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Guest Editors: Antonio Fernández-Nebro, Sara Marsal, Winn Chatham, and Anisur Rahman

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Editorial Board

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Editorial

Systemic Lupus Erythematosus: Genomics, Mechanisms, and Therapies

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Systemic lupus erythematosus (SLE) is a complex disease caused by complex interactions between genes and the environment (sex, age, hormones, smoking, infections, drugs, and abnormalities of both the innate and adaptive immune systems). To understand the mechanisms that regulate these interactions and the processes responsible for an immune system that is increasingly autoreactive, it is essential to definitively control lupus and related disorders.

Although the prevalence of SLE among East Asians is higher than among Europeans [1], most genomes wide association studies (GWAS) have been conducted on populations of European descent. Through multinational collaborations, these studies have achieved large sample sizes and considerable statistical power. Although the sample sizes of genetic studies in East Asians are generally much smaller than those in Europeans, some have yielded new candidate loci and copy number variations [2, 3]. However, in the last 3 years, the focus has clearly switched to GWAS in an attempt to discover new risk loci that may provide unique information in complex diseases. In this special issue on SLE, we have invited H. C. Chai et al. to review the genetic factors of SLE in the Malaysian population. In their paper, these authors emphasise that most of the polymorphisms investigated did not show significant associations with susceptibility to SLE among those of Malaysian descent, except for those polymorphisms occurring in MHC genes and genes encoding TNF-α, IL-1β, IL-1RN, and IL-6. Although this could be due to smaller sample sizes, the genetic heterogeneity of SLE among different ethnicities and gene-gene or gene-environment interactions could also lead to differences in SLE susceptibility.

It is increasingly recognised that subsets of B cells differ in function and that changes in the balance of these functionally distinct subsets may be relevant to the pathogenesis of SLE. In both a research paper and a review article, S. Koarada and colleagues describe a particular subset of B cells that do not express the Toll-like receptor homologue RP105. These RP105-negative B cells may expand in SLE and play a role in the pathogenesis of the disease. Toll-like receptors (TLRs) themselves may also be important in the pathogenesis of SLE. This applies particularly to TLR7 and TLR9, as discussed in the paper by G. Guggino et al.. TLRs are important for antimicrobial immunity, but TLRs could affect SLE through two major mechanisms. TLRs can be stimulated by exogenous antigens, such as viral RNA, which then stimulate resident immune cells [4]. Additionally, TLRs recognise endogenous self-antigens and initiate and propagate inflammation and autoimmunity [5].

TLRs are also expressed in some renal cells such as epithelial and mesangial cells. Mesangial cells have three main functions: filtration, support of glomerular capillaries, and the phagocytosis of apoptotic cells and immune complexes. An association between TLR9 and lupus nephritis has been reported in a murine lupus model and in human lupus, which indicates the possibility of crosstalk between innate immunity and autoimmunity [4, 6, 7]. Anti-dsDNA

antibodies are relevant in the development of lupus nephritis, but the mechanism by which they are nephritogenic is far from clear. In this special issue on SLE, G. Seret et al. propose that some types of anti-dsDNA antibodies stimulate mesangial cells to produce cytokines, chemokines, and matrix metalloproteinases and to induce proliferation and apoptosis, matrix protein accumulation, and chromatin accumulation and immune complex formation.

Free-radical-mediated reactions are implicated in SLE. Autoimmune conditions are associated with the increased activation of immune effector cells and the production of free radical species. The generation of neoantigenic determinants by free-radical-mediated reactions increases the antigenicity of DNA, LDL, and IgG, generating ligands for which autoantibodies show higher avidity [8]. However, the potential for oxidative stress to contribute to SLE pathogenesis remains largely unexplored in humans. In the present special issue, K. J. Li and colleagues have shown that deranged cellular bioenergetics and defective redox capacity in T lymphocytes and polymorphonuclear neutrophils are responsible for cellular immune dysfunction and are related to increased oxidative stress in active SLE patients.

Patients with SLE have an increased risk of cardiovascular disease compared with the general population, leading to increased cardiovascular morbidity and mortality. In the general population, the frequency of diastolic heart dysfunction increases with age, particularly in women and in patients suffering from arterial hypertension. Isolated diastolic heart dysfunction is often demonstrated in SLE [9] and is also related to patient age. However, autoimmune diseases are known to have a role in cases of unexplained diastolic failure. In this special issue, L. M. Blasco Mata et al. present an interesting paper that aims to identify autoimmune systemic diseases in subjects with recurrent unexplained diastolic heart failure. According to these authors, up to 11% of patients with recurrent unexplained diastolic heart failure exhibit autoimmune abnormalities.

Recent developments in several imaging techniques have improved the risk stratification of SLE patients with cardiovascular disease. In this special issue, S. C. Croca and A. Rahman have reviewed the use of various imaging techniques in the assessment of cardiovascular disease (CVD) risk in SLE. CVD is an important cause of morbidity and mortality in SLE, and the use of imaging to identify patients at risk of developing CVD before they develop symptoms is likely to become increasingly important.

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Review Article

Mesangial Cell-Specific Antibodies Are Central to the Pathogenesis of Lupus Nephritis

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Not only is nephritis a common complaint in systemic lupus erythematosus, but it is also the most life-threatening complication of the disease. Anti-double-stranded DNA antibodies (Abs), which are found in up to 80% of these patients, might be nephritogenic per se. That is, they may cross-react with mesangial cell (MC) surface proteins, such as alpha-actinin and annexin A2, they may cross-react with mesangial matrix protein such as laminine and fibronectin, or they may recognize chromatin material previously deposited in the glomeruli. The consequence of the binding of anti-MC Abs may be their internalization, which results in activation and proliferation of these MCs. In turn, these activated MCs are suspected of promoting immune complex formation by sequestering and thereby protecting chromatin from degradation. The present paper will explain the mechanisms through which such autoAbs may initiate nephritis.

1. Introduction

Systemic lupus erythematosus (SLE) is a nonorgan-specific autoimmune disease, the hallmark of which is a vast array of antiself antibodies (autoAbs), and, among them, the whole range of antinuclear Abs (ANAs). The ensuing immune complexes (ICs) settle in the tissues and thereby subsequently contribute to local damage.

Most organs are at risk of being involved in this process at one time or another, given that the course of the disease consists of sequential flares and remissions. Estimates of the prevalence vary from 20 to 150 cases per 100,000 individuals, with the highest frequency in Afro-Caribbeans, followed by Asians, and far less frequent in Caucasians [1]. The male-to-female ratio rises to 1 : 9 during child-bearing age but diminishes thereafter.

In fact, the pathophysiology of SLE is so complicated that its development implicates multiple genes and entails a number of environmental factors (recognized or unknown). With regard to the genetics, predisposing genes are associated with the innate as well as the acquired immune responses. Of these, SLE can involve the antigen- (Ag-) presenting DR2 and DR3 HLA class II molecules, the lymphocyte activation markers, components of the classical complement activation pathway, various features involved in the processing of ICs, and interferon (IFN) signaling cascade members [2].

Lupus nephritis (LN) predominates as a cause of mortality in SLE and displays several epidemiological particularities [3]. For example, there exists an ethnic susceptibility, in that it develops in 20% of Caucasian patients compared with 50% of Asian patients. Whereas SLE is, by and large, more frequent in females than in males, the susceptibility for LN in Caucasians reaches 50–60% in males compared with 20–35% in females. This complication arises usually within the first two years of the disease. Several gene polymorphisms have been claimed to favor LN (Table 1), and some SLE-specific autoAbs have been shown to recognize glomerular Ags (Table 2). Furthermore, it has been suggested that anti-double-stranded DNA (anti-dsDNA) Ab-induced renal failure could be linked to differences in the fine specificities of these autoAbs. Over several decades, a large body of work has been devoted to deciphering the anti-dsDNA Abs and to understand the deposition of anti-dsDNA/nucleosome ICs in the kidney, yet there are few reports available on

Table 1: Genes associated with lupus nephritis (LN) [4–10].

Gene	Function	Influence
CD48	Leucocyte adhesion	Protective effect
FcγRIIIA/IIA	Binding affinity	Susceptibility to SLE and LN
Kallikrein	Inflammation	Protective effect
IL-18	Inflammation	Susceptibility to LN
Myeloperoxidase	Inflammation	Susceptibility to LN
TLR9	Immune response	Susceptibility to LN
MBL2	Complement	Susceptibility to LN

Table 2: Glomerular targets for anti-double-stranded (ds)-DNA antibodies [11, 12].

Molecules that directly cross-react with anti-dsDNA antibodies	Cell type/glomerular matrix
Alpha-actinin	Mesangial cells
Annexin A2	Mesangial cells, epithelial cells
Ribosomal P protein	Mesangial cells, endothelial cells
Alpha-enolase	Mesangial cells, epithelial cells
Laminin	Glomerular matrix
Fibronectin	Glomerular matrix
Collagen	Glomerular matrix
Heparan sulfate	Glomerular matrix
Hyaluronic acid	Glomerular matrix

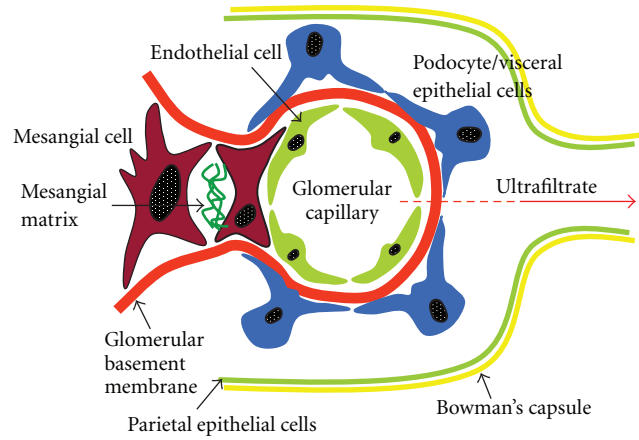

Figure 1: Schematic representation of the different cell types of the glomerular filtration barrier.

the recognition of glomerular structures, and even fewer studies on the recognition of mesangial cells (MCs). Our paper will, therefore, endeavour to provide glimpses into the mechanisms that may account for the development of nephritis in patients with SLE.

2. Mesangial Cells

2.1. Mesangial Cell Functions. Glomeruli are comprised of at least four cell types: MCs, endothelial cells (ECs), and podocytes plus parietal cells, both of an epithelial nature and the later shaping the Bowman's capsule (Figure 1). Filtration through the glomerular barrier is under the control of MCs plus podocytes, along with renal blood flow by contracting the GBM [13]. The glomerular blood-urine barrier superimposes three layers: fenestrae between adjacent ECs, the glomerular basal membrane (GBM), and the slit diaphragm mid podocytes.

The MCs are specialized smooth muscle cells, of which the contractility depends upon vasoactive molecules, such as angiotensin II and endothelin-1. They possess additional capabilities, including support of the glomerular capillaries. MCs synthesize and renew their own extracellular matrix, which is distinct from the GBM. The mesangial matrix is made up of fibronectin, collagen II, collagen IV, laminin, entactin, nidogen, and perlecan. The sialoglycoprotein fibronectin is located on the MC surface and is required for attachment of circulating components, including chromatin, to MCs and ECs. The other sialoglycoprotein laminin and the sulphated glycoprotein entactin are equally implicated in this event. Other important functions for MCs are their phagocytic capacity to take up apoptotic cells and their capacity to prevent accumulation of ICs by bringing into play nonspecific mechanisms, such as pinocytosis and phagocytosis, and specific mechanisms, such as receptor-dependent processes. Once activated, MCs secrete pro-inflammatory cytokines (e.g., interleukin (IL)-1, IL-6, IL-12, and IFN-γ), growth factors (e.g., transforming growth factor (TGF)-β and vascular endothelial growth factor), and metalloproteinases (e.g., metalloproteinase (MMP)-2 and MMP-9). All these effects are tightly regulated in normal cells and may be markedly altered by glomerular pathology.

2.2. Mesangial Cells and Kidney Diseases. A variety of ICs, which are lacking in normal mesangium, become detectable in the kidneys of patients with a variety of diseases, such as LN, IgA nephropathy (IgAN), C1q nephropathy, and mild postinfectious glomerulonephritis (GN). Such patients often present with hematuria, associated with proteinuria at the nephrotic syndrome stage. Much uncertainty surrounds abnormalities of MCs in ICs deposition. Several mechanisms are, in fact, supposed to prevent ICs access into the mesangium. They include the endothelial barrier itself, the effect of a protective glycocalyx, and the recycling capacity of the podocytes that express the neonatal receptor for IgG (FcRn) [14]. The immunoglobulin-specific MC receptor

remains a matter of debate, given that the mesangial Fc-gamma receptors are dispensable for kidney injury as well as for cellular activation [15]. Alternatively, nonconventional receptors have been proposed. On the front line of the pathophysiology of IgAN is the transferring receptor, referred to as CD71 [16–18]. The IgA-IgG/CD71 complexes are crucial [19], as suggested by the fact that blocking CD71 with a related monoclonal Ab (mAb) inhibits MC proliferation and cytokine production, namely, IL-6 and TGF-β. In addition, IgA and IgG are associated with the complement fraction C3 [20] and the mannose-binding lectin in the mesangium, highlighting the relevance of the complement lectin pathway to the development of such GNs.

Of note, proliferation of MCs and expansion of the mesangial matrix may take place in the absence of ICs. In this context, different forms of glomerular damage develop, namely, diabetic and amyloid nephropathies. In the course of diabetic nephropathy, elevated plasma levels of glucose contribute to the induction of nitric oxide synthase (iNOS), which in turn activate protein kinase C (PKC), mitogen-activated protein kinases (MAPK), and phosphatidyl inosytol-3 kinase/Akt [21]. As a result, fibronectin, collagen IV, and TGF-β synthesis are upregulated, leading to the development of fibrosis and resulting in end-stage renal failure. Actually, such is the usual outcome of a large number of GNs.

3. Mesangial Cells and Lupus Nephritis

3.1. Pathogenic Models. Anti-dsDNA Abs are relevant to the diagnosis of SLE and instrumental in the development of LN. However, the mechanism by which they contribute to the GN is far from clear, considering the fact that not all Abs to dsDNA are able to cause tissue damage to a similar extent. A popular view has been that GN results from ICs associating with nucleosomes released from apoptotic/necrotic cells that have bound anti-dsDNA/chromatin Abs. A wealth of evidence supports this simplistic model. For example, renal flares are preceded by a rise of the anti-dsDNA Ab levels in plasma and a reciprocal reduction in levels of free DNA [22]. In LN, the anti-dsDNA Ab/chromatin complexes are seen as electron-dense structures in the mesangial matrix and move to the GBM as soon as the disease is established [23]. An acquired renal DNase1 deficiency, coupled with chromatin sequestration by matrix protein accumulation, amplifies the process by offering more target Ags to anti-dsDNA Abs [24]. Nonetheless, this mechanism cannot be responsible for the whole process, since analysis of kidney-eluted IgG has revealed that those Abs binding to dsDNA represent as little as 10% of the total bulk of IgG [25]. Additional points to keep in mind are that only a minute fraction of anti-dsDNA Abs are pathogenic when transferred to experimental animals, and LN could develop in the absence of anti-dsDNA Ab. Last but not least, differences between nephritogenic and nonnephritogenic anti-dsDNA Abs are unrelated to structural differences in class, subclass, or avidity (Table 3). Rather, they consist of varying capacities to react with MC products in the absence of a DNA docking site [26]. The generation of nephritogenic Ab is incompletely understood and possibly results from an antigen-dependent stepwise process due to isotype switching and somatic mutations that would result in acquisition of cross-reactivity and high-affinity binding. Stimulation may be sustained by dsDNA along with a glomerular antigen or more probably shared epitopes. It is striking to observe that only one mutation can change the affinity, the cross-reactivity properties, and the kidney binding localization of a pathogenic anti-dsDNA Ab.

Accordingly, the concept has been put forward that anti-dsDNA Abs launch the GN process through cross-reaction with cell-surface and matrix components. So far, several glomerular Ags have indeed been suspected as serving as targets for anti-dsDNA Abs [11, 12]. To reconcile the theory of active cross-reactivity and the concept of passive IC deposition, we reasoned that neither is exclusive and speculated that both are ordered, in that Ab glomerular recognition precedes anti-dsDNA Ab/chromatin deposition [27, 30].

3.2. Histology. To account for so much variation in the clinical and histological patterns, the LN histopathological abnormalities have been classified into six classes. Based on the criteria proposed by the International Society of Nephrology/Renal Pathology Society (ISN/RPS) in 2003 [31], they include the morphology of the lesions, their mesangial, endothelial and epithelial extent, the Ab deposition, and the distinction between active and chronic lesions. Briefly, class I histopathological damage corresponds to mesangial deposits, but renal symptoms may be absent. Class II refers to mesangial proliferation, and mild proteinuria and microscopic hematuria characterize these patients. The renal prognostic value is often excellent but may evolve through mesangial and endothelial lesions [32, 33]. Class III and class IV imply glomerulus antibody deposition. In essence, class III LN (less than 50% of the glomeruli are impacted) manifest hematuria, proteinuria, nephritic syndrome, and occasionally hypertension. Class IV (more than 50% of the glomeruli) characterizes diffuse LN and comprises segmental and global forms, according to the severity of glomerular lesions. Hematuria, massive proteinuria, nephritic syndrome, and acute renal failure occur in 16% of class IV patients. Class V corresponds to immune-complex-derived membranous nephritis. The lesions display global or segmental distribution, although more than 50% of the capillary basement membrane is involved in either case. Clinical presentations include proteinuria (typically at a nephritic range), with hematuria but usually without renal insufficiency. Finally, class VI lesions correspond to the last stage of the disease, resulting from the alteration between flares and pauses, leading to overt renal failure, and substantiated by vascular sclerosis, tubulointerstitial scarring, and glomerular sclerosis. However, these clinical features are not well associated with the classification since, histologically, severe LN may be clinically silent. Besides these well-documented types of damage, SLE yields a broad variety of vascular lesions, which are neglected in the ISN/RPS 2003 classification.

Table 3: Nephritogenic and cross-reactive anti-dsDNA Ab properties [26–29].

	Non-nephritogenic anti-dsDNA Ab	Nephritogenic anti-dsDNA Ab	Cross-reactive anti-dsDNA Ab
Class	IgG, M and A	IgG mainly	IgG mainly
Somatic mutations	No	Yes	Yes
Affinity	Low	High	High
Cross-reactivity	No	Yes	Yes
Living cell internalization	No	Yes	suspected
Glomerular direct binding	No	Yes	Yes
Proteinuria	No	Yes	mainly

3.3. Mesangial Cells in Lupus Nephritis. Aberrant proliferation, apoptosis, and activation of MCs are common findings during LN. As a consequence, numerous genes have been demonstrated by immunohistochemistry and/or molecular biology to be upregulated during LN [34–38]. These include genes for survival and apoptotic factors (Bcl-2, Fas, FasL), chemokines that attract inflammatory cells (CCL5, CXCL1), inflammatory mediators (ROS, iNOS), proinflammatory type 1 cytokines (IFN-γ, IL-12, IL-6), mesangial matrix synthesis (fibronectin), collagen IV degradation (MMP-2 and MMP-9), and chromatin accumulation (DNase1 downregulation). MC pathogenicity could be attributed in part to anti-dsDNA activity since anti-dsDNA Abs stimulate MCs to produce chemokines (MCP-1, CCL-5), matrix metalloproteinases (MMP-2, MMP-9), reactive oxygen (iNOS), cytokines (IL-6, TGF-β), and lipocalin-2/NGAL [39, 40]. Although incompletely characterized, such effects are related in part to the activation of the PKC and MAPK pathways.

4. Autoantibodies and Lupus Nephritis

4.1. Antiglomerular Antibodies. ANAs may arise well before the development of overt disease, with a crescendo of more and more SLE-specific autoAbs being produced over 10 years [41]. The earliest ANAs are anti-Ro/Sicca Syndrome (SS)-A and anti-La/SSB Abs, on average 3.7 years before, followed by anti-dsDNA Abs, on average 2.2 years before, and the anti-Smith (Sm) ribonucleoprotein (RNP) Abs, on average 0.9 years before the advent of clinical symptoms. Intriguingly, the presence of anti-Ro/La/Sm RNP Abs and IgM anti-β2 glycoprotein I could well protect the patient from LN [42, 43]. On the other hand, high-titer and high-avidity anti-dsDNA Abs have been reported to be linked to active disease and suspected to be associated with LN.

Typically, ICs from patients suffering LN contain IgG, IgM, and IgA, along with the complement fractions C1q and C3. In 90% of the cases, IgG predominates over IgM and IgA which are associated with 60% of the IgG-containing ICs. These latter abnormalities are exceptional in diseases other than LN. With regard to fibrin and fibrinogen, they characterize crescent and necrotizing segments. Specificity analysis of Abs eluted from the kidneys unveils a broad range of reactivities. These are chromatin, α-actinin, collagen, entactin, fibrinogen, laminin, proteoglycan, phospholipids (PLs), myosin, RNP, and so on [25]. Similarly, microarray technology has distinguished two main clusters of serum IgM and IgG autoAbs in the serum of patients with LN, based on their specificities. One is directed to chromatin and the other to the glomerulus [44]. Their DNA dependence has been tested using DNase-1 pretreatment, and the results of these experiments indicated that 20% of the Abs binding to the glomeruli were DNA independent.

The observation that some anti-dsDNA Abs attach directly to renal tissues, and more particularly to MCs, raises the question as to whether or not any target Ag is specific for such LN-associated autoAbs. This issue has been addressed using several approaches. First, anti-dsDNA mAbs have been injected into nonautoimmune mice and shown to cause a LN-like disease [26, 45]. Similarly, immunization with a peptide for anti-dsDNA Ab can initiate LN in Balb/c mice [46]. Of note, site-directed mutagenesis of the nephritogenic anti-dsDNA mAb R4A alters not only its affinity to dsDNA, but also its cross-reactivity with glomerular Ags. Cross-reactivity can even shift from the glomerular to the tubular area [28]. Anti-dsDNA Ab point mutations may thus influence the evolution of LN over time. The second approach relied on glomerular-derived peptides which were examined for their interactions with anti-dsDNA Abs [47]. The third approach used human sera purified from LN patients and those which recognized human MCs as well [48]. This approach enabled the discovery of three main specific MC targets at 42, 63, and 74 kDa when using anti-dsDNA and non-anti-dsDNA purified Abs from these patients. DNase1 pretreatment did not affect their binding. Furthermore, purified antihuman MC Abs are likely to be internalized and thus able to encourage iNOS activation, MC proliferation, and matrix synthesis [12]. As recently documented [49], antihuman MC Abs are associated with 84% of active LN compared with 43% of inactive LN.

4.2. Antimesangial Cells Antibodies

4.2.1. Anti-α-Actinin Antibodies. Glomerular α-actinin is expressed on the surface of MCs and podocytes but not on that of the GBM. This actin-binding protein belongs to the superfamily of cytoskeletal proteins. It is comprised of four isoforms, and mutations in the fourth isoform can lead to focal and segmental glomerulosclerosis [50].

That α-actinin can be targeted by anti-dsDNA Abs has also been demonstrated. This is tied to the fact that injection of anti-dsDNA mAb into RAG-1-deficient mice induces

a proteinuria with glomerular deposits in these animals. Cross-reaction with α-actinin [26] or laminin-1 [51] provided the anti-dsDNA Abs with the capacity to impair the renal function. This view was supported by the finding that, once bound to MCs, anti-dsDNA R4A mAbs [29] upregulate the production of iNOS and proinflammatory chemokines [39]. Demonstration of the reality of pathogenic α-actinin, which is worthy of pursuit in the future, was thus reinforced by the observation that α-actinin-immunized normal mice mounted an anti-α-actinin Ab response first and then produced anti-α-actinin and anti-chromatin Abs, along with advancing stages of the LN-like disease [52]. In SLE patients, the anti-α-actinin Ab production culminates early at the initiation of the LN, but their titers drop dramatically after treatment is initiated, that is, when the disease activity is reduced. We must admit that, in contrast to the anti-dsDNA/chromatin activity, the results of the detection of these autoAbs are inconsistent [53–56]. Of interesting note, the anti-α-actinin response is related to the actin-binding site of α-actinin [54, 57].

4.2.2. Antiannexin A2 Antibodies. Annexin A2 is a calcium-dependent PL-binding protein expressed on the surface of phagocytic cells, such as macrophages, ECs, and MCs. This protein is pivotal in the regulation of MC proliferation, activation, apoptosis, and in coagulation by recruiting plasminogen and tissue plasminogen activator.

In LN, IgG, and C3, deposits colocalize with annexin A2 in the glomeruli but, surprisingly, not in the tubuli [12]. Annexin A2-dependence has been tested by gene silencing using RNA interference technology, as an attempt to establish that its downregulation prevents anti-dsDNA Ab binding, Ab internalization, and MC activation. Supporting this view, a positive antiannexin A2 Ab test is associated with active LN and thrombosis [12, 58]. The abnormality is related to the activation of the tissue factor on ECs and monocytes, which is in accord with the detection of anti-annexin A2 Ab in 40% of patients with the anti-PL syndrome.

4.3. Antimatrix Antibodies

4.3.1. Antilaminin Antibodies. Laminin belongs to the mesangial matrix. Laminin-1, which is the most abundant isoform, is derived from MCs. It is overexpressed and hence becomes detectable in the GBM during LN and at the periphery of end-stage sclerotic lesions [59]. It is, therefore, of no surprise that antilaminin Abs are found during LN and that their levels correlate with the disease activity and proteinuria [51]. Notwithstanding, they are not specific for SLE, being also detected in recurrent miscarriages, infertility and pemphigus. The main epitope recognized by antilaminin-1 Ab corresponds to the binding site of laminin to the basement membrane receptors.

4.3.2. Antifibronectin Antibodies. Fibronectin is absent from normal mesangial matrix but overexpressed in LN and colocalized with IgG/chromatin ICs in the mesangium. The prevalence of antifibronectin Abs ranges from 30 to 80%

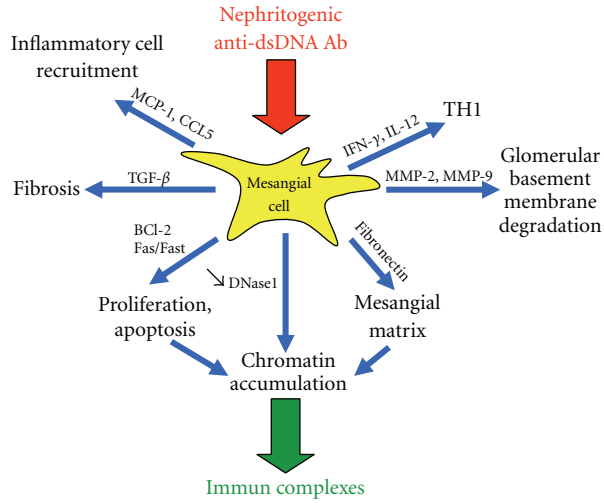

Figure 2: A working model of mesangial cell (MC) stimulation by nephritogenic anti-dsDNA Ab in lupus nephritis leading to accumulation of immune complexes.

in patients with SLE, and from 15 to 40% in those with rheumatoid arthritis and other systemic vasculitis. Although nonspecific for any disease, antifibronectin Ab levels correlate with activity in patients with SLE. In this regard, one of the most efficient drugs to treat LN, mycophenolate mofetil, prevents anti-dsDNA Ab-induced fibronectin production by MCs. In other words, the drug contributes to reduce IC deposition [40].

4.4. Miscellaneous Antibodies. Involved in the elongation step of protein synthesis when associated with the large ribosomal subunit, the ribosomal serine phosphorylated proteins P0, P1, and P2 appear on the membrane of multiple cells, including MCs and blood cells of patients with SLE. The reported prevalence of antiribosomal Abs varies from 5 to 45% in SLE, more often in Asian patients than in Caucasian and African patients [60]. In SLE, they are restricted to active disease, with kidney, hepatic, and neuropsychiatric complications. Once again, high-affinity anti-dsDNA Abs cross-react with ribosomal proteins.

Alpha-enolase appears on the surface of MCs and podocytes and in the tubuli from patients with LN. In this setting, it acts as a glycolytic enzyme and a receptor for plasminogen. The anti-α-enolase Ab test is positive in SLE patients but is not associated with LN and flares [61]. Alpha-enolase has been identified as an autoAg in other diseases, such as Behcet's disease, retinopathy, and severe asthma.

5. Conclusion

Whereas compelling evidence in LN suggests a pathogenic role for anti-dsDNA Abs, their detailed mechanisms of action are not restricted to IC formation. As illustrated in Figure 2, we propose that, among anti-dsDNA Abs, a minute fraction of anti-dsDNA Abs stimulate MCs to produce cytokines, chemokines, and matrix metalloproteinases important in the initiation of the inflammatory process. In addition, such

activation is associated with proliferation and apoptosis, matrix protein accumulation, and a reduction of DNase1 activity that would, in turn, contribute to the formation of anti-dsDNA Ab chromatin/ICs in the mesangium and later in the GBM that characterize severe LN. Furthermore, while the focus of this paper is on MCs, it should be mentioned that antigens could be displayed by other glomerular cells as well, including podocytes. As a consequence, the pathogenicity of these Abs would be enhanced by targeting more than one cell type.

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Review Article

Imaging Assessment of Cardiovascular Disease in Systemic Lupus Erythematosus

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Systemic lupus erythematosus is a multisystem, autoimmune disease known to be one of the strongest risk factors for atherosclerosis. Patients with SLE have an excess cardiovascular risk compared with the general population, leading to increased cardiovascular morbidity and mortality. Although the precise explanation for this is yet to be established, it seems to be associated with the presence of an accelerated atherosclerotic process, arising from the combination of traditional and lupus-specific risk factors. Moreover, cardiovascular-disease associated mortality in patients with SLE has not improved over time. One of the main reasons for this is the poor performance of standard risk stratification tools on assessing the cardiovascular risk of patients with SLE. Therefore, establishing alternative ways to identify patients at increased risk efficiently is essential. With recent developments in several imaging techniques, the ultimate goal of cardiovascular assessment will shift from assessing symptomatic patients to diagnosing early cardiovascular disease in asymptomatic patients which will hopefully help us to prevent its progression. This review will focus on the current status of the imaging tools available to assess cardiac and vascular function in patients with SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a multisystem, autoimmune disease and is one of the strongest known risk factors for atherosclerosis and coronary artery disease (CAD) [1, 2]. The range of cardiovascular disease (CVD) in SLE is broad and includes atherosclerosis, vascular inflammation, Raynaud's phenomenon, endothelial dysfunction, and a procoagulant tendency associated with antiphospholipid antibodies. The impact of SLE-associated CVD on both mortality and morbidity is impressive: the incidence of CAD is over 7 times greater in patients with SLE than in healthy controls, even when matched for cardiovascular risk factors [3]. Moreover, female patients with SLE between 35 and 44 years old have an incidence of myocardial infarction over 50 times greater than the observed in the Framingham dataset [4]. These findings account for the bimodal mortality pattern in SLE: an early peak (<1 year of diagnosis) associated with renal involvement and infection and a later peak (8 years after diagnosis) due to premature myocardial infarction [5]. In addition to an increased risk of CAD, patients with SLE are at greater risk for stroke, with a prevalence that can reach 20% and with a high recurrence rate and greater mortality than matched controls [6].

The reason why patients with SLE have a higher cardiovascular mortality and morbidity seems to be related to the presence of an accelerated atherosclerotic process [7, 8], which seems to be due to a complex interplay of traditional and lupus-specific risk factors [3, 9–11]. On the one hand, some of the factors contributing to an accelerated atherosclerosis may be associated with the disease itself: the systemic inflammation associated with poorly controlled SLE could contribute to plaque destabilization. On the other hand, patients with SLE have a high prevalence of traditional CVD risk factors [3, 10] such as hypertension, altered lipid profile [12], and impaired glucose tolerance, which to some extent result from chronic corticosteroid therapy [13]. However, not only has no unequivocal correlation been established between corticosteroid use and atherosclerosis in SLE, but some evidence seems to suggest an increased cardiovascular risk among patients who are under treated with steroids, thus implying that having poorer disease control is associated

with a higher vascular risk than steroid therapy *per se* [7]. The generally accepted notion is that systemic inflammation related to SLE contributes both to an accelerated atherosclerosis and plaque destabilization which in turn is the major cause of acute plaque disruption responsible for acute cardiovascular events such as myocardial infarction.

The relevance of accurate cardiovascular assessment in patients with SLE has been emphasized by recent studies that show that mortality associated with CVD has not improved over time, opposing the trend seen for other causes of mortality such as lupus nephritis [14, 15]. Several reasons can be hypothesised to explain this but one of the strongest is the poor performance of standard risk stratification tools (based on the Framingham risk equation) [16] in patients with SLE, which prevents an accurate assessment of the actual cardiovascular risk of the individual patient [9, 17]. It is, therefore, essential to find alternative ways to assess and identify patients with SLE at increased risk for CVD efficiently. Several imaging techniques have been studied as potential tools to assess these patients better, with particular emphasis in noninvasive screening tools aimed at detecting subclinical atherosclerosis.

This paper will focus on the current status of imaging assessment of cardiac and vascular function among patients with SLE. There are two possible roles for this type of assessment. One is to identify CVD in patients with suspicious symptoms or other good reasons to suspect CVD (e.g., heavy smoking). The more challenging role is to diagnose CVD in asymptomatic patients with few or no risk factors other than SLE itself. It is important to remember that though SLE is associated with an increased relative risk of developing CVD compared to healthy controls, the absolute risk of developing CVD in an individual patient remains small. For example, in a recent multicentre study of 1249 patients recruited within 15 months of the diagnosis of SLE and followed for up to 8 years, only 74 patients developed CVD [18]. Thus, it would be difficult to justify invasive or repeated imaging to screen for CVD in the majority of patients with SLE.

2. Assessing Cardiac Involvement

SLE-associated cardiac involvement can be divided into 4 groups: pericarditis/pericardial effusion, valvular disease, myocardial dysfunction, and coronary-artery disease (CAD). For the purpose of this paper, we will focus only on the last two groups.

2.1. SLE-Associated Myocardial Dysfunction. In SLE, myocardial dysfunction may be due to several features such as CAD, valvular disease, drug-related cardiotoxicity (e.g., cyclophosphamide and chloroquine), and lupus myocarditis. After the introduction of corticosteroid therapy, the prevalence of autopsy-identified SLE-related myocarditis decreased from 50%–75% [19] to 25%–30% [20]. However, clinically evident lupus myocarditis is identified in less than 10% of patients, showing the high prevalence of subclinical disease [21]. In fact, clinical manifestations of SLE-associated myocarditis are subtle and nonspecific. The fact that systolic function is preserved until late stages of the disease accounts for the low sensitivity of echocardiographic assessment [22]. Although still considered the gold standard for pericardial and valvular evaluation [22, 23], its use in lupus myocarditis diagnosis is limited. However, it can give some indication of left ventricle diastolic dysfunction through the presence of impaired myocardial relaxation, decreased compliance, and increased filling pressure [22]. Another way to assess cardiac function is through left ventricle angiography, both by the conventional method and by using Technetium-99m myocardial perfusion imaging (SPECT), which permits accurate assessment of left ventricle volume and function [24, 25]. However, these methods have largely been replaced by MRI imaging [26].

The definite diagnosis of lupus myocarditis is histological, with typical features being interstitial oedema, focal necrosis/fibrosis, and focal or diffuse inflammatory cellular infiltrates [27]. However, despite being the gold standard for diagnosis, endomyocardial biopsy cannot be used routinely or repeatedly, particularly in asymptomatic patients. Cardiac magnetic resonance (CMR) is sensitive to many of the changes that characterize lupus myocarditis, particularly through T2-weighted imaging (myocardial oedema) [28, 29] and early (EGE) and late (LGE) gadolinium-enhanced CMR [28, 30]. The combination of EGE, LGE, and T2 imaging sequences has been reported to have 76% sensitivity and 95.5% specificity for the detection of myocardial inflammation [28]. In addition, CMR is superior to other techniques in assessment of left ventricle size, function, and mass, provides high spatial resolution, is noninvasive and has high reproducibility and low intra and interobserver variability [26]. In a recent study, we carried out CMR and transthoracic echocardiography in 22 patients with SLE (11 patients with previous CVD and 11 age-sex matched controls) [30]. We found that CMR was more sensitive than echocardiography for the detection of myocardial changes, especially late gadolinium enhancement (LGE) in areas of previous infarction [30]. In contrast to a previous report [31], we did not find widespread small areas of LGE in the myocardial tissues of these patients. Mavrogeni et al. [32] reported LGE in 18/20 patients with autoimmune rheumatic diseases (three with SLE). Ten patients also had myocardial biopsies with a 50% agreement between biopsy and CMR results.

CT imaging is not considered an adequate tool for evaluation of cardiac muscle due to radiation exposure, movement artefacts and application of contrast media which prevents use in patients with renal failure and severe heart failure [33].

2.2. Coronary Artery Assessment. As stated before, SLE is associated with a significantly increased risk of CAD. The presence of CAD can be evaluated directly by coronary arteriography and indirectly by assessing left ventricle ejection function and ventricular wall motion through radionuclide ventriculography, echocardiography, SPECT, and CMR [22].

2D echocardiography is the most widely used method for routine assessment of left ventricle ejection fraction in patients with known CAD. Other methods, such as tissue Doppler imaging and 3D echocardiography have been proposed

as superior alternatives; however, they still have not replaced conventional echocardiography [22]. Turiel et al. [34] have proposed a global index of left ventricle function (TEI index) aimed at systolic and diastolic left ventricle function. However, its validity in SLE has yet to be shown. Stress echocardiography using either exercise or pharmacological stimulus can be a useful method for diagnosis and risk stratification in patients with suspected or known CAD [22].

Presently, several studies have shown the utility of MRI imaging in assessment of CAD—though not in patients with SLE [26, 28, 35]. Stress CMR (i.e., using dobutamine or adenosine) is an accurate method to identify ischemia-induced wall motion abnormalities, with a greater sensitivity (86% versus 74%) and specificity (86% versus 70%) than stress echocardiography [36]. In addition, perfusion defects can be identified with gadolinium-enhanced CMRI as well as positron emission tomography (PET) [37] and SPECT [25]. One additional benefit from PET imaging is the possibility of identifying stable plaques as a high uptake of contrast seems to be associated with a higher macrophage content which would correlate with the presence of intraplaque active inflammation [38]. However, the use of radioisotopes for PET limits its applicability.

Electron beam CT (EB-CT) can be used to quantify coronary artery calcification as a measure of coronary atherosclerosis. Asanuma et al. [7] compared EB-CT findings in 65 patients with SLE and 68 age-/sex-/ethnicity-matched controls. Mean calcification scores was significantly higher in patients than controls. After adjustment for cardiac risk factors including age, sex, smoking, hypertension, triglyceride, and homocysteine levels, patients with SLE were still 9.8 times more likely to have coronary calcification than controls. The reason for this was unclear. In a subsequent paper Kiani et al. [39] found coronary calcification in 43% of 200 women with SLE, but the only factors predicting this in multiple logistic regression analysis were age and body mass index. SLE disease activity was not associated with coronary calcification. CT angiography can be used to detect plaques in the coronary arteries. In a recent study [40], Ishimori et al. carried out both adenosine stress CMR and CTA in 18 female patients with SLE who had suffered chest pain within the previous six months and in 10 healthy control women. Eight patients with SLE, but no control subjects had abnormal perfusion on stress CMR. This was severe in 7 cases even though none of the patients had obstructive CAD detectable by CTA and only two of the 18 subjects had any CTA abnormalities. The perfusion defects were not characteristic of coronary artery disease [40]. Thus, it seems likely that stress CMR was detecting microvascular ischemia in patients with SLE though larger studies are required.

Invasive methods for assessing coronary circulation such as intravascular ultrasound (IVUS) and IVUS with virtual histology [41], optical coherence tomography [42], coronary angioscopy [43], and invasive MRI [44] may prove their usefulness in the future by allowing direct plaque imaging. However, presently their predictive value and impact on risk stratification is yet to be established.

3. Assessing Peripheral Vascular Involvement

Peripheral vascular involvement in SLE can be associated with active vasculitis, endothelial dysfunction and atherosclerosis. For the purpose of this paper, we will focus on the latter two.

3.1. Endothelial Dysfunction. The endothelium is the main regulator of vascular wall homeostasis. It regulates vascular tone and permeability, platelet and leukocyte adhesion and aggregation, and finally, vascular thrombosis. The term "endothelial dysfunction" describes a nonadaptive state of phenotypic modulation characterized by a loss or deregulation of the homeostatic mechanisms operative in healthy endothelial cells [45]. Current evidence suggests that endothelial dysfunction is an early event in atherogenesis and contributes to all the stages of plaque development [46]. Although there are currently no imaging methods that can effectively assess endothelial function, several functional methods have been developed to try and address this issue. The hallmark of endothelial dysfunction is an impaired endothelium-dependent vasodilatation [47]. Peripheral studies include flow-mediated vasodilatation assessment [47–49], forearm perfusion techniques, pulse wave analysis, and skin laser Doppler flowmetry [45]. Other potential markers of endothelial dysfunction correlate with circulating procoagulant, prothrombotic, and proinflammatory mediators, but with the exception of C-reactive protein, evidence for their independent predictive value is still lacking [45]. Although very few studies of this nature have been done in SLE [48, 49], attenuated flow-mediated dilation has been a consistent finding, suggesting the presence of impaired endothelial function in these patients even before overt cardiovascular disease is apparent.

3.2. Peripheral Vascular Assessment. The presence of common carotid artery intimal-medial thickening and discrete, nonobstructive carotid atherosclerosis has been shown to be independently associated with subsequent cardiovascular risk in several longitudinal studies [50, 51].

Ultrasound assessment of carotid atherosclerosis is an accurate, noninvasive method that allows for assessment of arterial wall thickness and degree of plaque. Manzi et al. [52] studied the prevalence of carotid atherosclerosis as measured by B-mode ultrasound in 175 women with SLE, finding that 40% had at least 1 focal plaque and that more than 20% had a at least one large plaque (>50% of the vessel diameter) or multiple plaques with at least one medium plaque (30%–50% of the vessel diameter). Patients with higher cumulative damage measured by the modified Systemic Lupus International Collaborative Clinics (SLICC) damage score were more likely to have plaque, even after excluding the cardiovascular components of the SLICC index. A strong association between duration of use and cumulative dose of corticosteroids was also found. Other groups, working independently have also found a prevalence of carotid plaque in the order of 40% in patients with SLE [8]. A longitudinal study from the Manzi group [53] assessed plaque progression in 217 female patients with SLE followed for 10 years using

Table 1: Overview and comparison of different imaging methods in atherosclerotic plaque assessment (IMT: intima-media thickness; CVD: cardiovascular disease).

Imaging method	Plaque characterization	Advantages	Disadvantages	Published data from patients with SLE
Carotid ultrasound	IMT and plaque in carotid arteries	No radiation rapid-convenient correlates with risk of future CVD	Interpretation is operator dependent. High frequency of plaque in Patients with SLE (clinical implications unclear)	Yes [22, 23, 54, 55, 58]
Magnetic resonance imaging (MRI)	Structure of myocardium quantification of lipid content	No radiation more sensitive than echo for myocardial change	Expensive use of gadolinium limited in patients with renal impairment motion artefacts. Lower spatial resolution in vascular assessment. Longer length of study time	Yes [2, 13, 18, 30, 33–36]
Computed tomography (CT)	Quantification of calcium, fibrous and lipid component	Noninvasive detection of vulnerable plaques	Motion artefacts. Contraindicated in renal impairment Low resolution	Yes [17, 41, 42]
Intravascular ultrasound-based methods	Plaque volume Luminal and vessel dimensions calcium content	Good penetration depth complements coronary angiography	Invasive lower spatial resolution	No
Positron emission tomography (PET)	Plaque macrophage content		Not established for widespread clinical use	Yes [26, 27]
Optical CT	Plaque microstructure (fibrous cap thickness measurement)	High spatial resolution	Invasive limited depth of penetration	No
Invasive MR	Plaque morphology and structure		Not established for widespread clinical use	No
Coronary angioscopy	Direct plaque surface visualization	Three-dimensional view of plaque	Superficial assessment of plaque. Risk of coronary occlusion	No

ultrasound. Progression of plaque occurred in 27% of patients and, overall, the mean increase in intima-media thickness was 0.011 mm/year. Plaque progression was greater in patients with SLE when compared with matched controls, suggesting that B-mode ultrasound may be a useful surrogate end point in SLE clinical management [53]. Importantly, this group went on to show, for the first time, that increase in IMT or the presence of plaque predicted increased risk of cardiovascular events [54]. They followed 224 women with lupus but no previous cardiovascular events. Over a 10-year followup period, 73 of them suffered either cardiac or cerebrovascular events. In multivariable analysis, higher IMT and presence of plaque at baseline predicted increased risk of cardiovascular events. For IMT, the hazard ratio was 1.24 (95% CI 1.04 to 1.48) per mm increase and the hazard ratio for presence of plaque was 5.97 (95% CI 1.52 to 23.38) [54]. Further enhancement of ultrasound assessment of carotid plaques can be achieved using integrated backscatter analysis of carotid-intima complex. This method has been shown to correlate with the calcium and collagen content of vascular wall, therefore noninvasively evaluating arterial sclerosis [55]. However, its usefulness in SLE has not been established [56].

High-resolution CT imaging has also been studied as an alternative and more accurate method of assessing carotid plaques. CT angiography has been shown to not only provide an accurate analysis of the degree of stenosis but also to correlate with histological findings of atheromatous plaques at the carotid bifurcation [57]. However, limitations associated with the use of contrast and radiation exposure are of concern.

A potential role for MRI imaging has emerged; as its use has much less limitation than CT methods [58], and there is a good correlation between them. In addition to assessing intimal and medial thickening, both methods yield information concerning the pattern of plaque calcification. Whether this correlates with the risk of embolic stroke is yet to be definitely established.

Presently, cutting edge, multimodal imaging research using animal models is aimed at determining ways to accurately assess plaque stability [59–61]. In animal models, factors like atherosclerotic plaque neovascularisation, thickness of fibrous plaque, lipid-rich necrotic-core, and macrophage content have been related to an increased plaque disruption risk. However, clinical implications in humans have not been established.

Table 1 summarises the different methods of imaging described in this paper with their advantages and disadvantages.

4. Conclusion

In summary, patients with SLE have a high risk of developing CVD. Despite their relevance, traditional reversible risk factors solely cannot account for the overall cardiovascular risk increase, which also depends on disease and treatment related issues. In this paper, we have described a number of technological advances that have enhanced the ability of clinicians to assess the myocardium, coronary arteries, and peripheral vessels in patients with CVD. For most of them, there is little or no information about use in patients with SLE. Some of these imaging techniques, for example, PET scanning and CT angiography, should clearly be reserved for patients with SLE with known CVD or very high CVD risk (based on traditional risk factors as well as the presence of SLE). Others, such as echocardiography and carotid ultrasound are convenient and noninvasive and could be used as screening tools in asymptomatic patients though it is still unclear how best to manage patients who have abnormalities on these tests. Perhaps the best way to use these imaging methods in the future will be in combination with assessment of traditional risk factors, disease activity measurements, and blood tests relevant to CVD [62]. This holistic assessment could then be used to identify patients who would benefit from more accurate but more invasive imaging methods such as cardiac MRI.

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Research Article

Deranged Bioenergetics and Defective Redox Capacity in T Lymphocytes and Neutrophils Are Related to Cellular Dysfunction and Increased Oxidative Stress in Patients with Active Systemic Lupus Erythematosus

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Urinary excretion of *N*-benzoyl-glycyl-*Nε*-(hexanonyl)lysine, a biomarker of oxidative stress, was higher in 26 patients with active systemic lupus erythematosus (SLE) than in 11 non-SLE patients with connective tissue diseases and in 14 healthy volunteers. We hypothesized that increased oxidative stress in active SLE might be attributable to deranged bioenergetics, defective reduction-oxidation (redox) capacity, or other factors. We demonstrated that, compared to normal cells, T lymphocytes (T) and polymorphonuclear neutrophils (PMN) of active SLE showed defective expression of facilitative glucose transporters GLUT-3 and GLUT-6, which led to increased intracellular basal lactate and decreased ATP production. In addition, the redox capacity, including intracellular GSH levels and the enzyme activity of glutathione peroxidase (GSH-Px) and *γ*-glutamyl-transpeptidase (GGT), was decreased in SLE-T. Compared to normal cells, SLE-PMN showed decreased intracellular GSH levels, and GGT enzyme activity was found in SLE-PMN and enhanced expression of CD53, a coprecipitating molecule for GGT. We conclude that deranged cellular bioenergetics and defective redox capacity in T and PMN are responsible for cellular immune dysfunction and are related to increased oxidative stress in active SLE patients.

1. Introduction

Systemic lupus erythematosus (SLE) is an archetype of systemic autoimmune disease that is characterized by diverse immune dysfunctions. SLE patients experience increased oxidative stress that is related to mitochondrial hyperpolarization and ATP depletion [1–4]. However, the molecular basis of this increased oxidative stress and its relationship to immune dysfunction in SLE patients remain unclear. Active immune cells such as lymphocytes and polymorphonuclear neutrophils (PMN) need constant energy for both basic housekeeping and specific immune functions such as tissue immigration, antigen processing/presentation, signal transduction, and other effector functions [5, 6]. The oxidation of glucose provides a major source of metabolic energy in mammalian cells. Glucose-6-phosphatase is an enzyme that is only present in mammalian liver and kidneys and plays an important role in producing glucose during periods of starvation [7]. Because the lipid bilayer on the mammalian cell surface is impermeable to the hydrophilic and polar glucose molecules, cellular uptake of glucose is only achieved through glucose carriers embedded in the bilayer. The glucose transporters include sodium-dependent cotransporters and facilitative glucose transporters (GLUT)

that facilitate glucose diffusion along a concentration gradient [8]. Each of the 14 isoforms of facilitative glucose transporters (GLUT-1 to GLUT-13 and HMIT-1), exhibits a different affinity for glucose and other hexoses. GLUT-1 and GLUT-3 possess a high affinity for glucose [9]. GLUT-1 is present at variable concentrations in many tissues and is believed to be responsible for basal glucose uptake [10]. GLUT-3 is mainly expressed in brain and circulating mononuclear cells [11]. GLUT-6 is only expressed in the spleen, leukocytes, and brain [12, 13]. There have been few studies on the relationship between glucose transporter expression and the bioenergetics of SLE immune-related cells reported in the literature.

Viora et al. [14] demonstrated that the intracellular reduction-oxidation (redox) state might affect lymphocyte proliferation and NK-mediated cytotoxicity. The glutaredoxin-glutathione system is the key player in redox regulation of the cells and is composed of NADPH, reduced-form glutathione (GSH), the flavoprotein glutathione peroxidase (GSH-Px), and glutathione reductase (GSSG-Rx) [15]. Because glutathione is present in all animal cells in high concentrations, it acts as the most important intracellular modulator for redox, cell proliferation, DNA synthesis, immune responses, and arachidonic acid metabolism [16, 17]. The antioxidant glutathione must be recaptured by γ-glutamyl-transpeptidase (GGT) [18]. Because CD53, a glycoprotein of the tetraspanin superfamily, can coprecipitate with GGT activity, elevated CD53 expression in an enhanced oxidative stress environment may prevent cell apoptosis due to oxidative damage [19]. In the present study, we hypothesized that deranged bioenergetics and defective redox capacity, especially in the immune-active cells, may be involved in the molecular basis of cellular immune dysfunction and increased oxidative stress in patients with active SLE. Our results support this hypothesis.

2. Patients and Methods

2.1. Patients and Controls. Fifty-four patients meeting the 2000 ACR revised classification criteria for SLE were enrolled in the study. An additional 52 age- and sex-matched healthy individuals comprised the normal control group. A second control group comprised of patients with non-SLE connective tissue diseases was created to enable us to compare the urinary excretion of $N\varepsilon$-HEL and 8-OHdG of such patients to that of SLE patients. The 11 patients in the second control group included patients with vasculitis ($n = 2$), Sjogren's syndrome ($n = 3$), rheumatoid arthritis ($n = 4$), antiphospholipid syndrome ($n = 1$), and systemic sclerosis ($n = 1$). Patients with SLE were divided into two groups. Forty-two patients with an SLEDAI score ≥ 6 were considered to have active SLE [20]. Twelve patients with scant clinical manifestations and who had normal levels of C3/C4 and anti-dsDNA were placed in the inactive SLE group. The glucose uptake of immune cells in the inactive SLE group was compared to that of cells from the active SLE group. The demographic, laboratory, and clinical data of all three groups are listed in Table 1. This study was approved by the Institutional Review Board and Ethical Committee, National Taiwan University Hospital, Taipei, Taiwan. Informed consent was obtained from each participant. Venous blood and 24-hour urine were collected from each participant for analysis.

2.2. Detection of 24-Hour Excretion of N-Benzoyl-Glycyl-Nε-(Hexanonyl)Lysine (Nε-HEL) and 8-Hydroxy-2-Deoxyguanosine (8-OHdG). We collected all urine excreted over a 24-hour period from 26 active SLE, 11 non-SLE, and 14 healthy volunteers in clean containers. Urinary $N\varepsilon$-HEL and 8-OHdG were quantified using commercially available kits (Japan Institute for Aging, Schizuoka, Japan). The concentration of urine creatinine (Ucre) was concomitantly measured. The excretion of $N\varepsilon$-HEL was calculated as pmol/mg Ucre, and 8-OHdG was calculated as pg/mg Ucre.

2.3. Isolation of T Lymphocytes and Polymorphonuclear Neutrophils from Peripheral Blood. Heparinized venous blood obtained from participants was mixed with one-quarter volume of 2% dextran solution (molecular weight, 464,000 daltons; Sigma-Aldrich Company, St. Louis, MO, USA) and incubated at 37°C for 20 min. The leukocyte-rich supernatant was collected and diluted with the same volume of Hanks' balanced salt solution. The cell suspension was layered on a Ficoll-Hypaque density gradient cushion (specific gravity 1.077, Pharmacia Biotech, Uppsala, Sweden) and centrifuged at 150 g for 30 min. The mononuclear cells (MNC) were aspirated from the interface and PMN were collected from the bottom. The RBC in the PMN suspension were lysed by incubation in 0.83% ammonium chloride solution chilled to 4°C for 10 min. To purify the T lymphocyte samples, the MNC suspension was positively selected by monoclonal antihuman CD3 antibody-coated microbeads and AutoMACS (Miltenyi Biotec, Bergisch, Gladback, Germany), as in our previous report [21]. PMN and T lymphocytes were more than 95% viable and pure, as confirmed by trypan blue dye exclusion and analysis with flow cytometry after staining with FITC-conjugated anti-CD16 (for PMN) or anti-CD3 (for T cell) antibody (Sigma-Aldrich).

2.4. Measurement of Intracellular Basal Lactate Levels in T and PMN. Intracellular basal lactate was measured using the method reported by Frauwirth et al. [22]. Briefly, we used enzymatic diagnostic kits (Sigma-Aldrich) to measure the intracellular basal lactate levels (mg/dL) in cell lysates (2×10^6 cells/mL) of T cells and PMN from normal and SLE patients. A detailed description of the procedures can be found in the manufacturer's instruction booklet.

2.5. Measurement of Intracellular ATP Levels in T and PMN. Intracellular ATP levels (nmole/10^6 cells/mL) in T and PMN lysates were determined by using ATP determination kits (Molecular Probes, Eugene, OG, USA). Detailed procedures are described in the manufacturer's instruction booklet.

Table 1: The demographic features and clinical data of normal, active, and inactive SLE groups.

Parameters	Normal ($N = 52$)	Active SLE ($N = 42$)	Inactive SLE ($N = 12$)
Age, years (mean ± S.D.)	27.3 ± 8.7	25.7 ± 10.4	25.6 ± 9.4
Gender (female : male)	47 : 5	50 : 4	11 : 1
Disease duration	—	3–9 years	4–8 years
SLEDAI score	—	6–13	0–5
Serum C3 (mg/dL)	91.1 ± 8.5	50.7 ± 11.1	87.4 ± 13.5
Serum C4 (mg/dL)	19.4 ± 2.4	8.8 ± 3.2	16.7 ± 2.9
Anti-dsDNA(IU/mL)	—	259.4 ± 30.7	107.7 ± 13.8
Medications			
Prednisolone	—	17.6 ± 10.7 mg/D	5.3 ± 6.2 mg/D
Azathioprine	—	50–100 mg/D	—
Hydroxychloroquine	—	400 mg/D	200–400 mg/D

2.6. Measurement of Spontaneous Glucose Uptake and Glucose Transporter Expression on T and PMN

(a) Measurement of Spontaneous Glucose Uptake of the Cells by ^{3}H-2-Deoxy-D-Glucose Incorporation Assay. Spontaneous glucose uptake of T and PMN was measured using the method described by Shikhman et al. [23]. Briefly, PMN (1×10^7 cells/mL) were incubated with 10 μL of radio-labeled ^{3}H-deoxy-D-glucose (specific activity, 15–25 Ci/mL; Roche Diagnostics, Indianapolis, IN, USA) at room temperature for 7 min. The cells were washed three times with cold PBS and then lysed with Cell Death Lysis buffer (Sigma-Aldrich). The radioactivity of the cells was detected by a β-counter.

(b) Measurement of Spontaneous Expression of Glucose Transporters, GLUT3, and GLUT6 on T and PMN by Flow Cytometry. The facilitative glucose transporters GLUT-3 and GLUT-6 are differentially expressed in different mammalian blood cells and brain tissues [11]. The spontaneous expression of GLUT-3 and GLUT-6 glucose transporters on T and PMN was ascertained by first staining with FITC-labeled monoclonal antibody against human GLUT-3 or GLUT-6 (Chemicon Company, Temecula, CA, USA) followed by FACSort flow cytometric analysis with 488 nm excitation (Becton-Dickenson, Franklin Lakes, NJ, USA).

2.7. Measurement of Intracellular GSH Concentration in T and PMN. Soluble cellular GSH concentration was measured using BIOXYTECH GSH-400 colorimetric assay kit (OXIS International Inc., Portland, OR, USA). Detailed procedures are provided in the manufacturer's instruction booklet. Briefly, the concentration of T and PMN in test samples was adjusted to 1×10^7 cells/mL and sonicated at 100 W for 60 s. Only the soluble cellular forms, GSH and glutathione disulfide, were detected by the kit. The detection limit of the assay is 0.5 μM/mL.

2.8. Determination of GSH-Px Enzyme Activity. We used BIOXYTECH GPx-340 colorimetric assay kits (OXIS International Inc.) to measure the GSH-Px enzymatic activity of T and PMN cell lysates. One milliunit (mU) of GSH-Px activity is defined as the activity that catalyzes the oxidation of 1 nmol NADPH/mL/min, using an extinction molar coefficient of $6.22 \times 10^6\ M^{-1} \cdot mL^{-1}$ for NADPH.

2.9. Determination of GSSG-R Activity. We used BIOXYTECH GR-340 colorimetric assay kits (OXIS International Inc.) to measure the GSSG-R enzyme activity of T and PMN cell lysates. The definition of 1 mU of GSSG-R enzymatic activity is the activity that catalyzes the reduction of 1 nmol $NADP^+$/mL/min.

2.10. Detection of GSH-Px mRNA Expression in T and PMN by RT-PCR. Total cellular RNA was extracted from 1×10^7/mL of T or PMN using an Ultraspec RNA isolation kit (Biotex Laboratories, Houston, TX, USA). Each extracted sample (5 μg) was reversely transcribed into cDNA by placement in 30 μL of reverse transcriptional buffer for 1 h at 42°C. The buffer contained 50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 0.5 mg oligo-dT primer, 0.5 mM dNTP, 32 U RNasin, 10 mM DTT, and 40 U MMLV reverse transcriptase (Promega, Madison, WI, USA) at pH 8.3. The reverse transcription products (5 μL) were added to a PCR buffer containing 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 100 ng forward primer, 100 ng reverse primer, 0.2 mM dTNP, 2 U DNA polymerase (Promega), and 5% DMSO. PCR was performed in a Hybaid OmniGene DNA thermocycler (Teddington, UK) with a program of denaturing at 95°C for 1 min, annealing at 50–58°C for 1 min, and primer extension at 72°C for 1 min. The amplification was carried out for 25–35 cycles. The reaction was stopped after a final extension at 72°C for 10 min followed by incubation at 25°C. The forward and reverse pair primers for human GSH-Px and glyceraldehyde-3-phosphate dehydrogenase (G3PDH, used as an internal control) are shown below:

GSH-Px: 5′-GGG GCC TGG TGG TGC TCG GCT-3′ (sense),
5′-CAA TGG TCT GGA AGC GGC GGC-3′ (antisense),

G3PDH: 5′-ACC ACA GTC CAT GCC ATC AC-3′ (sense),
5′-TCC ACC ACC CTG TTG CTG TA-3′ (antisense).

The amplified PCR products were 354 bp for GSH-Px and 452 bp for G3PDH.

2.11. Western Blot Analysis of GSH-Px Isomers. T and PMN at a concentration of 5×10^6/mL were lysed and electrophoresed in 10% SDS-PAGE. The distribution of GSH-Px isomers in cell lysates was detected by monoclonal antihuman GSH-Px antibody (MBL International, Woburn, MA, USA) and enhanced chemiluminescence protein detection kits (Amersham International Plc., Chalfont, Buckinghamshire, UK) after electrotransfer to a nitrocellulose membrane.

2.12. Determination of GGT Enzymatic Activity in T and PMN. We followed the method outlined by Carlisle et al. to quantify GGT activity in T and PMN [18]. Briefly, 1×10^6 cells were suspended in 1 mL of PBS containing 2.5 mM γ-glutamyl-*p*-nitroanilide and 60 mM glycyl-glycine at pH 7.2. After 90 min of incubation at 37°C, the cells were centrifuged and the absorbance of the supernatant was read at OD_{410} nm. The enzyme activity was calculated from the absorbance readings, and is expressed as μmol *p*-nitroaniline released/min/10^6 cells.

2.13. Detection of Surface-Expressed CD53 on T and PMN. The direct immunofluorescence antibody method, as reported by Pedersen-Lane et al., was employed to stain the CD53 surface expression on T and PMN [19]. We used FITC-labeled mouse monoclonal antibody against human CD53 purchase from BD Biosciences (San Jose, CA, USA) for this assay.

2.14. Statistical Analysis. All results are presented as mean ± S.D. The statistical significance of differences between groups was assessed by nonparametric Wilcoxon rank-sum tests using the commercially available software package: Stata/SE 8.0 for Windows. A P value ≤ 0.05 was considered to be statistically significant.

3. Results

3.1. Increased Oxidative Stress in Active SLE Patients Detected by Surrogate Urinary Biomarkers, Nε-HEL, and 8-OHdG Excretion. Oxidative stress can induce peroxidation of polyunsaturated fatty acid resulting in the production of *N*ε-HEL [24]. In addition, C-8 in the guanine residue of DNA is easily cleaved by hydroxyl free radicals. The production of 8-OHdG is the hydroxidative product of guanine after free radical hydroxylation [25]. Both *N*ε-HEL and 8-OHdG are excreted in the urine, and we used these oxidative products as biomarkers for oxidative stress. We measured the 24-hour urinary excretion of *N*ε-HEL, 8-OHdG and creatinine (Ucre) concomitantly. We found *N*ε-HEL secretion in active SLE patients was significantly higher than in non-SLE and normal individuals (Figure 1(a)). However, the excretion of 8-OHdG did not differ between the 3 groups (Figure 1(b)). These results suggest that *N*ε-HEL excretion is increased in active SLE and is consistent with the findings of other authors [1, 4]. Whether *N*ε-HEL urinary excretion in the non-SLE group is abnormal cannot be determined from our data due to the variety of diseases and the small sample size.

3.2. Deranged Cellular Bioenergetics with Increased Intracellular Basal Lactate Levels and Decreased ATP Production in SLE T Lymphocytes and PMN. We hypothesized that both deranged bioenergetics and defective redox capacity may attribute to increased oxidative stress in patients with active SLE. Accordingly, we measured the intracellular basal lactate levels and intracellular ATP production as indicators of cellular bioenergetics. As demonstrated in Figure 1(c), intracellular basal lactate levels in both SLE-T and SLE-PMN were higher than in normal cells. The ATP production in both T and PMN of SLE patients was below normal (Figure 1(d)). This may due to mitochondrial hyperpolarization in the preexcited immune cells of active SLE *in vivo* [3, 26]. Our results suggest that defective cellular bioenergetics of the immune active cells is one of the causes of increased oxidative stress in patients with active SLE.

3.3. Decreased Glucose Uptake and GLUT-3, and GLUT-6 Expression on T and PMN of SLE Patients. To determine whether the abnormal cellular bioenergetics of SLE immune active cells is related to decreased glucose uptake leading to high rate of lactate production and a subsequent induction of proapoptotic *Bcl-2* gene expression even under aerobic conditions [27–29], we detected cellular glucose-uptake by ^{3}H-2-deoxy-D-glucose incorporation. As shown in Figure 2(a) (T lymphocytes) and Figure 3(a) (PMN), the glucose uptake by SLE cells was significantly lower than normal cells regardless of active or inactive SLE status. These results suggest that decreased expression of facilitative glucose transporters GLUT-3 and GLUT-6 is congenital rather than acquired in SLE patients. This defect may contribute to deranged cellular bioenergetics in SLE patients.

3.4. Comparison of Redox Capacity in Plasma and Different Normal Blood Cell Populations. Cellular redox capacity is reduced in active SLE and is a major factor contributing to oxidative stress. Reduced-form glutathione is one of the most important endogenous molecules for modulating the redox state of all animal cells [15–17]. We measured the plasma GSH levels and intracellular GSH levels in different blood cell populations including T cells, PMN, RBC, and platelets of normal individuals (Figure 4). GSH is most abundant in the plasma and T lymphocytes and GSH levels of PMN > RBC > platelets (Figure 4(a)). The reduced form GSH is generated by the activity of GSH-Px on the oxidized form, GSSG [1]. GSH-Px enzymatic activity was higher in T and plasma than in PMN, RBC, and platelets, paralleling GSH levels in the normal blood subpopulations (Figure 4(b)). Although the total expression of GSH-Px mRNA in normal T and PMN did not differ in 2 cases shown in Figure 4(c), the composition and distribution of GSH-Px isomers in normal T and normal PMN are quite different, as determined by 10% SDS-PAGE analysis. As shown in Figure 4(d), T lymphocytes expressed mainly dimer (50 kDa) and tetramer (100 kDa) isomers rather than monomer (25 kDa) and trimer (75 kDa) isomers of GSH-Px. In contrast, PMN expressed mainly monomer and trimer isomers, rather than dimer and tetramer isomers, of GSH-Px. These results are compatible with those of

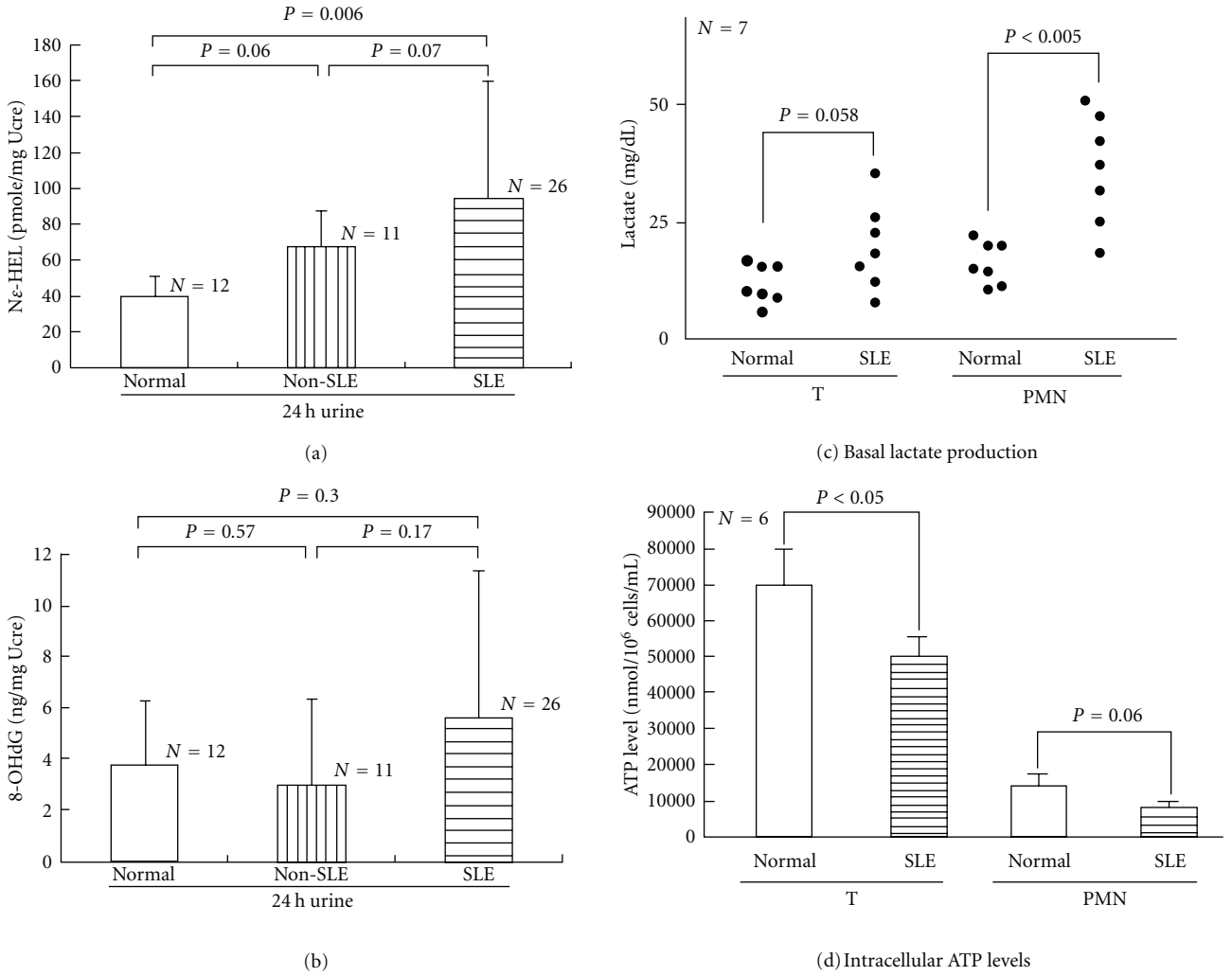


Figure 1: Comparison of 24-hour urinary excretion of *N*-benzoyl-glycyl-*Nε*-(hexanoyl)lysine (*Nε*-HEL) and 8-hydroxy-2-deoxyguanosine (8-OHdG), intracellular basal lactate levels, and ATP production of T lymphocytes and PMN from normal individuals, non-SLE patients, and patients with active SLE. (a) Urinary *Nε*-HEL excretion denoted by pmole/mg urine creatinine (Ucre). (b) Urinary 8-OHdG excretion denoted by ng/mg Ucre. (c) Intracellular basal lactate levels. (d) ATP production.

Misso et al. [30], who showed that normal neutrophils contain mainly monomer and trimer isoforms of GSH-Px. Because the GSH-Px enzymatic activity of T lymphocytes is much higher than that of PMN, we speculated that the dimer and tetramer isoforms of GSH-Px may possess more potent antioxidant activity than the monomer and trimer isoforms.

3.5. Defective Redox Capacity (GSH Levels, GSH-Px, and GSSG-R Activity) in Plasma, T, and PMN of SLE Patients. The plasma GSH levels in SLE patients was not different from that of normal individuals (Figure 5(a)). However, the GSH levels in SLE-T and SLE-PMN (Figure 5(b)), and the GSH-Px enzymatic activity in SLE-T were significantly lower than their normal counterparts (Figure 5(c)). The very low GSH-Px enzyme activity in PMN made it difficult to detect any differences between SLE and normal groups (Figure 5(c)). Unexpectedly, the distribution of the four GSH-Px isomers in SLE-T and SLE-PMN cells was not different from that of their normal counterparts (Figure 5(d)). The activity of GSSG-R, a redox-modulating enzyme that contains active dithiol moieties for protection and repair of protein sulfhydryls in oxidative stress situations [1], was not different between normal individuals and SLE patients (Figure 5(e)). To determine whether immunosuppressants such as glucocorticoids, hydroxyl-chloroquine, and azathioprine, or SLE disease activity *per se* affects the decreased redox capacity observed in SLE patients, we determined the redox capacity of T and PMN in 4 active SLE patients before and after effective treatment. We found that intracellular GSH levels in T and PMN of nontreated active SLE patients were defective in a manner similar to that of immunosuppressant-treated active SLE patients, but the low intracellular GSH levels in active SLE patients recovered after the immunosuppressant treatment were effective (Figures 5(f) and 5(g)). These results suggest that the reduced redox capacity of active SLE-T and

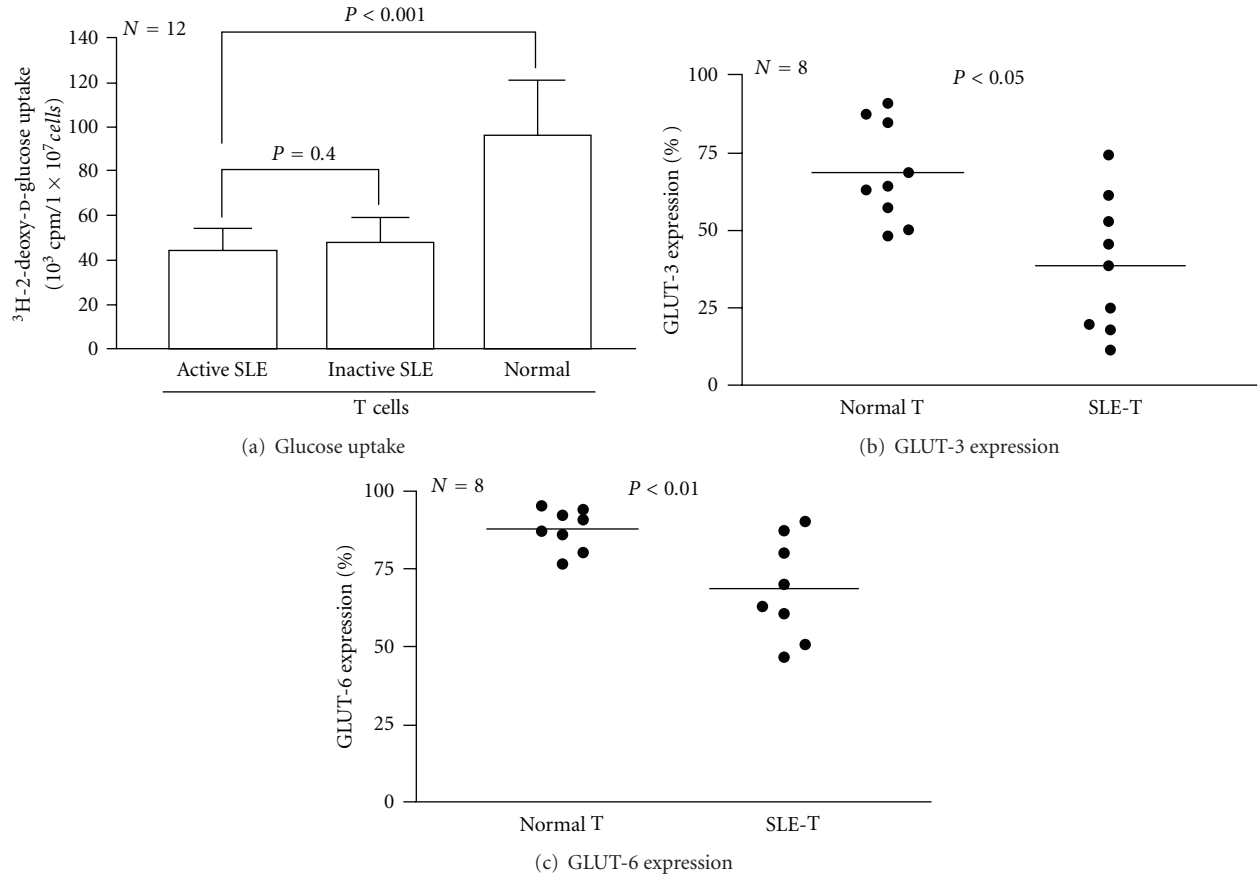


Figure 2: Comparison of glucose uptake and expression of glucose transporter type 3 (GLUT-3) and type 6 (GLUT-6) on T lymphocytes in normal and active SLE groups. (a) Glucose uptake by the cells detected by ^{3}H-2-deoxy-D-glucose incorporation after 24-hour incubation. (b) Expression of GLUT-3 on normal and active SLE T lymphocytes. (c) Expression of GLUT-6 on normal and active SLE T lymphocytes.

SLE-PMN originates from lupus disease activity, rather than the effects of immunosuppressants.

3.6. Comparison of GGT Activity and CD53 Expression on T and PMN of Normal and SLE Patients. Because GGT is crucial for enhanced antioxidant capacity through the recapture of glutathione molecules decreased intracellular GSH levels in SLE-T and SLE-PMN may be due to reduced GGT activity in the cells. GTT activity was below normal in both T and PMN from active SLE patients (Figure 6(a)). However, CD53 expression in SLE-T was not different from normal T (Figure 6(b), left panel). Unexpectedly, the CD53 expression in SLE-PMN was higher than in normal PMN (Figure 6(b), right panel). A representative case is shown in Figure 6(c). This may suggest that a compensatory mechanism is activated to limit oxidative damage in SLE-PMN [2, 4, 31] that results from the GSH-Px defect of the cells.

4. Discussion

T lymphocytes and PMN are very efficient cells for body defense. These cells need constant energy for basic housekeeping and specific actions against infections. Oxidative stress is increased in patients with active SLE [1–4]. Shah et al. [32] further demonstrated that the increased oxidative stress in SLE is related to Th1 cytokine IFN-γ and IL-12 and to disease activity. However, there have been no reports detailing the molecular basis of increased oxidative stress in active SLE. In the present study, we observed several interesting abnormalities of cellular bioenergetics and redox capacity in SLE-T and SLE-PMN. These include (a) increased intracellular basal lactate levels and decreased ATP production, (b) decreased glucose-uptake by these cells attributable to the defective expression of the glucose transporters, GLUT-3 and GLUT-6, (c) reduced redox capacity, including decreased intracellular GSH levels and enzymatic activity of GSH-Px and GGT, and (d) normal CD53 expression in SLE-T but increased expression in SLE-PMN. In addition, some other important contributory factors such as mitochondrial hyperpolarization, immune-mediated systemic tissue inflammation/damage, or accelerated atherosclerosis-mediated tissue hypoxia, may also be involved in the increased oxidative stress that occurs in patients with active SLE.

Glucose is a required energy source for many cells, particularly those in the immune system. Glucose is needed for oxidative and nonoxidative ATP production, anaerobic

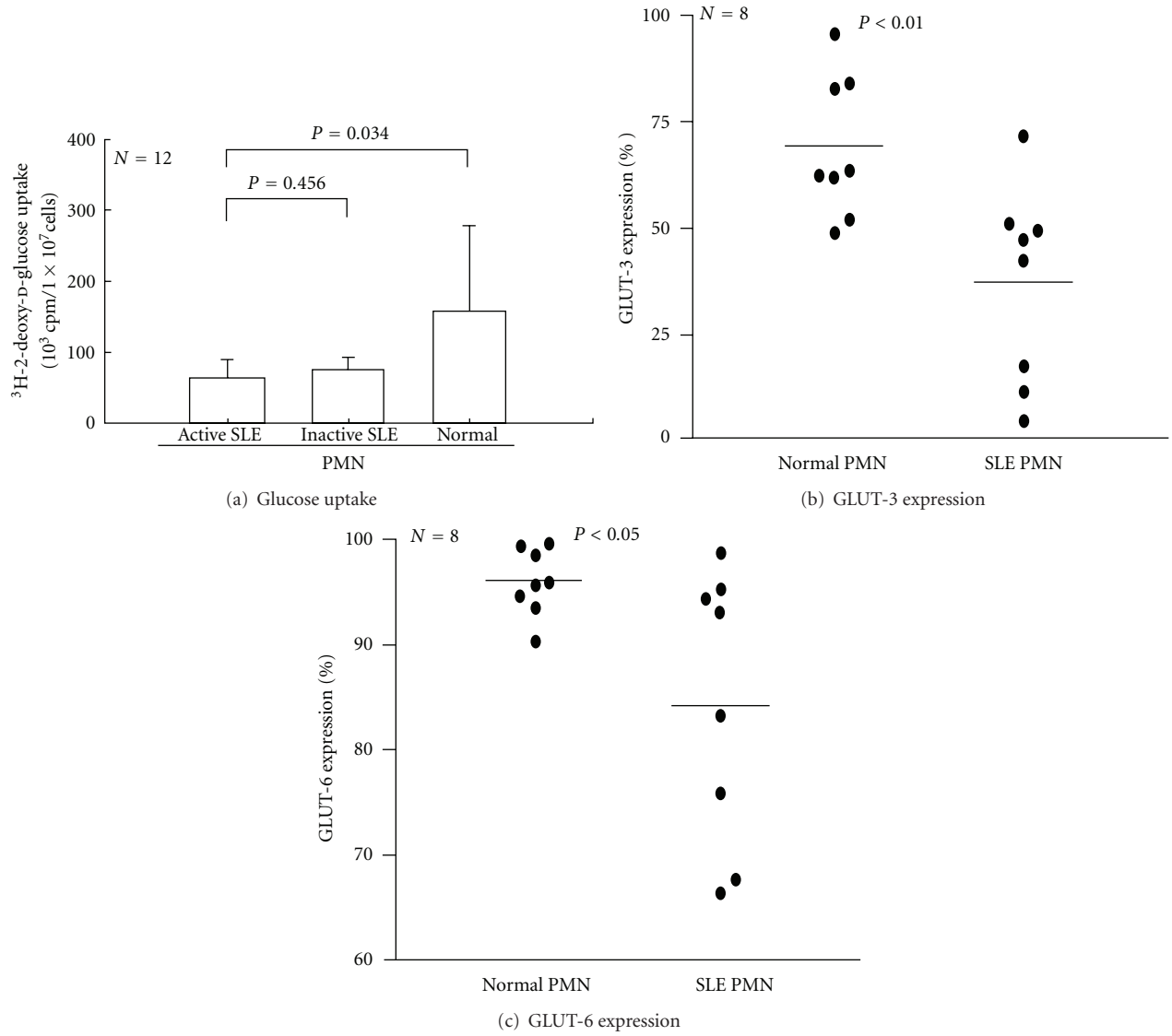
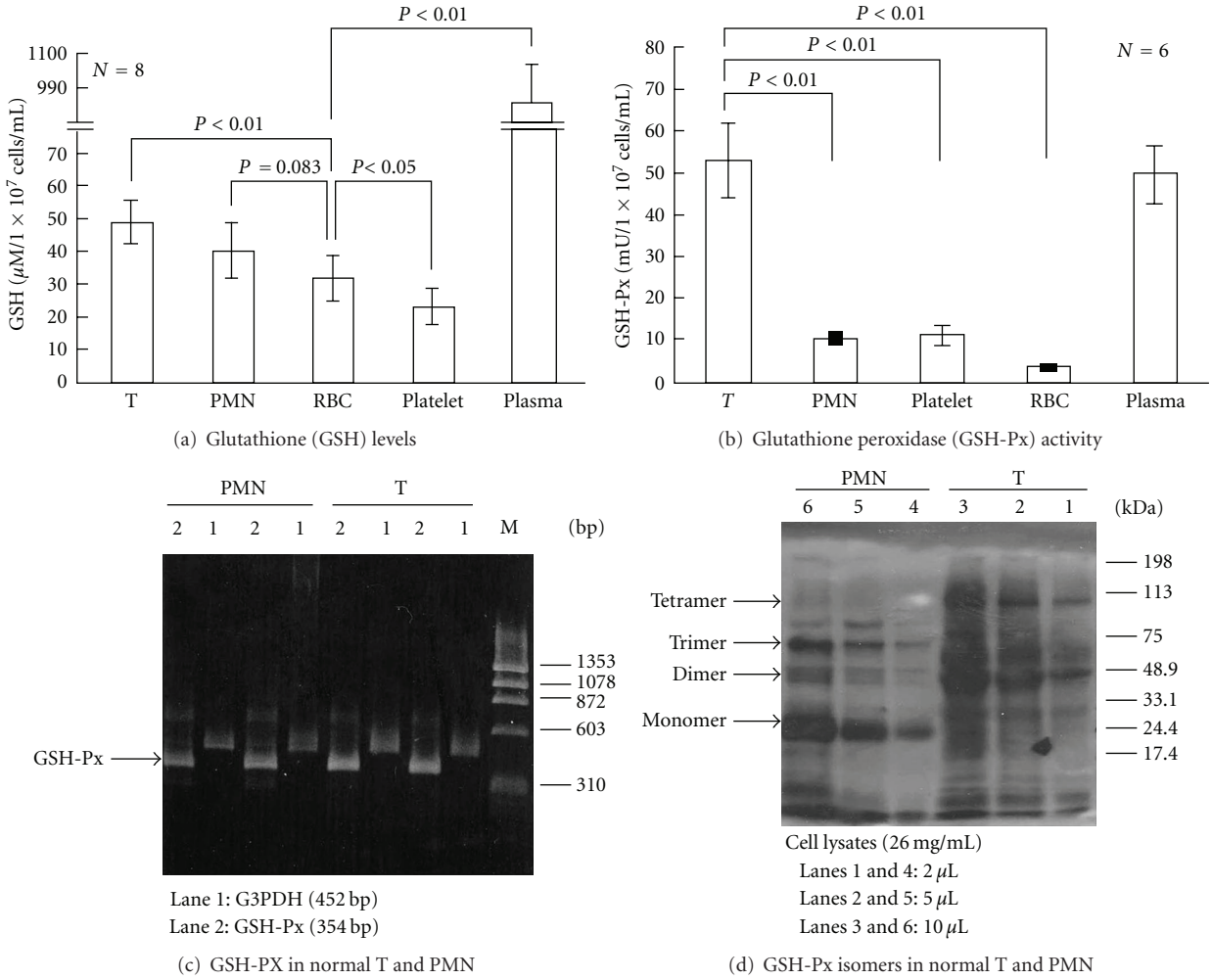


Figure 3: Comparison between normal and SLE-PMN glucose uptake and expression of glucose transporter type 3 (GLUT-3) and type 6 (GLUT-6). (a) Glucose uptake of the cells was detected by ^{3}H-2-deoxy-D-glucose incorporation after 24-hour incubation. (b) Expression of GLUT-3 on normal and active SLE-PMN. (c) Expression of GLUT-6 on normal and active SLE-PMN.

production of various sugar-containing macromolecules, and cell proliferation [6, 27–29]. Tan et al. [33] and Schuster et al. [34] demonstrated that after activation, PMN is critically dependent on glucose uptake and glycolysis for supplying the necessary energy to conduct effector functions. The bacterial products GM-CSF and phorbol myristate acetate enhance glucose uptake by sequential activation of neutrophilic protein tyrosine kinase C [32], p38 MAPK, and hypoxia-inducible factor pathways [34]. Fu et al. [13] further demonstrated that GLUT-1 and GLUT-3 expression provides cellular fuel for immune responses. Maciver et al. [29] showed that if glucose uptake is limited, glycolytic flux decreases to a level that no longer sustains viability, and the proapoptotic Bcl-2 family becomes activated, promoting cell death. This may lead to increased glycolytic capacity and a high rate of lactate formation from glucose even under aerobic conditions [27]. Our finding of decreased glucose uptake in SLE immune cells may be reflected in an increase of basal lactate levels and cell apoptosis in the patients. On the other hand, ATP generation is mainly derived from glucose metabolism via glycolysis or oxidative phosphorylation [35]. The elevated basal lactate levels observed in SLE PMN suggest an anaerobic metabolic state in these activated cells. In addition, compared to normal cells, SLE-T and SLE-PMN tend to have decreased ATP production (Figure 1(d)). This decreased ATP production reflects mitochondrial functions impaired by activation-induced cell death [36, 37] and decreased redox capacity in SLE [2, 4, 30]. Abnormal glucose bioenergetics in SLE cells owing to defective expression of GLUT-3 and GLUT-6 on the cell surface can lead to impaired immune functions in active SLE.



(a) Glutathione (GSH) levels

(b) Glutathione peroxidase (GSH-Px) activity

(c) GSH-PX in normal T and PMN

(d) GSH-Px isomers in normal T and PMN

Figure 4: Comparison of intracellular reduced-form glutathione (GSH) levels, enzyme activity, and gene expression of glutathione peroxidase (GSH-Px) in the plasma and different blood cell populations of normal individuals. (a) Intracellular GSH levels (μM/1 $\times$ 10^7 cells/mL) in T, PMN, red blood cells, platelets, and plasma of normal individuals. (b) GSH-Px enzyme activity (mU/1 $\times$ 10^7 cells/mL) in plasma and different blood cells of normal individuals. One milliunit (mU) of GSH-Px enzyme activity is the activity that catalyzes the oxidation of 1 nmol NADPH/mL/min. (c) Expression of GSH-Px mRNA in T and PMN of two normal individuals by RT-PCR, lane 1: G3PDH (452 bp, as internal control), lane 2: GSH-Px (354 bp). (d) A representative case demonstrating dose-response expression of GSH-Px isomers in a normal T and a normal PMN by Western blot. Three doses (2 μL in lanes 1 and 4; 4 μL in lanes 2 and 5; 10 μL in lanes 3 and 6) of cell lysates (protein concentration 26 mg/mL) were analyzed in Western blot probed by antihuman GSH-Px antibody. Four GSH-Px isomers are identified as monomer (25 kDa), dimer (50 kDa), trimer (75 kDa), and tetramer (100 kDa). Two normal samples exhibited a similar tendency.

Proinflammatory cytokines induce a variety of metabolic changes in the utilization of carbohydrates and fat [38]. IL-1β, TNF-α, and LPS effectively facilitate glucose uptake and modulate the expression of different glucose transporters in experimental cells [38–40]. SLE serum is thought to contain high levels of different Proinflammatory cytokines including TNF-α, IL-6, IL-12, IFN-α, and IFN-γ that may change glucose transporter expression and glucose metabolism [40–43]. We speculated that alterations in the intracellular bioenergetics of SLE immune cells are the result of long-term cell activation, which leads to production of a number of Proinflammatory cytokines. In clinical settings, abnormal redox states in the body fluid and blood cells of patients with some diseases, such as rheumatoid arthritis [44, 45], cardiovascular disorders [46, 47], and atopic asthma [48] have been reported. In the present study, we demonstrated that SLE-T and SLE-PMN have impaired intracellular redox capacity and increased oxidative damage. The defective redox capacity is due to SLE disease activity *per se* rather than the effects of immunosuppressive therapy. However, it is worth noting that any differences between the GSH levels of T and PMN in SLE patients and healthy individuals may be relevant to cell functions, but is unlikely to be relevant to the total ROS load. This is because any such differences would be masked by the large antioxidant capacity of plasma due to GSH, catalase, SOD, and numerous other free oxygen radical scavengers.

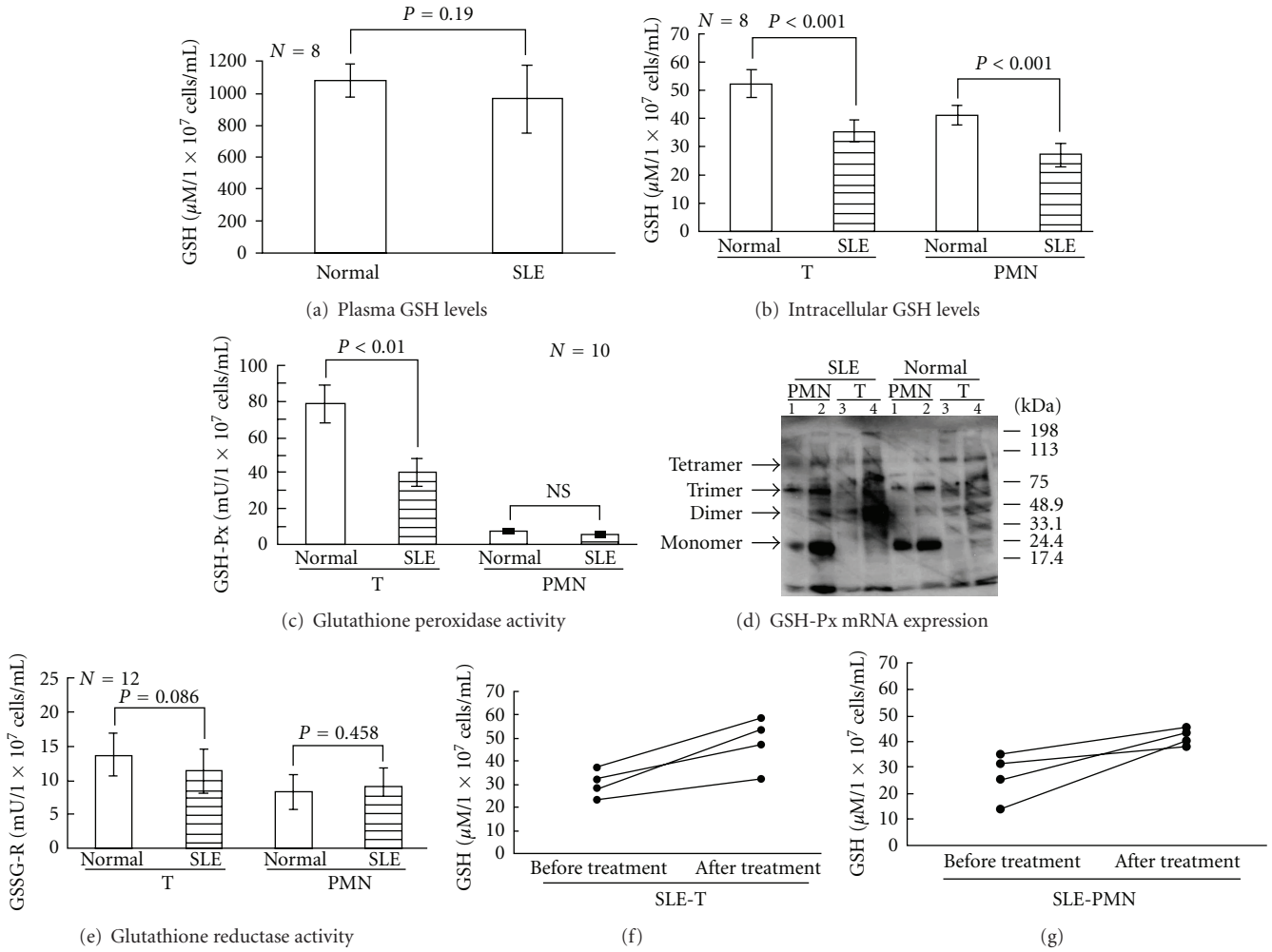
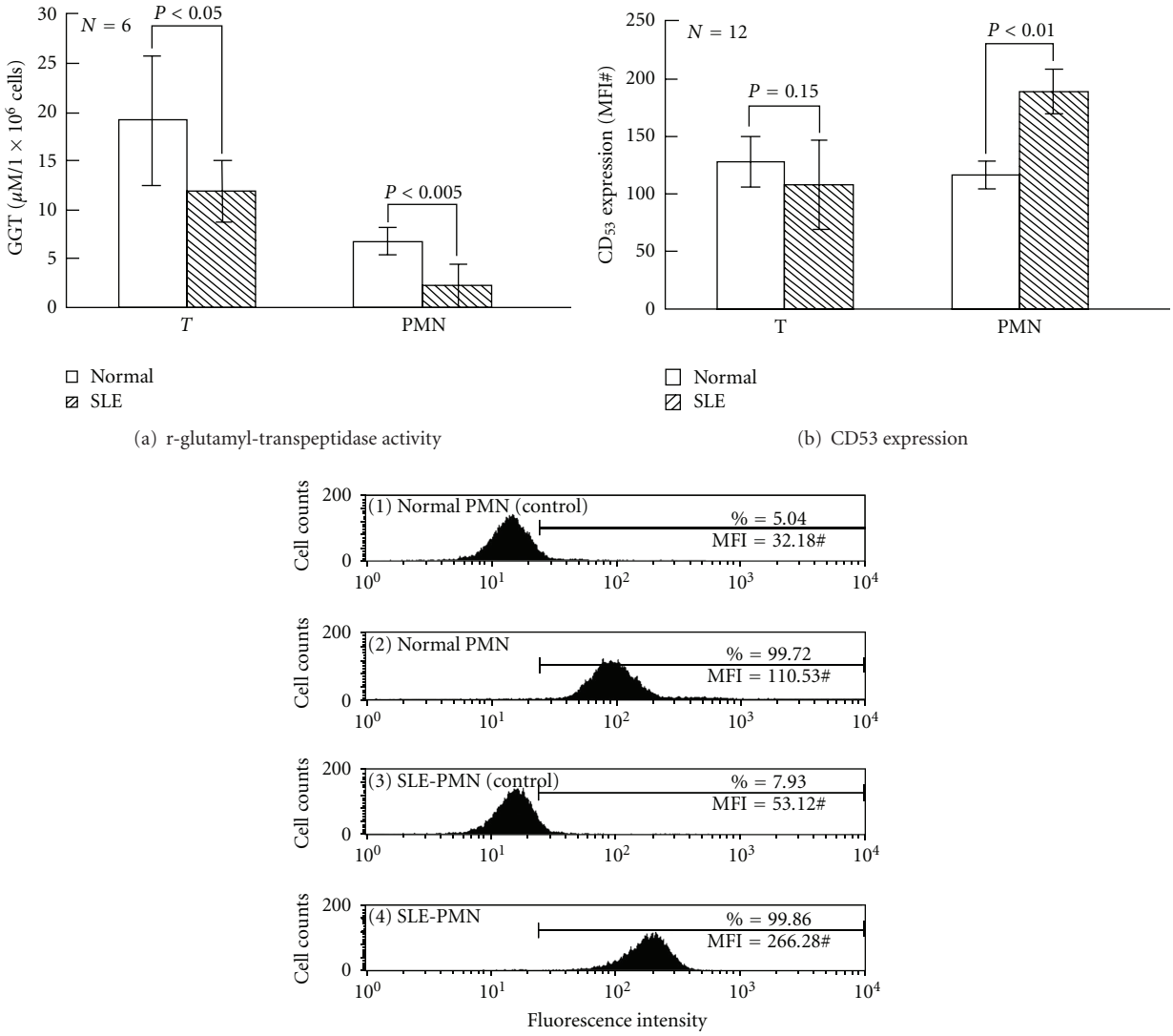


Figure 5: Comparison of plasma and intracellular GSH levels, glutathione peroxidase (GSH-Px) enzyme activity, GSH-Px isomer expression, and glutathione reductase (GSSG-R) enzyme activity in T and PMN from normal and active SLE groups. (a) Plasma GSH levels. (b) Intracellular GSH levels (μM/1 $\times$ 10^7 cells/mL) in T and PMN of normal and SLE patients. (c) GSH-Px (mU/1 $\times$ 10^7 cells/mL) enzyme activity in T and PMN of normal and active SLE group. One milliunit (mU) of GSH-Px enzyme activity is the activity that catalyzes the reduction of 1 nmol $NADP^+$/mL/min. (d) Western blot analysis of GSH-Px isomer distribution in two cases of T and PMN from 2 normal and 2 SLE patients. Both normal PMN and SLE-PMN contain mainly monomer (25 kDa) and trimer (75 kDa) isomers rather than dimer (50 kDa) and tetramer (100 kDa) isomers. In contrast, dimer (50 kDa) and tetramer (100 kDa) isomers were the main isomers found in normal and SLE-T cells. Lanes 1 and 2 are different cases of PMN. Lanes 3 and 4 are different cases of T. (e) Comparison of GSSG-R enzyme activity in T and PMN of normal and SLE group members. (f) Intracellular GSH levels in active SLE-T before and after effective treatment. (g) Intracellular GSH levels in active SLE-PMN before and after effective treatment.

The manipulation of cell redox states may become an alternative strategy for improving immune responses in some forms of cancer [17] and immune hyporesponsiveness states. Furthermore, Maurice et al. [44] demonstrated that an altered redox state is responsible for the hyporesponsiveness of rheumatoid synovial T cells. Supplementation of GSH with the glutathione precursor, *N*-acetyl-L-cysteine, enhances mitogen-induced proliferative responses and IL-2 production of synovial T lymphocytes. However, patients with atopic asthma [48], ischemic heart disease [46], and stroke [47] exhibit reduced redox capacity, but do not show distinct immune hyporesponsiveness to external stimuli. Wahl et al. [49] noted that chronically activated T cells such as SLE-T rely primarily on oxidative metabolism for ATP synthesis, suggesting that chronic antigen stimulation may be the basis for the metabolic abnormalities seen in SLE patients. Whether immune hyporesponsiveness in active SLE can be restored by supplementation with glutathione or its precursors is now under investigation. It would be interesting to directly measure the ROS generation in T and PMN and correlate it with autophagic activity, apoptosis, and other cellular functions in active SLE patients.

In conclusion, we found that impaired glucose bioenergetics and redox capacity in SLE-T and PMN are related to impaired cellular immune function and increased oxidative stress in active SLE.



(a) r-glutamyl-transpeptidase activity

(b) CD53 expression

(c) CD53 expression on PMN (an example)

Figure 6: Comparison of γ-glutamyl-transpeptidase (GGT) enzyme activity and CD53 expression in T and PMN of normal and SLE patients. (a) GGT enzyme activity. (b) Surface CD53 expression. (c) A typical case demonstrating surface CD53 expression on normal and SLE-PMN using flow cytometry. A similar tendency was seen in the cells of an additional 3 normal individuals and 3 SLE patients.

Conflict of Interests

The authors declare no competing financial interests.

Acknowledgments

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Research Article

Phenotyping of P105-Negative B Cell Subsets in Patients with Systemic Lupus Erythematosus

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This study aimed to investigate phenotype of RP105(−) B cell subsets in patients with systemic lupus erythematosus (SLE). Flow cytometry was used for phenotyping RP105-negaive B cell subsets. Based on CD19, RP105, and CD138 expression, RP105(−) B cells consist of at least 5 subsets of late B cells, including CD19(+)RP105(int), CD19(+) RP105(−), CD19(low) RP105(−) CD138(−), CD19(low) RP105(−)CD138(int), and CD19(low) RP105(−) CD138(++) B cells. Especially, CD19(+)RP105(int) and CD19(low) RP105(−)CD138(int) B cells are significantly larger than other RP105(−) B cell subsets in SLE. By comparison of RP105(−) B cell subsets between patients with SLE and normal subjects, these subsets were detectable even in normal subjects, but the percentages of RP105(−) B cell subsets were significantly larger in SLE. The phenotypic analysis of RP105(−) B cell subsets suggests dysregulation of later B cell subsets in SLE and may provide new insights into understanding regulation of B cells in human SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a typical systemic autoimmune disease characterized by production of various autoantibodies including anti-double-strand (ds) DNA antibodies from B cells [1–4]. Although the pathogenesis of SLE is not fully clarified, autoantibody-producing B cells play a pivotal role in developing autoimmunity in SLE [3, 5]. Therefore, understanding of human B cell biology in autoimmune diseases is an essential issue.

RP105 (CD180) is one of the homologues of Toll-like receptors (TLRs). RP105 expresses on mature B cells, macrophages, and dendritic cells (DCs) [6]. It has been reported that RP105 is associated with activation of B cells in mice and humans [7, 8]. RP105 also facilitates macrophage activation by mycobacterium tuberculosis lipoproteins through TLR2 [9]. However, we and other investigators have reported that RP105 negatively regulates the signal of TLR4 in DCs [10, 11]. Although the function of RP105 is still controversial and undefined, RP105 may affect activation and function of B cells in immune systems.

We have previously reported that enlarged population of RP105-lacking [RP105(−)] B cells in peripheral blood (PB) is an outstanding feature in patients with active SLE [12, 13]. Although RP105(−) B cells may be assigned to be subsets of activated late B cells with producing immunoglobulins (Igs) and anti-dsDNA antibodies [14], precise phenotype has not been examined yet.

Late B cells, including plasmablasts and plasma cells, play critical roles in humoral immune response and autoimmune diseases [15]. Comparison of the B cell subsets in healthy subjects with SLE patients could lead to relevant observations. The phenotypic analysis of subsets of RP105(−) B cells is helpful to understand the dysregulation of late B cells in SLE.

2. Materials and Methods

2.1. Patients and Agents. Patients with active SLE ($n = 15$) (14 women and 1 man, mean ± SD age: 41.2 ± 10.5 years) were enrolled in this study, who fulfilled at least 4 of the 11

classification criteria for SLE as defined by the American College of Rheumatology [16] and as updated in 1997 [17]. None of the active SLE patients was receiving immunosuppressive drugs at the time of examination. Age-matched 7 healthy volunteers joined as controls (6 women and 1 man, 38.2 ± 9.1 years). Written informed consent was obtained from all subjects prior to sample acquisition. The study protocol was approved by the Ethics Committees of Saga University, and the subjects' written consent was obtained according to the Declaration of Helsinki at the General Assembly in October 2008.

The following monoclonal antibodies (mAbs) were used in our studies fluorescein isothiocyanate- (FITC-) conjugated, phycoerythrin- (PE-) conjugated, or allophycocyanin- (APC-) conjugated antihuman CD19, FITC-conjugated or PE-conjugated antihuman RP105, FITC- or PE-conjugated anti-CD19, anti-CD20, anti-CD22, anti-CD24, anti-CD27, anti-CD28, anti-CD30, anti-CD31, anti-CD38, anti-CD40, anti-CD62L, anti-CD70, anti-CD72, anti-CD77, anti-CD79b, anti-CD80, anti-CD86, anti-CD95, anti-CD97, anti-CD126, anti-CD138, anti-CD147, anti-CD164, anti-CD200, anti-CD209, anti-CD267, anti-CD275, anti-CD279, anti-CCR7, anti-CXCR5 (CD185), anti-HLA-DR, anti-IgG, anti-IgM, anti-IgD, anto-TLR5, anti-TLR6, PE-conjugated anti-CD10, anti-CD21, anti-CD23, anti-CD25, anti-CD27, anti-CD28, anti-CD45RO, anti-CD69, anti-CD77, anti-CD122, anti-CD125, anti-CD132, anti-CD150, anti-CD152, anti-CD184 (CXCR4), anti-CCR2, anti-CCR10, anti-CX40, and anti-TLR2 were purchased from BD Bioscience (San Jose, CA, USA). The mAbs to human BCMA (B cell maturation antigen) (Vicky-1, rat IgG1), BAFF-R (B cell activating factor receptor) (11C1, mouse IgG1), and TACI (transmembrane activator and calcium modulator ligand interactor; CD267) (1A1, rat IgG2a) were obtained from ALEXIS Biochemical (Piscataway, NJ, USA). FITC- or PE-conjugated isotype-matched control mAbs were purchased from BD Bioscience. PerCP- (Peridinin chlorophyll protein-) conjugated CD138 was also obtained from BD Bioscience.

2.2. Flow Cytometric Analysis. Heparinized peripheral venous blood was obtained from patients with SLE. PB mononuclear cells (PBMCs) were separated immediately by centrifugation over Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). PBMCs were washed twice and resuspended at 1×10^6 cells/mL in staining buffer.

Direct immunofluorescence was carried out with PE- or FITC-conjugated antibodies against surface antigens and stained with FITC- or PE-conjugated anti-RP105, PerCP-conjugated anti-CD138, and APC-conjugated anti-CD19 mAbs. Irrelevant isotype-matched control antibodies were used to determine background fluorescence. These samples were analyzed with the saved setting of gate. More than 500 000 viable, antibody-labeled cells were identified according to their forward and side scattering, electronically gated, and analyzed on a FACScalibur flow cytometer (Becton Dickinson). Results were expressed as percent of positive cells or mean fluorescence intensity (MFI) using WINMDI software (http://facs.scripps.edu/software.html). The percentages of subsets of RP105(−) B cells (RP105(−) CD19(+) subset cells/CD19(+) cells%) were calculated.

2.3. Statistical Analysis. Statistical analysis was performed with the Mann-Whitney U test, the Wilcoxon signed rank test, or Student's t-test using SPSS software (SPSS Japan Inc., Tokyo, Japan); statistical significance was considered with a $P < 0.05$.

3. Results

3.1. RP105-Negative B Cells Consist of 5 Subsets of B Cells. In SLE patients, there is a large population of RP105(−) B cells [12]. Figure 1(a) shows a representative FACS profile of CD19 and RP105 expression on PBMCs from a patient with SLE and a normal subject. As reported in the previous studies [12, 13], a significant population of CD19(+) B cells from SLE patients lacked RP105. In this study, in conjunction with RP105, we used the B cell marker CD19 and plasma cell marker CD138 in an attempt to subdivide RP105(−) B cells further.

We identified 4 populations of B cells in the panel of CD19 and RP105. The populations were CD19(+)RP105(+) B cells (subset 0) and three RP105(−) B cell subpopulations, CD19(+)RP105(int; intermediate) (subset 1), CD19(+) RP105(−) (subset 2), and CD19(low)RP105(−) B cells (presubset). The population of CD19(low)RP105(−) B cells (presubset) was (subset 3), CD19(low)RP105(−)CD138(int) (subset 4), and CD19(low)RP105(−)CD138(++) (subset 5) B cells according to CD138 expression levels after gating presubset (Figure 1(b)). Collectively, we identified at least 5 subsets of RP105(−) or (low) B cells.

The percentages of subsets in PB of each patient and normal subject were shown in Figure 1(c). Among these subsets, the populations of subset 1 (10.4 ± 4.4%) and 3 (7.1 ± 5.8%) B cells were larger than other subsets (subset 2; 5.0 ± 3.5%, subset 4; 3.8 ± 4.2%, subset 5; 1.6 ± 1.7%). All RP105-negative B cell subsets of SLE patients were significantly increased compared with normal subjects.

3.2. Phenotype of RP105-Negative B Cell Subsets in SLE. In order to characterize these subsets, we analyzed various antigens on B cells (Figures 2 and 3). Subset 0, 1, and 2 B cells were positive for CD20, but in subset 3, 4, and 5 B cells, CD20 expression decreased. Although subset 0, 1, and 2 B cells expressed CD21, CD22, and CD24, subset 3, 4, and 5 B cells lost CD22 completely, and expression levels of CD21 and CD24 were lower compared to subset 0, 1, and 2 B cells. CD23 expression was low only in subset 4 and 5 B cells.

On the other hand, CD38 and CD27 expressions were the lowest on subset 0 B cells. The levels of CD38 and CD27 gradually increased from subset 1 to subset 4 and 5 B cells. While subset 0, 1, and 2 B cells expressed surface IgD and IgM, subset 3, 4, and 5 B cells had lower levels of surface Igs. All the subsets of RP105(−) B cells expressed HLA-DR. However, the levels of HLA-DR gradually decreased. Low expression of CD25 was detectable in subset 0, 1, 2, and 3 B cells.

CXCR5 (CD185), a homing receptor, presented in subset 0, 1, and 2 B cells, but it disappeared in the subset 3, 4, and

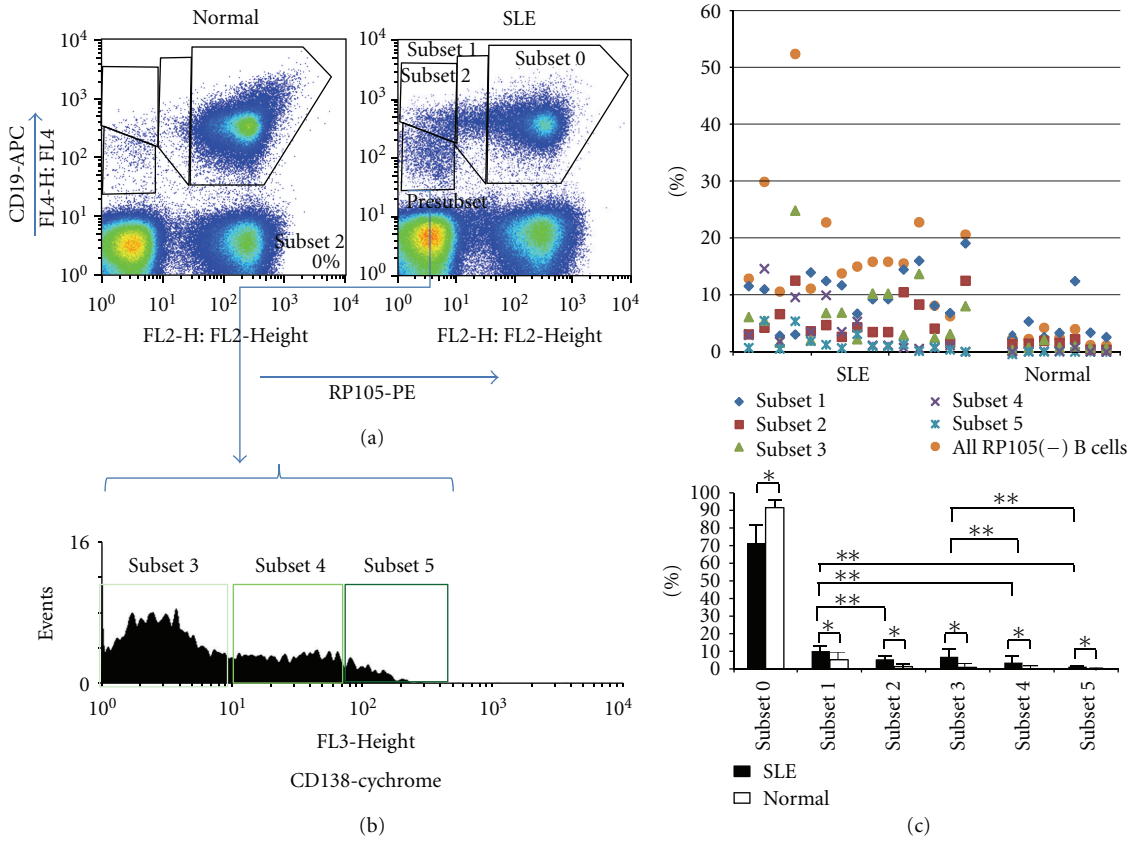


Figure 1: Subsets of RP105(−) B cells. (a) Representative flow cytometric profiles of RP105 expression on CD19(+) B cells from an active SLE patient and a normal subject. The population was subdivide (1) subset 1; CD19(+)RP105(int), (2)subset 2; CD19(+)RP105(−), and (3) presubset; CD19(low)RP105(−). (b) CD138 levels after gating CD19(low)RP105(−) presubset. The presubset cells were further subdivided into 3 subpopulations, subset 3; CD138(−), subset 4; CD138(int) and subset 5; CD138(++). (c) The percentages of subsets in PB of each patient and normal subject. $^{*}P < 0.05$, $^{**}P < 0.05$.

5 B cells. The expression of CXCR4 (CD184) was the highest in subset 0 B cells but was lost in subset 4 and 5 B cells.

More interestingly, of BAFF receptors, BAFF-R expression was higher in subset 0, 1, and 2 B cells, but BCMA expression was conversely higher in subset 3, 4, and 5 B cells.

From subset 0 to subset 2 B cells, CD72, CD79b, and CD200 expressions were found, but those were lower on subset 3, 4, and 5 B cells. CD86, CD95, CD97, and CD126 were positive on subset 3, 4, and 5 cells. CD1a, CD1b, CD10, CD40, CD77, and CD80 were low or negative in all subsets. CD31, CD49d, and CD45RA were constantly positive on all subsets (data not shown).

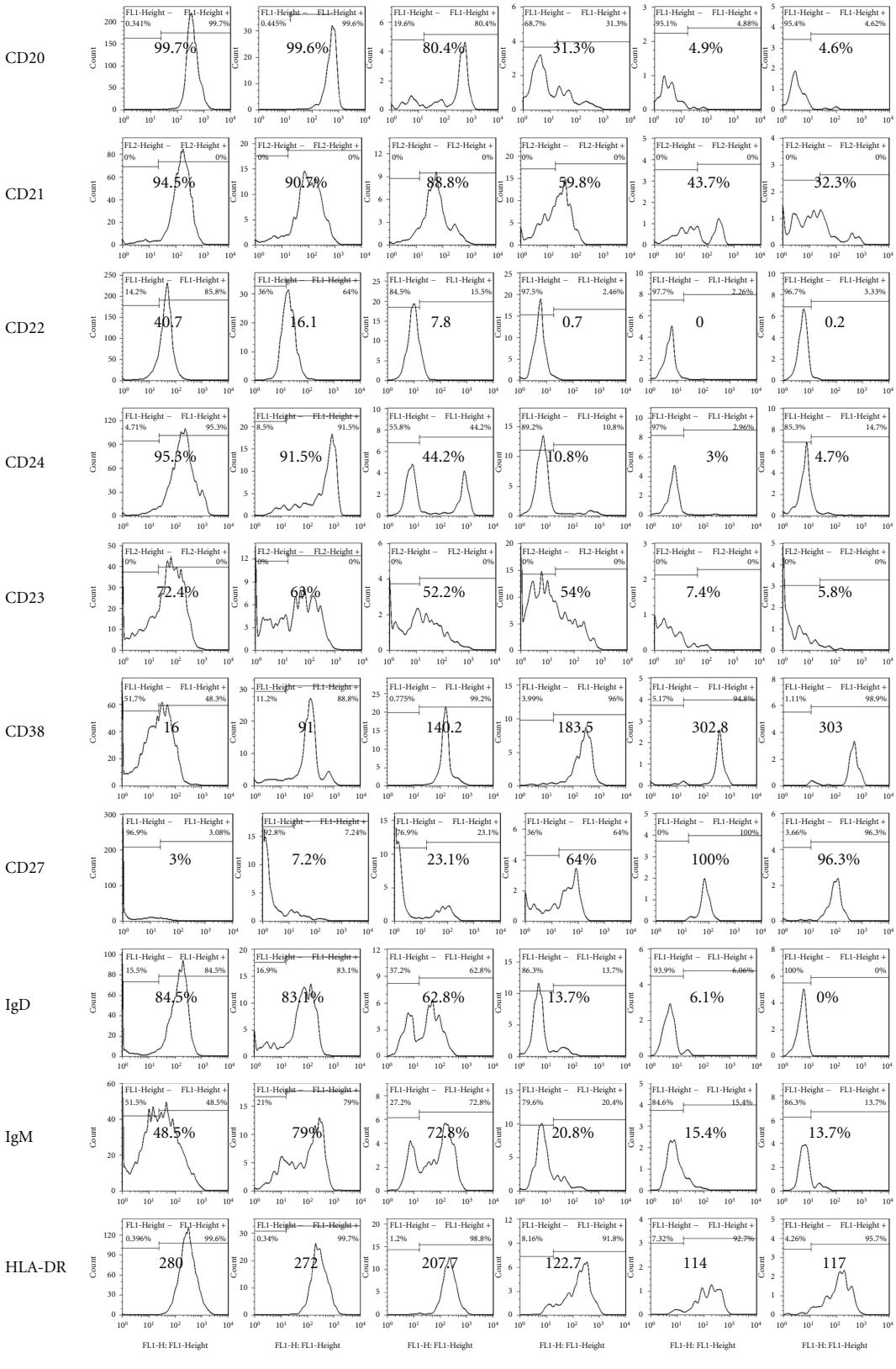
We investigated and summarized the phenotype of RP105(–) B cell subsets in patients with active SLE. RP105(–) B cells consist of at least 5 subsets of late B cells, including CD19(+)RP105(int), CD19(+) RP105(–), CD19(low)RP105(–)CD138(–), CD19(low) RP105(–)CD138 (int), and CD19(low)RP105(–)CD138(++) B cells. The phenotypic analysis of RP105(−) B cells suggests mature phenotype of these RP105(−) B cells, CD20(low or lost) CD22(low or lost) CD27(high) CXCR5(low or lost).

3.3. Phenotype of RP105-Negative B Cell Subsets in Normal Subjects. We analyzed whether our finding of subsets of RP105(−) B cells is valid in healthy subjects. Although circulating RP105(−) B cells are very rare in healthy subjects, subsets of RP105(−) B cells were identified. Therefore, comparison of the identified B cell subsets in healthy subjects with SLE patients could lead to relevant observations.

Virtually, patterns of phenotype of RP105(−) B cells from normal subjects seemed similar to those from SLE patients. However, the expression levels of several antigens were significantly different between SLE patients and normal subjects (Figure 4). In subset 3, 4, and 5, levels of CD38 and HLA-DR of SLE patients were significantly higher than those of normal subjects ($P < 0.05$). On the other hand, in subset 4 and 5, levelsof CD95 were lower in SLE patients compared with normal subjects ($P < 0.05$).

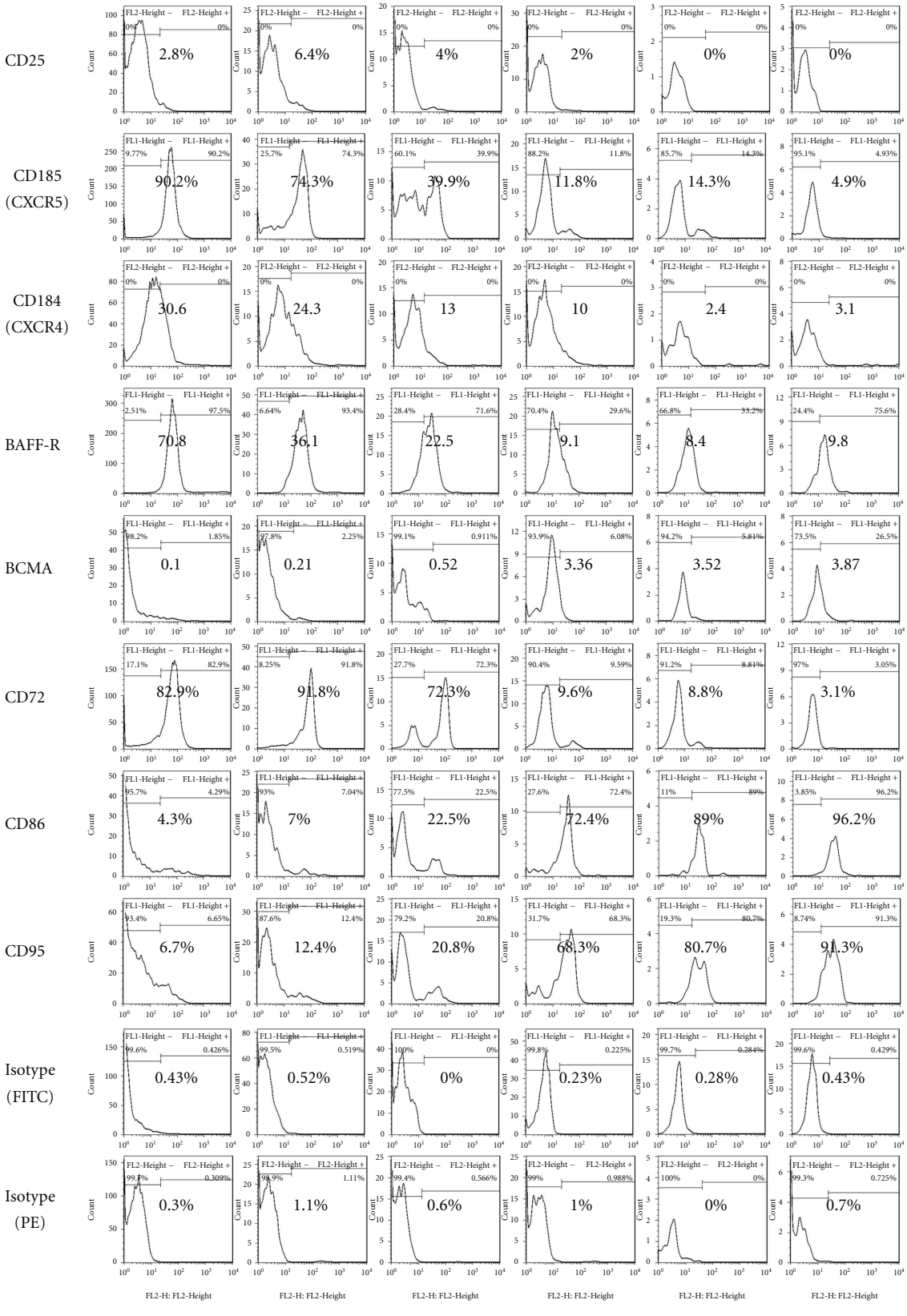
4. Discussion

RP105 (CD180) is mainly expressed on mature B cells and regulates B cell function in humans and mice [6]. Enlarged population of RP105(−) B cells was remarkable in active patients with SLE [12]. In the past studies we suggested that RP105(−) B cells are activated and well-differentiated B cells. However, precise phenotype of RP105(−) B cells has not been elucidated. We described here phenotype of human late



(a)

Figure 2: Continued.



(b)

Figure 2: Flow cytometric analysis of various antigens on and in B cell subsets from a patient with SLE. Positive cell ratio or MFI (mean fluorescence intensity) was shown in flow cytometric profiles.

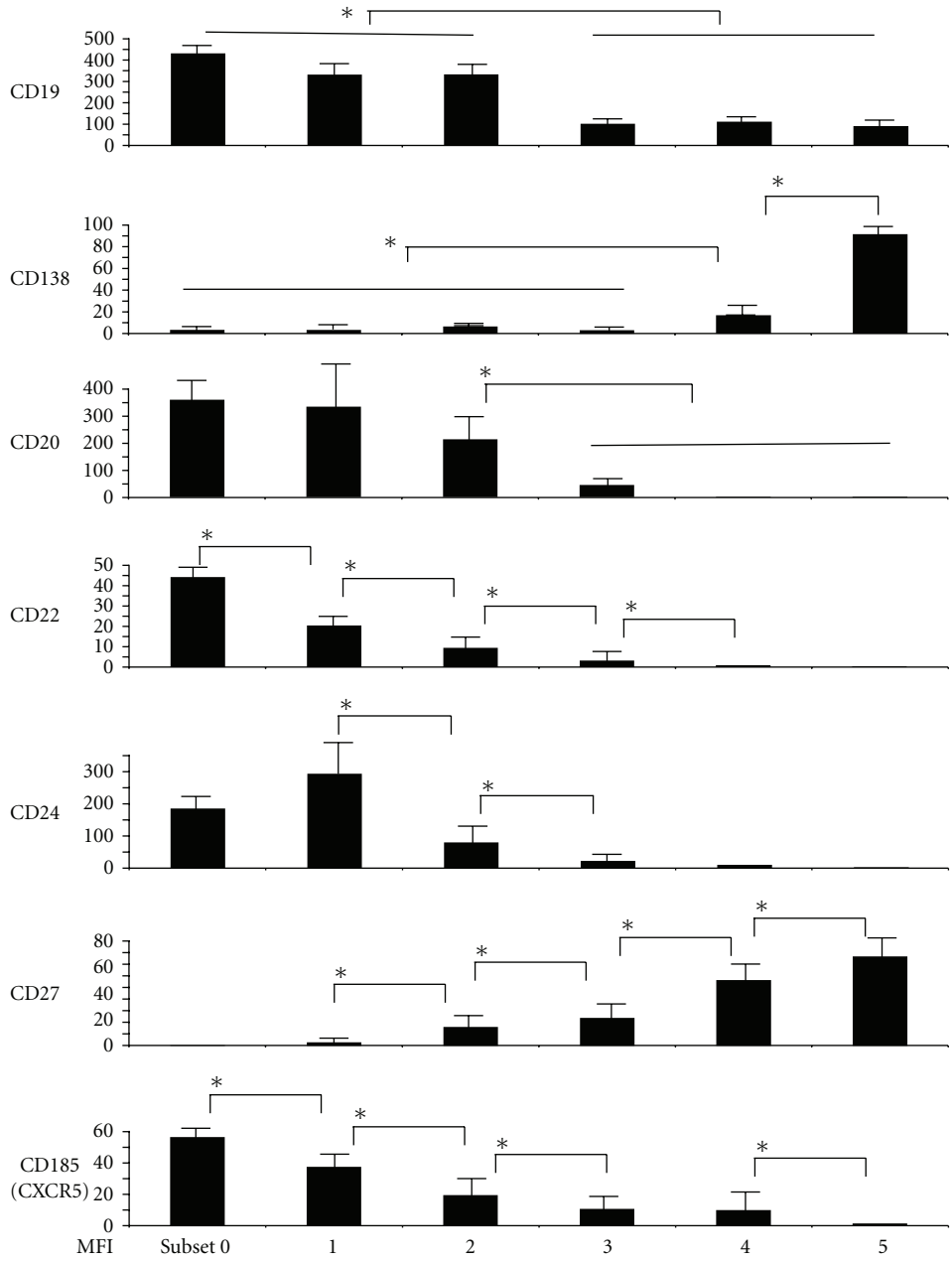


Figure 3: Expressions of important B cell markers (MFI) on RP105(−) B cells (subset 1–5) and RP105(+) B cells (subset 0). The levels of CD19, CD20, and CD24 were significantly lower in subset 3, 4, and 5 B cells compared to subset 0, 1, and 2 B cells ($P < 0.05$). CD27 and CXCR5 expression was significantly different: ($P < 0.05$ subset 0 versus 1, subset 1 versus 2, subset 2 versus 3, subset 3 versus 4, and subset 4 versus 5).

B cells more precisely using markers of CD19, RP105, and CD138.

We identified 5 subpopulations of RP105(−) B cells with different phenotype that are categorized as follows: subset (1) CD19(+)RP105(int), (2) CD19(+)RP105(−), (3) CD19(low) RP105(−)CD138(−), (4) CD19(low)RP105(−)CD138(int), and (5) CD19(low)RP105(−)CD138(++) B cells. Each subset of RP105(−) B cells showed different phenotype of B cells.

IgD and CD38 were classically used to subdivide PB B cells into four quadrants, IgD(+)CD38(−) naïve B cells (Bm1 and Bm2), IgD(+)CD38(+) Pre-GC B cells (Bm2'a and Bm2'b), IgD(−)CD38(+) GC B cells (Bm3 and Bm4), and IgD(−)CD38(−) memory B cells (Bm5) [18]. According to this classification system, subset 3, 4, and 5 of RP105(−) B cells, expressing CD38 and lacking surface IgD, may be tentatively considered to belong to GC B cells. Although GC B cells are CD10(+), CD20(++), and CD27(−), the RP105(−) B cells lost CD10 and CD20, but express CD27. Therefore, several subsets of RP105(−) B cells do not correspond with classically categorized GC type B cells.

CD27 expression is commonly used as an exclusive marker for human memory B cells [18]. Although memory

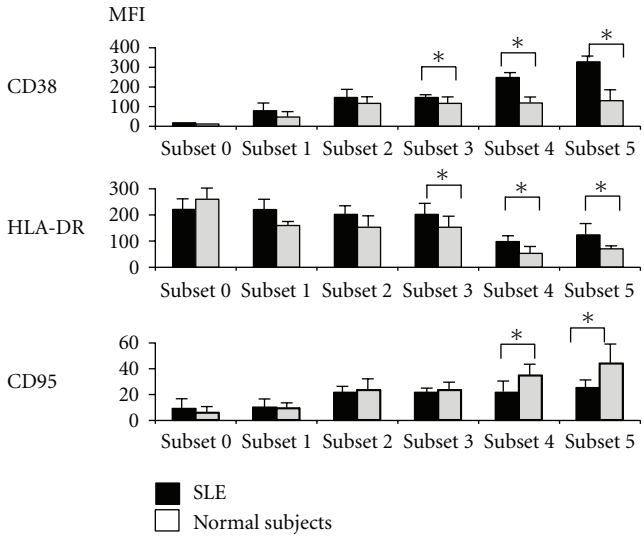


Figure 4: Comparison of surface expression of various antigens with significant difference of mean of MFI on RP105(−) B cell subsets between SLE patients and normal subjects.

B cells express CD27 and lack IgD and CD38, 5 subsets of RP105(−) B cells clearly expressed CD38. Accordingly, RP105(−) B cells are not memory type B cells. Several previous reports have shown that CD27-bright plasma cells increasehighly in PBMC from patients with active SLE and that the frequency is useful in evaluating disease activity with a significant correlation with SLEDAI and autoantibodies [19, 20]. We presented CD27 expression of RP105-negative B cell subsets in Figures 2 and 3. CD27 expression was gradually increased from subset 0 to subset 5. It has been reported that CD27 expression is upregulated during B cells differentiation into plasma cells [21]. Therefore, higher expression of CD27 on B cells is also a marker of differentiation towards plasma cells [21]. Based on CD20, CD27, and CD38 expressions, RP105(−) B cells correspond neither to germinal center B cells nor memory B cells.

It is reported that in vitro human activated B cells and plasmablasts/plasma cells express CD19, whose levels, however, are lower on plasmablasts and plasma cells compared to activated B cells [22]. Actually, in this study, CD19 expression was lower in subset 3, 4, and 5 B cells.

Regarding homing receptors, differentiated plasma cells are characterized by a disappearance of CXCR5, a progressive reduction in CXCR4 [22]. From subset 0 to subset 5 B cells, CXCR5 and CXCR4 levels became decreased. The expression levels of these molecules also suggested that RP105(−) B cell subsets may belong to the cells during the process towards plasma cell formation.

RP105(−) B cells have abundant intracellular Igs [12] but lost surface Igs. Therefore, subset 3, 4, and 5 B cells may be assigned as to be plasmablasts or plasma cells, preparing to secret Igs. However, due to lacking CD138, subset 3 B cells may be assigned as plasmablasts not as plasma cells. On the other hand, subset 4 B cells may be assigned as preplasma cells due to intermediate CD138 expression and subset 5 B cells as PB plasma cells with bright CD138.

Collectively, each subset represents the step of B cell differentiation towards plasma cells. However, to confirm each steps comparison between PB and bone marrow plasma cells is required. Further analysis of in vitro culture of B cells that develop and differentiate into plasma cells with morphological change, functional studies, and expression of transcription factors is also important. In our results, we present the existence of increased various late B cell subsets in SLE patients.

We performed phenotype analysis in healthy subjects. The results were shown in Figures 1 and 4. Although circulating RP105(−) B cells are very rare, we found the subsets of RP105(−) B cells even in healthy subjects. The comparison of the identified B cell subsets in healthy subjects with SLE patients showed that, virtually, phenotype of RP105(−) B cells from normal subjects seemed similar to those from SLE patients. Interestingly, the expression levels of several antigens were different between SLE and normal subjects. However, the importance of the expression levels of antigens for pathophysiology in SLE is not unclear in this study. Therefore, further analysis of the antigens of RP105(−) subsets found in SLE patients with various activity and other systemic rheumatic diseases should be required.

Recently, it has been reported that increased circulating CD138(int) B cells, producing autoantibodies, are related to autoimmunity in MRL/lpr lupus mice [15]. RP105(−) B cells showed similar phenotype with CD138(int) B cells: (1) intermediate expression of CD138, (2) larger cell size and increased granularity [12], (3) cytoplasmic Ig and IgM expression. Thus, CD19(low)RP105(−)CD138(int) B cells may be the human counterparts of CD138(int) B cells in mice. These results suggest possible similar mechanisms in dysregulation of B cells in human and murine autoimmune diseases.

Further studies will be required to determine mechanisms of appearing, differentiation, and proliferation of RP105(−) B cell in human SLE. It is possible that inappropriate enlarged population of various subsets of RP105(−) B cells in PB is greatly related to pathophysiology in SLE.

Conflict of Interests

No conflict of interests has been declared by the authors.

Acknowledgments

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Clinical Study

Systemic Lupus Erythematosus and Systemic Autoimmune Connective Tissue Disorders behind Recurrent Diastolic Heart Failure

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Diastolic heart failure (DHF) remains unexplained in some patients with recurrent admissions after full investigation. A study was directed for screening SLE and systemic autoimmune connective tissue disorders in recurrent unexplained DHF patients admitted at a short-stay and intermediate care unit. It was found that systemic autoimmune conditions explained 11% from all of cases. Therapy also prevented new readmissions. Autoimmunity should be investigated in DHF.

1. Introduction

Diastolic heart failure (DHF) is a clinical syndrome in which patients have symptoms and signs of heart failure (HF), normal left ventricular ejection fraction (LVEF), and evidence of diastolic dysfunction. Recurrent DHF episodes are rare when recognized triggers are corrected and therapy is appropriate. However, it remains unexplained in some patients with recurrent admissions after full investigation. There are no systematic data available upon recurrent DFH, and few reports provide an underlying diagnosis. Interestingly, Schwagten described DFH as a first presentation of mixed connective tissue disease [1].

Inflammation and autoimmunity are currently receiving attention as mechanisms for DHF [2–6]. Impaired myocardial dysfunction [7–11] has been confirmed in Systemic Lupus Erythematosus (SLE). Nevertheless, autoimmunity is not routinely tested in DHF patients. There are not previous reports in the literature which investigate autoimmunity as the underlying cause of DHF.

2. Objectives

The main purpose of the study attempts to identify SLE or systemic connective tissue disorders (SCTD) in recurrent unexplained DHF patients. The secondary point was length of readmission-free time under appropriate therapy.

3. Patients and Methods

3.1. Definition of Case and Study Population. Case was defined as unexplained recurrent DHF whom fulfilled SLE or STCD classification criteria.

3.2. Definition of Unexplained Recurrent DHF. DHF was defined as (1) symptoms and signs of heart failure described in the guidelines published by the American Heart Association [12] and European Society of Cardiology [13]; (2) LVEF over 55% and altered diastolic filling pattern; and (3) absence of hypokinesis or ventricle dilation, on echocardiogram.

Recurrent was considered two or more admissions within last year under appropriate treatment and absence of precipitant factors. Patients had received diuretics and angiotensin converter enzyme inhibitors. Hydric balance, salt intake, blood pressure, atrial fibrillation, or valve involvement were not triggers and must be stable at admission.

Unexplained implied unidentifiable cause after investigation, including routine blood count, complete serum and urine biochemistry, troponin and thyroid hormones, chest X-rays, continuous electrocardiography monitorization and new echocardiography. Infection, coronary ischemia, anemia, hypothyroid or hyperthyroid state were ruled out. Pulmonary embolism was investigated, even in anticoagulated patients, by angioCT scan when D-dimmer was positive or there was suggestive echocardiography.

3.3. Diagnosis of Autoimmune Condition. Common SLE/SCTD symptoms and signs were evaluated. STCD comprised APS, Sjögren Syndrome (SS), Mixed Connective Tissue Disease (MCTD), and Undifferentiated Connective Tissue Disease (UCTD). Physicians were guided by a nonvalidated internal consensus questionnaire, which contained 17 items (see the appendix). Clinical pattern was accepted as probable SLE/SCTD when fulfilled 3 or more items.

Immunological tests were only requested when this clinical pattern was presented and accepted after two repeated positive determinations. Immunology tests included antinuclear antibodies (ANAs), anticardiolipin antibodies (aCL), anti-β2-glycoprotein I antibodies (anti-β(2) GPI), anti-DNA antibodies, thyroglobulin autoantibodies (anti-TGB) and thyroid peroxidase autoantibodies (anti-TPO). Anti-TGB and anti-TPO were decided due to given association between rheumatic heart disease and autoimmune thyroid disease [14].

Recurrent unexplained DFH patients whom fulfilled probable SLE/SCTD with positive immunology were revaluated by a trained physician on systemic autoimmune connective tissue disorders. Moreover, this physician only selected those cases whom fulfilled correct diagnosis and evident SLE/APS/SCTD classification criteria [15, 16]. Alarcon-Segovia [17] and European Consensus Group [18] classification criteria were used for MCTD and SS, respectively. UCTD was restrictively considered, for research purposes, when clinical and immunological features did not fulfilled classification criteria for those conditions but usual clinical practice actually suggested disease and recommended further followup expecting new criteria in the outcome or investigations. Patients with similar profile but insufficient data were not included, although they will be briefly commented.

3.4. Study, Protocol, and Phases. A prospective observational study was performed between July 2007 and November 2009 at a short-stay and intermediate care unit (UARH) of the University Hospital Santander, Spain. The study was divided in Phase I, Phase II, and Phase III (Figure 1), using an identical evaluation protocol.

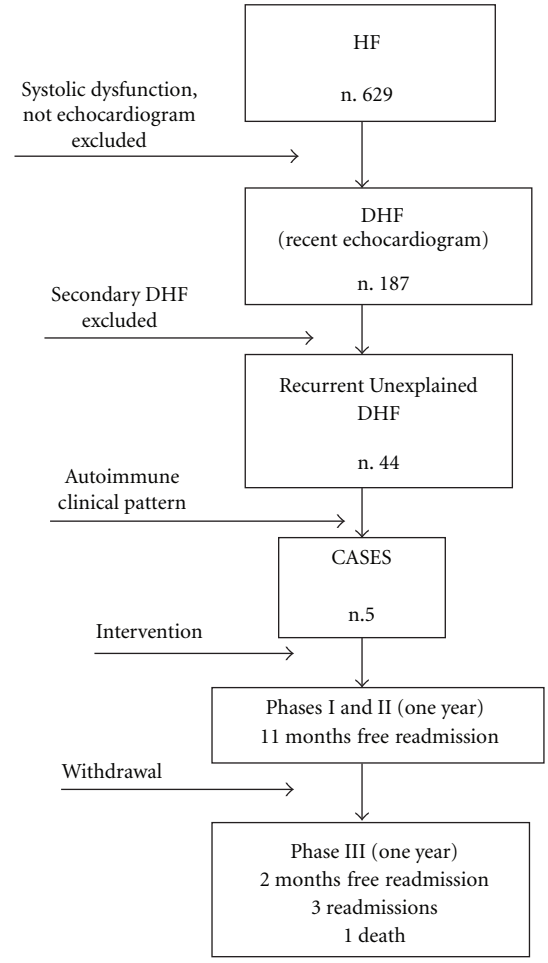

Figure 1: Protocol and phases. HF: Heart failure, DHF: Diastolic heart failure.

Phase I was conducted between July 2007 and September 2008, for main objective. Cases were recruited from consecutive admitted heart failure patients. Systolic dysfunction and absence of recent echocardiography were exclusion criteria. History, clinical findings, laboratory results, and imaging were integrated by the clinicians in charge, which finally made the diagnosis of unexplained recurrent DHF. Second screening was subsequently directed for SLE/SCTD in those DHF patients, who underwent questionnaire and regular followup with immunology tests. Those who fulfill probable SLE/SCTD criteria were revaluated by an expert physician, who finally considered cases when patients with correct diagnosis met classification criteria for SLE/APS/SCTD.

Phase II was conducted until October 2008, for secondary objectives. Cases were treated and outcome was observed. Detailed regimens will be discussed in results. Free-admission time, readmission rate, and therapeutic changes by other specialists were registered.

Phase III was conducted between October 2008 and November 2009. Cases were referred to respective competent departments (Cardiology, Hematology, Rheumatology, and Internal Medicine) for specialized long-term followup. We

Table 1: Cases characteristics ($n = 5$).

Age	74 years (65 y–80 y)
Gender	100% female (5)
Ethnicity	White South European
Allergies or urticaria	80% (4)
Polyarticular rheumatism	100% (5)
Thyroid problems	60% (3)
Miscarriages and obstetric morbidity	20% (1)
Raynaud	40% (2)
Livedo reticularis	60% (3)
Leucopenia	40% (2)
Previous positive autoantibody tests	80% (4)
Antithyroid autoantibodies positive	40% (2)
Autoimmune condition previously diagnosed	0% (0)
Secondary Hypertension	80% (4)
Rheumatic mitral lesion	40% (2)
Atrial fibrillation	100% (5)
Pulmonary thromboembolism	20% (1)
Autoantibodies positive	100% (5)
ANAs positive >1/340	80% (4)
aCL positive	40% (2)
SLE	40% (2)
APS	20% (1)
UCTD	60% (3)

observed outcome and registered therapy and diagnosis changes. Free-readmission and readmission rate time were also listed again.

4. Results

4.1. Phase I. 629 heart failure patients were admitted in Phase I. Only 187 patients had prior echocardiogram and showed nonsystolic heart failure (30%); 442 patients were excluded. From them, 44 patients were classified as recurrent and unexplained DHF (Figure 1).

Finally, 5 patients (11%) fulfilled the definition of case (Table 1).

4.2. Cases Description

4.2.1. Case 1. Case 1 was an 80-year-old woman with past history of penicillin allergy, secondary hypertension, polyarticular rheumatism in adolescence, thyroid surgery in adulthood, and current mild renal failure, with noninvestigated hematuria. She had been diagnosed osteoarthritis because of joint pains, joint effusion on knees, and ANA 1/160, by rheumatology department ten years ago. She had been followed due to rheumatic double mitral lesion by cardiology department for 13 years, but she had not needed interventions and recent LVEF was normal. She referred Raynaud phenomena, chronic asthenia, malar rash and photosensitivity, which have been improved with age; she had livedo reticularis, joints effusions on wrist, knees and ankles. She had shown chronic leucopenia (<4000/mL) on past blood counts. Immunology tests demonstrated myeloperoxidase-anti-neutrophil-cytoplasmic antibodies (MPO-ANCA) > 100, and anti-β(2)GPI 24. She fulfilled diagnosis criteria and 5 classification criteria for SLE, with antiphospholipid antibodies. MPO-ANCA may be observed until 25% of SLE patients, with activity correlation. Renal, heart, and thyroid disease were not invasively assessed searching more criteria.

4.2.2. Case 2. Case 2 was a 67-year-old woman, with past history of metamizol allergy, late onset hypertension, and asthma. She had been diagnosed with amiodarone-induced hyperthyroidism, although she had showed repeated high titers of anti-TPO. Amiodarone was only given for some months and definitively discontinued. She had started being followed due to rheumatic double mitral lesion by cardiology department last year after one episode of heart failure admission. She had undergone anticoagulant therapy. She had rashes, photosensitivity, chronic symmetric distal polyarticular joint pain and swelling, Raynaud phenomena, asthenia, and cognitive impairment. She presented livedo reticularis and joint effusions on physical examination, and erythema was confirmed in followup. Brain CT scan demonstrated multiple lacunar infarcts, although acenocoumarol levels were adequate. Immunology tests showed ANA > 1/1280 but aCL and repeated anti-TPO were negative. Libmann-Sacks endocarditis was not found on repeated echocardiography. She fulfilled diagnosis criteria and 4 classification criteria for SLE.

4.2.3. Case 3. Case 3 was an 80-year-old woman, with past history of metamizol allergy, constrictive pericarditis, and heart failure. She referred facial rashes, significant photosensitivity, Raynaud phenomena, sicca syndrome, relapsing asthenia periods, and swelling and joint pains on wrists, knees, and ankles. Dermatological and joint signs were confirmed on examination. Blood account showed leucopenia. Initial immunology tests demonstrated ANA 1/320 and anti-β(2)GPI 33, which remained repeatedly positive. Echocardiogram showed a 70 mm left auricle and diastolic dysfunction. Case-resembled SLE or SS, but she was classified as UCTD with antiphospholipid autoantibodies, due to absence of sufficient criteria.

4.2.4. Case 4. Case 4 was a 67-year-old woman, with intricate past history. She suffered one miscarriage at second pregnancy trimester and an antenatal death due to preterm fetus. Then, she developed an episode of 9-month-length unexplained fever and joints pain when she was 42 years old. She had suffered two episodes of labeled transient ischemic attack, when she was 51 and 54 years old. However, the second one consisted of new onset headache and sensitivity loss during 3 months. Finally, she was diagnosed with recurrent periorbital rash and hypothyroidism by Allergy Department in last year. She referred recurrent rash flairs, eye dryness, and

Raynaud phenomena. She had residual periorbital erythema, a round bright hyperkeratosic pink plaque on ankle, and livedo reticularis. Immunology tests showed ANA 1/1280 and anti-TPO 507. The only echocardiographic hallmark was a 48 mm left auricle and diastolic dysfunction. Case resembled SLE/ APS/SS, but she was classified as UCTD, in the absence of sufficient criteria.

4.2.5. Case 5. Case 5 was an 80-year-old woman with rheumatic double mitral lesion which required prosthetic replacement when she was 68 years old. However, she underwent new replacement due to unexplained early valve dysfunction two years later. She had been admitted after second surgery in multiple occasions because of heart failure, although repeated echocardiography showed both normal valve and LVEF. She had been also evaluated due to polyarthritis, uveitis, anemia, and ANA 1/320 five years ago, but without definitive conclusions. ANA was repeated and remained positive (1/320). She was classified as UCTD.

4.3. Phase II. All of cases have been treated on angiotensin enzyme converter inhibitors, acenocoumarol, and diuretics, which were continued.

Azathioprine 50 mg pd. was given to Case 1. Initial methylprednisolone bolus (1.5 g), azathioprine 50 mg pd., and hydroxychloroquine 200 mg pd. were given to Case 2. Hydroxychloroquine 200 mg pd. was selected for the other three cases. Diuretics were reduced in all of cases. Case 2 required increasing diuretics after complete discontinuation of treatment by Surgery Department, 6 months later. Moreover, methimazole was discontinued in Case 2 but thyroid hormones continued normally during Phase II. Echocardiograms were not repeated again at Phase II.

There were not new admissions due to DHF within Phases I and II. The admission-free time was, respectively, 11, 26, 11, 5, and 5 months. Case 1 was readmitted because of pulmonary embolism two months after diagnosis, and she was reclassified as SLE and secondary APS.

4.4. Phase III. We refer all of cases to Cardiology, Rheumatology, and Internal Medicine Departments. SLE and UCTD diagnosis were criticized but APS were accepted. Patients did not received an alternative diagnosis, and we understand that they were considered false-positive autoantibody cases. Immunosuppressive agents were discontinued. Cases 1, 2, and 3 readmitted. The admission-free mean time was two months after therapy changes. Cases 1 and 2 needed Intensive Care Unit in the first readmission. Case 1 died due to heart failure and retroperitoneal hemorrhage after a long three-month admission. Case 2 was readmitted again 7 months later.

5. Discussion

Systemic autoimmune connective tissue disorders explained 11% from recurrent unexplained DFH admissions in this study.

DHF is determined by abnormal diastolic filling of left ventricle. It may be precipitated by hemodynamic stress from cardiovascular (e.g., tachycardia, hypertension) or other origin (e.g., infection, anemia). DHF usually is easily managed when triggers are corrected; therefore, recurrences are not expected. Nevertheless, absence of evident cause may be followed by new episodes. We usually observe unexplained recurrent DHF episodes in patients whom accumulate suggestive data of autoimmune background. We believe that chronic uncontrolled systemic inflammation hinders adequate diastolic filling as other studies reported [10, 19]. However, few studies attempt to investigate underlying autoimmunity in heart failure patients.

The case profile was an elderly woman with past history of allergies, rashes, chronic polyarticular involvement, thyroid disease, secondary hypertension, rheumatic mitral lesion, and ANA positive. Surprisingly, no case had presented rheumatic fever features in childhood. SLE and APS cause similar valve involvement [20]. On the other hand, Hashimoto's thyroiditis has been already linked to rheumatic heart disease [14], so we could speculate about possible unrecognized systemic autoimmune diseases. Thus, we believe rheumatic description on echocardiography should be taken cautiously and should be further investigated.

In the same way, we think that elderly age of cases demonstrated delayed diagnosis and evolution of uncontrolled inflammation. Although young patients may display more autoimmune activity and diastolic dysfunction, overt heart failure is unlikely to expect, as in nonautoimmune patients. We did not found DHF patients under 50 years old. Therefore, elder patients should not be excluded without an appropriate history. With respect of this, osteoarthritis or hypertension must be dated in order to decide whether they are primary or consequence of previous inflammation.

Moreover, specific therapy seemed to prevent readmission and discontinuation of medications. Inversely, withdrawal was dramatically associated to readmissions after long symptom- free periods.

Therefore, we could state that autoimmunity, particularly SLE/SCTD, should be always considered within DHF patients.

Study was designed to answer whether autoimmune connective tissue disorders explained recurrent episodes, but not for every episode or other autoimmune diseases. Perhaps, affected males or vasculitis should be investigated following different protocols. We cannot conclude that autoimmune DFH patients fulfill the proposed profile. In fact, we found one patient with bronchiolitis and vasculitis, who did not satisfy our criteria. We did not know if DHF or even any type of heart failure should be always investigated. We noticed *a posteriori* that study excluded 6 similar patients. As well as, 8 excluded patients showed rheumatic heart disease.

Likewise, it does not answer if immunology tests are valid or/and cost-efficient although clinical pattern was absent. Prevalence studies for underlying autoimmune diseases are needed. On other hand, other DHF causes should be investigated.

Finally, the study design did not include controls to compare admission-free mean times. Conclusions on therapy remain intuitive.

We tried to explain the events on Phase III. Phase III intended congruency among physicians and second opinion for patients. Unfortunately, we did not expect the dramatic outcome of Cases 1 and 2. We searched reasons on the classification criteria misuse by inexpert and strict physicians. Patients were informed about possible changes but they complained after discontinuation of a regimen felt as beneficial. Ethical issues and consensus should be improved on new experimental clinical research about autoimmunity.

6. Conclusions

Autoimmunity is an important mechanism of diastolic dysfunction. Autoimmune connective tissue disorders should be considered in patients with recurrent DHF. Specific therapy may improve patients and prevent readmissions. Elderly patients should not be excluded from investigations. Rheumatic heart disease should be reconsidered in suspicion of systemic disease. More studies are warranted for clarifying prevalence of underlying autoimmunity, for systematic exposition of other DHF causes, and for cost-effectiveness of immunological tests.

Conflict of Interests

The authors declared that there is no conflict of interests.

Appendix

Questionnaire

SYMPTOMS

✓	CLINIC DATA
1	**AGE**
	Age 15–45 years-old
	Age >45–55 years-old
2	**VASCULAR RISK FACTORS**
	Not
	Yes
3	**Pill adverse efects**
	Yes
	Not
4	**DVT/PE**
	Yes
	Not
5	**Migrain**
	Yes
	Not
6	**Cognitive impairment**
	Yes
	Not
7 (no.)	**Miscarriages**
	Yes
	Not
8	**Obstetric complications**
	Yes
	Not
9	**Familiar background (SLE, etc.)**
	Yes
	Not
10	**Dermatology** (erythema, photosensitivity, aphtae, alopecia, Raynaud)
	Yes
	Not
11	**Joint involvement**
	Yes
	No

PHYSICAL EXAMINATION

✓	SIGNS
12	**LÍVEDO RETICULARIS**
	Yes
	Not
13	**FACIAL ERYTHEMA**
	Yes
	Not
14	**MOUTH ULCERS**
	Yes
	Not
15	**ALOPECIA**
	Yes
	Not
16	**PERIUNGEAL LESIONS**
	Yes
	Not
17	**ARTHRITIS HANDS/FEET**
	Yes
	Not

REQUEST AUTOANTIBODIES

APS (*)	**APA, ANA**
SLE	ANA, C3,C4,Ch50
Vasculitis	ANA, ANCA p y c
ANA +	anti-DNA, Anti Ro, anti La
APA +	Repeat 6 weeks

(*) APS: when at least 2 items (no. 1–9, 12)

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Review Article

Genetic Risk Factors of Systemic Lupus Erythematosus in the Malaysian Population: A Minireview

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SLE is an autoimmune disease that is not uncommon in Malaysia. In contrast to Malays and Indians, the Chinese seem to be most affected. SLE is characterized by deficiency of body's immune response that leads to production of autoantibodies and failure of immune complex clearance. This minireview attempts to summarize the association of several candidate genes with risk for SLE in the Malaysian population and discuss the genetic heterogeneity that exists locally in Asians and in comparison with SLE in Caucasians. Several groups of researchers have been actively investigating genes that are associated with SLE susceptibility in the Malaysian population by screening possible reported candidate genes across the SLE patients and healthy controls. These candidate genes include *MHC* genes and genes encoding complement components, TNF, FcγR, T-cell receptors, and interleukins. However, most of the polymorphisms investigated in these genes did not show significant associations with susceptibility to SLE in the Malaysian scenario, except for those occurring in *MHC* genes and genes coding for TNF-α, IL-1β, IL-1RN, and IL-6.

1. Introduction

Systemic lupus erythematosus (SLE) is the prototypical autoimmune disease that is characterized by autoantibody production, complement activation, and immune complex deposition leading to diverse clinical manifestations and target tissue damage. The prevalence of SLE is estimated to be between 40 and 400 cases per 100,000 individuals [1]. While the precise etiology of SLE still remains vague, genetic predisposition and environmental and hormonal factors are deemed to play important roles in its pathogenesis. Severity, acquisition risk, and clinical manifestations of this disease can vary by ethnicity, geography, and sex, with a prevalence that is higher in women during their childbearing ages and some non-European populations such as African Americans, Hispanics, and Asians [2, 3].

In Asians, the prevalence of SLE generally falls within 30–50/100,000 individuals. SLE is more frequent among Chinese communities in Asia than it is in India and tropical Africa [4]. Malaysia is a multiracial country. In the peninsular, the Malays (55.1%), Chinese (24.3%), and Indians (7.4%) represent the largest ethnic groups. A prevalence of 43/100,000 individuals in Malaysia has been reported [5, 6]. Likewise, Chinese have the highest prevalence of SLE in Malaysia (57/100,000), followed by Malays (33/100,000) and Indians (14/100,000) [7, 8]. The overall 5-year and 10-year survival rates were reported as 82% and 70%, respectively [5], whereas the overall mortality rate was 20.2% [9]. Renal involvement is highest among the Malaysian patients [5]. However, the major cause of death in Malaysian SLE patients was reported to be from infection [9].

Pathogenesis of SLE is associated with functional deficiency of multiple immunologic components, including the innate immune system, altered immune tolerance mechanisms, hyperactivation of T and B cells, reduced ability of immune complexes and apoptotic cell clearance, and defects in multiple immune regulatory networks [10]. The failure of these mechanisms could be due to the influence of variants within SLE susceptibility genes. To date, many different genes have been found to contribute to disease susceptibility. In a small proportion of patients (<5%), a single gene could become the key player for this disease [11]; however, multiple genes have been implicated in most patients. It is estimated that at least four susceptibility

genes or loci are needed for the development of the disease [12]. The susceptibility genes most extensively studied are within the major histocompatibility complex (MHC). It is believed that human leukocyte antigen (HLA) class II gene variants are very important. The introduction of genome-wide association studies (GWASs) has not only helped us to support the findings from previous candidate gene studies, but also unveiled many other novel genetic loci that may be important. Candidate genes that have been recently discovered can be clustered into three main groups: (i) *IRF5*, *STAT4*, *TNFAIP3,* and *TREX1* which are involved in innate immune response including TLR/interferon signalling pathway; (ii) *HLA-DR*, *PTPN22*, *PDCD1*, *LYN*, *BLK,* and *BANK1* which are involved in immune signal transduction of B, T, and antigen-presenting cells; (iii) *C2*, *C4*, *FCGRs*, *CRP,* and *ITGAM* which are involved in immune complex clearance mechanism [13, 14].

The genetic information obtained from GWAS has allowed many researchers to investigate specific variants for particular genetic loci using a variety of approaches such as RFLP-PCR, tetra-primer ARMS-PCR, and real-time genotyping PCR. This in turn has enabled the replication of these experiments and confirmed those associated polymorphisms with SLE in different populations. Genetic heterogeneity is common among populations in SLE, especially between Caucasians and Asians. For instance, *PTPN22,* which demonstrated significant association with SLE in Caucasians, was not found to be associated with some ethnicities in Asia [15]. The identification of genetic heterogeneity may enhance our understanding of mechanisms that lead to SLE pathogenesis in certain populations and subsequently may permit more precise diagnosis, prognosis, and treatment for the patients.

In Malaysia, researchers have been actively looking into the genetic risk factors of SLE in the multiracial population for the past 15 years. These efforts have generated a considerable amount of data that have been useful contributions to enriching global statistics and knowledge of SLE. PCR-based methods were mainly used in these studies. In this paper, the association of several candidate susceptibility genes with SLE in Malaysian population will be discussed and summarized (Table 1). In addition, genetic heterogeneity in SLE susceptibility observed in different ethnicities will be discussed.

2. Candidate Genes

2.1. Major Histocompatibility Complex Genes. The major histocompatibility complex (MHC), which contains human leukocyte antigen (*HLA*) genes, is a large genomic region located on chromosome 6. HLA antigens and genes have long been associated with SLE, and this can be dated back to 1971, when Grumet et al. [33] reported a possible relationship. Of the several classes of HLA, HLA class II genes seem particularly important in SLE. They encode cell-surface antigen-presenting proteins that present antigens to T cells and in turn stimulate the multiplication of T-helper cells and production of antibodies by B cells. HLA class II genes have also been associated with the presence of certain autoantibodies such as anti-Sm, anti-Ro, anti-La, anti-nRNP, and anti-DNA antibodies, which have been useful biomarkers in SLE diagnosis. HLA-DR2 has been reported to be consistently associated with SLE in both Caucasian and Asian populations [34, 35]. HLA class III genes, particularly those encoding complement components C2 and C4, may also confer increased risk for SLE in different ethnicities

In Malaysia, Azizah et al. [16] reported significant association of HLA-DR2, -DQB1∗0501, and -DQB1∗0601 with SLE in Malays. A significant positive association of DR2 and DQB1∗0501 with renal involvement and DR8 with alopecia in Malays was also described in their study. For the investigation of the role of *HLA* genes in autoantibody expression, they found significant association of DQB1∗0601 with anti-Sm/RNP, DR2 with anti-Ro/La, and DR2, DRB1∗0501 and ∗0601 with anti-dsDNA. The same group of researchers also carried out similar study on Chinese population and suggested that DQB1∗0102, DQB1∗0501, ∗0601, and DPB1∗0901 were significantly associated with SLE [17]. Clinically, a strong association of DR2 and DQA1∗0301 with renal involvement and DQA1∗0102 with alopecia was reported. In contrast to Malays, DQA1∗0102 and DQA1∗0301 were observed to be strongly associated with anti-Ro/La and anti-dsDNA, respectively, in Chinese. Earlier on, Doherty et al. [18] reported that HLA-DRw15 and DQw1 were observed to be significantly associated with SLE among Southern Chinese in Malaysia and most prevalent in patients with lupus nephritis and cutaneous manifestation.

A recent comprehensive study conducted by Mohd-Yusuf and coworkers [19] in Malaysia revealed that HLA A∗1101, 1102, DRB5∗01-02, DQB1∗05, DRB3∗0101, 0201, 0202, 0203, 0301, and DQB1∗0301, 0304 were significantly associated with SLE in Malaysians. In addition, DRB1∗0701 and DRB4∗0101101, 0102, 0103 alleles were significantly increased in the Malay SLE patients, whilst DRB1∗1601-1606 (DR2 subtype) and DRB5∗0101, 0102, 0201, 0202, 0203 alleles were significantly higher in Chinese SLE patients. The investigation revealed that these two different sets of DR alleles may be specific and representative for the two ethnic groups in this SLE cohort and that DQB1∗05 could be the common HLA susceptibility allele in the Malaysian SLE population.

2.2. Complement Components. The complement system is mainly involved in innate immunity, whereby it helps to remove cellular debris from foreign and apoptotic cells. The links between complement system activity with SLE have been reported since the 1980s. Mutant C4 genes have been mostly reported in Caucasian families, but are still uncommon in other populations. Apart from Caucasians, the presence of C4A null allele (C4aQ0) was also observed in Chinese and Japanese with SLE by Dunckley et al. [36]. In the Malaysian scenario, none of the mutations located at exons 13, 20 and 29 of C4 gene, as well as the null alleles, was found to be significantly associated with SLE [20]. The same situation was also observed in Malaysian Southern Chinese by Doherty et al. [18]. However, a synergistic effect

Table 1: Summary of associations between candidate genes and SLE susceptibility in the Malaysian population.

Gene	Ethnicity	Cases	Controls	Allotype/minor allele frequency Cases	Allotype/minor allele frequency Controls	*P* value	RR/OR	Reference
HLA								
DR2	Malays	56	59	48 (85.7%)	36 (61%)	0.03#	3.83	[16]
DQB1∗0501	Malays	56	59	27 (48.2%)	10 (16.9%)	0.0036#	4.56	[16]
	Chinese	70	66	19 (27.1%)	5 (7.6%)	0.003#	4.55	[17]
DQB1∗0601	Malays	56	59	20 (35.7%)	5 (8.5%)	0.0048#	6.00	[16]
	Chinese	70	66	28 (40%)	9 (13.6%)	0.006#	4.22	[17]
DQA1∗0102	Chinese	70	66	61 (43.6%)	44 (33.3%)	0.032#	3.39	[17]
DPB1∗0901	Chinese	70	66	22 (31.4%)	6 (9.1%)	0.02#	4.58	[17]
DRw15	Chinese	87	66	37.9%	10.6%	<0.006#	5.2	[18]
DQw1	Chinese	88	63	75.0%	57.1%	<0.006#	5.2	[18]
A∗1101, 1102	Malaysian	160	107	33.05%	18.69%	0.0002#	2.147	[19]
DRB5∗0101, 0102, 0201, 0202, 0203	Malaysian	160	107	56.52%	41.90%	0.0014#	1.802	[19]
DRB3∗0101, 0201, 0202, 0203, 0301	Malaysian	160	107	42.03%	71.43%	0.000#	0.290	[19]
DQB1∗05	Malaysian	160	107	37.80%	20.61%	0.0000#	2.341	[19]
DQB1∗0301, 0304	Malaysian	160	107	12.80%	32.89%	0.0000#	0.300	[19]
DRB1∗0701	Malays	61	49	14.13%	4.76%	0.0356#	3.291	[19]
DRB4∗0101101, 0102, 0103 (not DR53N)	Malays	61	49	47.83%	19.05%	0.0001#	3.896	[19]
DRB1∗1601-1606	Chinese	99	58	14.46%	3.79%	0.0030#	4.090	[19]
DRB5∗0101, 0102, 0201, 0202, 0203	Chinese	99	58	51.81%	34.92%	0.0040#	2.003	[19]
C4								
2bp insertions (+TC) at codon 1213 in exon 29	Malaysian	130	130	0	0	—	—	[20]
1bp deletions (−C) at codon 811 in exon 20	Malaysian	130	130	0	0	—	—	[20]
1bp deletion (−C) at codon 522 in exon 13	Malaysian	130	130	0	0	—	—	[20]
2bp deletions (−GT) at codon 497 in exon 13	Malaysian	130	130	0	0	—	—	[20]
Null alleles	Malaysian	130	130					
C4A∗Q0				0	2	—	—	
C4B∗Q0				2	0	—	—	
C4A gene with long C4B gene	Chinese	85	63	57.6%	68.3%	—	—	[18]
C4A gene with short C4B gene	Chinese	85	63	67.1%	76.2%	—	—	[18]
C4A or C4B gene deletion	Chinese	85	63	34.1%	27.0%	—	—	[18]
C4X	Chinese	85	63	41.2%	28.6%	—	—	[18]
C1q								
C1qA-Gln186 (C > *T*)	Malaysian	130	130	0	0	—	—	[21]
C1qB-Gly15 (G > *A*)	Malaysian	130	130	0	0	—	—	[21]
C1qB-Arg150 (C > *T*)	Malaysian	130	130	0	0	—	—	[21]
C1qC-Gly6 (G > *A*)	Malaysian	130	130	0	0	—	—	[21]
C1qC-Arg41 (C > *T*)	Malaysian	130	130	0	0	—	—	[21]
C1qA-Gly70 (G/A)	Malaysian	130	130	47	45	0.660	1.081	[21]
C1qC-Pro14 (T/C)	Malaysian	130	130	75	79	0.254	0.789	[21]
TNF								
TNF-α−308 G/A	Chinese	70	59	37	20	0.003#	1.42	[22]
	Malaysian	100	100	42	22	0.0064#	2.1507	[23]

Table 1: Continued.

Gene	Ethnicity	Cases	Controls	Allotype/minor allele frequency		*P* value	RR/OR	Reference
				Cases	Controls			
TNF-β +252 A/G	Malaysian	100	100	117	111	0.5446	1.1303	[23]
FcγR								
FcγRIIA (H131R)	Chinese	175	108	0.40	0.45	0.3200	0.83	[24]
	Malays	50	50	0.34	0.37	0.7676	0.88	[24]
FcγRIIIB (NA1 or NA2)	Chinese	183	100	0.347	0.32	—	—	[7]
	Malays	55	50	0.38	0.38	—	—	[7]
CD28								
IVS3 +17 T/C	Malaysian	100	100	46 (23)	41(20.5)	0.5446	1.1584	[25]
CTLA-4								
Exon 1 (+49 A/G)	Malaysian	130	130	155 (0.60)	151 (0.58)	0.722	0.94	[26]
Promoter site (−1722 T/C)	Malaysian	130	130	90 (0.35)	103 (0.40)	0.238	0.81	[26]
Promoter site (−1661 A/G)	Malaysian	130	130	27 (0.10)	37 (0.14)	0.182	1.43	[26]
Promoter site (−318 C/T)	Malaysian	130	130	19 (0.07)	18 (0.07)	0.865	1.06	[26]
3′-UTR (+6230 A/G)	Malaysian	130	130	34 (0.13)	22 (0.09)	0.117	1.63	[26]
IL								
IL-1β−511 C/T	Malaysian	100	100	96	139	<0.05[#]	0.4051	[27]
IL-1β +3954 E1/E2	Malaysian	100	100	49	77	<0.05[#]	0.5184	[27]
IL-1RN	Malaysian	100	100					[28]
IL-1RN∗1				196 (96%)	180 (90%)	0.019[#]	2.667	
IL-1RN∗2				6(3%)	18 (9%)	0.012[#]	0.313	
IL-4 third intron RPI/RPII	Malaysian	100	100	54 (27%)	55 (27%)	0.9106	0.9751	[8]
IL-6 −174 G/C	Malaysian	100	100	53 (26.5%)	95 (47.5%)	0.0000136[#]	0.3985	[29]
IL-10 −1082 G/A	Malaysian	44	44	8 (9%)	12 (13%)	0.342	—	[30]
IL-10 −824 C/T	Malaysian	44	44	55 (62.5%)	53 (60%)	0.757	—	[30]
IL-10 −597C/A	Malaysian	44	44	55 (62.5%)	53 (60%)	0.757	—	[30]
ACE I/D dimorphism	Malaysian	170	190	117 (34.4%)	138 (36.8%)	0.5938	0.9201	[31]
RANTES-28 C/G	Malaysian	130	130	14	19	0.3684	0.7219	[32]
SDF-1 3′ UTR G801A	Malaysian	130	130	116	132	0.1601	0.7811	[32]

[#]-significant association, RR: relative risk, OR: odds ratio, —not studied.

of C4 deletions and HLA-DRw15 in conferring disease susceptibility was detected.

The other complement component of particular importance in SLE is C1q. Individuals having a congenital genetic deficiency of C1q gene could develop SLE-like symptoms at more than 90% prevalence [37, 38]. Various mutations in C1q have been reported, including nonsense mutations, missense mutations and single nucleotide polymorphisms (SNP). It is conceivable that these may lead to failures in the synthesis of intact C1q molecules leading to abnormal immune responses. While C1q deficiency was reported to be associated with SLE in Turkish and Mexican subjects [39, 40], no association was observed between any of the mutations [at C1qA-Gln186 (C > T), C1qB-Gly15 (G > A), C1qB-Arg150 (C > T), C1qCGly6, (G > A), and C1qC-Arg41 (C > T)], or SNPs [at C1qAGly70 (G/A), and C1qC-Pro14 (T/C)] within C1q and SLE in the Malaysia [21].

2.3. Tumour Necrosis Factor . Tumour necrosis factor (*TNF*) genes are situated at the short arm of chromosome 6. TNF proteins are a group of low-molecular-weight cytokines that mediate inflammation processes. TNF-alpha (TNF-α) protein, also known as cachectin, has been frequently investigated. It plays an important role in the regulation of immune cells, stimulation of apoptotic cell death, and induction of inflammation. Cytokine imbalances are believed to be drivers of certain autoimmune diseases, including SLE. The first biallelic *TNF-α* gene polymorphism was reported by Wilson et al. [41], which involved a single base change from G to A at the position -308 in the promoter region of the gene. A meta-analysis study revealed that the 308-A/G functional promoter polymorphism association was inconsistent. However the risk genotype A/A and risk allele A were associated with SLE in European populations but not in Asian or African populations [42]. The other member of TNF family is TNF-beta (TNF-β), known as lymphotoxin. The biallelic polymorphism in intron 1 of *TNF-β* gene is believed to influence TNF-α production and has been associated with SLE in both Caucasian and Asian populations [43–45].

Risk allele A of TNF-α −308 was associated with SLE in Malaysian cohorts as reported by Azizah et al. [22] and Chua et al. [23], in conjunction with a significant increased frequency of A/G heterozygotes in patients. The TNF-β +252 polymorphism in intron 1 did not feature in SLE susceptibility [23].

2.4. Fc Gamma Receptors. Fc gamma receptors (FcγRs) are present on the surface of most effector cells of the immune system and involved in mediation of phagocytosis, immune complex clearance, antibody-dependent cell-mediated cytotoxicity and stimulation of inflammatory cells [46].

FcγRIIa is the most widely distributed member of FcγR, and *FcγRIIA* gene may occur in two allelic forms that can cause single amino acid residue modification at position 131. FcγRIIa-R131 has a relatively lower affinity for human IgG2 that causes less ability to process and clear immune complexes effectively. Thus it was suggested as a disease susceptibility factor for SLE, as observed in a meta-analysis that involved European, African, and Asian populations [47]. However many studies actually did not show association between polymorphism of FcγRIIA and SLE susceptibility in their populations [48], including Malays and Chinese in Malaysia [24].

FcγRIII is encoded by two distinct but highly homologous genes: *FcγRIIIA* and *FcγRIIIB*. A SNP (T to G substitution) in *FcγRIIIA* that results in a valine (V) substitution for phenylalanine (F) at amino acid residue position 158 has been correlated with SLE in Asians [35]. As for FcγRIIIB, the polymorphism may occur as neutrophil antigen 1 (NA1) or 2 (NA2). Study by Yap and coworkers [7] showed no association between FcγRIIIB-NA polymorphism and SLE in Malay and Chinese patients in Malaysia. This was in agreement with other reports in Caucasian SLE patients. They were also able to detect a Chinese SLE patient with NA-null, which is a consequence of a FcγRIIIB gene deficiency or deletion.

2.5. T-Cell Receptors. CD28 and CTLA-4 are receptors on T-cell surfaces that have opposite effects on T cells. CD28 is a costimulatory molecule which is responsible for T-cell proliferation, cytokine production, and the prevention of T-cell anergy [49], whereas CTLA-4 maintains the immune response at physiological level by regulating the activity of CD28 and T-cell activation.

Few studies have been carried out to investigate the association of *CD28* gene polymorphism with SLE susceptibility. A study performed on the Malaysian population demonstrated no association between CD28 IVS3 +17T/C SNP and SLE susceptibility, although the frequency of T allele and its corresponding homozygous was the highest among the population [25]. In contrast to CD28, there have been more reports of the association of CTLA-4 polymorphisms with SLE, both in Caucasians and Asians. CTLA-4 promoter (−1722 T/C) polymorphism and (+49 A/G) polymorphism from exon-1 were found to have their TC and GG genotypes, respectively, being significantly associated with SLE in Asian populations [35, 50]. However, polymorphisms in *CTLA-4* gene (+49A/G at exon 1, −1722T/C, −1661A/G and −318C/T at promoter sites and +6230A/G in 3′-untranslated region) were not reported to be important in Malaysian SLE patients [26].

2.6. Interleukins. Interleukins are a group of cytokines, the majority of which are secreted by helper T cells, monocytes, macrophages, dendritic cells, natural killer cells, and B cells. They are mainly involved in promoting the development and differentiation of T and B cells and activation of natural killer cells.

Interleukin-1 (IL-1) is a polypeptide encompassing IL-1 alpha (IL-1α) and IL-1 beta (IL-1β). *IL-1* gene is located on chromosome 2, and genes encoding IL-1α and IL-1β are in close proximity to each other. The defective production of IL-1 has been implicated in development of SLE since 1983 [51]. However, not many studies have been conducted to investigate the association between *IL-1* gene polymorphisms and susceptibility to SLE. According to Chua et al. [27], SLE

patients in Malaysia are susceptible to IL-1β −511 C/T polymorphism, with the C allele and its corresponding homozygous exhibiting a higher risk to SLE. These findings differed from a report by Parks et al. [52] that showed T allele had more potential to confer risk of SLE in African Americans. In Taiwan, no association between IL-1β −511 C/T polymorphism and SLE was observed [53]. In a similar study carried out by Chua and coworkers [27], a significant correlation of another IL-1β polymorphism (+3954 E1/E2 in exon 5) with SLE susceptibility in Malaysian population was noted, with E1 allele rather than the E2 at higher frequency among patients. This was also the case in Columbian SLE patients but not in the Taiwanese [53, 54].

The secretion and activity of IL-1 are tightly counterbalanced by IL-receptor antagonist (IL-1ra), which competitively binds to the same receptor as IL-1. *IL-1RN* gene, which encodes IL-1ra, is also situated on chromosome 2. The dysregulation of IL-1 production by IL-1ra will cause abnormal inflammatory activity that leads to subsequent tissue damage, which is the characteristic pathogenesis of SLE. In as much as IL-1ra may contribute to the occurrence of SLE, many studies have been done to investigate the association of polymorphisms in *IL-1RN* gene with SLE susceptibility. Polymorphism in IL-1ra is always characterised by variable numbers of an 86-bp tandem repeat in the intron 2 that may functionally affect three potential protein binding sites: an α-interferon silencer A, a β-interferon silencer B, and an acute-phase response element [55]. The first study to correlate this polymorphism with SLE susceptibility was done on Caucasians in 1994, and carriage of IL-1RN∗2 was reported to be associated with severity rather than susceptibility to SLE [56]. In Malaysia, however, the risk allele associated with SLE susceptibility in SLE patients was IL-1RN∗1 instead. The IL-1RN∗2 allele displayed an inverse association [28].

Interleukin-4 (IL-4) is secreted by T-helper type-2 cells and responsible for proliferation and differentiation of B and T cells, as well as production of antibodies. *IL-4* gene is located on human chromosome 5, and the study of the impact of its polymorphisms on SLE susceptibility is not as popular as other candidate genes. An IL4 haplotype −590C/−33C/9241G/14965C was significantly associated with SLE in Taiwan Chinese population [57]. Another study in Taiwan revealed the association of IL-4 −590T/C and intron 3 VNTR (variable number of tandem repeats) poly-morphisms with the presence of certain clinical manifestations in SLE patients [58]. In the Malaysian cohort that was studied, the VNTR variants within intron 3 of *IL-4* gene were not associated with SLE susceptibility [8].

Interleukin-6 (*IL-6*) gene, located on chromosome 7, is another interleukin gene of interest that has been studied and associated with the susceptibility of SLE. IL-6 promoter polymorphism (−174 G/C) is commonly investigated as a risk factor in SLE. While a study on Malaysian population found a significant correlation between homozygous G genotype and SLE susceptibility, none of the studies in Taiwan, Iran, and Portugal reported the association of this polymorphism with SLE in their populations [29, 59–61].

Interleukin-10 is also believed to play an important role in the pathogenesis of SLE. Various polymorphisms in IL-10 promoter region have been reported to display significant association with SLE susceptibility [62–64]. An earlier study reported that the IL10.G microsatellite alleles in IL-10 promoter region had significantly higher frequency in Caucasian SLE patients [64]. A study investigating the relationship of three SNPs in *IL-10* gene promoter (−1082G > A, −824C > T, and −597C > A) with SLE susceptibility in Malaysian population revealed that haplotype frequencies rather than genotypes or alleles were more important [30].

2.7. Other Genes. The role of angiogenic-converting enzyme (*ACE*) gene I/D dimorphism in susceptibility to SLE in the Malaysian population was illustrated by Lian and coworkers [31]. *ACE* gene, located on the q arm of chromosome 17, produces protein which is an important player in the renin-angiotensin system and kallikrein-kininogen system [65]. Dysregulation of ACE could lead to vascular damage, particularly in kidneys of SLE patients. In that study, no significant difference was observed in the distribution of I and D alleles between cases and healthy controls although ID heterozygote did show significant association with SLE [31]. This finding was in accordance with what was reported in African-American and European-American populations [66] but contradicted those in Japanese and Slovakian populations, whereby I and D alleles were found, to be significantly associated with SLE, respectively [66, 67].

Polymorphisms at position 28 of the regulated on activation, normal T cell expressed and secreted (*RANTES*) gene promoter region and position 801 in 3′ UTR of stromal cell-derived factor 1 (*SDF-1*) gene were also analysed by Lian et al. [32]. Again, both polymorphisms did not show significant association with SLE in Malaysia and similar observations were also reported in Mexican and Han Chinese populations [68–70].

3. Conclusion

Most studies conducted in Malaysian SLE patients did not exhibit significant association of the candidate genes with susceptibility, safe for a few which are within the human MHC. There are several pertinent reasons for these findings. Firstly, this could be due to smaller sample sizes as it is often difficult to obtain large numbers of SLE patients within a medical centre or hospital within a relatively short period of time when such studies are undertaken. So far, there has yet to be a long term or longitudinal national study on this enigmatic disease. The second reason may be that given the complexity of SLE and the dynamic nature of the disease, there may well be different sets of genes and biological players that assume various roles during the precipitation and pathogenesis of SLE from predisposition to actual onset and resultant progression. The genetic heterogeneity evident in different SLE patients of various ethnicities could also be attributed to the inheritance of different ancestral genotypes that impact upon the development and/or progression of this disease [13]. Gene-gene and gene-environment interactions could also confer differences in susceptibility to or be protective against a particular disease in different

populations or ethnic groups. It is hoped that with larger and better defined patient sets and appropriate controls, more comprehensive genetics and systems biology approaches, and better technologies, we will be able to gain a better understanding of SLE and insight into ways of managing this most enigmatic and challenging of autoimmune diseases.

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Review Article

RP105-Negative B Cells in Systemic Lupus Erythematosus

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Systemic lupus erythematosus (SLE) is a multisystem disease characterized by B cells producing autoantibodies against nuclear proteins and DNA, especially anti-double-strand DNA (dsDNA) antibodies. RP105 (CD180), the toll-like receptor- (TLR-) associated molecule, is expressed on normal B cells. However, RP105-negative B cells increase in peripheral blood from patients with active SLE. RP105 may regulate B-cell activation, and RP105-negative B cells produce autoantibodies and take part in pathophysiology of SLE. It is possible that targeting RP105-negative B cells is one of the treatments of SLE. In this paper, we discuss the RP105 biology and clinical significance in SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a prototypical multisystem disease characterized by dysfunction of T cells and polyclonal B-cell activation [1–4]. Although the pathogenesis of the autoimmune mechanisms in SLE is not fully understood, B cells producing autoantibodies against nuclear proteins and DNA, especially anti-double-strand DNA (dsDNA) antibodies, may be responsible for tissue damages as effector cells [5].

To date, the effective methods of target therapy for autoantibody-producing B cells for itself have not been established yet. RP105 (CD180), the toll-like receptor- (TLR-) associated molecule, is expressed on normal B cells [6]. We reported that RP105-negative B cells, that lacked RP105 expression on the surface, increase in peripheral blood from patients with active SLE [7]. It was proved that RP105-negative B cells produce autoantibodies, including anti-ds-DNA antibodies, in vitro [8].

These results suggest that RP105 may function as a negative regulator of B-cell activation. Recently, it was proved that RP105 is a negative regulator for TLR-4 in immune reactions in dendritic cells (DCs) [9, 10]. RP105 also regulates TLR-8 and TLR-9 responses and functions to limit autoreactive B-cell activation [11]. Moreover, it is possible that in the condition of high levels of BAFF (B cell-activating factor belonging to the TNF family) and APRIL (a proliferation-inducing ligand), RP105-negative B cells maintain autoreactivity in autoimmune diseases through BCMA (B-cell maturation antigen). Therefore, RP105-negative B cells are one of the target cells for treatment of SLE. In this paper, we discuss the RP105 biology and clinical significance in SLE.

2. RP105 (CD180) Biology

A type I transmembrane receptor, RP105 (radio protective MW105), was originally discovered as a surface marker of B cells in mice and humans [6, 12]. However, the molecules also express on monocytes [7], macrophages, and dendritic cells. RP105 has leucine-rich repeat (LRR) motifs, similar to the other TLRs with extracellular LRRs. The LRR is a motif involved in protein-protein interaction [13]. The molecules having LRRs take part in the recognition of exogenous pathogens and subsequent activation of the immune system in diverse species [14, 15]. The TLR was first identified in Drosophila [16] and has a function of triggering innate defenses against bacterial and fungal pathogens. In humans, LRR molecules are also important in the defense against pathogens.

The fact of structural similarity of the extracellular domain of RP105 to TLRs suggests that RP105 senses pathogen invasion [17]. RP105 ligation by anti-RP105

antibodies transmits an activation signal leading to B-cell proliferation, resistance against radio-induced apoptosis, and costimulatory molecule expression of CD86, in mice [6]. However, RP105 lacks a conserved intracellular signaling domain (Toll-IL-1 receptor domain; TIR domain) and has a very short cytoplasmic tail, 11-amino-acids [18]. RP105 forms a complex with soluble protein MD-1, making heterodimer complex on surface of B cells [6, 17, 19]. MD-1 is essential for expression of RP105 on cell surface, similar to MD-2 for TLR-4. Although signaling molecules binding to tail of RP105 have not been identified yet, RP105 ligation triggers Lyn, Bruton tyrosine kinase (BTK), mitogen-activated protein kinase (MAPK), and NF-kB activation. The results of RP105- and MD-1-deficient mice showed that activation of B cells, including antibody production, CD86 expression, and proliferative response to LPS, was reduced [19].

On the other hand, it have been recently reported that RP105 has a negative regulatory function for TLR-4/MD-2 signal [9, 10]. We also investigated the role of RP105 in the development of collagen-induced arthritis (CIA). RP105-deficient mice accelerated the onset of arthritis and increased severity. The results indicate that RP105 regulates the antigen-presenting cell function and Treg development [10]. Interestingly, B cells activated with anti-RP105 antibodies show growth arrest and apoptosis upon antigen receptor signaling [20].

Collectively, in mice, RP105 may be considered to regulate the growth and death of B cells. However, in human, it has not been clarified yet which function is predominant. The information of RP105 obtained mainly from mice; there had been little information in human. Therefore, we started the study of RP105 in human SLE, in which autoantibody-producing and activated B cells play pivotal roles.

3. RP105 in SLE

SLE is a typical multisystem disease of unknown etiology [21] characterized by dysfunction of T cells and B cells. Together with the production of autoantibodies against nuclear proteins and DNA, polyclonal B-cell activation is one of outstanding features of SLE [21]. Therefore, we had estimated that B cells producing autoantibodies, which avoid apoptosis and maintain their activated state in peripheral blood, may have abnormal expression of RP105.

In contrast to murine RP105, little was known about the molecule in humans. The human homologue of RP105 has been identified and its mAb also established in 1998 [12, 22]. Therefore, it is important to clarify the role of RP105 on B cells in human SLE. Staining of PBMCs from SLE patients and normal subjects was performed with anti-human CD19 and RP105 antibodies, and analyzed using FACScan. It has been shown that RP105 is expressed on virtually all mature B cells from normal subjects. Interestingly, however, we found that the number of RP105-negative B cells was dramatically increased in SLE patients [7].

It has been also suggested that the disease activity of SLE, SLE-DAI scores, was related to the number of RP105-negative B cells. Serial changes of RP105-negative B cells from active SLE patients were analyzed individually after treatment. The percentages of RP105-negative B cells decreased as the disease turned inactive. To know B cell function, serum levels of IgG were measured. The levels were correlated with the percentage of RP105-negative B cells. These results suggest that the emergence of RP105-negative B cells in the peripheral blood closely relates to the disease activity and B-cell function of SLE.

RP105-negative B cells disappeared in the peripheral blood from inactive patients treated with corticosteroids [2]. It is estimated that RP105-negative B cells seem to be more sensitive to corticosteroids than RP105-positive B cells [2]. The effects of dexamethasone on the apoptosis of RP105-negative B cells in vitro were examined. RP105-negative B cells underwent spontaneous apoptosis, compared to RP105-positive B cells. Dexamethasone induced apoptosis of RP105-negative B cells, although in contrast, apoptosis of RP105-positive B cells were not induced. This result may illustrate in vivo phenomena, clearance of RP105-negative B cells, in patients after treatment with corticoids [2].

In patients with SLE, ANA in serum is generally positive. Therefore, ANA-negative SLE is very rare [23]. However, ANA-negative SLE seems to be a subpopulation of SLE, and the diagnosis may be difficult in patient without immunological disorders [24–31]. We present that RP105-negative B cells were increased in the peripheral blood of two patients with ANA-negative SLE [32]. The numbers of RP105-negative B cells were associated with disease activity even in ANA-negative SLE patients [32]. Without significant serological markers for SLE, examination of RP105 on B cells may be useful in evaluation of activity. Later, the patients turned out to be serologically positive, including ANA, dsDNA, and anti-Sm antibody.

4. Pathophysiology of RP105-Negative B Cells in Autoimmune Diseases

The most important issue was to clarify the functional roles of RP105-negative B cells in SLE. RP105-negative B cells and -positive B cells from patients with SLE were separated using a cell-sorter and analyzed production of IgG and IgM antibodies in vitro. Spontaneous IgG and IgM antibodies from RP105-negative B cells in vitro were enhanced by a mitogen, staphylococcus aureus Cowan I (SAC), or IL-6. Surprisingly, RP105-negative B cells but not RP105-positive B cells produced IgM and IgG class anti-dsDNA antibodies in the culture with activated T cells by anti-CD3 antibodies and IL-10 [8]. These results suggest at least partially that the population of RP105-negative B cells may include pathogenic autoreactive B cells subset(s).

It has been shown that B cells from RP105-deficient mice were hyporesponsive to TLR-4 and TLR-2 stimulation [33, 34], Divanovic et al. showed that RP105 directly interacts with TLR-4 and negatively regulates TLR-4 signaling by experiments using transfectant cells and RP105-deficient mice-derived DCs [9]. These results are suggestive to consider the function of RP105 in human SLE. It is possible

that loss of RP105 induced the dysregulation of TLRs and maintain autoreactive response of B-cell hyperactivation and production of auto-antibodies and result in autoimmunity, ultimately.

5. Characterization and Phenotypes of RP105-Negative B Cells

To characterize RP105-negative B cells in SLE patients, a multicolor analysis was performed using a flow cytometry [7]. RP105-negative B cells expressed higher levels of CD95 and CD86 but not CD80. RP105-positive B cells showed no expression of these molecules. Although RP105-positive B cells expressed low levels of CD38, RP105-negative B cells expressed higher levels of CD38. Surface IgD and IgM levels were low on RP105-negative B cells; however, RP105-negative B cells lacked intracellular IgM but about a half of the RP105-negative B cells had intracellular IgG. RP105-negative B cells can produce class-switched Ig (IgG) although it is still stored in the cytoplasm of B cells. RP105-negative B cells were distinct from CD5-positive cells, since almost all RP105-negative B cells were negative for CD5. Moreover, RP105-negative B cells are CD20-negative and CD28-positive. Therefore, RP105-negative B cells may belong to late B cells. Also CD138 expression was dull (submitted). The phenotype of RP105-negative B cells was summarized as CD95-positive, CD86-positive, CD38-bright, IgD-negative, IgM-dull, intracellular Ig-positive, large sized B cells, which is consistent with the phenotype of activated and effector B cells differentiated into plasma cells. However, so far, this phenotype of B cells had not been reported. RP105-negative B cells may consist of subsets between early plasmablasts and plasma cells. However, another possibility that RP105-negative B cells emerge only in autoimmunological pathogenic state could be proposed.

6. RP105-Negative B Cells in Various Rheumatic Diseases

The expression of RP105 on peripheral blood B cells from patients with various types of rheumatic diseases was examined by a flow cytometer [35]. The percentages of RP105-negative B cells varied among the diseases. The numbers of RP105-negative B cells in the peripheral blood of patients with Sjogren's syndrome (SS) and dermatomyositis (DM) were increased. In other rheumatic diseases, including rheumatoid arthritis (RA), systemic sclerosis (SSc), angiitis syndrome, Behçet's disease, mixed connective tissue disease (MCTD), and polymyositis (PM), the numbers of RP105-negative B cells were slightly increased compared with normal subjects but the levels were low.

Although, clinically, DM and PM (except for the presence of skin manifestations) are similar, two diseases are etiologically different [36–38]. The involvement of humoral immune mechanisms in DM and cellular immunity in PM are proposed. The levels of RP105-negative B cells in the peripheral blood of active patients with DM and PM were analyzed by flow cytometry [39]. The percentage of RP105-negative B cells in PM was low, and increased RP105-negative B cells were found in DM. Interestingly, bronchoalveolar lavage fluid from a DM patient contained a large number of RP105-negative B cells. The increase of RP105-negative B cells is a marker of DM, and B cell activation in DM but in PM seems to be pathophysiologically different.

Because it was well established that B cells from SS patients are in a polyclonally activated state [40], it is conceivable that a large proportion of the RP105-negative B cells existed in SS patients [41]. RP105-negative B cells from SS patients produced IgG and IgM spontaneously in vitro. The production of Ig was enhanced by SAC or IL-6. Salivary glands from some SS patients were found to have lymphoid follicles whose germinal centers consisted of RP105-negative B cells. A larger proportion of B cells infiltrating the area other than lymphoid follicles was also negative for RP105. RP105-negative B cells may be associated with the inflammation and tissue damage of the salivary glands.

We have demonstrated that the RP105-negative B cells were significantly increased in SLE, SS, and DM in which B cell activation is postulated to be involved. Also, the RP105-negative B cells were located in the impaired organs, such as lung or salivary gland and related to autoimmunological inflammation. These results suggest that RP105-negative B cells may be one of the targets of treatment with refractory autoimmune diseases with organ involvements.

7. Future Biologic Agents in the Treatment of SLE, Targeting RP105-Negative B Cells

For treatment of SLE, one of the targeting B cell therapies is rituximab (anti-CD20 antibodies) [42], which has shown effective response in SLE and RA patients. If possible, the most promising therapy of SLE may be a strategy of targeting autoantibody-producing B cells. Because RP105-negative B cells produce anti-dsDNA antibodies, the therapy of targeting RP105-negative B cells may be one of the possible therapies for SLE. For this purpose, we are studying the identification of the antigens specific for RP105-negative B cells using DNA microarrays [43]. Differential gene expression between RP105-negative and RP105-positive B cells was analyzed and surface expression of antigens specific for RP105-negative B cells was confirmed by a flow cytometry. Although several antigens were identified, BCMA was one of the most interesting antigens. RP105-negative B cells from SLE patients showed more preferential expression of BCMA compared with BAFF-R than normal subjects and were possibly regulated by BAFF/APRIL. These results also suggest that BCMA may be one of the target for elimination of RP105-negative B cells to treat SLE. The data of BCMA expression suggest that RP105-negative B cells from active SLE patients may have predisposition toward survival response in high levels of BAFF and/or APRIL in vivo via BCMA.

Because the nonspecific immunosuppressive therapies induce significant adverse effects and disability in autoimmune diseases, novel safer and more effective therapies

specific for pathogenic cells or molecules are required. Our results suggest that blocking agents to the signals between BAFF/APRIL and their receptors, especially the passage via BCMA may be effective. Prevention of emerging or depletion of RP105-negative B cells may be an additional option for the treatment of SLE patients. Further studies will be required to determine whether targeting RP105-negative B cells could inhibit autoantibody production and regulate autoimmune events. Our results in human SLE would provide a new insight of potential mechanism and intervention of B cells in autoimmune diseases.

8. Conclusions

RP105 may regulate B-cell activation, and RP105-negative B cells produce autoantibodies and take part in pathophysiology of SLE. Autoantibody-producing RP105-negative B cells are considered as one of the therapeutic targets in SLE. Moreover, the targeting RP105-negaive B cells would be a specific treatment for pathogenic cells, and the efficacy and safety should be confirmed in further studies.

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Review Article

Are Toll-Like Receptors and Decoy Receptors Involved in the Immunopathogenesis of Systemic Lupus Erythematosus and Lupus-Like Syndromes?

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In this paper we focus our attention on the role of two families of receptors, Toll-like receptors (TLR) and decoy receptors (DcR) involved in the generation of systemic lupus erythematosus (SLE) and lupus-like syndromes in human and mouse models. To date, these molecules were described in several autoimmune disorders such as rheumatoid arthritis, antiphospholipids syndrome, bowel inflammation, and SLE. Here, we summarize the findings of recent investigations on TLR and DcR and their role in the immunopathogenesis of the SLE.

1. Introduction

Systemic lupus erythematosus (SLE) and lupus-like syndromes are multiorgan autoimmune diseases whose pathogenesis is multifactorial. Genetic and environmental factors, together with abnormalities of both the innate and the adaptive immune system, are involved. Although faulty activation of autoreactive B lymphocytes and autoantibodies is the hallmark of SLE, other cell subsets are involved in the pathogenesis of the disease. In this context dendritic cells (DC) or other antigen presenting cells (APC) as well as B and T lymphocytes appear to be involved through the activation of autoantibodies, the complement pathway, as well as cytokines and chemokines which in turn activate different effector mechanisms. Recently studies demonstrated an important role of molecular mechanisms of innate immunity in the cascade of events that contribute to the disease [1]. In this paper we focus our attention on the role of two families of receptors such as TLR and DcR involved in the generation of SLE and lupus-like syndromes in human and mouse models.

2. Relevance of TLR in SLE and Lupus-Like Syndromes

Toll-like receptors (TLR) are pattern recognition receptors capable of recognizing specific pathogen-associated molecular patterns (PAMPs) conserved among microorganisms as a part of innate immune system. There are currently twelve known TLR in mammals, which identify common constituents of invading pathogens including double-stranded and single-stranded RNA, unmethylated CpG DNA, bacterial lipopolysaccharide (LPS), lipoproteins, and flagellin [2]. TLRs' binding with several exogenous and/or endogenous ligands activates numerous transcription factors including activating protein-1 (AP-1), nuclear factor-κB (NF-κB), and some of the interferon regulatory factors (IRFs) [3]. A number of adaptor molecules are involved in the TLR signaling

pathway, including myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein, TIR domain-containing adapter inducing IFN-β (TRIF), and TRIF-related adaptor molecule. TLR are expressed by a great variety of cell types including professional immune cells, for example, DC as well as nonprofessional immune cells, for example, synovial fibroblast-like cells and epithelial cells [4, 5]. Their common theme, however, is to recognize infections and to induce signaling pathways which ultimately lead to the expression of inflammatory mediators and the induction of an immune response. Innate signals were found to play a crucial role in the development of autoimmune nephritis [6]. Given the association between TLR and autoimmunity, a great deal of work has been directed toward understanding how these receptors act in disease progression. Studies have shown two different ways to describe the role of TLR in autoimmune disease. The presence of exogenous antigens, like viral ss-RNA, can stimulate TLR that can activate resident immune cells to initiate and propagate inflammation and autoimmunity, or the TLR can recognize endogenous self-antigens and generate an aggressive autoimmune response in tissues. A crucial point in the generation of autoimmune disease is represented by defective apoptotic cell clearance, common among SLE patients, that can lead to development of antinuclear antibodies [7]. A number of studies have hypothesized that inefficient clearance of apoptotic debris triggers nucleic acid-binding TLR, which induce the B-cell response and subsequent antinuclear antibodies (ANA) production. Studies have demonstrated that as many as one million cells die each second by apoptosis in the human body as a part of normal tissue turnover, although, usually, this phenomenon does not cause autoreactivity [8, 9]. In fact nuclear and cytoplasmic material is sequestered and digested trough autophagy that constitutes an important catabolic mechanism; therefore a failure in autophagy blocks the removal of apoptotic bodies and influences the immunogenicity of death cells [9]. Moreover, if we consider that an apoptotic cell contains a lot of modified self-antigens, the connection between the clearance of apoptotic bodies and autoreactive activation is quite plausible [8, 10, 11]. In 2006 Gaipl et al. demonstrated that an impaired clearance of dying cells may explain accumulation of apoptotic cells in SLE tissues contributing to the development of SLE in humans and mice by several mechanisms [11]. Autoreactive B cells in SLE internalize immune complexes or apoptotic material containing nucleic acids that activate TLRs, causing increased expression of the BAFF receptor TACI and increasing tissue damage mediated by autoantibodies [12–14].

Recently, two TLR, namely, TLR7 and 9 have been connected to both human and mouse models of SLE and lupus-like syndromes where they act synergistically with BCR to induce B-cell proliferation [3, 15, 16]. In addition, treatment of lupus-prone mice with a dual inhibitor of TLR-7 and TLR-9 leads to the reduction of autoantibody production and amelioration of disease symptoms [17]. This consideration suggests that aberrant activation of a number of TLR pathways may lead to the initiation and/or perpetuation of SLE. TLR7 and TLR9 blockers, such as antimalarials like hydroxychloroquine, have been used to treat SLE for many years because it can block activation of TLRs by inhibiting endosome maturation. More recently other drugs have been developed to activate or inhibit TLRs [3].

Experiments conducted in two different murine strains have demonstrated that injection of syngeneic late apoptotic thymocytes into wild-type B6 mice led to anti-dsDNA and antihistone antibody production whereas injection into MyD88$^{-/-}$ mice had no effect, suggesting that TLR stimulation is important in development of anti-dsDNA antibodies in situations of late apoptotic cell excess. The DNA-binding TLR9 has also been heavily studied in connection with murine lupus in MRL*lpr/lpr* strains. TLR9 deficiency in some lupus models including MRL*lpr/lpr* mice can lead to reductions or alterations in antichromatin antibodies; however, TLR9 deficiency paradoxically leads to disease exacerbation in many experimental models [15].

TLR7 has been shown to play an important role in several mouse models of SLE [6, 17–19]. The interaction between TLR7 and ligands directly activates DCs and B cells supporting the expansion of effector T and B lymphocytes specific for autoantigens. In addition, TLR7 activation could be involved in breaking peripheral tolerance mediated by Tregs, important to generate protection against pathogenic autoreactive immunity [18]. Furthermore, TLR7 activation by exogenous and endogenous TLR7 ligands impairs Treg generation and function. Hackl et al. demonstrated that TLR ligands have differential effects on Treg generation. TLR7 and similarly TLR9 ligands but not TLR4 ligand (e.g., LPS) reduced de novo generation of Tregs from naïve T cells. This effect appears to be mediated by IL-6 with a minor role for IFN-γ and IL-4 in inhibiting Treg generation in the presence of TLR7 ligand, which is in accordance with a recent report describing the influence of Th1-/Th2-polarizing cytokines on Treg differentiation [20]. IL-6 inhibits conversion of naïve T cells into Tregs and supports Th17 differentiation [21–23]. Expression of ROR$\gamma\tau$ and IL-17 mRNA in FOXP3$^+$ T cells generated in the presence of TLR7 ligand suggested the possibility of a Th17 regulatory cells [24]. Tregs were originally believed to be a stable Th-cell lineage but since then, several studies have clearly shown that Foxp3 expression can be downregulated in subpopulations of natural as well as induced Tregs allowing conversion into Th1, Th2, or Th17 effector cells under the influence of polarizing cytokines *in vitro* and in inflammatory environments *in vivo* [24]. In summary, TLR7-modified immunoregulation by Tregs contributes to the breakdown of peripheral tolerance and development of autoimmunity in SLE, where activation of TLR7 by endogenous ligands was shown to play a role in the pathogenesis. New therapeutic approaches for SLE and lupus-like syndromes would be better directed on modifying Foxp3 expression by interfering with TLR7 activation or by blocking downstream effector cytokines such as IL-6.

TLR4 is another TLR thought to be involved in the pathogenesis of glomerulonephritis in SLE. LPS-mediated immune responses are mediated through Toll-like receptor 4 (TLR4) on the target cells. Overexpression of TLR4 in mice has led to autoimmune glomerulonephritis and lupus-like disease [25], suggesting that LPS-mediated TLR4 signaling plays a pivotal role in lupus nephritis [26].

TLR4 as well as the commensal flora has been shown to be essential for the production of anti-dsDNA and the immune complex-mediated glomerulonephritis in transgenic mice expressing surface gp96 [25]. Lee et al. demonstrated that the activations of immature APC and autoreactive B cells in anti-dsDNA Tg mice by TLR4 signaling correlated with an increase in production of IFN-γ, IL-10, and anti-dsDNA production. Therefore, discovery and development of novel TLR4 and LPS antagonists may be a new paradigm for the therapy of SLE [26].

Various studies concerning autoimmune diseases in lupus-prone mouse models and from genome-wide association studies in human lupus patients and *in vitro* studies with cells obtained from patients showed a correlation between nucleic acid-binding TLR and the progression and severity of lupus and other autoimmune diseases [27]. Although there has not been a new drug approved for the treatment of lupus, current investigation regarding the targeting of TLRs and their downstream effectors is promising and therefore merits further investigation. The accumulation of evidence pointing towards the involvement of TLRs in autoimmunity has opened the door for potential therapeutic interventions directed to modulate Toll-like receptors expression and their signaling pathways [15].

3. Decoy Receptor Role in Humans and Murine SLE-Like Models

Decoy receptors are "silent scavengers" of CC chemokines and cytokines, which play a key role in damping inflammation and tissue damage [28]. Decoy receptors recognize certain inflammatory cytokines with high affinity and specificity, but are structurally incapable of signaling or presenting the agonist to signaling receptor complexes. They act as a molecular trap for the agonist and for signaling receptor components [29]. Inhibition of T cell death could modify clonal shrinkage after clonal expansion during an immune response, resulting in the survival of abnormal self cross-reactive T cells and, potentially, autoimmune disease.

At present, the DcR6 receptor, expressed on endothelial cells, hematopoietic stem cells (HSC), megakaryocytes, mast cells, and DC, is the focus of intense investigation in the field of inflammation since it binds many inflammatory CC chemokines (CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL14, CCL22, and weakly CCL17), without triggering any signals in target cells [28]. Interestingly, although previous studies have always focused on the anti-inflammatory role of DcR6 in relationship with its capacity to scavenge circulating proinflammatory CC chemokines, recent studies in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis, have suggested that DcR6 functions may be different and can change depending on the setting of the inflammatory conditions. In EAE models, DcR6-deficient mice showed a significantly lower immune response with a reduced inflammatory leukocyte infiltration in the spinal cord and, consequently, a decreased demyelization [28].

Recent studies have focused their attention on another decoy receptor: DcR3. It is a tumor necrosis factor receptor family member and is a secreted protein that can enhance cell survival by interfering with multiple apoptosis pathways. It is a decoy receptor for the Fas ligand (FasL) and can inhibit FasL-induced apoptosis.

DcR3 seems to function like the soluble Fas (sFas) in terms of binding FasL and competing with membrane Fas. Hyperproduction of DcR3 may be involved in the acquisition of autoimmunity (DcR3 and T-cell activation). In 2007 Han et al. demonstrated that DcR3 overexpression could lead to a lupus-like syndrome [30]. Since then, several reports have identified DcR3 as a possible parameter and risk factor for SLE and that its elevated levels in serum can contribute to enhancing T cells activation in SLE. SLE patients showed significantly high serum level of DcR3, and the mean serum DcR3 level was higher for those with active disease (SLE disease activity index (SLEDAI) > 10) compared with that in patients with inactive disease (SLEDAI < 10). In addition soluble DcR3-Fc enhanced T-cell proliferation and increased interleukin-2 (IL-2) and IFN-γ production via co-stimulation of T cells in response to cell death. Moreover, enhanced T-cell reactivity to DcR3-induced costimulation was demonstrated in lymphocytes from patients with SLE, suggesting that the elevated serum DcR3 may be associated with enhanced T-cell activation *in vivo*. It is likely that the DcR3-Fc-induced T-cell proliferation is via interaction between DcR3 and LIGHT (the cognate receptor). Previous studies have demonstrated that DcR3 could bind to LIGHT and transducer costimulatory signals into both human and murine T cells [31]. It is still not clear whether there is any particular T-cell subset responding to DcR3-induced costimulation, and what effector function will develop after triggering by DcR3.

Normal T cells express low levels of DcR3 [32], and healthy individuals have near-background serum levels [33]. DcR3 expression is augmented in activated T cells [32], thus probably represent a fine-tuning mechanism to balance the need for clonal expansion and subsequent massive activation-induced cell death of T cells. A dysfunction of this balance due to failed AICD might lead to pathologic consequences, such as autoimmune disease including SLE. Studies conducted with DcR3 transgenic mice with actin promoter-driven expression of human DcR3 demonstrated these mice to manifest a lupus-like syndrome after 5-6 months of age, thus, suggesting that DcR could play a role in the complex intertwinement of mechanism that drive the pathogenesis of lupus-like syndromes. The hypothetical role of decoy receptors in lupus-like syndromes could be different to that reported in experimental Mtb infection [34]; in systemic autoimmune disease the increase of decoy receptors, binding more chemokines, could amplify the recruitment of activated cells (neutrophils, macrophages, lymphocytes, dendritic cells, etc.) increasing the inflammatory state of the tissue damaged by autoimmune reactions.

4. Concluding Remarks

The pathogenesis of SLE and lupus-like syndromes in humans and mice is multifactorial. Several factors can contribute to the immunopathogenesis of SLE and lupus-like

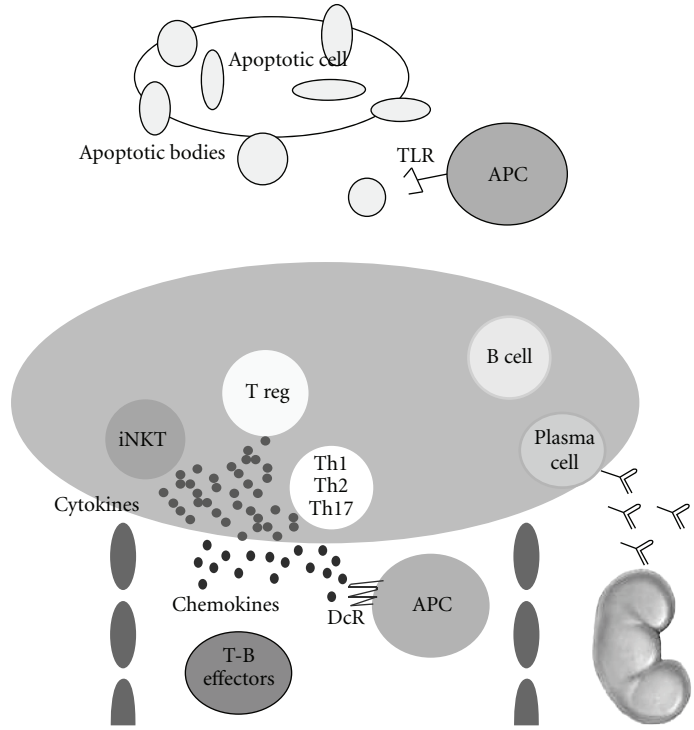

Figure 1: Mechanisms involved in the pathogenesis of SLE and lupus-like syndromes. Different cells and receptors contribute to the activation of B cells secreting autoantibodies; these Immunoglobulins cause tissue damage.

syndromes (sex, age, hormones, infectious background, environmental factors, drugs, abnormalities of both innate and adaptive immune system). Here, we draw your attention to two kinds of receptors associated with innate immunity as players in the pathogenesis of SLE and lupus-like syndromes: TLRs (7, 9, and 4) as possible receptors of autoantigenic molecules and decoy receptors as molecules able to propagate autoimmune inflammation acting in synergy to cause autoimmune inflammations (Figure 1). Our paper, highlighting the new players in this autoimmune disorder, could offer the opportunity to design new immunotherapeutical approaches for systemic autoimmune diseases.

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