopulmonary disease.

4.3. Europe. Gender differences in SLE and nephritis were studied by two separate groups in Greece. The more recent retrospective study by Stefanidou et al. sought to analyze the prevalence of the most relevant clinical features of SLE in a sample of male patients as well as the incidence of the main causes of morbidity in a 5-year period after the diagnosis. Another goal was to investigate the impact of gender on expression and morbidity of SLE. Data were collected from the medical records of 59 male and 535 female patients with SLE who were diagnosed at the hospitals in the region of Thessaloniki. Several differences in the expression and morbidity of the disease were found in relation to the gender of the patient. Male patients had a higher prevalence of thromboses, nephropathy, strokes, gastrointestinal tract symptoms, and antiphospholipid syndrome when compared with female patients, but tended to present less often with arthralgia, hair loss, Raynaud's phenomenon, and photosensitivity as the initial clinical manifestations. The rates of nephropathy were 27.1% in males versus 16.1% in females ($P = 0.002$, OR = 2.806, 95% CI = 1.462–5.382). Specific details about class of nephritis or progression to ESRD and dialysis were not provided. The rates of thrombosis were 20.3% versus 4.7% ($P < 0.001$, OR = 5.832, 95% CI = 2.698–12.608), and stroke 8.5% versus 0.9% ($P < 0.001$, OR = 12.289, 95% CI = 3.176–47.55). During the 5-year followup, positive associations were noted between male gender and the incidence of tendonitis, myositis, nephropathy, and infections, particularly of the respiratory tract [13].

Voulgari et al. published a study of 489 Greek patients who visited the Rheumatology Department of the University Hospital of Ioannina, Greece, between 1981 and 2000. Four hundred and twenty-one were female (86%), while 68 were male (14%). There were no significant differences in the mean age at presentation, mean age at disease onset, duration of the disease, or in duration of followup between men and women. At the time of diagnosis, there were no major differences in major organ involvement; during followup, men had a greater frequency of renal involvement in younger patients. Eight men (14%) and 25 women (7%) developed chronic renal failure, while 3 men (5%) and women (1%) developed end-stage renal disease. No other major organ differences were observed between genders [14].

4.4. North America. The study of SLE within the United States Military presents a unique opportunity for examining clinical variables in the period before diagnosis. In addition, the demographics of the United States Military increase the expected proportion of male SLE patients. All military personnel have readily accessible health care and are required to receive regular physical exams, minimizing the impact of health care disparities and, to a lesser extent, of socioeconomic and cultural differences. The US Department of Defense serum repository contains samples from active duty personnel at entry into the military and, on average, every other year thereafter. Arbuckle et al. used this data to make comparisons between demographic, serologic, and clinical manifestations, as well as to identify factors associated with the manifestations of disease during the clinical period after the initial presentation to diagnosis of lupus. A cohort was assembled of 130 individuals diagnosed with SLE while on active duty in one of the United States uniformed services, with the goal of identifying potential subsets within the patient population [3].

Sixty-five of the patients were women; among the entire population 62% were African American (AA) and 26% were European American (EA). Asians and Hispanics comprised three and 9%. Individual gender subgroups contained sufficient numbers to permit separate analyses for AA and EA men and women. No difference in the time of the occurrence of the first criterion to diagnosis was based upon ethnicity alone; however, the time from this first manifestation to diagnosis was significantly shorter for males when compared with females. Evaluation of AA males in whom the diagnosis was made 0.17 years after the presence of their first clinical criteria suggests that this difference was due almost exclusively to AA males. Eighty-seven percent of AA males met SLE diagnostic criteria less than six months after their initial presentation criteria compared with 42% of AA females ($P = 0.001$; OR = 9.2). Manifestations at presentation and during followup were identified for AA males and compared to others (AA females and EAs) in order to assess for clinical variables that might contribute to the rapid onset observed in AA males. AA males had more anti-RNP antibodies, developed more nephritis and had less cutaneous manifestations than AA females and EAs. African American males were much more likely to present with nephritis, pleuritis, and/or seizures than others [3].

In another US study, Crosslin and Wiginton utilized hospital discharge data collected during a seven-year period to determine the effect of gender on SLE comorbidities and disease severity. Patients were hospitalized in the Dallas-Fort Worth metropolitan area between 1999 and 2005 and had a diagnosis of SLE. The sample consisted of 14,829 patients with SLE, 10% of which were male. Differences between males and females for disease severity, age, length of stay in the hospital, total hospital charges, and number of autoimmune diseases were studied. Disease severity was measured with the SLE comorbidity index, which weights 14 conditions in SLE. Male patients had significantly greater disease severity compared with female patients, while female patients had more autoimmune diagnoses compared with male patients. Male patients were more likely to have cardiovascular and renal comorbidities compared with female patients. Female patients had significantly greater odds of diagnoses of urinary tract infection, hypothyroidism, depression, esophageal reflux, asthma, and fibromyalgia. Males had greater odds of acute and chronic renal failure and nephritis, specifically, as well as thrombocytopenia, CHF, and arrhythmias. These findings corroborated those of previous studies of male severity of renal involvement in SLE [15].

Most recently, Tan et al. published a study of comparing male and female patients with SLE in the Hopkins Lupus Cohort. The cohort consisted of 157 men (66.2% white,

33.8% African American) and 1822 women (59.8% white, 40.2% African American).The mean followup was 6.02 years (range 0–23.73). Men were more likely than women to have hypertension, thrombosis, renal, hematological, and serological manifestations. Specifically increased in males were lymphopenia, positive anti-Sm, direct Coombs, lupus anti-coagulant, low C3, and anti-dsDNA. Men experienced increased rates of end-organ damage including neuropsychiatric, renal, cardiovascular, peripheral vascular disease, and myocardial infarction, and to have died. Women were more likely to have malar rash, photosensitivity, oral ulcers, alopecia, Raynaud's phenomenon, or arthralgia [16].

5. Gender Differences in Disease Manifestations: Pediatric Lupus

In the pediatric SLE population, as previously stated, the gender difference in numbers of patients is much reduced, to a ratio of three to one females to males before puberty. Several studies have reported that children have often an aggressive clinical course with more frequent renal involvement as compared to adults. Boys were reported to have a higher prevalence of severe renal disease and poorer outcome, but other reports did not confirm these findings [17]. Al-Mayouf and Sonbul sought to determine the influence of gender and age of onset on the outcome in Saudi children with SLE [17]. Outcome measures included SLICC/ACR, renal disease requiring dialysis, or transplant and death related to SLE. Patients were classified based on age at disease onset into early onset (<5 years) and late onset (>5 years). Eighty-nine patients (76 female and 13 male) were included, with median disease duration of 5 years. Twelve patients had early-onset disease. There was no difference in the mean age, age at diagnosis, disease duration, and followup between the different groups. Logistic regression analysis showed significant association of high SLICC/ACR score with early-onset disease and male gender, while renal disease requiring dialysis and renal transplant was associated significantly with male gender independently of age of disease onset. Death related to SLE was influenced by early-onset disease. Male children and early-onset disease of this cohort had poorer outcome [17].

6. Molecular Mechanisms That May Contribute to Gender Bias in Lupus: Estrogen and Its Receptors

As above, sex hormones are probably partly responsible for the higher occurrence of autoimmune disorders given the female predominance in autoimmune diseases. However, studies have found that sex hormone levels in patients with autoimmune disorders are not significantly different from patients without autoimmune disorders, indicating that other gender-associated differences including hormone regulation and effects on cytokine production, along with chromosomal factors, contribute to the high female predominance in these diseases compared with men [18]. Thus, the relationship of sex hormones increasing serum levels of certain cytokines and the estrogen receptor may be important in disease development [19].

Estrogen's primary effects are mediated via estrogen receptors alpha and beta (ER α/β) that are expressed on most immune cells. ERs are nuclear hormone receptors that can either directly bind to estrogen response elements in gene promoters or serve as cofactors with other transcription factors. ERs have prominent effects on immune function in both the innate and adaptive immune responses. Genetic deficiency of ERα in murine models of lupus results in significantly decreased disease and prolonged survival, while ERβ deficiency has minimal to no effect in autoimmune models [20]. These two isoforms of ER are able to modulate the cytokine production of various key target cells of the immune system.

ERα is expressed in most immune cells both at baseline and at increased levels after estrogen is given, in particular on antigen presenting cells. It can be detected in thymocytes, bone marrow nonhematopoietic cells, T cells, B-cell precursors, and circulating B cells, as well as dendritic cells (DCs) [20]. Estradiol can modulate lymphocyte cytokine production, cytokine receptor expression, and activation of effector cells. Estrogens favor the Th2 immune response, and enhanced interferon-γ (INFγ), TNFα, TGFβ, interleukin (IL)-1, IL-5, IL-4, and IL-10 production. Estrogen causes a proliferation of M2 macrophages, and myeloid-derived suppressor cells, further amplifying the Th2 immune response [18]. Estrogen and prolactin are both capable of stimulating autoreactive B cells, promoting the failure of immune tolerance and secretion of autoantibodies [19].

Regarding DCs, Oertelt-Prigione found that exposure of immature murine DCs to estrogen increased their IL-6, IL-8, and MCP-1 production, but most importantly enhanced their stimulatory capacity on T lymphocytes, and another group demonstrated estrogen's role in enhancing the differentiation of DCs from bone marrow in vivo. Estrogen-driven upregulation of MHC II in the dendritic cells, enhancement of proinflammatory cytokine production, and an interaction between estrogen and MHCII expression regulation have also been confirmed in animal models [21].

Further evidence of sex hormones influence on systemic autoimmunity is derived from lupus animal models (NZB X NZWF1). In this model females develop disease earlier than males and die at younger age. Oophorectomy delays the disease onset while castrated males suffer of early and more severe disease; treatment with estrogens and prolactin causes early mortality. In comparison, mice treated with the estrogen receptor antagonist tamoxifen have a mild disease and a longer life [19]. In a recent murine study ERα KO genotype was bred onto three different murine lupus-prone strains, NZB/NZW f1, MRL/lpr, and NZM2410. In each of these three strains, the lack of ERα significantly attenuated disease. In NZB/NZW mice, the lack of ERα resulted in decreased autoantibody levels, while in NZM2410 and MRL/lpr mice, autoantibodies, if anything, were increased; the primary impact of ERα deficiency appeared to be on the response of the kidney to immune injury. When used in murine models of lupus, ER inhibitors were reported to have beneficial effects on lupus disease expression [20].

Additional details about estrogen-induced modulation of cytokine production in SLE mediated by the estrogen receptor and of the various aspects of estrogen receptor signaling in this disease, estrogen receptor subtypes, their structure, and the mode of action of estrogens by gene activation and via extranuclear effects are outlined in a recent review by Kassi and Moutsatsou [22].

7. Gender Differences in Pathogenesis

7.1. Genetics. As SLE affects more females than males, it is unexpected that the disease in males would be more severe. Given this predominance of the disease in females and its association with higher estrogen in both males and females, Hughes et al. sought to clarify why affected men often experience more severe disease by examining sex-specific genetic effects among SLE susceptibility loci. The group of male patients in this study was noted to have twice the risk of renal disease (OR = 1.70 (95% CI = 1.34 to 2.17, $P = 1.2 \times 10^{-5}$)) and more likely to have thrombocytopenia (OR = 2.26 (95% CI = 1.62 to 3.15, $P = 5.7 \times 10^{-7}$)) [23]. They investigated differences in allelic frequency between men and women using 18 previously identified independent autosomal genetic susceptibility loci for SLE. Genotyping was performed on over four thousand patients with SLE and nearly the same number of healthy controls. Sex-specific genetic association analyses and cumulative genetic risk scores for SLE in each individual were calculated to examine aggregate differences in sex-specific genetic risk. The genetic risk for SLE was significantly higher in males than females; more specifically, the frequency of two risk alleles in the HLA locus was significantly higher in males (rs3131379: OR male-female 1.37 (95% CI = 1.14 to 1.66), $P = 0.0010$; rs1270942: OR male-female 1.40 (95% CI = 1.16 to 1.69), $P = 0.00046$). This was also the case for an SNP in IRF5 (rs2070197: OR male-female 1.23 (95% CI = 1.01 to 1.49), $P = 0.039$). There was no difference in the risk allele frequencies in the control group between men and women ($P = 0.39$, 0.52 and 0.64, for rs3131379, rs1270942, and rs2070197, resp.).

7.2. Hormones. In many clinical and experimental scenarios, the incidence and the rate of progression of non-lupus-related renal diseases are influenced by multiple gender-dependent factors, such as kidney and glomerular size, differences in glomerular hemodynamics, and direct effects of sex hormones on renal tissue and signal pathways such as the renin-angiotensin-aldosterone system and signal molecules (e.g., nitric oxide, reactive oxygen species, cytokines, and growth factors) [24]. It has been shown that the main female hormone, 17β estradiol, is capable of inhibiting inflammatory and proapoptotic processes and protecting the renal tissue. In contrast, the male hormones, testosterone and dehydroepiandrosterone, have the opposite effect. Hormonal manipulation by male or female castration changes the course of renal disease progression and confirms the influence of the sex hormones. Female gender is therefore considered a protective factor in many kidney diseases, such as primary glomerulonephritis, autosomal dominant polycystic kidney disease (ADPKD), and hypertensive nephropathy [24].

Studies in patients with chronic kidney diseases have also shown that men have a more rapid disease progression and that with age, men exhibit greater decrements in renal function and increased glomerular sclerosis than women [25]. Women with several nondiabetic renal diseases such as membranous nephropathy, IgA nephropathy, and polycystic kidney disease present with a slower progression [25]. Thus, men appear to be at greater risk for renal injury than are women, though the exact reasons have not yet been established. It has been suggested that sex hormones mediate the effects of gender on chronic renal disease, through the interaction with the renin-angiotensin system, the modulation of nitric oxide synthesis, and the down regulation of collagen degradation. Androgens may contribute to continuous loss of kidney cells though the stimulation of programmed cell death which is activated in several chronic kidney diseases. Studies in vitro indicate that androgens prime a Fas/FasL-dependent apoptotic pathway in kidney tubule cells. The mechanisms to cell death which are primed by androgens may interact with others occurring in several conditions leading to the loss of renal cells. These findings are consistent with a role for androgens to promote chronic renal injury in men [25]; however, none of these findings have been directly connected to SLE renal disease.

Although the gender effect of dimorphism in lupus nephritis development has been often attributed to sex hormones as above, XXY males have nearly a 14-fold higher risk of developing SLE than 46 XY males, indicating that X-linked genes may also be risk factors for SLE in humans [26].

7.3. Toll-Like Receptors. Located at Xp22.2, Toll-like receptor 7 and its functionally related gene *TLR8* encode proteins that play critical roles in pathogen recognition and activation of innate immunity; they recognize endogenous RNA-containing autoantigens and when stimulated promote the expression of type I IFN, a pivotal cytokine in the pathogenesis of SLE. Animal models have demonstrated a connection between X-linked gene overexpression and TLR7. The BXSB strain of mice spontaneously develops an autoimmune syndrome with features of SLE; males are affected much earlier than females. Genetic analysis of the F1 hybrids of male BXSB mice with other lupus-prone mice demonstrated that the accelerated development of SLE in male BXSB mice is linked to the Y chromosome of the BXSB strain. This genetic abnormality present in BXSB Y chromosome has thus been called *Yaa*, Y-linked autoimmune acceleration [27].

The Yaa mutation was shown to be a consequence of a translocation from the telomeric end of the X chromosome and onto the Y chromosome [27]. Based on the presence of the gene encoding TLR7 in this translocated segment of the X chromosome, the possible role of TLR7 in the activation of autoreactive B cells and the development of SLE, the TLR7 gene duplication has been proposed to be the etiological basis for the Yaa-mediated enhancement of disease. Studies of Yaa and non-Yaa double bone marrow chimeric mice have demonstrated that anti-DNA autoantibodies are selectively

produced by B cells bearing the Yaa mutation, and that T cells from both Yaa and non-Yaa origin efficiently promote anti-DNA autoantibody responses [27].

Amano et al. additionally demonstrated that the Yaa mutation causes defective development of marginal zone B cells in BXSB mice, suggesting a role for the marginal zone B cells in the generation of pathogenic autoantibodies in SLE [28]. Shen et al. have identified the association of a TLR7 SNP with SLE in 9274 Eastern Asians with a stronger effect in males than female subjects (odds ratio, male versus female = 2.33 (95% CI = 1.64–3.30) versus 1.24 (95% CI = 1.14–1.34); $P = 4.1 \times 10^{-4}$). Their data established a functional polymorphism in type I IFN pathway gene TLR7 predisposing to SLE, especially in Chinese and Japanese human male subjects [26].

8. Summary and Conclusions

The impact of gender in SLE renal disease has been assessed thus far, mostly with chart reviews and retrospective analyses. In order to further identify and clarify the true and significant differences in pathogenesis, prognosis, and long-term outcome, more systematic and prospective studies should be undertaken. Both nonrenal (Table 1) and renal manifestations (Table 2) have been compared in male versus female patients in several cohorts. It seems clear that both in the adult and pediatric lupus populations, male patients have greater disease severity, including rapid clinical progression to diagnosis, progression to renal injury and failure, and greater renal-related morbidity. Separation by gender for future studies of treatment outcomes might serve to identify which of the many existing and competing treatment strategies have the greatest benefit for each group, and to further identify which subgroups should be targeted for aggressive treatment at diagnosis.

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Review Article

Pulmonary Arterial Hypertension in Systemic Lupus Erythematosus: Current Status and Future Direction

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Pulmonary arterial hypertension (PAH) is commonly associated with connective tissue diseases (CTDs) including systemic sclerosis and systemic lupus erythematosus (SLE). The prevalence of PAH in SLE is estimated to be 0.5% to 17.5%. The pathophysiology of PAH involves multiple mechanisms from vasculitis and *in-situ* thrombosis to interstitial pulmonary fibrosis which increases pulmonary vascular resistance, potentially leading to right heart failure. Immune and inflammatory mechanisms may play a significant role in the pathogenesis or progression of PAH in patients with CTDs, establishing a role for anti-inflammatory and immunosuppressive therapies. The leading predictors of PAH in SLE are Raynaud phenomenon, anti-U1RNP antibody, and anticardiolipin antibody positivity. The first-line of diagnostic testing for patients with suspected SLE-associated PAH (SLE-aPAH) involves obtaining a Doppler echocardiogram. Once the diagnosis is confirmed by right heart catheterization, SLE-aPAH patients are generally treated with oxygen, anticoagulants, and vasodilators. Although the prognosis and therapeutic responsiveness of these patients have improved with the addition of intensive immunosuppressive therapies, these treatments are still largely unproven. Recent data put the one-year survival rate for SLE-aPAH patients at 94%. Pregnant women are most at risk of dying due to undiagnosed SLE-aPAH, and screening should be considered essential in this population.

1. Introduction

Pulmonary arterial hypertension (PAH) is a complex and devastating disease. PAH is defined as an increase in mean pulmonary arterial pressure (mPAP) ≥ 25 mmHg at rest, pulmonary artery wedge pressure (PAWP), or left ventricular end diastolic pressure ≤ 15 mmHg and increased pulmonary vascular resistance (PVR) [1]. PAH can be idiopathic (IPAH), heritable, drug, or toxin induced or associated with human immunodeficiency virus infection, portal hypertension, congenital heart diseases, schistosomiasis, or chronic hemolytic anemia. It can also be associated with varied connective tissue diseases (CTDs) such as systemic sclerosis (SSc), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), or mixed connective tissue disease (MCTD). These PAH-associated conditions are categorized in the World Health Organization (WHO) Group 1 PAH classification [1, 2].

While the pathophysiologic pathways linking PAH to SLE have not been adequately explored, this paper will address the key research findings and available data on this subject, as derived from an extensive literature review. PAH disease progression is characterized by narrowing of the pulmonary arterial bed due to extensive endothelial, adventitial and smooth muscle dysfunction. Genetic, environmental, and other predisposing conditions, including vasodilator and vasoconstrictor imbalance, inflammatory and uncontrolled immune response, and an imbalance between proliferation and apoptosis [3, 4], lead to constrained blood flow, potentially resulting in increased pulmonary vascular resistance. Patients with unrecognized PAH or those who are not yet treated progress to right ventricular dilatation and failure, which can ultimately lead to death.

Recent intensive immunosuppressive and vasodilator therapies have shown a lot of promise in treating SLE-associated PAH (SLE-aPAH). Recent data reveal that one-year survival rate was notably higher (at 94%) in SLE-aPAH patients when compared to that for SSc-aPAH patients

Table 1: REVEAL registry demographic and diagnostic comparison.

	IPAH	CTD	SLE-aPAH	SSc-aPAH
Total # of patients	1251	641	110	399
Patients newly diagnosed at enrollment (%)	14	15	14	16
Age (years)	50.1 ± 17.5	57.1 ± 13.7	45.5 ± 11.9	61.8 ± 11.1
Sex, (#)				
Female	987	578	104	353
Male	264	63	6	46
Race (%)				
White	74.8	71.8	37.4	83.9
African-American	11.7	16.5	31.8	10.9
Hispanic	8.3	7.5	17.8	3.6
Other	5.2	4.2	13.1	1.6
Raynaud phenomenon (%)	1.4	26.5	13.6	32.6
Renal insufficiency (%)	3.9	6.9	4.6	8.7
Time between diagnostic RHC and enrollment (months)	41.1 ± 44.1	27.2 ± 29.9	34.4 ± 39.1	24.2 ± 24.1
BNP (pg/mL)	245.6 ± 427.2	432.8 ± 789.1	263.8 ± 338.8	552.2 ± 977.8
DLCO (%)	63.6 ± 22.1	44.9 ± 18	53.3 ± 19.5	41.2 ± 16.3
Immunosuppressive therapy (%)	1.3	11.9	22	6.8
Alive at 1 year (%)	93	86	94	82

(at 82%) [5, 6]. The hospitalization rates were also significantly lower in SLE-aPAH patients. Although the prognosis and therapeutic responsiveness of these patients have improved relative to the better understood SSc-associated PAH patients (SSc-aPAH), these therapies are still unproven and require further study.

2. Prevalence and Demographics

The prevalence of all PAH has been estimated at 15 cases per million (adults) according to the national French registry [7]. Studies from France and Scotland estimated the prevalence of CTD-associated PAH (CTD-aPAH) to be 2.3 and 10 cases per million, respectively, within their general population [7, 8]. The prevalence of PAH in SLE is estimated to be 0.5% to 43% in some older studies [9–12] and 0.5% to 17.5% in two newer French studies [13, 14]. The estimated prevalence range is wide, caused by multiple factors such as varied population groups, lack of a uniform PAH definition, and different diagnostic approaches (echocardiogram versus right heart catheterization (RHC)) [9–14]. In a large community-based lupus cohort from the United Kingdom ($n = 288$), the prevalence of SLE-aPAH was 4.2%. However, the UK study used echocardiogram, which tends to yield estimated systolic pulmonary artery pressures that can differ significantly from the "gold standard", RHC [9].

The Registry to Evaluate Early and Long-term Pulmonary Arterial Hypertension Disease Management (REVEAL) is a 54-center longitudinal US based registry for patients with PAH. It has the largest cohort of patients ($n = 2,967$) with PAH confirmed by RHC. The registry included 641 patients with CTD-aPAH, of which 110 patients had SLE-aPAH, including approximately 15 patients with newly diagnosed SLE-aPAH. Table 1 provides a comparative analysis of demographic and diagnostic features of the IPAH, CTD-aPAH, SLE-aPAH, and SSc-aPAH patients observed in the registry. Patients with SLE-aPAH were younger compared to other CTD-aPAH patients. Both SLE-aPAH and CTD-aPAH patient groups were comprised predominantly of women who had similar body mass indices.

Cohort studies, other than REVEAL, have similarly confirmed the SLE-aPAH patients' demographics: patients are predominantly females of child-bearing age, from 18 to 40 years, with a female to male ratio of 10 : 1. The majority of patients with SSc-aPAH in the REVEAL cohort were white (84%), compared with only 37% of SLE-aPAH patients. Approximately one-third of patients with SSc-aPAH and MCTD-aPAH were reported to suffer from Raynaud phenomenon, compared with 14% of patients with SLE-aPAH ($P < 0.0001$). Although other studies have estimated the prevalence of Raynaud phenomenon in SSc and SLE to be as high as 90% and 45%, respectively, the registry revealed low numbers for both SLE and SSc due to underreporting of this data in the REVEAL registry [6, 11, 15–18].

Two large cohort studies have examined the differences in treatment of SLE-aPAH versus SSc-aPAH. SLE-aPAH patients were more likely to receive immunosuppressive therapies in both US and UK cohorts. In the REVEAL cohort (US), 22% of SLE-aPAH patients received immunosuppressive therapy versus 6.8% of SSc-aPAH patients. Due to the different therapeutic approaches, nearly four times as many UK based cohort patients with SLE-aPAH received immunosuppressive therapy [5, 6].

3. Pathobiology of Systemic Lupus Erythematosus-Associated Pulmonary Hypertension

Although a causal relationship between SLE and PH has not been established, the various elements of SLE, from vasculitis

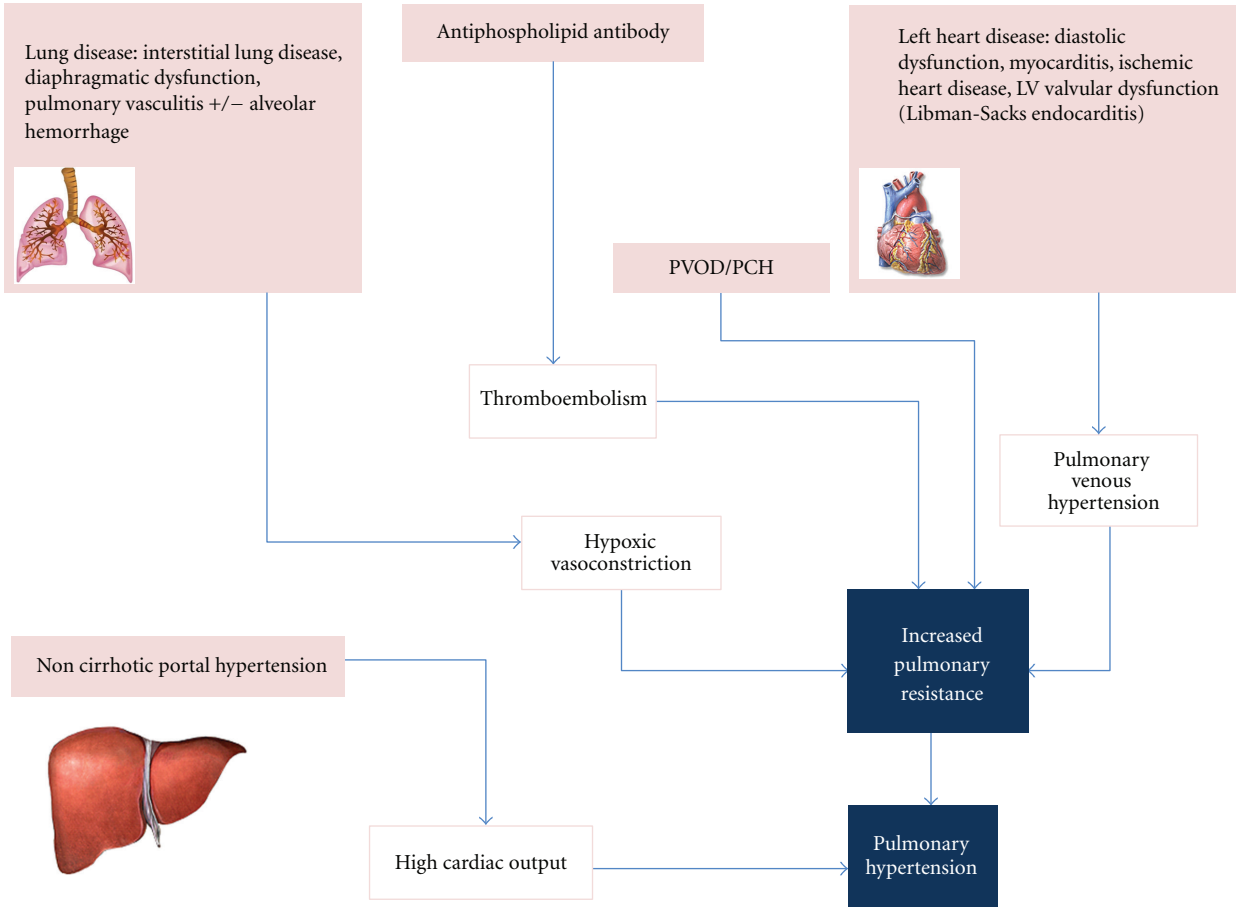

Figure 1: Pathophysiology of pulmonary hypertension in systemic lupus erythematosus. Pulmonary venoocclusive disease (PVOD); pulmonary capillary hemangiomatosus (PCH); left ventricle (LV).

and *in-situ* thrombosis to interstitial pulmonary fibrosis, can lead to endothelial and smooth muscle proliferation and damage of the pulmonary vasculature resulting in PH [16–18, 28]. Increased pulmonary vascular resistance may result from multiple mechanisms in patients with SLE-aPAH, including hypoxia due to lung disease (hypoxic vasoconstriction), pulmonary venous hypertension due to left heart disease, antiphospholipid antibody predisposing to *in-situ* thrombosis or acute/chronic thromboemboli, high output state from non cirrhotic portal hypertension, and pulmonary venoocclusive disease (PVOD)/pulmonary capillary hemangiomatosis (PCH) [29–35] (refer to Figure 1).

Autopsy findings in multiple reports suggest multifactorial mechanisms for SLE-aPAH. Vascular pathologic findings in patients with SLE-aPAH are similar to those in patients with IPAH, including the plexiform lesions, muscular hypertrophy, and intimal proliferation [36]. In addition, studies have shown an imbalance between vasoconstrictors and vasodilators in SLE-aPAH, with higher levels of endothelin-1 [37] and thromboxane A2 and an inhibition of prostacyclin production by endothelial cells. It should be noted that these imbalances (elevation of thromboxane A2 and inhibition of prostacyclin) have not been shown specifically in SLE patients and are extrapolated from IPAH and SSc data. Another key mechanism involves immunoglobulin and complement deposition in the arterial walls [38, 39]. Tables 2 and 3 summarize the pathology and the key causative mechanisms in pulmonary arterial hypertension due to systemic lupus erythematosus [40–64].

Table 2: Pathology of systemic lupus erythematosus associated pulmonary hypertension.

Pathological changes in arteries, arterioles and veins
(i) Medial hypertrophy
(ii) Chronic intimal fibrosis
(iii) Periadventitial fibrosis
(iv) Alteration of elastic laminae
(v) Necrotizing fibrinoid arteriopathy
(vi) Aneurysmal dilatation and plexiform lesions
Pathological changes in Thrombotic Arteriopathy
(i) Intimal eccentric fibrous thickening
(ii) Luminal occlusion with recanalization
(iii) Plexiform lesions coexistent with intimal thrombotic lesions in some arteries
(iv) Concentric laminar intimal fibrosis not present

Table 3: Key causative mechanisms of PAH in systemic lupus erythematosus.

Mechanisms similar to IPAH patients
(i) Overactivation of transcription factors (hypoxia inducible factor-1 alpha and Nuclear Factor of activated T lymphocytes)
(ii) Decreased expression of certain voltage gated potassium channels
(iii) *De novo* expression of the antiapoptotic proteins
Mechanisms involving inflammation and autoimmunity
(i) Chronic inflammation caused by viral infections and autoimmune diseases, leading to the migration of monocytes, neutrophils, mast cells, and dendritic cells to the structurally damaged pulmonary artery
(ii) Invasion of the elastic lamina, stimulating the release of chemokines, cytokines and growth factors
(iii) Resultant vascular remodeling, collagen deposition, and uninhibited proliferation of endothelial cell
Immune dysregulation mechanism
(i) Decreased percentage of $CD4^+/CD25^+$ T cells, diminished regulation by regulatory T cells and B cells, and stimulated signals to B cells
Pathology involving autoantibodies
Antiendothelial cell antibodies (AECA)
(i) AECA prevalence ranges from 15% to 80%
(ii) AECA levels are increased in active SLE, in particular in patients with nephritis, PH and vascular injuries.
(iii) AECA enhances release of endothelin-1
(iv) Binding of AECA or immune complexes may augment release of interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α)
Antiphospholipid antibodies (aPL)
(i) Present in 40% of patients with SLE
(ii) aPLs activate the endothelial cells, monocytes, and platelets leading to a prothrombotic state
Other autoantibodies in SLE-associated PAH
(i) Antinuclear antibody (ANA) invariably present
(ii) >25% prevalence of ribonuclear protein (RNP)
(iii) 50% to 80% prevalence of rheumatoid factor (RF)

Immune and inflammatory mechanisms may play a significant role in the pathogenesis or progression of PAH, especially in patients with connective tissue diseases, establishing a role for anti-inflammatory/immunosuppressive therapy. The inflammatory hypothesis in PAH has been validated in multiple studies, due to the finding of infiltration of macrophages and lymphocytes in the plexiform lesions. Similarly, the finding of IgG and complement in the pulmonary artery walls lends support to the immune hypothesis. Furthermore, in support of the immune mechanism, researchers have found elevated serum levels of proinflammatory cytokines and overexpression of growth factors in diseased pulmonary arteries of severe PAH patients [4, 40, 49, 52].

Figure 2 is a diagrammatic representation of the role of inflammation and dysregulated immune response in the development of PAH in SLE [52].

4. Clinical Features

The most common presenting symptoms of SLE-aPAH are dyspnea, chest pain, dry cough, and fatigue. The onset of PH in patients with SLE does not correlate with disease duration or the degree of extrapulmonary manifestations of the illness, and patients may present with PH in advance of their diagnosis of SLE. Physical findings of elevated jugular venous pressure, fixed S2, murmurs of tricuspid or pulmonic insufficiency, liver enlargement ascites, and lower extremity edema occur as a consequence of right ventricular strain, enlargement, or failure.

The study by Lian et al. [65] examines the predictors contributing to SLE-aPAH as shown in Table 4. By using univariate and multivariate regression models, the authors identified the leading predictors of PAH in SLE to be Raynaud phenomenon, anti-U1 RNP antibody, anticardiolipin antibody positivity, and serositis (statistically significant in the univariate regression model only), noted as § in the table. Echocardiography to evaluate pulmonary artery pressure and right heart function is recommended in SLE patients with these leading independent predictors. Additionally, patients with SLE-aPAH tend to have a high SLE disease activity index score.

The extrapulmonary symptom of Raynaud phenomenon, one of the major predictors of PAH in SLE, is present in 75% of patients with SLE-aPAH and in 10%–45% of all patients with SLE [11, 17, 18].

Pleural effusions are uncommon in pulmonary arterial hypertension. In a study of 89 patients with PAH associated with CTD, Luo et al. [66], demonstrated that 39.3% of the patients had trace to small and bilateral pleural effusions including 37.5% of the patients (6 out of 16) with SLE-aPAH. When compared with the patients without pleural effusions, the patients with pleural effusions had significantly higher mean right atrial pressures and lower cardiac indices.

Exercise intolerance is common in patients with SLE. However, the assessment of these patients can be confusing and difficult because the intolerance could also be

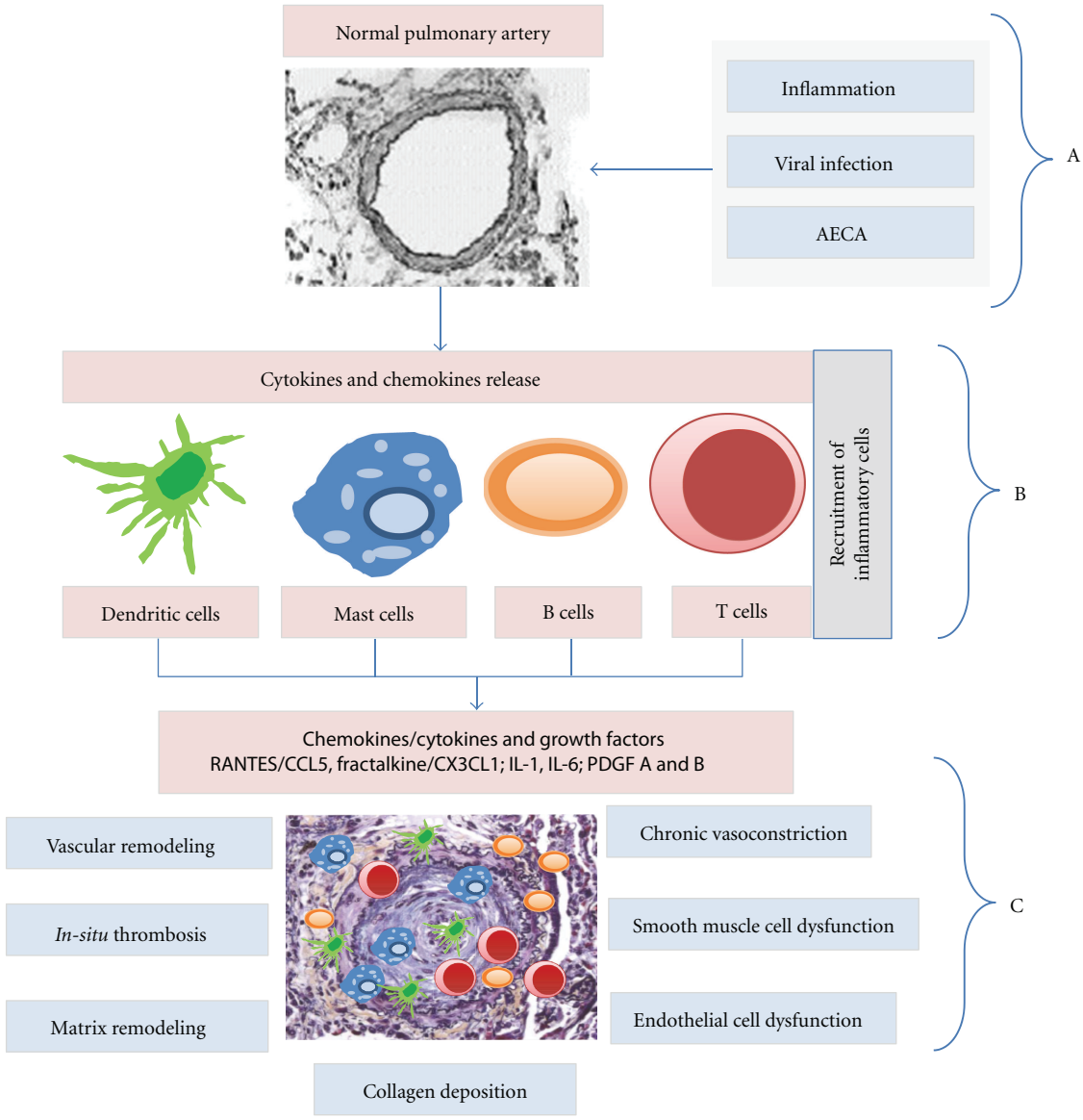

Figure 2: Role of inflammation and Dysregulated immune response in the development of PAH in SLE. (A) Viral infection, AECA, and other agents damage the normal pulmonary endothelium. (B) Increase in chemokine/cytokine concentrations as a result of endothelial injury, leading to recruitment of dendritic cells, mast cells, B cells, and T cells. (C) Infiltration of the small, and medium-sized pulmonary arteries by the dendritic cells, mast cells, B cells and T cells, resulting in dysregulated angiogenesis. AECA: antiendothelial cell antibodies; RANTES: regulated upon activation, normal T cell expressed and secreted; CCL5: chemokine Ligand 5; CX3CLI: chemokine Ligand 1 [Fractalkine]; IL-1: interleukin-1; IL-6: interleukin-6; PDGF: platelet derived growth factor.

attributed to other concomitant conditions such as physical deconditioning, arthritis/arthralgias, obesity, myopathy or neuropathy [39].

5. Antiphospholipid Syndrome

Antiphospholipid syndrome (APS), defined as the continuous presence of antiphospholipid antibody (aPL) with arterial, venous, or small vessel thrombosis, with or without recurrent pregnancy losses, can occur in association with SLE. The high prevalence of antiphospholipid antibodies in SLE-aPAH patients is well known, occurring in 83% of patients with SLE-aPAH and in 30% to 50% of patients with SLE without PAH, compared to 7% in patients with systemic sclerosis. These antibodies have been reported in 10% to 15% of IPAH patients who may be at risk for developing an underlying CTD, such as SLE, later on in the disease course.

Pathogenic aPLs activate the endothelial cells, monocytes, and platelets leading to a prothrombotic state. Patients with these antibodies are more susceptible to developing thrombotic arteriopathy and therefore require a careful assessment for chronic thromboembolic pulmonary hypertension (CTEPH). Increased levels of circulating endothelin-1 have been reported in patients with aPL, possibly contributing to vasoconstriction and PAH [47–51]. Patients with

Table 4: Possible risk factors for the development of PH in systemic lupus erythematosus.

(i) Female gender
(ii) Isolated reduction in diffusion
(iii) Raynaud phenomenon §
(iv) Serositis §
(v) Renal disease
(vi) Digital gangrene
(vii) Cutaneous vasculitis/livedo reticularis
(viii) Rheumatoid factor
(ix) Anti-U1 RNP §
(x) Anticardiolipin antibodies §
(xi) Antiendothelial cell antibodies

APS and SLE with high levels of aPL also have increased prevalence of valvular disease (Libman-Sacks endocarditis) which can contribute to pulmonary venous hypertension.

6. Diagnosis

PAH may be suspected due to findings on routine chest radiography and/or 12-lead electrocardiogram, obtained in the evaluation of dyspnea. Computerized tomography of the lung to rule out pulmonary parenchymal abnormalities is not recommended in the absence of abnormalities on physical exam, routine chest radiograph, or pulmonary function testing. The first-line of diagnostic testing for patients with suspected SLE-aPAH involves obtaining a Doppler echocardiogram to look for elevations in estimated pulmonary artery pressure and/or tricuspid valve insufficiency. The estimation of pulmonary artery pressure (PAP) by Doppler echocardiography (DE) does not necessarily correlate with the measurement of PAP obtained directly by RHC [67–69]. During the RHC, vasodilator agents such as nitric oxide, epoprostenol or adenosine may be used to identify vasoreactivity. DE may, in some instances, be used in lieu of the RHC to follow patients while on therapy; right ventricular parameters such as tricuspid annular plane systolic excursion (TAPSE) and right ventricular fractional area change could be useful indices for evaluating right ventricular function. RHC is required to confirm the diagnosis and assess the severity of PH and also to provide definitive assessment while on therapy. Most patients in this SLE-aPAH population are not vasoreactive and calcium channel blocker therapy has not proven to be beneficial. The mPAP of >30 mmHg during exercise is no longer considered to be part of the definition of PAH as the normal baseline mPAP for exercise had not been established.

Other studies to consider as part of the evaluation for secondary causes of PH, even in a patient with known SLE, include polysomnography to evaluate for sleep disordered breathing, testing for human immunodeficiency virus, hepatitis serology, pulmonary function tests (the finding of an isolated defect in diffusing capacity for carbon monoxide on lung function testing may be an early predictor of SLE-aPAH [70]), and ventilation perfusion scan to evaluate for acute or chronic thromboemboli.

7. Treatment

SLE-aPAH patients are generally treated with therapies such as oxygen, anticoagulants, calcium channel blockers, and vasodilators, similar to the therapeutic interventions for patients in WHO Group I. However, no single therapeutic regimen has been shown to be fully effective in treating SLE-aPAH. The vasodilators employed are selective and nonselective endothelin receptor antagonists (ETRAs), phosphodiesterase-5-inhibitors (PDE-5-I), and oral, inhaled, subcutaneous, or intravenous prostanoids [1].

The key findings in the vasodilator trials (summarized in Table 5) show improvement in exercise capacity, hemodynamic parameters, New York Heart Association Functional Class, increase in time to clinical worsening, and a trend towards improved quality of life in CTD-aPAH patients. The number of patients with SLE-aPAH in these trials was small, and most studies did not perform subgroup analysis for SLE-aPAH patients. As a result, no definitive conclusion can be drawn for this subgroup of patients. However, one study by Badesch et al., on behalf of the SUPER study group (SUPER 1), performed a posthoc analysis to study the efficacy of sildenafil on CTD-aPAH patients ($n = 278$) of which 23% had SLE-aPAH. This double-blinded study showed significant improvement in pulmonary hemodynamics, exercise capacity, and WHO functional class with 20 mgs of Sildenafil over a 12-week period [22].

As discussed earlier, patients with aPLs are more susceptible to *in-situ* thrombosis and thrombotic arteriopathy and should be screened for chronic thromboembolic pulmonary hypertension (CTEPH). Once diagnosed, the CTEPH patients require different modalities of treatment [71–75].

Another important condition seen in patients with PH associated with SLE is mitral and aortic valvular pathology (referred to as Libman-Sacks endocarditis) causing regurgitation and leading to pulmonary venous hypertension. The precise incidence has not been determined and effective treatment is unavailable [76–81].

As discussed in the pathology section, SLE-aPAH results from sustained pulmonary vasoconstriction leading to luminal obliteration of small and medium-sized pulmonary arteries, due to the formation of plexiform lesions and *in-situ* thrombosis. In addition, inflammatory and dysregulated immune components play a major role in the pathogenesis of PAH in SLE leading to therapy with anti-inflammatory glucocorticoids and immunosuppressive therapies, a subject of on-going investigation [19–21, 23–27, 82–84].

Table 5 delineates the various treatment modalities and their respective outcomes in patients with SLE-aPAH. In the studies summarized in the table, all patients had RHC for diagnosis of PAH. It should be noted that the immunosuppressive therapy trials to date have been small nonrandomized, observational, retrospective, uncontrolled studies (with historical controls) and case reports, whereas the vasodilator treatment studies are mostly randomized controlled studies with a small number of SLE-aPAH patients. Additional comprehensive and controlled trials are needed to test the effectiveness of immunosuppressive therapies in the SLE-aPAH patients.

Table 5: Treatment modalities and respective outcomes for SLE-aPAH patients. Mean pulmonary artery pressure (MPAP) in mmHg; pulmonary vascular resistance (PVR) in Woods units; 6 minute walk distance (6MWD) in meters; age in years; New York Heart Association Functional class (NYHA FC); Average (avg.).

Studies	Drug/design	Patients and baseline characteristics	Outcome
Intensive Immunosuppressive therapy (IIT) trials			
Miyamichi-Yamamoto et al. [19]	IIT: IV cyclophosphamide + oral glucocorticoids + vasodilator therapy (VT) Observational cohort study from a single center with historical control	(i) 8 patients with SLE-aPAH (ii) MPAP = 39.5 ± 9.2 (iii) PVR = 8.75 ± 5.43 (iv) NYHA FC = I, II, III (v) 6MWD = 442 ± 54 (vi) Age = 42 ± 8	IIT: (i) Significantly decreased MPAP (ii) Tended to decrease PVR (iii) Normalized hemodynamics in a few patients. IIT + VT improved the pulmonary hemodynamics and long-term prognosis of patients with CTD-aPAH.
Jais et al. [20]	IIT: IV cyclophosphamide + glucocorticoids ± VT Retrospective, uncontrolled study	Rx with IIT / Rx with IIT + VT N = 13: 9 / 4 MPAP: 48 ± 12 / 58 ± 10 PVR: 8.6 ± 3.5 / 14.3 ± 1.3 NYHA FC: II, III / III, IV 6MWD: 347 ± 80 / 381 ± 71 Age: 31 ± 10 / 38 ± 9	(i) SLE-aPAH patients with less severe disease may respond to treatment with IIT. (ii) For patients with more severe disease, VT should be started, possibly in combination with IIT. (iii) These retrospective and uncontrolled data need to be confirmed by randomized controlled trials.
Sanchez et al. [21]	IV cyclophosphamide ± glucocorticoids Retrospective study	(i) 13 patients with SLE-aPAH (ii) MPAP (avg.) = 54 (iii) PVR (avg.) = 19 (iv) NYHA FC = II, III (v) 6MWD (avg.) = 370 (vi) Age (avg.) = 29	(i) Of the responders [R] 62% had SLE. (ii) R's had a significantly improved 6MWD and hemodynamic parameters. (iii) R's had a better survival than non responders [NR].
Oral agents: endothelin receptor antagonists (ETRAs) and phosphodiesterase-5-inhibitors (PDE-5-I)			
Badesch et al. [22]	Sildenafil 20 mg, 40 mg, 80 mg 12 week, double-blind study (SUPER-1)	(i) 19 patients with SLE-aPAH (ii) MPAP = 47 ± 11 (iii) PVR = 10.13 ± 5.45 (iv) NYHA FC = II, III, IV (v) 6MWD = 342 ± 76 (vi) Age = 53 ± 15	In patients with PAH-aCTD, sildenafil improves exercise capacity, hemodynamic parameters (at the 20 mg dose), and NYHA FC after 12 weeks of treatment.
Galiè et al. [23]	Sildenafil 20 mg, 40 mg, 80 mg Double-blind placebo-controlled trial	(i) 19 patients with SLE (ii) MPAP = 52.75 ± 14 (iii) PVR = 11.95 ± 6.29 (iv) NYHA FC = II, III, IV (v) 6MWD = 344 ± 82 (vi) Age = 49 ± 15	Sildenafil improves exercise capacity and hemodynamics in patients with symptomatic PAH. SLE-aPAH subgroup analysis was not done.

Table 5: Continued.

Studies	Drug/design	Patients and baseline characteristics	Outcome
Rubin et al. [24]	Bosentan Double-blind placebo-controlled trial	(i) 16 patients with SLE (ii) MPAP = 55 ± 16 (iii) PVR = 12.68 ± 8.48 (iv) NYHA FC = III, IV (v) 6MWD = 330 ± 74 (vi) Age = 49 ± 16	Statistically significant improvement in exercise capacity, NYHA FC and increase in time to clinical worsening.
Subcutaneous, inhaled, and intravenous prostanoids			
Oudiz et al. [25]	Subcutaneous treprostinil Double-blind placebo-controlled trial	(i) 25 patients with SLE (ii) MPAP = 52 ± 2 (iii) NYHA FC = II, III, IV (iv) 6MWD = 280 ± 13 (v) Age = 54 ± 2	Improved exercise capacity, dyspnea fatigue symptoms, hemodynamics and trend toward improved quality of life.
Olschewski et al. [26]	Inhaled Iloprost Randomized placebo-controlled trial	(i) 35 patients with CTD (ii) MPAP = 52.8 ± 11.5 (iii) PVR = 12.86 ± 4.88 (iv) NYHA FC = III, IV (v) 6MWD = 332 ± 93 (vi) Age = 51 ± 13	(i) Statistically significant benefit in combined endpoint of 10% improvement in 6MWD and FC improvement and absence of clinical deterioration. (ii) No subgroup analysis done for SLE.
Robbins et al. [27]	Intravenous epoprostenol Case series	(i) 6 patients with SLE (ii) MPAP = 57 ± 9 (iii) PVR = 14 ± 7 (iv) NYHA FC = III, IV (v) Age = 26–35	Dramatic improvement in FC and marked improvement in hemodynamics.

Intensive immunosuppressive therapy (IIT) is defined as an intravenous (IV) bolus of cyclophosphamide 500–600 mg/m^2 monthly for 3–6 months in addition to oral glucocorticoids 0.5–1 mg/kg/day for 4 weeks followed by a slow taper. In the three studies [19–21] highlighted in the table, variations of above mentioned doses and time periods of administration of cyclophosphamide and oral or intravenous glucocorticoids were used.

8. Other Therapies

Atrial septostomy and lung or heart-lung transplantation may be an option for some patients with SLE-aPAH who have failed maximized medical therapy and continue to have disease progression (acceptance for transplant maybe predicated upon quiescence of other systemic manifestations of SLE) [85]. IPAH patients have a better prognosis than SLE-aPAH patients. Most patients with SLE-aPAH are women of a child bearing age, and due to the high maternal mortality in this group, screening for PH in pregnant mothers is recommended [86–90].

9. Survival

The one- and three-year survival rates for SLE-aPAH are 78% and 74% respectively [2, 5]. While the one-year survival rate of SSc-aPAH patients is similar to that of SLE-aPAH patients, the three-year survival rate is much lower at 47%. The REVEAL cohort of patients with SLE-aPAH had a one-year survival rate of 94% [6]. The advanced therapy including immunosuppression given to SLE-aPAH patients early in the course of disease may account for the improved survival rates, despite similar abnormalities in baseline pulmonary hemodynamics in both patient groups. However, if other respiratory disorders coexist with PH, the prognosis is similar to that of patients with SSc-aPAH.

Quadrelli et al. [91] examined 90 SLE necropsies and found 97.8% to have some pleuropulmonary involvement but not necessarily related to SLE. The most frequent lung finding was bacterial bronchopneumonia (a contributing cause of death) in 90% followed by pleuritis in 88%. Four out of 90 patients had findings of pulmonary hypertension (4.4%). In another study [92], pulmonary arterial hypertension was the third most common cause of death, after infection and lupus manifestations other than renal involvement. The patients in the latter study were on higher doses of corticosteroids preceding their death.

10. Conclusions

Connective tissue disease-associated PAH has historically had a poor prognosis with a one-year survival rate of

45% in patients with SSc-aPAH. Recent survival rates of all CTD-aPAH have improved, in part due to the advances in therapies, although these modalities require further study. To date, most of the research has focused on SSc-aPAH, leaving insufficient data on the other CTD-aPAH. Jais, Sanchez, and colleagues [19–21, 82] have studied SLE-aPAH and the effect of intensive immunosuppressive therapy on the survival rates. The linkage between intensive immunosuppressive therapy and improved survival rates is not yet conclusive due to the paucity of randomized placebo-controlled studies. These studies are difficult to conduct because there are few patients with this disease who are not already on therapy. However, patients should be treated aggressively with immunosuppressive and anti-inflammatory therapies, coupled with vasodilator therapy due to progression of disease. In certain cases, an initial combination of intensive immunosuppressive and vasodilator therapies may be used [93, 94].

To date, there have not been consensus recommendations for screening for PH in patients with SLE. However, young women of child bearing age are most at risk of dying due to undiagnosed SLE-aPAH during pregnancy, delivery, and post partum. Therefore, screening should be considered essential in this population. Patients with anti-U1 RNP antibody, anticardiolipin antibody, and Raynaud phenomenon should also be seriously considered for screening, given the high correlation between these predictors and PH.

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