TYPE I INTERFERON IN THE PATHOGENESIS OF SYSTEMIC LUPUS ERYTHEMATOSUS

By

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UNIVERSITY OF FLORIDA

2008
© 2008 Haoyang Zhuang
To my parents and my sister, for their endless love.

谨以此文献给我最亲爱的爸爸妈妈和姐姐
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<td>Antibody-dependent cellular cytotoxicity</td>
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<td>ANCA</td>
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<td>APC</td>
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<td>C reactive protein</td>
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<td>ECLAM</td>
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<td>GN</td>
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<td>HCV</td>
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<td>Interferon α receptor</td>
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<td>IRF</td>
<td>Interferon regulatory factor</td>
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<td>ISGF</td>
<td>IFN-stimulated gene factor</td>
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<td>ISRE</td>
<td>IFN-stimulated response element</td>
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<td>IVIG</td>
<td>Intravenous immunoglobulin</td>
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<td>LPS</td>
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<td>MDA5</td>
<td>Melanoma differentiation–associated gene 5</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
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<td>nRNP</td>
<td>Nuclear ribonuclear protein</td>
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<td>NSAIDs</td>
<td>Nonsteroidal anti-inflammatory drugs</td>
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<td>OAS</td>
<td>2’5’-oligoadenylate synthetase</td>
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<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
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<tr>
<td>PDC</td>
<td>Plasmacytoid dendritic cell</td>
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<tr>
<td>PIP</td>
<td>Proximal interphalangeal</td>
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<tr>
<td>PKR</td>
<td>Protein kinase R</td>
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<tr>
<td>Poly (I:C)</td>
<td>Polyinosinic–polycytidylic acid</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
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<td>RD</td>
<td>Repression domain</td>
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<td>RIG-I</td>
<td>Retinoic acid–inducible gene I</td>
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<td>SIS</td>
<td>SLE Index Score</td>
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<td>SLAM</td>
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<td>SLE</td>
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<td>SLEDAI</td>
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<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<td>SOCS</td>
<td>Suppressors of cytokine signaling</td>
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<td>STAT</td>
<td>Signal Transducers and Activators of Transcription</td>
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<td>TGF β</td>
<td>Transforming growth factor β</td>
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<td>TLR</td>
<td>Toll like receptor</td>
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<td>TICAM</td>
<td>Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule</td>
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<td>TIR</td>
<td>TLR–interleukin-1 receptor</td>
</tr>
<tr>
<td>Term</td>
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<td>TIRAP</td>
<td>Toll-interleukin 1 receptor (TIR) domain-containing adapter protein</td>
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<td>TNF α</td>
<td>Tumor necrosis factor α</td>
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<tr>
<td>TMPD</td>
<td>2,6,10,14 tetramethylpentadecane</td>
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<tr>
<td>TRAM</td>
<td>Toll-receptor-associated molecule</td>
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<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-β</td>
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<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
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<td>YAA</td>
<td>Y chromosome-linked autoimmune acceleration</td>
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Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease with multiple organ involvements. Cytokines, such as type I interferon (IFN-I) play an important role in the immune system, including regulating the activation and survival of B and T cells, up-regulation of MHC II expression, and acting as adjuvant in antibody production.

In this study, we have shown that the production of IFN-I was increased in peripheral blood mononuclear cells compared to normal controls or patients with other autoimmune disease. The elevated production of IFN-I was associated with the autoantibodies against dsDNA, Sm/RNP, Ro/La and was negatively associated with anti-phospholipid antibody. The increased IFN-I production was also correlated with disease activity and renal involvement.

Paradoxically the major IFN-I producing cells, namely plasmacytoid dendritic cells (PDCs) were decreased in SLE patients when compare to normal controls. We have shown that the decreased number of PDCs was also correlated with certain autoantibody production. We found a subset of monocytes which were CD14+CD71+ in lupus patients. These cells were correlated with IFN-I production.

At last we show that over production of IFN-I could lead to a lupus like disease. We studied two patients with an inbalanced translocation of chromosome 9 and 21 with 3 copies of
IFN-I cluster. These patients exhibit a lupus like disease as well as increased IFN-I production and decreased PDC number as other SLE patients.

In conclusion, we found IFN-I was important in the pathogenesis of SLE and that CD14⁺CD71⁺ monocytes were responsible for the IFN-I production. Over expression of IFN-I was sufficient to induce a lupus like disease.
CHAPTER 1
INTRODUCTION

Autoimmunity and Autoimmune Disease

As early as 1904, Paul Ehrlich used the term autoimmunity to signify an immune response against self and introduced the phrase “horror autotoxicus” (1). However the concept of autoimmunity was not fully accepted until 1960. Over the years, it has become recognized that autoimmunity is not an uncommon occurrence and is not necessarily detrimental. Thus, an important distinction must be drawn between autoimmunity, which is indicated by the presence of immune response to self antigen, (e.g. the formation of autoantibodies) and autoimmune disease, which occurs when autoimmunity leads to an inflammatory response resulting in tissue injury. Autoimmunity may be asymptomatic and it cannot be assumed that an autoimmune response implies the existence of autoimmune disease.

Systemic Lupus Erythematosus

Systemic Lupus Erythematosus 101

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease with multiple organ involvement. It is characterized of the production of autoantibodies against components of the cell nucleus and cytoplasm. Currently there is no single diagnosis of SLE, and the SLE is defined for research purposes as the presence of any 4 out of following 11 criteria: malar rash, dicoid rash, photosensitivity, oral ulcers, arthritis, serositis, renal disorder, neurologic disorder, hematologic disorder, immunologic disorder (anti-dsDNA, Sm, phospholipid antibodies), and antinuclear antibodies (2).

SLE primarily affects young women and the peak of incidence occurs between the ages of 15 and 40 (2). In this age range the ratio of affected women to men is around 10:1 whereas before or after this period the ratio is around 4:1, suggesting that estrogen plays a role in the
The disease is more common among African Americans, Asians, and Hispanics whereas the prevalence is lower in Caucasians (around 1 in 2000). The prevalence in non-Caucasian females is as high as 1 in 200.

The frequency of autoimmune disease in first degree relatives of SLE patients is significantly higher than in non-relatives, consistent with a genetic contribution. However, it does not appear to follow the classical Mendelian pattern (4). Environmental factors, such as sunlight and infections, also play important role in the pathogenesis of SLE.

**Genetics of SLE**

Genetics factors play important roles in the pathogenesis of SLE. Twin studies show that monozygotic twins have a 24-58% risk of developing SLE compared to only 2-5% for dizygotic twins (5). An individual with an affected first-degree relative is 20 times more likely to develop autoimmune disease (6, 7).

Several genome wide linkage scans have been performed in families and large cohorts of lupus patients. Although none of the loci revealed to be associated with lupus have been reproduced in all the studies, seven regions show strong evidence of linkage in at least 2 studies. These regions include 1q22-24(8, 9), 1q41-42(8), 2q32-37(9), 4p16(8, 9), 6p11-21(8-10), 11p13(11), 12p11(8, 9), 12q24(9), 16q12-13(10, 11) and 19q13(8). The studies suggest potential candidate genes for further studies. The lack of consistency among studies may be due to the fact that SLE is a heterogeneous disease or maybe related to different ethnic compositions of the study population.

The most studied locus is 1q22-24, which was identified in many populations (8, 9). Studies have been focused on the low affinity Fcγ receptor gene *CGR2A* and *B*, *FCGR3A* and *B*, which locate on Chromosome 1. FcγRIIa (CD32) is widely expressed on monocytes, macrophages, neutrophils, dendritic cells and platelets. A single nucleotide polymorphism (SNP)
results in a histidine (H)/arginine (R) switch at amino acid position 131 leading to a much lower binding affinity for IgG2, and potentially a lower capacity to clear IgG2 containing immune complexes. Some studies showed homozygous RR individuals have higher susceptibility to SLE (12). FcγRIIIa (CD16) is expressed primarily on monocytes, macrophages, NK cells, and some T-cells. A valine (V) to phenylalanine (F) switch at amino acid position 158 results in a reduction in binding affinity for IgG1 and IgG3 as well as immune complexes containing these isotypes (13). Some studies suggest the 158F allele is associated with lupus nephritis (14) and homozygous FF SLE patients have a higher risk for nephritis compared with the homozygous VV patients (15, 16). In addition to the F158 allele, F176 allele which has been shown to be associated with increased risk of SLE in both African American and European American (17, 18). FcγRIIIb is a stimulatory receptor expressed primarily on neutrophils. It exhibits a copy number polymorphism. However, the association between the FcγRIIIb copy number and SLE has been controversial (19). Another candidate gene on 1q23 is C reactive protein (CRP). CRP is an acute phase protein which is up-regulated by IL-6 and inflammation. It binds to CRP receptor on macrophages and enhances phagocytosis as well as complement binding to foreign antigens. In some studies this CRP polymorphism is also associated with SLE (20, 21).

Major histocompatibility complex (MHC) molecule was found to be associated with lupus 20 years ago (22). The human MHC region locates on Chromosome 6p11-21. It spans 3.6 Mb of DNA and contains over 200 genes, divided into the class I region containing HLA A, B and C, the class II region containing HLA DR, DQ and DP and the class III region containing a variety of immunological genes including tumor necrosis factor (TNF) and complement components C2 and C4. The association between MHC haplotype and lupus is highly consistent. An association of HLA DR2 and DR3 with SLE has been reported in several populations, with a relative risk for
the development of disease of approximately two to five (23, 24). A special combination of DR2/DR3 heterozygous individual has a moderate risk of developing SLE. The HLA class II genes are also associated with the production of certain autoantibodies such as anti-Sm (small nuclear ribonucleoprotein, as well as anti-Ro, anti-La, anti-nRNP, and anti-DNA antibodies (25-27). C4 protein is transcribed from two genes, C4A and C4B, and also exhibits a copy number polymorphism. The C4A null allele (C4AQ*0) has been associated with SLE in some studies. C2Q*0 is carried on a rare DRB1*1501 containing MHC haplotype and homozygote of C2Q*0 is associated with SLE development, yet there is no evidence for heterozygote of C2Q*0 with higher risk for lupus (28-30).

More recent studies showed increased type I interferon expression in SLE (31, 32). Interferon regulatory factor 5 (IRF5) is an interferon regulated transcription factor that plays an important role in the production of the proinflammatory cytokines TNFα, IL-12, and IL-6 in response to toll-like receptor stimulation. IRF5 has a complex splicing pattern. There are three alternative 5′UTR exons (exon1-A, exon1-B, and exon1-C). The three alternative 5′UTRs lead to the translation of several different isoform named V1–V12. SNP rs2004640 (G<T) has an alternative splicing site on exon1-B and leads to the expression of unique isoforms including V2, V9 and V10 which derived from exon1-B (33). The SNP rs2004640 is the strongest association ever reported with SLE (34). Another genetic variant (rs2280714) has been shown to regulate the overall expression level of IRF5 (35). It has failed in analysis of association with lupus but is linked with the risk allele of rs2004640 (36).

**Environmental Factors**

Environmental factors can trigger autoimmune disease in susceptible hosts, as illustrated by the initiation of disease by sun exposure in a subset of lupus patients. The wavelengths most likely to induce lupus fall in the UV range: UVC (200-290 nm), UVB (290-320 nm), and UVA
(320-400 nm). UVB irradiation is mostly absorbed in the upper layers of the epidermis, whereas the longer wavelength UVA is able to reach the dermis. UV exposure can induce apoptosis and release of immune mediators, activating of resident dendritic cells and T cells (37). It has been suggested that expression of certain self-antigens, such as Ro60/Ro52, on the surface of the apoptotic cells may lead to antibody-mediated inflammatory responses that could play a role in the pathogenesis of skin rashes in lupus (37, 38).

The importance of environmental factors is further illustrated by the induction of a murine lupus syndrome by the hydrocarbon pristane (see Animal Models, below), which appears to act, in part, through the induction of Type I interferon (IFNα and IFNβ) production (39). Many other chemicals and drugs have been implicated as triggers of autoimmunity or autoimmune disease. Procainamide, hydralazine, chlorpromazine, methyldopa, quinidine, minocycline, and nitrofurantoin all have been associated with the induction of antinuclear antibodies and in cases antineutrophil cytoplasmic antibodies (ANCA) as well as in the pathogenesis of “drug-induced lupus”, most frequently manifested by serositis (inflammation of the pleura or pericardium) and arthritis (40-42). Silica is recognized as a precipitating factor for scleroderma (43, 44), cigarette smoke may aggravate rheumatoid arthritis (45), and trichloroethylene is thought to promote lupus in animal models and possibly humans (46). Other chemical agents implicated in the pathogenesis of autoimmunity include heavy metals such as mercury, gold, and cadmium, pesticides, herbicides, hydrazine, and certain dyes (47) (48).

Infections also are implicated in the pathogenesis of autoimmune disease. The classic example is rheumatic fever, which is thought to be a consequence of cross-reactivity or “molecular mimicry” between antigens carried by certain strains of streptococci and self-antigens of the heart. A variety of parasitic (e.g. schistosomiasis, Chagas disease) (49, 50),
bacterial (e.g. *Helicobacter pylori*, staphylococci, salmonella, Lyme borreliosis) (51, 52), mycobacterial (e.g. tuberculosis, leprosy) (50), and viral (e.g. cytomegalovirus, Epstein-Barr virus, hepatitis C, coxsackievirus, parvovirus B19) (53, 54) infections can be complicated by autoimmunity. Proposed mechanisms include molecular mimicry and the chronic over-production of cytokines, such as interferon-α. Indeed, therapy with IFNα can itself lead to the development of autoimmune diseases such as autoimmune thyroiditis and SLE.

**Autoantibodies in SLE**

The immunological hallmark of SLE is the production of autoantibodies. These autoantibodies include antibodies against RNA-protein complexes (e.g. anti-Sm, RNP, Ro/SS-A, and La/SS-B antibodies) and DNA-protein complexes (e.g. anti-double stranded DNA, anti-histone, anti-chromatin antibodies). The target antigens are found mainly in the cell nucleus, although in some cases (e.g. anti-ribosomal antibodies) they may be cytoplasmic.

Over 95% of the SLE patients are positive for antinuclear antibodies (ANA), however the specificity of ANA test for SLE is only around 70% (55). Normal individuals have an increased frequency of ANA production with aging. Patients with scleroderma, polymyositis, and Sjogren’s syndrome usually are ANA positive as well (55).

Anti-dsDNA, anti-Sm, anti-ribosomal protein, and anti-RNA helicase A antibodies are relatively specific for SLE (55). This makes specificities useful diagnostically. In some patients the titer of anti-dsDNA antibodies increases with disease activity, making anti-dsDNA antibody a useful marker for monitoring lupus flares and the response to therapy (56).

**Type III Hypersensitivity and SLE**

Tissue damage in autoimmune diseases can occur through several mechanisms, which are analogous to three of the classical types of hypersensitivity reactions: Type II (caused by
autoantibodies reactive with cell surface or matrix antigens), Type III (caused by immune complexes), and Type IV (delayed type hypersensitivity, mediated by T cells).

Autoantibodies can cause disease by forming networks of autoantibodies bound to their antigens (immune complexes). The antigen-antibody complexes can deposit in tissues, causing inflammatory lesions. Studies of serum sickness led to the first description of an immune complex disease (57). Serum sickness is manifested by fever, glomerulonephritis, vasculitis, urticaria, and arthritis, appearing 7-21 days after primary immunization or 2-4 days after secondary immunization with a foreign protein. Two consequences of immune complex formation are complement fixation and binding to Fc or complement receptors on phagocytes. Clearance is facilitated by the binding of immune complexes to C3b receptors (CR1) on erythrocytes, which retain the complexes in the circulation until their removal by the reticuloendothelial cells of the spleen or liver.

Immune complex formation is a normal process, which removes foreign antigens from the circulation. Removal of immune complexes by phagocytes bearing Fc or complement receptors prevents their deposition at other sites. The efficiency of uptake of immune complexes by either Fc receptors or CR1 is proportional to the number of IgG molecules associated with the complex.

Immune complexes can activate either the classical or the alternative complement pathway. The classical pathway plays a major role in maintaining immune complexes in a soluble form, preventing their deposition in tissues. C3b bound to the solubilized immune complexes promotes their clearance by the erythrocyte complement receptor CR1. If the rate of immune complex formation exceeds the ability to clear these complexes via Fc receptors and CR1, the immune complexes can deposit within tissues, leading to inflammation. This efficient immune complex transport and removal by Fc and complement receptors can be overwhelmed, however, leading
to tissue deposition and immune complex disease. This situation may result from overproduction of immune complexes, blockade of phagocytosis by the reticuloendothelial system, or complement depletion resulting in inefficient solubilization of immune complexes.

SLE is the prototype of human immune complex disease. Tissue damage in lupus is mainly caused by immune complexes containing autoantibodies to soluble antigens. Autoantibodies containing immune complexes, especially anti-double stranded DNA antibodies, are selectively enriched in the renal glomeruli of patients with lupus nephritis and are thought to play a critical role in establishing the inflammatory response. Immune complex deposition in the kidney leads to proliferative glomerulonephritis and effacement of the normal glomerular architecture. As is the case in serum sickness, active lupus nephritis frequently is associated with hypocomplementemia. In addition to the kidneys (glomeruli), immunoglobulin and complement deposits are found in blood vessels (vasculitis), skin (rashes), nervous system, and other locations. Preformed immune complexes may become trapped in the glomerular filter or immune complexes may develop \textit{in situ} as a consequence of the interaction of cationic antigens (e.g. histones) with heparan sulfate glycosaminoglycan in the glomerular basement membrane.

**Clinical Manifestations**

As mentioned above, SLE is a complex systemic disease. At onset it might just affect one organ system with additional symptoms in other organs appearing later on. The disease typically follows a relapsing-remitting course. Systemic symptoms include fatigue, malaise, weight loss and anorexia and are frequently prominent.

Almost all SLE patients experience arthralgias and myaligias and eventually develop arthritis. The most common site of swelling joins includes proximal interphalangeal (PIP) and metacarpophalangeal (MCP) of the hands, wrists, and knees. Arthritis in lupus is usually non-erosive and symmetric which is different from rheumatoid arthritis.
The malar rash (referred as “butterfly rash” which is an erythematous rash over the cheeks and bridge of the nose) is a typical SLE cutaneous manifestation. A more diffused maculopapular rash can appear on any part of the body and usually exacerbated by UV or sun light exposure. These rashes can be reversed without scarring. Discoid rashes are different kinds of lesions which are circular with an erythematous rim, raised and scaly with follicular plugging and telangiectasia. It usually results in depigmentation and scar. Vasculitic skin lesions are common. These include subcutaneous nodules, ulcers, purpura, and infarcts of skin or digits.

Renal manifestations are seen in the majority of SLE patients. Although most SLE patients have immune complex deposited in glomeruli, not all of them have clinical nephritis. Patients can be asymptomatic, or may present with proteinuria and edema (nephritic syndrome) or with hematuria, pyuria, and urinary casts (nephritic syndrome). Renal involvement can be severe, and in fact renal failure is one of the major causes of death.

Any part of the nervous system including the brain, spinal cord, cranial, and peripheral nerves can be involved in SLE. Involvement of the central nervous system usually occurs with active disease although mild mental dysfunction is more common and can be seen in the absence of disease activity. Nervous manifestations include seizures, psychosis, headaches, focal infarcts, and many others. Depression and anxiety are frequent.

Some SLE patients exhibit vasculitis and thrombosis. It is believed that thrombosis is associated with production of anti-phospholipids antibodies, which promote coagulation. Anemia, thrombocytopenia, and leucopenia (or lymphopenia) is a common finding in SLE patients. Pericardial pain is the most frequent symptom of cardiac lupus. Pleurisy and pleural effusions are not rare; however the most common cause of pulmonary infiltrates in patients with SLE is infection.
Measurement of Disease Activity

Measurement of disease activity in SLE is challenging because of the complexity of the disease manifestations. Almost any organ may be affected. Clinical symptoms of SLE can mimic the appearance of many other diseases. There is no single marker that adequately reflects disease activity.

Several systems have been developed to assess disease activity in SLE. The most commonly used ones are the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI), the British Isles Lupus Assessment Group (BILAG), the Systemic Lupus Activity Measures (SLAM), the European Consensus Lupus Activity Measurement (ECLAM), and the SLE Index Score (SIS) by National Institute of Health.

SLEDAI is the most commonly used disease activity measure. It was initially proposed in 1992 by Bombardier et al in Canada (58). It is an index that weights the importance of different organ involved. There are 24 organ criteria, each weighted from 1 to 8 points. The sum of the total score falls between 0 and 105 with a higher score representing more active disease. It is relatively easy to perform and is reliable and reproducible. However, the SLEDAI system does not take into account severity already exist or partial improvement of a particular manifestation. The SLEDAI also does not account for subjective symptoms such as fatigue or myalgia.

The SLAM index was designed to assess the severity of disease (59). 32 criteria are divided into 11 organs systems and a numeric score (1-3) is given according to the severity of manifestation of a particular organ, and the possible score is as much as 86. Although the SLAM is considered a measurement of severity, it gives equal weight to mild and serious manifestation.

The BILAG was developed by Symmons et al in 1988 (60). It is a comprehensive scoring system for assessing both current lupus disease activity and changes in that activity since the patient was last seen. It is based on the intention of the physician’s intention to treat. The newest
version of the BILAG was released in 2004. There are eight systems: general, mucocutaneous, neurological, musculoskeletal, cardiorespiratory, vasculitis, renal and haematological. A score is calculated for each system depending on the clinical features present and whether they are new=4, worse=3, the same=2 or improving=1 in the last 4 weeks compared to the previous measures. Scores for eight different organ systems are calculated separately.

Current Treatment

Treatments for autoimmune disease are diverse and in recent years the options have increased rapidly. The majority of systemic autoimmune diseases, such as SLE, are treated with immunosuppressive medications. Immunosuppressive medications can be categorized by mode of action.

Anti-inflammatory agents

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been used since the 1800s when salicin was extracted from willow bark (1828) and sodium salicylate (1875) and aspirin (1899) were synthesized. A large number of these drugs, which either selectively or non-selectively inhibit the enzyme cyclooxygenase (COX, a synthetic enzyme for prostaglandins), are currently in use for the treatment of inflammatory disease. Although most of their anti-inflammatory properties derive from the inhibition of prostaglandin synthesis, at high doses there is inhibition of the transcription factor NFκB, a key mediator of inflammatory cytokine production. Corticosteroids have a more potent effect on NFκB and consequently a greater anti-inflammatory effect.

The anti-inflammatory properties of cortisone were discovered by Hench in 1949 (61). Corticosteroids are a mainstay of therapy for many systemic autoimmune diseases, including SLE, rheumatoid arthritis, and inflammatory myopathies such as polymyositis. Corticosteroid therapy also is used for the treatment of some of the more serious organ specific autoimmune
diseases, such as autoimmune hemolytic anemia, autoimmune thrombocytopenia, multiple sclerosis, and Goodpasture’s disease. Corticosteroids reduce inflammation by multiple mechanisms of action. One major action is enhanced transcription of an inhibitor of NFκB called IκB. IκB dimerizes with NFκB, inhibiting the production of inflammatory cytokines mediated by this transcriptional pathway (62). In addition, corticosteroids promote the differentiation of a subset of anti-inflammatory macrophages that produce the cytokine IL-10 (63).

Antimalarials

Antimalarials have been used for the treatment of SLE and rheumatoid arthritis (RA) since the early 1900s. The precise mechanism of action remains uncertain, but they have been shown to inhibit cytokine (IL-1 and IL-6) production in vitro (64). The antimalarials pass freely through cell membranes at neutral pH, but in acidic environments, such as endosomes, they become protonated and can no longer diffuse freely. This leads to concentration of the drug within endosomes and the collapse of endosomal pH gradients (65). It has been proposed that the inhibition of endosomal acidification interferes with antigen processing or alternatively, that there is an effect on the interaction of microbial substances such as unmethylated CpG DNA or uridine-rich RNA with endosomal Toll-like receptors (TLR9 and TLR7/TLR8, respectively) (66). In addition to SLE and RA, antimalarials are used in the treatment of juvenile rheumatoid arthritis, Sjogren’s syndrome, and inflammatory myopathies.

Anti-cytokine agents

The development of TNF α inhibitors in the 1990s ushered in a new era of therapy of autoimmune disease using “biologicals” capable of interfering with the interactions between cytokines and their receptors. The initial clinical use of TNF inhibitors such as etanercept (a soluble recombinant TNF receptor II linked to the Fc portion of human IgG1) (67), infliximab (a chimeric human-mouse anti-TNFα monoclonal antibody) (68), and adalimumab (a fully
humanized monoclonal antibody against TNFα) (69) in RA demonstrated that although multiple cytokines may be involved in disease pathogenesis (in RA, IL-1 and IL-6 in addition to TNFα), inhibitors of a single cytokine pathway may show therapeutic efficacy. In addition to RA, TNFα inhibitors are used for treating inflammatory bowel disease, psoriasis and psoriatic arthritis, and are being tested in sarcoidosis, Wegener’s granulomatosis, pyoderma gangrenosum, SLE, and Behcet’s disease. Usage of TNFα inhibitor in SLE is controversial since TNFα has been shown to have inhibitory effect on IFN production.

Anti-TNF therapy is only the tip of the “biological iceberg”. Recombinant IL-1 receptor antagonist (anakinra) has been approved for the treatment of RA and numerous other cytokine antagonists are currently in clinical trials or under development.

**Methotrexate**

Methotrexate is a folic acid analog used extensively for the treatment of RA. It appears that its ability to inhibit dihydrofolate reductase is not responsible for its efficacy in RA. Instead, activity may be related to effects on aminomimidazole-carboxamide-ribotide-transformylase, leading to the release of adenosine, a potent anti-inflammatory molecule that inhibits neutrophil adherence to fibroblasts and endothelial cells (70). Methotrexate inhibits IL-1 and increases the expression of TH2 cytokines (e.g. IL-4), leading to decreased production of TH1 cytokines (e.g. interferon γ) (71).

**Anti-T Lymphocyte therapy**

T cells play a key role in the pathogenesis of Type IV autoimmune reactions and also are critical for generating the T cell-dependent autoantibodies mediating Type II and Type III autoimmune diseases. Consequently, considerable effort has gone into the development of therapeutic agents that selectively or non-selectively target T lymphocytes. Drugs that target primarily T cells include cyclophosphamide, azathioprine, cyclosporin A, tacrolimus, and the
biological CTLA4-Ig. Cyclophosphamide is an alkylating agent that substitutes alkyl radicals into DNA and RNA. The drug is inactive by itself, but is converted to an active metabolite responsible for its immunosuppressive effects. It is used for the treatment of lupus nephritis and other life-threatening complications of SLE (72).

Azathioprine is a purine analog that inhibits the synthesis of adenosine and guanine. Like cyclophosphamide, it is converted to an active metabolite (6-mercaptopurine), which inhibits the division of activated B and T cells. Azathioprine is used in the treatment of RA, SLE, autoimmune hepatitis, inflammatory myopathy, vasculitis, and other autoimmune disorders (73).

Unlike cyclophosphamide and azathioprine, cyclosporin and tacrolimus (FK506) have immunosuppressive effects that are highly selective for T cells. Both agents interfere with the phosphatase calcineurin, ultimately leading to the inhibition of the activation of the transcription factor NFAT (nuclear factor of activated T cells). Cyclosporin binds to the intracellular protein cyclophilin and tacrolimus to a protein called FK binding protein. The cyclosporin-cyclophilin and tacrolimus-FK binding protein complexes bind to calcineurin, preventing its activation by intracellular calcium, preventing the activation of NFAT (74). Although used most frequently to prevent transplant rejection, these agents have been shown to have activity in the treatment of RA, SLE, and certain forms of vasculitis (75).

The CTLA4 (CD152) molecule is an inhibitory receptor expressed by activated T cells that blocks the co-stimulatory interaction between CD80 or CD86 on the surface of antigen-presenting cells and CD28 on T cells. It acts by binding CD80/CD86 with greater affinity than CD28. CTLA4 is expressed late in T cell activation, and serves to turn off the activated state. CTLA4-Ig (abatacept) is a recombinant chimera of CTLA4 and the Fc fragment
of IgG1. CTLA4-Ig/abatacept is used for the treatment of rheumatoid arthritis and is active in mouse models of lupus (73). Clinical trials in SLE patients are in progress.

**Anti-B Lymphocyte therapy**

Rituximab is a cytotoxic chimeric human-mouse monoclonal antibody with a high affinity for CD20, a pan-B cell surface antigen. It was developed originally for the treatment of B cell lymphomas (76). The killing of B cells by rituximab is thought to depend on both the specific recognition of B cells by this monoclonal antibody and natural killer (NK) cell-mediated antibody-dependent cellular cytotoxicity (ADCC) of those cells. There is considerable evidence that the interaction of B cell-bound monoclonal antibodies with NK cell CD16 (FcγRIIIA) is a critical event leading to ADCC following treatment with rituximab (77). Rituximab has been shown to have activity in a variety of autoimmune diseases associated with autoantibody production, including RA, SLE, polymyositis/dermatomyositis, Sjogren’s syndrome, and cryoglobulinemic vasculitis (78).

**Intravenous immunoglobulin (IVIG)**

IVIG is a preparation of human immunoglobulin pooled from thousands of healthy individuals. It was originally developed for replacement therapy in humoral immunodeficiency syndromes, but has more recently become an important therapeutic modality in severe autoimmune disorders, such as thrombocytopenic purpura, autoimmune hemolytic anemia, neuroimmunological diseases such as Guillain-Barre syndrome, SLE, certain forms of vasculitis, and polymyositis/dermatomyositis. The mechanism of action remains unclear, but IVIG may block the function of Fc receptors expressed by phagocytes of the reticuloendothelial system and also induces FcγRIIB (inhibitory Fc receptor) expression on infiltrating macrophages in the K/BxN model of RA (79). An additional mode of action may involve the presence of anti-idiotypic antibodies that block the antigen combining sites of pathogenic antibodies (80).
The duration of action is limited by the metabolism of serum immunoglobulin, and generally IVIG is regarded as a temporary measure that is followed by more definitive therapy.

**Animal Models of SLE**

Spontaneous SLE models have offered an appropriate starting point to investigate the effects of environmental agents on genetic susceptibility and autoimmune disease progression.

The NZB/W model was the first murine model described for lupus nephritis. It is the F1 hybrid of the New Zealand Black mice which develop autoimmune hemolytic anemia and New Zealand White mice which spontaneously develop mesangial glomerulonephritis (GN) late in life. The F1 hybrid termed NZB/W mice has disease closely resembled human lupus, including the production of autoantibodies (ANA, anti-chromatin, anti-dsDNA) and the deposition of the immune complexes on the glomerular basement membrane, result in a severe glomerulonephritis. Genetic analysis has revealed three major susceptibility loci on chromosomes 1, 4 and 7 related with SLE (81).

MRL/\textit{lpr} mice develop an autoimmune disorder characterized by marked hypergammaglobulinemia, production of autoantibodies, and very impressive lymphadenopathy. This results from an \textit{ETn} retrotransposon insertion into the Fas gene which encodes a protein important for apoptosis (82). The defective apoptosis leads to the massive CD3\textsuperscript{+} CD4\textsuperscript{−} CD8\textsuperscript{−} cell proliferation. There also are some similarities with human cutaneous lupus including microscopic changes and IgG deposits at the dermoepidermal junction (83). The inflammatory response in the salivary glands also makes the MRL/\textit{lpr} mice a model useful for studying Sjogren’s syndrome (84).

Unlike other mouse model and human SLE, male, but not female BXSB mice develop severe form of lupus. This strain was created by crossing male SB/Le mice and female C57BL/6J mice. A mutant gene located on the BXSB Y chromosome, designated Yaa (Y
chromosome-linked autoimmune acceleration), is responsible for the acceleration of the disease observed in male BXSB mice. Male BXSB mice exhibit lymph node enlargement, autoimmune hemolytic anemia, hypergammaglobulinemia, splenomegaly, production of autoantibodies and severe glomerulonephritis (85). Recently it was found that the phenotype of mice with the Yaa mutation results from translocation of a 4-megabase portion of the X chromosome to the Y chromosome, leading to an increase in the expression of several genes that are normally X linked, including TLR7. It can be shown that the autoimmune defect is due to the extra copy of TLR7 in these mice (86).

Intraperitoneal injection of pristane (2,6,10,14 tetramethylpentadecane, TMPD) can induce lupus like syndrome in normal mice (87). Pristane-treated BALB/c and SJL mice develop autoantibodies characteristic of SLE, including production of autoantibodies, a severe glomerulonephritis with immune complex deposition, mesangial or mesangiocapillary proliferation, and proteinuria. Noticeable production of anti Sm/RNP and anti Su antibody and “interferon signature” is seen in TMPD treated mice which closely resembled human SLE (87).

**Type I Interferon**

Type I interferons (IFN-I), including multiple IFNα subtypes, IFNβ, IFNω, and IFNτ, have many immune effects. IFN-I (IFNβ) was first identified in fibroblasts by Isaacs and Lindeman in 1959 and termed “viral interference factor” (88, 89). However, Type I IFNs also are produced by leukocytes and other cells and it has been recognized that in addition to antiviral effects, IFN-I has protean effects on immune function and cell survival. IFN-I induces MHC class II expression on antigen presenting cells (APCs), promotes dendritic cell (DC) maturation, and the survival of activated T cells and antigen-activated B cells. Additionally, IFN-I can either promote or inhibit apoptosis (90).
All IFN-I species bind to a single receptor composed of two subunits, IFNAR1 and IFNAR2 (91, 92). Interactions of Type I IFNs with the IFNAR cause reciprocal transphosphorylation of the non-receptor tyrosine kinases Tyk2 and Jak1, leading to receptor phosphorylation and phosphorylation of transcription factor Stat1 and Stat2. IFNAR1 interacts with Tyk2 and IFNAR2 with Jak1, Stat1, and Stat2 (93). Phosphorylation of Stat1/Stat2 leads to the activation of interferon regulatory factors (IRFs), such as IRF-9, to form the heterotrimeric complex IFN-stimulated gene factor 3 (ISGF3). The ISGF3 then binds to upstream regulatory consensus sequences of IFN-α/β-inducible genes (IFN-stimulated response elements, or ISRE) and initiates transcription of a group of interferon inducible genes (ISGs) (94) (Fig. 1-3).

Up to 100 ISGs have been identified. Among them, a group of secondary anti-viral response genes, such as the 2’5’-oligoadenylate synthetase (OAS) family, RNase L, dsRNA dependent protein kinase (PKR), and the GTPase Mx1 (protein product MxA) are best studied (95). MxA is a large GTPase that interferes with viral transcription and replication (96). Mx1 gene expression is tightly regulated by IFN-I but not other cytokines, making it a good candidate for estimating IFN-I level. Additionally it has been reported that the level of the MxA protein (determined by ELISA) can be used as a very sensitive and selective bioassay for Type I IFN levels (97).

**Induction of IFN-I**

IFN-I is a key mediator of host defense to viral and bacterial infection. At least three pathways had been shown to induce the production of IFN-I. These pathways utilize distinctive adapter proteins: MyD88, TRIF, IPS-1 and TBK-1 (98, 99).

**Toll-like Receptors Dependent Pathways**

TLRs are pattern reorganization receptors recognizing molecules that are shared by groups of related pathogens (patterns). Thirteen TLRs have been identified in mammals (100), and these
receptors are the first line of host defense and play an important role in innate immunity. Each of the TLRs responds to a specific ligand or ligands. TLR 1, 2, 4, 5, 6, and 11 are expressed on cell surface, and detects lipids, lipoproteins or peptidoglycans shared by extracellular pathogens including bacteria, fungi or protozoa. TLR 7, 8 are intracellular and can be activated by GU-rich ssRNA. Intracellular TLR9 is activated by unmethylated DNA which is distinguishable from mammalian methylated DNA. Whether TLR3 express on cell surface or intracellular is debatable, and it binds to double stranded RNA and its analog Polyinosinic–polycytidylic acid (poly (I:C)) (PIC). TLR8/10 is expressed in humans, but not mice, and the ligand for TLR10 remains unknown. TLRs 11, 12, and 13 are expressed in mice but not humans (100-102).

All TLRs express TLR–interleukin-1 receptor (TIR) domains which will undergo dimerization and conformational changes upon activation. TIR domains can bind to several specific adaptors, such as TRIF (TICAM1), TRAM (TICAM2), TIRAP (Mal), and MyD88.

Plasmacytoid dendritic cells, known as professional IFN-I producing cells, express TLR7 (both mouse and human)/TLR8 (human) and TLR9 but not TLR2, TLR3, TLR4, or TLR5 (103). Unmethylated CpG DNA motif stimulates TLR9, and TLR9-induced IFN production is MyD88 dependent but not TRIF dependent. Lysosomal inhibitors such as chloroquine block TLR9 induced IFN-I production, indicating the reorganization of TLR9 ligand involvement of endosome (104). In addition, single-stranded RNAs rich in GU or U sequences also stimulate IFN-I production through interactions with TLR7 and/or TLR8. Imidazoquinolones, such as imiquimod, also engage these receptors, stimulating IFN-I production. The activation of TLR7/8 is also dependent on MyD88 (Fig 1-1) (66, 105). Upon activation, TRL7/8/9 recruits and bind to an adaptor protein myeloid differentiation primary response gene 88 (Myd88) after dimerization of TIR. MyD88 in turn forms a complex with members of the IL-1 receptor–associated kinase
(IRAK) family (IRAK1 and IRAK4) and TRAF6, which lead to the activation of IkB kinases (IKKs) IKKa and IKKβ. Phosphorylated IKKs degrade NF-κB inhibitor IkB, allowing nuclear factor NF-κB to translocate into the nucleus (106, 107). Transcription factor IRF7 forms a complex with activated MyD88, and then can be phosphorylated by IRAK1 though the MyD88 dependent pathway leads to the production of IL-6, TNFα, and IFN-I (107).

In addition to TLR7/8 and TLR9, activation by ligands of TLR3 and TLR4 can induce the production of IFN-I as well. TRL3 uniquely signals through adaptor protein TRIF, and it is independent of MyD88. TRIF interacts with TBK1 and IKKi which leads to the activation of transcription factor IRF3 (108). TRIF also mediates NF-κB activation through non-TIR region with PIP1 as well as TRAF6. TLR4 can signal through both MyD88 dependent and independent pathway (109).

Activation of TLRs leads to initial production of IFNβ, which in turn binds to the Type I IFN receptor (IFNAR), acting in an autocrine manner to induce the expression of a set of secondary antiviral response genes, such as *MxA/Mx1, Irf7 and Oas*. Expression of these genes is tightly regulated by IFN-I (94).

**Toll-like Receptor Independent Pathway**

It’s been increasingly clear that cytosolic RNA helicases, retinoic acid–inducible gene (RIG-I) and melanoma differentiation–associated gene 5 (MDA5, also called Helicard) can induce IFN-α/β induction independent from TLRs (110-112). RIG-I contains a extreme C-terminal repression domain (RD), two caspase recruitment domains (CARD) in the N terminus. In the absence of appropriate ligands, RIG-I is self locked in an autorepressed conformation through binding of RD with CARD though linker region between the RNA helicase domains III and IV (cis repression) (113, 114). Upon activation, RIG-I and MDA5 can bind to their RNA
ligand, and leads to a conformation change and dimerization of RIG-I, and become associated with CARD to mitochondrial MAVS (also known as IPS-1, VISA or Cardif). This triggers activation of the protein kinases TBK1 and IKKε, and in turn activates transcription factors IRF3 and IRF7, leading to IFN-α/β expression. It also activates NF-κB and AP-1 through IKKα/β/γ. RIG-I and MDA5 are upregulated by IFN-α/β through a positive feedback mechanism (115, 116).

RIG-I recognizes cytosolic uncapped ssRNA with 5′-triphosphates which is generated by some virus such as influenza, Sendai virus, vesicular stomatitis virus (VSV), rabies virus, and viruses of the Flaviviridae family, including hepatitis C virus (HCV)(117), whereas MDA5 might recognize viruses with protected 5' RNA ends, such as in picornaviruses (112, 118, 119). Some studies suggested MDA5, but not TLR3, seems to be the dominant receptor for polyI:C in vivo (120).

LGP2 is a RIG-I/MDA5 inhibitor which is a RNA helicase that is homologous to RIG-I and MDA5, but lacks CARD domains. It acts in similar fashion as the RD domain of RIG-I that blocks the RIG-I activity by binding with CARD. The RIG-I/MDA5 pathway is essential for IFN-α/β production by fibroblasts, conventional splenic and thymic DCs and macrophages, but not PDCs (113, 114, 121).

**Crosstalk of Type I Interferon and Other Cytokines**

IFN-α/β is expressed in low levels constitutively. Basal IFN-α/β expression is required for rapid production of cytokines in response to viral infection; it also appears to be required for efficient IFN-γ signaling (122). Not only are IFNAR-/- cells unresponsive to IFNα/β, but they are defective in IFN-γ responses as well (123). Because these cells fail to induce dimerization of STAT1, it is thought that IFNAR signaling induced by spontaneous low levels of IFN-α/β, (which is not enough to activate downstream signaling events), provides docking sites on
tyrosine residues of IFNAR1 for more efficient STAT1 recruitment after stimulation through the IFN-γ receptor (IFNγR). This cooperation is dependent on the interaction between the IFNAR1 chain and the IFNGR2 chains on caveolar membrane domains, which along with the increased STAT1 recruitment leads to at least a ten fold increase of IFN-γ signaling (124). IFN-α/β signaling also upregulates IFN-γ production by T cells favoring the induction and maintenance of Th1 cells (124). Conversely, pretreatment of IFN-γ increases signaling through IFNAR by upregulating STAT1 expression (124). Other studies suggest that upregulation of ISGF3 may enhance IFN-I signaling (125). The effect of STAT1 depends on the activation of tyrosine kinase Syk (126). Thus, type I and II IFNs reciprocally influence both the production of one another and signaling through the respective receptors.

Type I IFNs can also influence the expression and function of a number of other cytokines. Basal levels of IFN-α/β enhance IL-6 signaling by providing docking sites for STAT1 and STAT3 on the phosphorylated IFNAR1 (127). In addition, IFN-I stimulates production of anti-inflammatory cytokines, such as transforming growth factor β (TGF-β) (128), and IL-1 receptor antagonist (129).

Besides favoring the production Th1 cytokines, it also inhibits Th2 cytokines IL-4 and IL-12 signaling by inducing SOCS-1 expression (130). A low concentration of IFN-α/β also upregulates expression of the high-affinity IL-12Rβ2 subunit on CD4+ T cells and IL-15 expression by DCs, causing strong and selective stimulation of memory CD8+ T cells.(131) IL-10 reduces IFN-I signaling by suppressing the phosphorylation of STAT1 favoring Th2 polarization (132). Similarly, reciprocal suppression of IFN-α/β and TNF-α production has been reported. It has been proposed that the relative abundance of these two cytokines may influence the type of autoimmune disease. For example, IFN-α/β predominantly leads to lupus and TNF-α
leads to RA (133). The successful treatment of RA with TNFα inhibitors and the fact that these drugs can worsen SLE supports possibility (134, 135). However, the role of TNF-α in lupus is controversial, since other studies have shown that TNF-α may also have a proinflammatory effects in lupus. In fact, there are also reports of beneficial effects of TNF-α inhibitor in lupus patients (136).

**Type I Interferon is Involved In Pathogenesis of SLE**

Several studies have suggested that IFN-I is involved in the pathogenesis of SLE. Patients treated with IFNα for malignant carcinoid syndrome or hepatitis C infection sometimes develop antinuclear antibodies or even overt SLE (137-139). In the course of IFNα therapy of carcinoid tumors, as many as 22% of patients develop ANA, 8% develop anti-dsDNA antibodies, and 0.7% develops lupus (139). Serum levels of IFNα correlate with anti-dsDNA antibody levels and disease activity in SLE and increased IFN-I expression is seen in lupus skin lesions (140).

Moreover, recent studies point to an IFN-I gene expression “signature” associated with SLE (141, 142). Our laboratory has reported that the intraperitoneal injection of pristane in BALB/c and other non-autoimmune strains of mice results in the production of autoantibodies characteristic of SLE, such as anti-Sm, anti-nRNP, anti-dsDNA, and anti-ribosomal P as well as immune complex-mediated glomerulonephritis resembling lupus nephritis (87). We have found recently that IFN-I inducible gene expression is greatly up-regulated in pristane treated mice (39).

Interestingly, TLR4 deficient mice are less susceptible to pristane induced lupus and TLR7 deficient mice are highly resistant, arguing that TLR signaling may play a role in the pathogenesis of dysregulated IFN-I production in lupus (Lee et al, submitted).
Subsets of Dendritic Cells (DCs) Produce IFN-I

DCs are professional antigen presenting cells that serve as “nature’s adjuvant”. Immature DCs reside in the periphery and after interacting with a maturation stimulus, such as TLR ligands or TNFα, migrate to secondary lymphoid tissues where they undergo maturation (143, 144). Maturation is accompanied by the down-regulation of phagocytic activity and a concomitant upregulation of MHC class I and class II expression (145, 146). Thus, DCs mature from cells specialized in “antigen sampling” to cells specialized for antigen presentation to naïve T cells (147). DC precursors derived from bone marrow migrate to the periphery where they sample antigen. Once they capture antigen and receive an activation signal, they migrate to secondary lymphoid organs. Immature DCs express low levels of MHC I and II molecules, the co-stimulatory molecules CD80 and CD86, and adhesion molecules important for their interactions with T cells. They also express certain chemokine receptors, such as CCR1, CCR2, CCR5, CCR6, CXCR1, and CXCR2, which mediate their migration into sites of inflammation where the ligands for these receptors, such as MIP-1 and CCL5 (RANTES), are expressed.

Human blood contains two major subsets of DC: CD11c (-) CD123 (+) plasmacytoid dendritic cells (PDCs) [also BDCA-2 (CD302) and BDCA-4 (CD304) positive] and CD11c (+) CD123 (-) myeloid dendritic cells (MDCs) [also BDCA-1 (CD301) positive]. They lack lineage markers of T cells (CD3), B cells (CD19), monocytes (CD14), and NK cells (CD56) (147, 148). Some studies have shown that CD14+ monocytes can serve as MDC precursors, differentiating into MDC under the influence of GM-CSF and IL-4 (149). Monocytes also differentiate into DC when incubated with GM-CSF and IL-3, or SLE serum, and this is thought to be due to IFN-I (150, 151).
Although most, if not all, nucleated cells are capable of producing IFN-I, the existence of a minor population of cells in the peripheral blood that produces large amounts of IFN-I was recognized about 20 years ago (152). These “interferon producing cells” (IPCs) have only recently been shown to be PDCs (153). In response to viral infection or oligonucleotides containing unmethylated CpG motifs, PDCs produce ~1000-fold more IFN-I than most other cell types (153). However, MDCs also can produce large amounts of IFN-I in response to intracellular viral infections (154-156). MDCs produce IFN-I following electroporation or lipofection of the double-stranded (ds) RNA analog PIC in amounts comparable to that of PDCs stimulated with CpG DNA (154). Unlike PDCs, which are activated following endosomal uptake of bacterial DNA or ssRNA in a chloroquine-dependent manner, MDCs are activated following cytoplasmic (non-endosomal) recognition of dsRNA mediated by the cytosolic enzyme protein kinase R (PKR) and/or DAI (154, 157).

DCs also express different subsets of surface receptors mediating innate immunity. Specifically, PDCs express large amounts of TLR7 and TLR9, whereas MDCs express TLR3, TLR4, and TLR8 (147). Thus, PDCs are stimulated preferentially by viral GU-rich single-stranded RNA and unmethylated CpG DNA, whereas MDCs are stimulated preferentially by dsRNA and LPS. In view of the correlation between TLR expression and responsiveness of different types of DCs to microbial stimuli, the differential expression of TLRs is likely to play a significant role in the regulation of IFN-I production by DCs (147). There is evidence that signaling from TLRs can vary depending on the cell type or maturation state (144).

Once activated, immature DCs down-regulate CCR1, CCR2, CCR5, CCR6, CXCR1, and CXCR2, and up-regulate CCR7, a receptor for CCL21 (SLC) and CCR19 (ELC) (147). SLC is produced by high endothelial venules in the secondary lymphoid organs and ELC is expressed in
T cell zones of the secondary lymphoid tissues. Thus, mature MDCs and PDCs migrate to SLC and ELC, which is mediated in part by increased expression of CCR7. Strikingly, although immature PDCs express high levels of CCR7, a maturation signal is required to couple CCR7 to cell migration (148). MDCs respond to CCL2/MCP-1, CCL20/MIP-3α, whereas PDCs do not respond to these chemokines, although they express the corresponding chemokine receptor (158). Thus, not only must the appropriate receptor be expressed in order for DC migration to take place, but the signal must be coupled to cell migration by mechanisms that remain poorly defined.
Figure 1-1. Toll-like receptor signaling pathway and induction of type I interferon. Engagement of TLR3 by dsRNA, TLR7/8 by ssRNA or TLR9 by CpG DNA in endosomes and of TLR4 by LPS at the cell surface mediates IFN-α/β production. TLR3 utilizes a TRIF-dependent pathway, whereas TLR7/8 induces a MyD88-dependent pathway. TLR4 can signal through both TRIF and MyD88 pathways. The activation of adaptor protein TRIF and MyD88 can in turn activate transcription factor NFκB and IRF3, which induces the production of IL-6, TNFα and type I interferon.
Figure 1-2. Production of IFN-I induced by TLR independent pathway. Engagement of RNA with the cytosolic RNA helicases RIG-I and MDA5 induces activation of RIG-I/MDA5, and allow CARD disassociate with RD, and CARD in turn interacts mitochondrial adaptor MAVS (IPS-1). MAVS then activates NFκB through IKKα/β/γ with cooperation of RIP and FADD. It also induces IRF3 phosphorylation through TBK-1/IKKe. In addition, MAVS also activates MAPK and AP-1. Translocation of these transcription factors to the nucleus then induce Ifnb1 transcription.
Figure 1-3. Type I interferon signaling. IFN-I bind to heterogeneous interferon receptor composed by IFNAR1 and IFNAR2, which induces conformation change of IFNAR and recruits adaptor protein Jak1 and Tyk2. Phosphorylation of Jak1 and Tyk2 in turn recruits STAT1/2. IRF9 phosphorylates STAT1 and STAT2 and form a complex with these molecules which translocates to nucleus and bind to RSRE9, inducing the transcription of downstream ISGs including Mx1, OAS etc.
CHAPTER 2  
MATERIAL AND METHOD

Subjects

Subjects seen at the University of Florida Center for Autoimmune Disease were classified as having SLE, scleroderma, Sjogren’s syndrome, polymyositis/dermatomyositis, rheumatoid arthritis, mixed connective tissue disease, or undifferentiated connective tissue disease using established criteria (2). Patient demographics are summarized in Table 2-1. Disease activity was assessed by SLEDAI and renal disease was scored as described (58). Subjects were questioned about viral or bacterial infections within 2 weeks and medication use. Self-reported ethnicity of each subject’s grandparents was recorded along with the subject’s birth date. These studies were approved by the University of Florida Institutional Review Board.

Reagent

Polyinosinic-polycytidylic acid [poly (I:C)] and LPS (Salmonella minnesota Re 595) were from Sigma-Aldrich (St. Louis, MO). CpG oligodeoxyribonucleotide (ODN #2006,) was from Coley Pharmaceutical Group (Wellesley, Massachusetts) and CpG oligodeoxyribonucleotide (ODN #2216, 2395, 2336) are from Invivogen (Carlsbad, CA). Serum-free lymphocyte medium AIM-V and LMG-3 was from Invitrogen Life Technologies (Carlsbad, California). Antibodies against Lin-FITC (a cocktail of anti-CD3, -CD14, -CD16, -CD19, -CD20, and -CD56), CD123-PE, CD11c-APC, HLA-DR-PerCP were from BD-Biosciences (San Diego, CA). Anti-CD14-FITC, anti-CD71-PE, anti-CD36-APC, anti-CD33-APC, anti-CD45RA-PB, anti-CD163-biotin, anti-CD62L-AF750, anti-CD64-FITC, anti-CD16-APC-Cy7 antibodies are from eBioscience (San Diego, CA), anti-CD117-APC antibody is from Biolegend (San Diego CA), anti-BDCA-2- FITC, anti-BDCA-4-APC, anti-CD123-APC antibodies are from Miltenyi Auburn CA)
**RNA Preparation**

Peripheral blood from SLE patients, patients with other autoimmune disease, and healthy controls was collected into PAXgene blood RNA tubes and total RNA was isolated using the PAXgene RNA kit (Qiagen, Valencia, CA). In other experiments, cultured or fragmented peripheral blood cells were lysed in Trizol reagent (Invitrogen) and RNA were isolated as manufacture’s instruction. RNA (1 μg) was treated with DNase I (Invitrogen) to remove genomic DNA and reverse transcribed to cDNA using Superscript First-Strand Synthesis System (Invitrogen) for RT-PCR.

**Real-Time PCR**

Gene expression was determined by real-time PCR using SYBR green (Applied Biosystems, Foster City, CA). Primers used in these experiments are listed in Table 2-2.

One μL of cDNA was added to a reaction mixture containing 3 mM MgCl₂, 1 mM dNTP mixture, 0.025 U of Amplitaq Gold, SYBR Green dye (Applied Biosystems), optimized concentrations of specific forward and reverse primers, and DEPC-treated water in a final volume of 20 μl. Amplification conditions were as follows: 95°C (10 min), followed by 45 cycles of 94°C (15 sec), 60°C (25 sec), 72°C (25 sec), with a final extension at 72°C for 8 minutes. Transcripts were quantified using the comparative \(2^{-\Delta\Delta Ct}\) method. In some experiments, Mx1 and β-actin expression levels were determined by real-time PCR with LUX primers and platinum PCR supermix kit (Invitrogen). Amplification conditions were as follows: 50°C (2 min), 95°C (2 min), followed by 45 cycles of 95°C (15 sec), 55°C (30 sec), 72°C (30 sec), and a final extension at 72°C for 8 minutes.
Effect of Corticosteroids and Antimalarials on Cytokine Production

PBMCs from healthy controls were prepared within 2 hours of collection from heparinized blood by Ficoll-Hypaque density gradient centrifugation. They were cultured for 6 hours at 1.5-2.5 \times 10^6 /ml in the presence or absence of dexamethasone (0.1 or 10 \mu g/ml) or hydroxychloroquine (0.1 or 10 \mu g/ml) or were pre-incubated with dexamethasone or hydroxychloroquine for 1 hour before stimulation with LPS (100 ng/ml), poly (I:C) (50 \mu g/ml), loxoribine (50\mu g/ml) or CpG ODN #2006 (10 \mu g/ml) for 6-hours. RNA was extracted using TRIzol, and gene expression was measured as above.

In other experiments, PBMCs were cultured with 0.01, 0.1 or 1 \mu g/ml of dexamethasone for 6, 16, 48 hours. 50\mu l of cells were used in the following flow cytometry. The rest of cells were lysed in Trizol and culture medium was collect for ELISA.

Serological Testing

ANA were tested by immunofluorescence using HeLa cells as substrate (serum dilution 1:160) and Alexa 488-conjugated goat anti-human \gamma + light chain antibodies (1:50 dilution, Molecular Probes, Eugene, OR). Cells were mounted in Vectashield containing DAPI (Vector Laboratories, Burlingame, CA) to stain DNA. Anti-nRNP, -Sm, -Ro (SS-A), and -La (SS-B) autoantibodies were detected by immunoprecipitation of [35S] labeled K562 (human erythroleukemia cell) extract as described (159). Anti-dsDNA, anti-RNP, -Sm, -Ro (SS-A), and -La (SS-B) autoantibodies antibodies were quantified by ELISA.

Flow Cytometry

Heparinized blood was processed within 4 hours and mononuclear cells were isolated on Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ), washed, and resuspended in PBS + 3% fetal bovine serum. The equivalent of 0.1 ml of starting whole blood was stained with saturating amounts of antibodies (BD Biosciences). For dendritic cell characterization, this
consisted of Lin-FITC (a cocktail of anti-CD3, -CD14, -CD16, -CD19, -CD20, and -CD56), anti-CD123-PE, anti-HLA-DR-PerCP, and anti-CD11c-APC. Following incubation for 30 min at 4°C in the dark, the cells were rinsed in PBS and fixed in 2% paraformaldehyde. Samples were run on a four-color FACSCalibur flow cytometer (BD Biosciences). At least 100,000 events were acquired per sample. In parallel, the red blood cells from 100 μl of whole blood was lysed (BD FACS Lysing Solution), fixed in 2% paraformaldehyde, and used to obtain a total white blood cell count and differential (lymphocytes, monocytes, and neutrophils) by forward and side scatter characteristics. The absolute monocyte count was calculated. These results showed excellent correlation with results from the clinical laboratory. Data were analyzed with FCS Express V2 (DeNovo Software, Thornhill, Ontario). Size gating was used to eliminate any residual neutrophils or RBCs, which were typically less than 15%. MDCs and PDCs were defined as Lin−, HLA−, DR+ cells that were either CD11c−, CD123+, (PDCs) or CD11c+, CD123− (MDCs). Absolute numbers of dendritic cells and monocytes were calculated as a percentage of the complete blood cell count.

**Karyotype Analysis**

Metaphase chromosome spreads were prepared from PHA-stimulated PBMC cultures following standard cytogenetic procedures. Chromosome preparations were banded utilizing a standard GTG-banding procedure. Metaphase imaging and karyotype production were facilitated by computer assisted methods (CytoVision software, Applied Imaging). Twenty metaphase cells were analyzed for each family member studied.

**Cell Enrichment**

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation. Monocytes, B cells and plasmacytoid dendritic cells (PDCs) were positively selected by anti-CD14, anti-CD19 or anti-CD304 (BDCA4) labeled magnetic
microbeads, respectively. The purity of the positively selected cell fractions was typically 90% or greater.

**Flow Cytometric Cell Sorting**

PBMCs were isolated on Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ), washed and resuspended in 1ml of RPMI1640 with 10% fetal bovine serum. Cells were stained with anti-CD14-FITC (BD science) and anti-CD71-PE (ebioscience). CD14⁺CD71⁺ and CD14⁺CD71⁻ cells were sorted with FACS Vantage SE Turbosort (BD Bioscience).

**Electron Microscopy**

Sorted CD14⁺CD71⁺ or CD14⁺CD71⁻ cells were fixed in 2% paraformaldehyde and 1% glutaraldehyde in Tyrode buffer over night. After washing with tyrode buffer, fixed cells were washed with 0.1 M Na cacodylate pH=7.4 for 15 min, stained with 2% osmic acid in 0.1 M Na cacodylate buffer at 4°C for 1 hour. TAAB mixture consists TAAB: Dodecenylsuccinic anhydride: Methyleneic anhydride (Sigma-Aldrich, Inc, St. Louis, MO) 48:19:33 were prepared in advance. Stained cells were dehydrated in graded concentration of ethanol, and immersed in 1:1 TAAB: ethanol for 1 hour, 2:1 TAAB: ethanol overnight and 100% TAAB for 2 hours, and 100% TAAB with 2,4,6-Tris(dimethylaminomethyl)phenol (dmp-30) (Sigma) for 1.5 hours before embedded in TAAB with dmp-30 and polymerized at 60°C overnight. Blocks were trimmed, sectioned to 800-900 nm, placed onto 150 mesh copper grids and allowed to air dry for 30 minutes. Sections were floated in filtered alcoholic 8% uranyl acetate for 4 min and washed with 50% ethanol and allowed to dry for 30 min. Grids were stained with one drop of lead citrate/grid for 60 seconds and washed with distilled water and before drying. Sections were viewed with Zeiss EM10 electron microscope (Carl Zeiss, Inc., Peabody MA) and images were captured by Finger Lakes digital camera (Finger Lakes Instrumentation, LLC., Lima, NY)
**Statistical Method**

All tests were done with Prism 4.03 (GraphPad software). Difference among categories was tested with one way ANOVA or student t test. Significance was reached when P<0.05.
Table 2-1. Patient demographics

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¹ Includes Sjogren’s syndrome (13), polymyositis/dermatomyositis (6), scleroderma (9), undifferentiated connective tissue disease (5), rheumatoid arthritis (2), positive ANA (27), and miscellaneous diagnoses (20)

² methotrexate, azathioprine, cyclophosphamide, or mofetil mycophenolate
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CHAPTER 3
RESULTS AND DISCUSSION: ASSOCIATION OF ANTI-NUCLEOPROTEIN AUTOANTIBODIES WITH TYPE I INTERFERON PRODUCTION IN SYSTEMIC LUPUS ERYTHEMATOSUS

Introduction

Most SLE patients produce autoantibodies against ribonucleoproteins, such as the Sm/RNP, Ro (SS-A), and La (SS-B) antigens, or deoxyribonucleoproteins such as chromatin (160-162), often to the exclusion of other cellular antigens. It is unclear why nucleoproteins are selectively targeted for an autoimmune response in this disease. We have reported that the RNA components of the Sm/RNP and Ro60 antigens, U1 RNA and Y1-5 RNAs, respectively, stimulate the production of Type I interferons (IFN-I) and induce dendritic cell maturation, hallmarks of the adjuvant effect (163).

The Type I interferons IFNα and IFNβ promote dendritic cell maturation, primary and memory B cell responses, and are powerful adjuvants (164). Precursors of plasmacytoid dendritic cells (PDCs), the primary IFN-I producing cells, represent ~0.5% of peripheral blood mononuclear cells (PBMCs) (165, 166) and express IL-3 receptors (CD123) and MHC class II, but not CD11c or other lineage (lin) markers (165, 167). Myeloid dendritic cell (MDC) precursors (CD11c+, CD123−, class II+, lin−) also circulate (168) and undergo maturation in response to IFN-I (169, 170). The maturation state of MDCs is a key determinant of whether antigen presentation is tolerogenic as in the case of self-antigens presented by immature MDCs or immunogenic, as in the case of foreign antigens presented by MDCs activated by Toll-like receptor (TLR) ligands (171). Double-stranded (ds) RNA and certain single-stranded (ss) RNAs are ligands for TLR3 and TLR7/8, respectively (66, 105, 172, 173) and cellular DNA sequences have recently been shown to activate TLR9 (174). Engagement of Toll-like receptors by these nucleic acid ligands stimulates IFN-I production through MyD88-dependent and
MyD88-independent pathways. Since nucleoproteins carry endogenous TLR ligands in the form of their associated nucleic acids, it was of interest to see if autoantibodies against the Sm/RNP, Ro (SSA), and chromatin (dsDNA) autoantigens are associated with high levels of IFN-I.

**Results**

**Increased IFN-I Production and Low Peripheral Blood Dendritic Cells in SLE**

As total IFN-I consists of 14 IFN\(\alpha\) isoforms plus IFN\(\beta\), the specificity of ELISA depends on the specificities of the detection antibodies (175). Measurement of the expression of IFN-I inducible genes such as Mx1 by real-time PCR permits an estimate of total IFN-I production and has been shown previously to correlate with IFN-I levels (176). We first examined the stability of mRNA from PBMCs to verify that degradation did not occur during processing. Mx1 and \(\beta\)-actin mRNA from blood collected in PAXGene tubes was stable for 8 hours at room temperature and was largely intact after 5 days at room temperature (Fig. 3-1A). When processed immediately, Mx1 expression levels were similar in blood collected in heparinized tubes vs. PAXGene tubes (Fig. 3-1B). However, when blood collected in heparinized tubes was incubated at room temperature, there was a time-dependent increase in Mx1 expression suggesting that the PBMCs became activated after blood collection (Fig. 3-1B). The increase was nearly 3-fold at 3 hours of incubation. For this reason, gene expression in this study was measured using blood collected in PAXGene tubes.

Mx1 expression was increased up to 7000-fold in a subset of SLE patients in comparison with healthy controls and patients with other autoimmune conditions (\(P < 0.0001\), ANOVA; post-test comparison data shown in the figure) (Fig. 3-1C). Expression was higher than the mean of healthy controls in 22\% of patients and higher than the mean + 2 S.D. (= 642 units) in 22\% of SLE patients. Expression of a second IFN-I regulated gene, OAS, correlated well with Mx1 (\(P <\)
0.0001 Pearson’s correlation test) (Fig. 3-1D). In addition, the Mx1 expression in PBMCs correlated well with levels of mRNA for the Type I interferon IFNβ ($R^2 = 0.68$, $P < 0.0001$ Pearson’s correlation test) (Fig. 3-1E). In other experiments, we found that Mx1 expression was induced in K562 cells by IFN-I but not by IL-6, TNFα, or IFNγ (data not shown), verifying its specificity for IFN-I.

We next examined the numbers of circulating PDC precursors, the major IFN-I producing cell type. Peripheral blood PDC counts (CD123+, CD11c−, lin− cells) were greatly reduced in SLE patients ($n = 126$) vs. healthy controls ($n = 66$) as were the MDC counts (CD123−, CD11c+, lin− cells) ($P < 0.0001$ for both PDCs and MDCs, unpaired t-test) (Fig. 3-2A-B). About half (52%) of lupus patients had low PDC and MDC counts defined as values lower than 95% of controls (abnormal < 0.66 PDCs/μl or < 1.1 MDCs/μl) whereas 20% had values lower than the mean of healthy controls. PDC and MDC counts correlated highly with one another ($P < 0.0001$, Pearson’s correlation test) (Fig. 3-2C). In contrast, monocyte counts were comparable in SLE vs. healthy controls (Fig. 3-2D). Taken together, these data indicate that SLE is associated with high IFN-I production and low numbers of circulating PDC and MDC precursors.

**IFN-I and PDC/MDC Abnormalities are not Explained by Medication Use**

The high IFN-I (Mx1) expression and the low numbers of circulating PDCs and MDCs could be a consequence of the disease itself or of therapy. The two most common medications used in our lupus cohort were antimalarials (mainly hydroxychloroquine) and corticosteroids (mainly prednisone). In comparison with patients not receiving antimalarials, those receiving antimalarials did not have higher or lower PDC/MDC counts or Mx1 expression (Fig. 3-3A). Overall comparison of SLE patients and controls also did not reveal differences attributable to corticosteroid treatment, but when the SLE patients were considered separately from controls,
both absolute PDC (P = 0.04, unpaired t-test) and MDC (P = 0.01, unpaired t-test) counts were lower in steroid-treated patients than in patients not receiving steroids, suggesting that corticosteroids could have a modest effect (Fig. 3-3B). There also was a trend toward higher Mx1 expression in steroid-treated patients (P = 0.06, unpaired t-test). Thus, although the low PDC/MDC counts were not primarily due to corticosteroid therapy, steroids may have a small effect or else the patients with low PDC/MDC counts may be more likely to receive steroids.

To further explore the effects of pharmacotherapy, PBMCs from healthy controls were pre-incubated for 1 hour with dexamethasone or chloroquine before adding LPS, poly (I:C), or CpG oligodeoxyribonucleotide. As shown in Fig. 3-3C, dexamethasone (0.1-10 μg/ml) had no effect on either basal (unstimulated) or TLR ligand-stimulated Mx1 expression. However, as expected (177) expression of IL-6 (Fig. 3-3C) and TNFα (not shown) mRNA decreased in a dose-dependent manner, suggesting that dexamethasone inhibited TLR4 signaling via the MyD88/NFκB dependent pathway more than signaling via the TRIF/IRF-3 pathway. These in vitro results are consistent with the in vivo data (Fig. 3-3B). Chloroquine (0.1-10 μM) also had no effect on LPS- or poly (I:C)-stimulated IFN-I (Mx1) or IL-6 production (Fig. 3-3D). However, IFN-I and IL-6 production stimulated by CpG DNA (TLR9 ligand) were inhibited in a dose-dependent manner as expected (104).

**Increased Mx1 and Low PDC/MDC Counts Are Associated With a Subset of Autoantibodies**

In view of the association between exogenous IFNα administration and antinuclear antibodies, we investigated whether increased endogenous IFN-I production and low PDC/MDC counts were associated with autoantibody production. SLE patients who were anti-Sm/nRNP autoantibody positive had higher Mx1 expression than those who were negative; similarly, patients with autoantibodies against the Ro (SSA) or La (SSB) ribonucleoproteins had higher
IFN-I levels than those without (P = 0.0025, ANOVA; post-test comparison data shown in the figure) (Fig.3-4A). When Mx1 expression was sorted and the SLE patients were divided into four equal groups (group 1 = lowest Mx1, group 4 = highest), the number of patients with anti-Sm/RNP/Ro/La autoantibodies increased progressively, further suggesting that higher Mx1 expression was associated with a higher likelihood of having autoantibodies against ribonucleoproteins (not shown). Autoantibodies to dsDNA also were associated with high Mx1 (P = 0.02, unpaired t-test) (Fig. 3-4B). In striking contrast, Mx1 expression was negatively associated with anti-phospholipid antibodies (APLA, P < 0.0001, unpaired t-test) (Fig. 3-4C), suggesting that increased IFN-I production was characteristic only of the subset of patients producing autoantibodies against RNA-protein (anti-Sm, -RNP, -Ro/SSA, or -La/SSB) or DNA-protein (anti-dsDNA) antigens.

Similarly, anti-dsDNA, -nRNP and -Sm antibodies were associated with low circulating MDC counts (P = 0.02 and P = 0.03, respectively, unpaired t-test) whereas anti-phospholipid antibodies were not (P = 0.16) (Fig. 3-5). Strikingly, anti-nRNP/Sm autoantibodies (but not anti-dsDNA or anti-phospholipid antibodies) also were associated with low PDC counts (P < 0.0001). These data suggest that the association of autoantibody production with increased IFN-I expression and decreased circulating PDC/MDC precursors is selective for ribonucleoprotein (Sm, RNP, Ro60, La) and deoxyribonucleoprotein (dsDNA) antigens.

**Relationship of Mx1 Expression and Dendritic Cell Counts to Disease Activity**

Clinical significance of the abnormal IFN-I production and low PDC/MDC counts was evaluated using the number of ACR SLE criteria met (a measure of extent of disease) and the presence or absence of renal involvement. There was no apparent relationship between Mx1 expression and number of ACR criteria met or the presence of renal disease (Fig. 3-6). In contrast, low PDC/MDC counts were strongly associated with more extensive disease and renal
involvement (Fig. 3-6). Thus, the disappearance of PDCs and MDCs from the peripheral blood is associated with more severe immune complex disease.

**Discussion**

Autoantibodies to small ribonucleoproteins, such as the U1, U2, U4-6, and U5 small nuclear ribonucleoproteins (snRNPs) and the cytoplasmic Ro60 (Y1-Y5) ribonucleoproteins are strongly associated with systemic autoimmune diseases such as SLE and Sjögren’s syndrome (161, 162, 178). Over half of lupus patients produce autoantibodies against one or more of these antigens and about 70% produce anti-dsDNA antibodies (179). Titers of these autoantibodies can be extraordinarily high, sometimes approaching 20% of the total immunoglobulin (180). The reasons for the striking propensity of lupus patients to produce autoantibodies against nucleoprotein antigens remain poorly defined despite intensive study. We recently reported that U1 RNA and the Y1-5 RNAs are immunostimulatory and others have shown that eukaryotic DNA may be stimulatory as well (181), suggesting that one explanation for the high frequency of autoantibodies against nucleoproteins may that they carry “endogenous adjuvant” in the form of immunostimulatory nucleic acids. Adjuvant activity is operationally defined as the capacity to induce proinflammatory cytokines, notably IFN-I or IL-12 and to promote dendritic cell maturation as indicated by increased expression of CD80, CD86, class II, and CD40 and homing to lymphoid tissues (148, 164, 182).

The present findings add support to the idea that the production of autoantibodies against nucleoprotein autoantigens is associated with evidence of adjuvant activity. As compared with autoantibody negative SLE patients, patients who produced anti-RNP/Sm or anti-Ro60 autoantibodies had increased Mx1 and OAS gene expression, markers of the production of IFN-I (Fig. 3-1). Expression of IFNβ was increased as well (Fig. 3-1). These changes were specific for
patients with anti-nucleoprotein autoantibodies, as anti-phospholipid autoantibody positive patients had significantly lower IFN-I expression than negative patients. Increased expression of IFN-I inducible genes has been noted previously in SLE (141, 142, 183) and increased production of the proteins has been reported as well (184-186), though this is seen less consistently. The difficulty in detecting IFN-I proteins in the serum of SLE patients may relate to interference of rheumatoid factors with sandwich-type ELISAs as well as the binding of circulating IFN-I to IFN-I receptors, the expression of which is widespread. Because the 14 or more Type I IFNs all interact with the same receptor (187), measurement of Mx1 gene expression by real-time PCR affords the means of specifically assaying the bioactivity of all Type I IFN proteins, offering significant advantages over the measurement of serum IFN-I levels using sandwich ELISAs. This approach has been validated in patients treated with exogenous IFNα (176) and we confirmed a strong correlation between the expression of Mx1 and the expression of other IFN-I inducible genes (Fig. 3-1).

The increased Mx1 expression seen here in ~20% of adult lupus patients differs from previous reports that virtually all children (95%) and adults (77%) with SLE over-express IFN-inducible genes (141, 142). This likely reflects primarily differences in data analysis. In the microarray studies, increased gene expression was defined as greater than the expression in healthy controls (141, 142). Here, increased Mx1 was defined as greater than the mean + 2 S.D. of the normal group. Using the former definition, ~60% of our SLE patients had increased Mx1, a figure approximating what was reported previously. An alternative explanation is that the lower prevalence of high IFN-I seen here was related to mRNA degradation. We think this is unlikely because the Mx1, OAS, IFNα2, and IFNβ expression levels correlated with each other and all were compared with β-actin expression. It would therefore be necessary to postulate that the
degradation was specific for interferons and IFN-I inducible genes. Moreover, all blood samples in the present study were collected in PAXgene tubes to minimize RNA degradation. The effectiveness of this approach is illustrated by Fig. 3-1A. Indeed, collection of blood in heparinized tubes is a potential source of bias (Fig. 3-1B) that may have contributed to the high frequency of elevated IFN-I inducible gene expression reported in prior studies.

Along with IFN-I production, dendritic cell activation is a hallmark of the adjuvant effect (182). Although we have not shown directly that the production of autoantibodies against the U1 and Y1-5 ribonucleoproteins or against dsDNA is associated with dendritic cell activation, the deficiency of circulating PDC and MDC precursors in SLE (Fig. 3-2) is likely an indirect measure of dendritic cell activation. Activated PDCs home to lymphoid tissues (188) where they produce IFN-I (166), which then promotes MDC maturation (127, 150). Like PDCs, MDCs acquire chemokine receptors mediating homing to secondary lymphoid tissues (189). This may explain the strong correlation between the numbers of circulating MDCs and PDCs (Fig. 3-2C). Similar mechanisms may account for the PDC/MDC deficit in HIV infection (190). Although it has been suggested that certain medications, notably corticosteroids, can cause the number of circulating PDCs to fall (191, 192), standard doses of antimalarials or oral corticosteroids did not have a major effect on either Mx1 expression or the numbers of circulating dendritic cell precursors, as SLE patients had higher Mx1 expression and lower PDC and MDC counts than healthy controls regardless of whether or not they were treated with these medications (Fig. 3-3). It is worth noting, however, that the corticosteroid doses reported to depress PDC counts (192) were substantially higher than the usual doses of oral prednisone. In preliminary studies, we have confirmed that high dose methylprednisolone (1 gram IV daily for 3 days) decreases Mx1 expression and PDC/MDC counts (data not shown). However, in patients receiving a daily dose
of up to 60 mg of prednisone orally, the low PDC/MDC counts probably reflect either abnormal production or increased homing to other sites. Circulating PDC/MDC precursors originate in the bone marrow and are mobilized by G-CSF and other cytokines (193). It remains to be determined whether the low numbers of circulating PDCs and MDCs in lupus reflects deficient production of G-CSF or other cytokines or abnormally rapid PDC/MDC maturation and recruitment to the periphery.

A major question raised by our findings is whether increased IFN-I production is a primary stimulus of the production of autoantibodies to nucleoproteins or a secondary event resulting from the endosomal processing of the nucleic acids associated with these antigens. Certainly, it has been shown that therapy with IFNα is associated with autoimmune phenomena, including antinuclear antibody production and the induction of overt autoimmune diseases such as autoimmune thyroiditis and SLE (139, 194). However, the specificities of the autoantibodies appearing during IFNα therapy have not been investigated systematically, and it is unclear whether there is a preference for nucleoprotein antigens. Conversely, in view of the ability of U1 and Y1-5 RNA as well as unmethylated CpG DNA motifs to stimulate IFN-I production, it is possible that the increased production of IFN-I seen in patients who produce autoantibodies to nucleoproteins is secondary to the activation of dendritic cells due to the endosomal uptake of these antigens (163). In this case, conditions that enhance the exposure of activated MDCs to nucleoproteins, perhaps in the form of blebs (195) from apoptotic cells, may be critical to the generation of autoantibodies. The two possibilities are not mutually exclusive. Mechanisms exist to prevent the activation of B cells upon exposure to endogenous chromatin and dendritic cells might also be susceptible to desensitization under appropriate conditions. Exposure to either endogenous or exogenous IFNα could lead to partial activation of dendritic cell precursors
and/or monocytes, thereby bypassing this checkpoint. When these cells encounter nucleoprotein autoantigens such as the U1 or Y1-5 ribonucleoproteins, they may undergo further maturation into fully immunostimulatory MDCs capable of inciting an immune response against peptides derived from self-antigens. In this scenario, exposure of partially activated MDCs or monocytes to immunostimulatory small RNAs derived from self-antigens may act as a positive feedback mechanism promoting autoimmunity, which is directed mainly against the protein components of these antigens. Importantly, the nucleic acid components of autoantigens may also act on B cells to promote autoantibody production. For instance, the interaction of cellular DNA from chromatin with TLR9 acts in concert with B cell receptor interactions to promote B cell activation. Similarly, since B cells express TLR7/8, the likely receptor for U1 and Y1-5 RNA (163), it is likely that the RNA components of self antigens also can promote B cell activation. Thus, IFN-I and endogenous nucleic acids with immunostimulatory properties may act together to promote autoantibody production and the formation of immune complexes, leading to immune complex disease. The association of high Mx1 expression and low numbers of circulating PDCs and MDCs with both extent of disease and lupus nephritis (Fig. 3-6) is compatible with this model.

In conclusion, immunostimulatory effects of the nucleic acid components of autoantigens on dendritic cell activation/maturation and IFN-I production may help explain the high prevalence of autoantibodies against nucleoprotein antigens in SLE, raising the possibility that therapy directed against IFN-I might lower autoantibody levels and alleviate disease. Very recent studies in mice support the potential value of this approach (196).
Figure 3-1. Increased expression of IFN inducible genes in SLE. A) Stability of PBMC RNA in PAXgene tubes. Whole blood was incubated in PAXgene tubes at room temperature for 2 hours to 5 days and then processed. B) Mx1 expression in heparinized blood. Blood samples were collected either in sodium heparin tubes or PAXgene tubes and incubated at room temperature for 0 to 6 hours before isolation of RNA for quantification of Mx1 expression by real-time PCR. C) Mx1 expression in SLE patients and controls. Mx1 expression (real-time PCR) in SLE patients (n = 88) was increased in comparison with healthy controls (n = 57) and patients with other diagnoses (n = 82). Levels were higher in SLE vs. controls and other diagnoses (P < 0.0001, ANOVA; P < 0.001 SLE vs. Control; P < 0.001 SLE vs. Other (Bonferroni’s multiple comparison test). Dotted line shows the mean of healthy controls + 2 S.D. (642 cells/μl). NS, not significant. D) Correlation of Mx1 and OAS expression. Mx1 gene expression correlated strongly with expression of the IFN-I inducible gene OAS (real-time PCR) in a group of 54 consecutively tested patients from the original 88 SLE patients (R^2 = 0.18, P < 0.0001 Spearman rank correlation test). E) Correlation of Mx1 and IFNβ expression. Mx1 expression correlated strongly with expression of IFNβ (real-time PCR) in a group of 38 consecutively tested SLE patients (R^2 = 0.68, P < 0.0001 Spearman rank correlation test).
Figure 3-2. Reduced circulating PDCs and MDCs in SLE. Circulating PDC and MDC counts were analyzed in 33 controls and 76 SLE patients by flow cytometry. PDCs were defined as lineage- (CD3, CD14, CD16, CD19, CD20, CD56); HLA-DR+, CD123+, CD11c-. MDCs were defined as lineage-, HLA-DR+, CD123-, CD11c+. A) and B) Absolute PDC and MDC counts. The dotted lines indicate the 5th percentile of healthy control counts (0.66 cells/μl and 1.1 cells/μl for PDCs and MDCs, respectively). C) Correlation of the absolute PDC and MDC counts (P < 0.0001, Pearson’s correlation test). D) Absolute monocyte counts. Monocyte counts in SLE patients vs. controls were not significantly different.
Figure 3-3. Effect of medications on Mx1 expression in SLE patients. A) Effect of antimalarial therapy. Peripheral blood PDC and MDC counts and Mx1 expression in patients treated with antimalarials (hydroxychloroquine or quinacrine), patients not taking antimalarials, and controls. B) Effect of corticosteroid therapy. Peripheral blood PDC and MDC counts and Mx1 expression in SLE patients treated with corticosteroids vs. patients not taking steroids and controls. In each of the figures, the difference between the three groups was analyzed by ANOVA. Bonferroni’s test was used to measure the inter-group differences. C) In vitro effect of corticosteroids. PBMCs were pre-treated with dexamethasone (0, 100 ng/ml, or 10 g/ml) for 1 hour prior to stimulation with poly (I:C) (PIC, 50 g/ml), LPS (100 ng/ml), or CpG ODN (10 μg/ml) for 6 hours. Expression of Mx1 and IL-6 was measured by real-time PCR normalized to -actin. D) In vitro effect of antimalarials. PBMCs were pre-treated with chloroquine (0, 100 nM, or 10 mM) for 1 hour prior to stimulation with PIC, LPS, or CpG ODN and measurement of Mx1 and IL-6 expression as in C.
Figure 3-4. Increased Mx1 expression correlates with autoantibody production. A) Autoantibodies to ribonucleoproteins. Mx1 expression was increased in individuals with either anti-nRNP/Sm or anti-Ro (SS-A)/La (SS-B) (immunoprecipitation assay) vs. individuals without any of these specificities (P = 0.0025, ANOVA; P < 0.01 anti-nRNP/Sm vs. control; P < 0.05 anti-Ro/La, Bonferroni’s multiple comparison test). B) Anti-DNA autoantibodies. Mx1 expression also was increased in patients with anti-dsDNA antibody (ELISA, P = 0.02, unpaired t-test). C) Anti-phospholipid antibodies. Mx1 expression was negatively associated with the presence of anti-phospholipid antibodies (IgG or IgM anti-cardiolipin by ELISA or lupus anticoagulant, P < 0.0001, unpaired t-test).
Figure 3-5. Decreased dendritic cell counts correlate with autoantibody production. Absolute peripheral blood counts of myeloid dendritic cells (mDC, left) and plasmacytoid dendritic cells (pDC, right) were determined (flow cytometry) in patients who were positive or negative for anti-dsDNA antibodies (Top), anti-RNP/Sm antibodies (middle), or anti-phospholipid antibodies (APLA, bottom). MDC counts were significantly lower in anti-dsDNA and anti-RNP/Sm positive patients than in autoantibody negative patients (P = 0.02 and P = 0.03, respectively, unpaired t-test). PDC counts were significantly lower in anti-RNP/Sm positive patients than in autoantibody negative controls (P < 0.0001, unpaired t-test).
Figure 3-6. Relationship of Mx1 expression, PDC and MDC counts to disease severity. Mx1 expression (real-time PCR) and PDC/MDC counts (flow cytometry) were correlated with extent of disease (number of SLE criteria met) in 66 SLE patients (Spearman). Mx1 expression and PDC/MDC counts in SLE patients with and without renal involvement were compared by unpaired t-test.
CHAPTER 4
RESULTS AND DISCUSSION: CD71+ MONOCYTES IN PERIPHERAL BLOOD FROM LUPUS PATIENTS PRODUCE INTERFERON α/β

Introduction

Most lupus patients produce abnormal high levels of interferons (IFN) α and β as indicated by the up-regulation of a group of IFN-I-stimulated genes (ISGs) (141, 142, 183, 197). This “interferon signature” correlates with active disease, renal involvement, and the production of lupus-associated autoantibodies such as anti-dsDNA and anti-Sm/RNP. Therapeutic use of recombinant IFNα is associated with a variety of autoimmune disorders including SLE (139). Moreover, a lupus-like disease with excess IFN-I production was seen in two patients with a chromosomal translocation resulting in trisomy of chromosome 9p, which carries the IFN-I gene cluster (198). These observations suggest that Type I interferons (IFN-I) could be involved in the pathogenesis of SLE.

Further evidence for a direct pathogenic role of IFN-I comes from the observation that experimental lupus induced by tetramethylpentadecane (TMPD) is greatly attenuated in mice lacking the Type I interferon receptor (199). Although plasmacytoid dendritic cells (PDCs) are the most efficient IFN-I producing cells (153, 166), studies of TMPD lupus indicate that immature monocytes bearing the marker Ly6C are responsible much of the IFN-I (200). These cells have low phagocytic activity, express high levels of TLR7, and respond well to TLR7 ligands, but only weakly to TLR9 ligands [(200) and PY Lee, et al., Submitted].

A monocyte phagocytosis defect affecting the clearance of dying cells has been reported in about half of lupus patients (201-203). This has been attributed to defects in the phagocytes themselves as well as serum factors (203, 204). Since the immature interferon-producing monocytes in TMPD-lupus also are poorly phagocytic (200), we hypothesized that similar cells in the peripheral blood of SLE patients might be responsible for the observed monocyte
phagocytosis defect. Although Ly6C is not expressed by human monocytes, increased numbers of circulating CD71+ (transferrin receptor 1+) monocytes are seen in SLE (205). CD71 is expressed on rapidly dividing cells and is generally expressed at only low levels in non-proliferating cells (206). Thus, CD71 may be a marker for relatively immature/proliferating monocytes. In the present study, we show that in SLE patients, circulating monocytes bearing CD71 produce significant amounts of IFN-I and may be the human equivalent of the Ly6C\text{hi} monocyte subset in murine lupus.

**Results**

**Low PDC Numbers Correlate with High Interferon Levels in SLE**

In normal individuals, circulating PDCs express endosomal TLR7 and TLR9, and upon stimulation by virus or other ligands, produce large amounts of IFN-I (182, 207). Consistent with previous reports (197, 208), we found that PDCs are depleted from the blood of lupus patients compared with healthy controls (Fig. 4-1A-B). Using expression of the IFN-I inducible gene Mx1 as a measure of total IFN-I expression (mainly IFN\(\alpha\) and IFN\(\beta\)), we found a negatively relationship between Mx1 expression and circulating PDCs (Fig. 4-1C). It has been suggested that this apparent paradox may be explained by sequestration of the IFN-I producing cells in affected tissues, such as the skin lesions of SLE patients (209, 210). However, we found that peripheral blood mononuclear cells (PBMCs) from SLE patients expressed significantly higher levels of IFN\(\alpha\)2 than those of healthy controls (\(P < 0.001\), Mann Whitney test) (Fig. 4-2A). Interestingly, when PBMCs from SLE patients were fractionated into CD14+ and CD14- populations using magnetic beads, the CD14+ subset exhibited higher IFN\(\alpha\)2 expression than the CD14- subset (\(P < 0.01\), Mann-Whitney). Similarly, PBMCs from SLE patients expressed high levels of IFN\(\beta\) mRNA, a significantly higher fraction of which was found in the CD14+ subset than in the CD14- subset (\(P < 0.05\), Mann-Whitney) (Fig. 4-2B). In contrast, depletion of PDCs
had little effect on the level of IFNβ mRNA in lupus PBMCs. PBMCs and CD14+ cells from healthy controls also expressed IFNβ, but the level was about 20-fold lower than that seen in lupus PBMCs (Fig. 4-2C). In contrast to the SLE patients, IFNβ mRNA seemed to be associated with PDCs in majority.

**CD14+ Cells from SLE Patients Produce IFN-I in Response to TLR Ligands**

Endogenous and exogenous RNA ligands can stimulate TLR7 to induce IFN-I production. In lupus PBMCs, TLR7 expression was significantly higher than normal controls but not patients with other autoimmune disease (Fig 4-3A) (One way ANOVA, P<0.05). Interestingly CD14+ monocytes from lupus patients expressed TLR7, but in lower level compared to B cells (Fig 4-3B). Normal CD14+ monocytes express TLR7 in very low level (data not shown). IRF7, a transcription factor involved in TLR signaling, was expressed higher in monocytes compare to non-monocytes (Fig 4-3C). Flow cytometry revealed a significant stronger CD14 expression on lupus monocytes compared to normal individuals (Fig. 4-3D). These results indicated abnormal TLR signaling in SLE monocytes. Along this line, we stimulated PBMCs, CD14+ monocytes and CD14- fraction with TLR3, 4, 7, 9 ligands PIC, LPS, loxoribine, or CpG 2216 for 6 hours. As expected, CD14+ monocytes from lupus patients responded to LPS challenge and produced cytokines such as IL-6 (Fig 4-3E). LPS stimulation also induced IFNβ expression by lupus monocytes (Fig. 4-3F). Interestingly, lupus monocytes also responded to stimulation of loxoribine, a TLR7 ligand. Loxoribine stimulated IFN-I production in lupus monocytes was higher than LPS induced response (Fig. 4-3F) whereas IL-6 production in response to LPS was higher than loxoribine (Fig. 4-3E). CpG2216 induced IFN-I production in CD14- cells (presumably dendritic cells and B cells), whereas it did not stimulate cytokine production above baseline in CD14+ monocytes (Fig. 4-3F).
Circulating CD71⁺ Monocytes in SLE Express IFN-I

In agreement with a previous study (205), we found increased expression of CD71 on the surface of CD14⁺ monocytes from a subset of SLE patients vs. healthy controls (P = 0.01, Mann-Whitney test) (Fig. 4-4A-C). The % of monocytes positive for CD71 negatively correlated with the number of circulating PDCs (Fig. 4-4D) and correlated positively with the expression of IFNβ mRNA expression in PBMCs and with interferon-inducible gene (Mx1) expression, a marker of IFN-I protein level (P = 0.001 and P = 0.01 respectively, Spearman’s test) (Fig. 4-4E,F).

To directly examine IFN-I production by the CD14⁺CD71⁺ subset, we isolated these cells by FACS sorting. Both CD14⁺CD71⁺ and CD14⁺CD71⁻ cells expressed more IFNβ mRNA than CD14⁻ cells, but the highest levels were seen in CD14⁺CD71⁺ monocytes (p = 0.02, one-way ANOVA) (Fig. 4-5A). Moreover, by electron microscopy the sorted CD14⁺CD71⁺ cells exhibited typical monocytoid morphological features more consistent with the features of immature monocytes than of more mature phagocytes (Fig. 4-5B). The extensive rough endoplasmic reticulum typical of PDCs was not seen in this cell population. Longitudinal analysis of CD71⁺ monocytes and Mx1 gene expression provided further evidence that CCD71⁺ monocytes produce physiologically significant amounts of IFN-I (Fig. 4-5C). As shown in Figure 4-5C, the number of CD71⁺ monocytes closely paralleled total IFN-I levels over time. This was not explained by an induction of CD71⁺ expression by IFN-I, as monocytes cultured in vitro with IFNα failed to upregulate CD71 (data not shown).

Increased CD14⁺CD71⁺ Monocytes in Patients with Anti-U1A Antibodies

SLE patients who make autoantibodies against ribonucleoproteins such as the U (anti-Sm/RNP) and Y (anti-Ro) ribonucleoproteins have higher IFN-I levels than patients who do not produce these antibodies (197). Circulating CD71⁺ monocyte numbers were higher in
anti-U1A (anti-RNP) positive SLE patients than in anti-U1A negative patients (P = 0.01, Mann-Whitney test) (Fig. 4-6). In contrast, there was no difference in the number of circulating PDCs and myeloid dendritic cells (MDCs) (Fig. 4-6 B-C).

**Phenotype of CD14⁺CD71⁺ Cells**

To further characterize the surface properties of CD14⁺CD71⁺ monocytes, we analyzed additional monocyte markers by flow cytometry (Fig. 4-7). The expression of HLA-DR, and CD64 (FcγRI), and CD62L was significantly increased on the CD14⁺CD71⁺ population vs CD14⁺CD71⁻ cells (Fig. 4-7A,B). Expression of PDC marker CD123 and myeloid marker CD11c was not upregulated on these cells (Fig. 4-7D and data not shown). The CD14⁺CD71⁺ population are negative for the PDC markers BDCA-2, BDCA-4 or MDC marker BDCA-1 (Data not shown). These data suggested that a population of activated (CD71⁺, HLA-DR⁺, CD62⁺, CD64⁺) monocytes produces substantial amount of IFN-I in SLE and that these cells do no express high levels of surface markers characteristic of dendritic cells (CD123, CD11c)

**Discussion**

There is increasing evidence that IFN-I is involved in the pathogenesis of SLE, but the source of the high levels of IFN-I in lupus is unknown. Although many cell types can produce type I IFNs in response to viral infection, PDCs are the most efficient IFN-producing cells (153, 166). Although individual PDCs can generate up to 1000-fold more IFNα then other cell types in response to viral infection, the frequency of circulating PDCs is greatly decreased in SLE patients (197, 208). Nevertheless, the expression of IFN-I is significantly higher in SLE peripheral blood than in normal controls (141, 142, 183, 197). The presence of PDCs in SLE patients’ skin lesions has been invoked as an explanation for this paradox (209, 210). However, recent data in TMPD-induced murine lupus indicate that most of the IFN-I is derived from an
immature Ly6C\textsuperscript{hi} monocyte population (200). The present study was an effort to determine whether a similar cell type might be involved in IFN-I production in human lupus. As human monocytes do not express Ly6C, additional surface markers were evaluated. We show here that CD14\textsuperscript{+}CD71\textsuperscript{+} monocytes produce a significant amount of IFN-I in SLE and may be the human equivalent of Ly6C\textsuperscript{hi} monocytes in murine lupus. Depletion of these cells from PBMC populations using magnetic beads greatly reduced the amount of IFN\textalpha and IFN\textbeta mRNA whereas purified CD14\textsuperscript{+}CD71\textsuperscript{+} cells exhibited higher levels of IFN\textbeta gene expression than CD14\textsuperscript{+}CD71\textsuperscript{-} or CD14\textsuperscript{-} cells. Moreover, the number of circulating CD71\textsuperscript{+} monocytes correlated with IFN\textbeta and Mx1 expression by PBMCs, whereas the number of PDCs correlated inversely. These data challenge the concept that PDCs are exclusively responsible for the increased level of IFN-I in SLE.

**Production of IFN-I by CD71\textsuperscript{+} Monocytes in SLE**

In murine TMPD-induced lupus, the development of glomerulonephritis and autoantibodies against the Sm/RNP and dsDNA antigens requires signaling through the Type I IFN receptor (199). Much of the IFN-I driving these autoantibodies appears to be derived from a population of immature (Ly6C\textsuperscript{+}) monocytes (200). Elimination of dendritic cells by diphtheria toxin treatment of CD11c-diphtheria toxin receptor (CD11c-DTR) transgenic mice has little effect on the ability of TMPD to induce IFN-I production, whereas elimination of monocytes using clodronate loaded liposomes nearly abrogates TMPD-induced IFN-I production (200). The IFN-I producing monocytes have an immature phenotype (Ly6C\textsuperscript{hi}CD11b\textsuperscript{+}Mac3\textsuperscript{+}Moma2\textsuperscript{+}Sca1\textsuperscript{+}) and although short-lived, are rapidly replenished from the bone marrow. Unfortunately, other than CD11b, these markers are not expressed on human cells, necessitating the evaluation of additional surface markers for the identification of a putative human equivalent. The human CD14\textsuperscript{+}CD71\textsuperscript{+} IFN-I producing cells, like the cells found previously in mice, appear to be immature monocytes.
CD71 is found in proliferating cells, suggesting that the circulating CD14⁺CD71⁺ cells may have been exported recently from the bone marrow. In addition, CD14 is down-regulated during terminal differentiation of monocytes to either macrophages or dendritic cells. It is unclear at this point whether, as in the mouse model (203), the CD14⁺CD71⁺ cells are somehow prevented from undergoing maturation. However, high IFN-I levels may help maintain their phenotype because they lose CD71 expression in culture *in vitro* within 4 hours, whereas the CD71⁺ phenotype can be maintained in the presence of IFNa (data not shown).

Although there is considerable overlap between monocytes and dendritic cells (25,26) (211, 212), we think that the interferon producing CD14⁺CD71⁺ cells are more closely akin to monocytes than to PDCs. These cells are strongly HLA-DR⁺, but surface markers characteristic of PDCs (CD11c and CD123) are not expressed at higher levels than seen on CD14⁺CD71⁻ cells (Fig. 4-7 and data not shown). Moreover, by electron microscopy (Fig. 4-5B), these cells have the typical appearance of immature monocytes rather than the extensive rough endoplasmic reticulum and eccentrically placed nucleus typical of PDCs (153). Although PDCs are considered by some investigators to be the main source of IFN-I in SLE (213), a significant amount of IFN-I can be produced by non-PDCs from SLE peripheral blood (150). Monocyte derived “IFN-DCs” express high levels of TLR7 and produce IFNa, as do the CD14⁺CD71⁺ cells, but unlike the latter subset, these cells down-regulate CD14 expression (214). While we cannot exclude the possibility that the CD14⁺CD71⁺ IFN-producing cells are IFN-DCs, the fact that monocytes from SLE patients are more strongly CD14⁺ than those from healthy controls (Fig. 4-3C) and that the expression was similar on CD71⁺ vs. CD71⁻ cells (Fig. 4-4) further supports the viewpoint that the CD14⁺CD71⁺ cells are immature monocytes.
Mechanism of IFN-I Production

The striking correlation between the level of IFNβ mRNA on the one hand and Mx1 expression on the other with numbers of circulating CD71+ monocytes (Fig. 4-D-E) and the inverse relationship between the numbers of circulating PDCs and Mx1 expression (Fig. 4-C) raises the possibility that the much of the interferon responsible for increased IFN-inducible gene expression in lupus PBMCs may be produced locally (i.e. in the circulation). Unfortunately, it is difficult to evaluate the contribution of IFN-I produced at distant sites, such as lupus skin lesions (209, 210). At present, it is not known how the CD14+CD71+ cells are triggered to produce IFN-I. In mouse models, the importance of signaling via TLR7 is becoming increasingly clear (86, 215, 216). It is noteworthy, therefore, that the RNA components of the common lupus autoantigens Sm/RNP (U1 RNA) and Ro60 (Y RNAs) are TLR7 ligands capable of stimulating IFN-I production in vitro (163). It has been reported that U1 RNA exclusively stimulates IFN-I production in CD14+ monocytes but not PDCs (217). Consistent these observations, we found a strong association of anti-U1-A (RNP) autoantibodies with the presence of increased numbers of CD71+ monocytes (Fig. 4-7). In addition, we found that expression of TLR7 is expressed on lupus monocytes at levels approaching the expression of this molecule on B cells (Fig. 4-3). Preliminary study showed that level of TLR7 in lupus monocytes was higher than on control monocytes (data not shown). Stimulation with TLR7 ligands was able to induce IFN-I production in lupus monocytes (Fig. 4-3F). Interestingly, we also found that IRF-7 is expressed at high levels in monocytes from SLE patients vs. healthy controls (Fig. 4-3C). IRF-7 is an interferon-inducible transcription factor that interacts with and is activated by MyD88/TRAF6 and is absolutely required for IFN-I production (218, 219). It is expressed constitutively at high levels in PDCs (157, 220), but also is expressed in monocytes where it is thought to play a role in terminal differentiation into macrophages (221-223). The high level of IRF-7 expression in
CD14+ cells from SLE patients suggests that, like PDCs, these cells may be poised to respond to ligands capable of interacting with endosomal TLRs (TLR7, 8, and 9). It is of some interest, therefore, that the activation of TLR9 by its ligands occurs in transferrin receptor (CD71)-positive endosomes and leads to IFNα production (224). Further studies are needed, however, to define the role if any of endosomal TLRs in activating IFN-I production by CD14+CD71+ cells.
Figure 4-1. Reduced plasmacytoid dendritic cells in SLE and correlation between CD14+CD71+ monocytes. A) Flow cytometry of PDCs from SLE patients and health controls. PDCs were characterized as Lin- HLA-DR+CD11c-CD123+ cells. B) frequency of PDCs was decreased in SLE patients (n=) compare to healthy controls (n=) (p<0.01) C. Negative correlation between frequency of CD14+CD71+ monocyte and PDCs (p<0.05)
Figure 4-2. Production of IFN-I by CD14+ cells. PBMCs from SLE patients or healthy were isolated from fresh peripheral blood by Ficoll. CD14+ cells and CD19+ cells were enriched by magnetic beads respectively. RNA were isolated by Trizol reagent and reverse transcribed into cDNA. Expression of A) IFN-α2 or B) IFN-β in lupus patients and C) IFN-β in healthy controls were determined by real time PCR.
Figure 4-3. SLE monocytes express high level of TLR7, IRF7 and CD14 and produce IL-6 as well as IFNβ in response to loxoribine stimulation. A), TLR7 expression in monocytes from SLE patients, patients with other autoimmune disease and normal controls. (One way ANOVA). B, C), TLR7 and IRF7 expression in PBMCs, CD14+ monocytes, CD14- cells, CD19+ B cells and CD19- cells determined by real time PCR. D, CD14 expression determined by flow cytometry in normal controls and SLE patients without steroid treatment. E, F), PBMCs, CD14+ monocytes and CD14- cells were stimulated with PIC (50μg/ml), LPS (100 ng/ml), loxoribine (50 μg/ml) or CpG2216 (10μg/ml) for 6 hours. IL-6 and IFNβ expression was measured by real time PCR.
Figure 4-4. Increased CD14+ CD71+ monocyte in SLE and correlation of IFN-I production. A,B) Flow cytometry showing increased CD14+CD71+ monocyte in SLE. Upper panels, flow cytometry of monocytes in normal controls (left) and SLE patients (right). Lower panel, isotype control. Results is a representative staining of over 60 of such experiments. C) percentage of CD71+ cell of total monocytes was increased in SLE patients compare to normal controls (unpaired t-test, p<0.005). D) correlation between frequency of CD14+CD71+ monocytes and PDCs. E, F) The percentage of CD71+ monocyte was positively correlated with IFNβ and Mx1 expression (Spearman’s test, p=0.003, p=0.01 respectively). Expression of IFNβ and Mx1 in whole blood was determined by real time PCR.
Figure 4-5. Production of IFN-I by CD14+CD71+ monocytes. PBMCs were isolated by Ficoll. CD14+CD71+ or CD14+CD71- monocytes were enriched by cell sorting. IFNβ expression was detected by real time PCR. A), IFNβ expression in CD14+CD71+ monocytes, CD14+CD71- monocytes and CD14- cells. B), Electron microscopy of CD14+CD71+ monocytes. C), Longitudinal study of Mx1 expression and CD14+CD71+ monocytes in one lupus patients.
Figure 4-6. Percentage of CD71+ cells correlates with anti-U1A antibody production. A), CD71+ monocytes counts were significantly higher in patients with anti-U1A antibody compared to anti-U1A negative patients. B,C), MDC or PDC counts were not associated with anti-U1A antibodies.
Figure 4-7. Characterizations of CD14+CD71+ monocyte in SLE blood. Surface expression of HLA-DR, CD64, CD62L and CD11c were determined by flow cytometry. For each individual marker, MFI was shown on the left and histogram was shown on the right.
CHAPTER 5
RESULTS AND DISCUSSION: LUPUS-LIKE DISEASE AND HIGH INTERFERON LEVELS CORRESPONDING TO TRISOMY OF THE TYPE I INTERFERON CLUSTER ON CHROMOSOME 9P

Introduction

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by antinuclear antibodies (ANAs) and immune complex deposition in the kidneys and other organs (225). Susceptibility is conferred by multiple genes in a manner consistent with a threshold liability model, whereas disease onset may be influenced by environmental triggers (226, 227). Hereditary complement deficiency, defective complement receptor function (228), and Fc receptor polymorphisms (229) are among the genetic factors contributing to SLE.

Recent interest has focused on the role of type I interferons (IFNs), such as IFN and IFN. Patients with SLE exhibit increased levels of IFN in the serum (184, 186). Moreover, in the peripheral blood mononuclear cells (PBMCs) of SLE patients, IFN-inducible genes are expressed at increased levels (141, 142, 183, 197). Therapeutic use of IFN induces ANAs or even overt SLE in some individuals (139, 194), suggesting that IFN may play a causative role. It is unknown whether the increased IFN production in SLE reflects a genetic defect or the action of unidentified environmental factors.

Type I IFNs, produced by most cell types in response to viral infection, link innate immunity with adaptive immunity (172, 230, 231). The 14 IFN genes, 4 IFN pseudogenes, and single IFN and IFN genes are clustered on the short arm of chromosome 9 within a genomic duplication (187, 232, 233). Decreased IFN production by cells with only 1 copy of the type I IFN cluster may promote neoplasia (234). The present study addresses the question of whether individuals with 3 copies of the type I IFN cluster produce high levels of type I IFN due to a gene-dosage effect, resulting in increased susceptibility to SLE.
Results

Pedigree

There have been more than 150 reports of individuals with a partial or complete trisomy of Chromosome 9 (235). About half of the cases of a partial trisomy of Chromosome 9 were the result of a 3:1 segregation of a balanced parental translocation (236). We herein describe a family in which 4 of the members had a balanced translocation of Chromosome 9 to Chromosome 21, referred to as 9;21, while 2 individuals had an unbalanced translocation resulting in trisomy of 9p, the chromosomal region encoding the type I IFN cluster (Figure 5-1). The proband subject (designated UB1), who had an unbalanced translocation and trisomy of 9p, was evaluated for joint pain and ANA positivity. His similarly affected half-brother (subject UB2) was also evaluated, along with 3 relatives who had balanced translocations (subjects B1, B2, and B3) (Figure 5-1).

Case Reports

Subject UB1, a 28-year-old white man with dysmorphia, had developmental delay, protuberant ears, micrognathia, clubbed feet, bilateral syndactyly of the second and third toes, bilateral camptodactyly of the third and fourth metacarpophalangeal (MCP) joints, smooth transverse palmar creases, and profound mental retardation, as well as a history of grand mal seizures since age 10 years that were treated with phenytoin and carbamazepine. His karyotype was 46,XY,-21,+der(9),t(9;21)(q22.3;q21)mat, rendering him partially monosomic for the proximal third of the long (q) arm of Chromosome 21, and partially trisomic for the short (p) arm of Chromosome 9 as well as for the proximal portion of the q arm of Chromosome 9 (Figure 5-2A).

The subject was evaluated for polyarthralgias and progressive joint deformities as well as a positive test result for ANAs (>1:1,280, speckled), and he was observed to have faint malar
erythema, livedo reticularis of the legs, and multiple reducible subluxations of the proximal interphalangeal and MCP joints, with ulnar deviation bilaterally. Radiographs revealed nonerosive subluxation of the MCP joints and boutonniere deformity of the third digit of the right hand, suggestive of Jaccoud's arthropathy. Blood cell counts and renal function were normal. The erythrocyte sedimentation rate was 51 mm/hour. Anti-RNP autoantibodies and IgM-anticardioplin antibodies (33 IgM phospholipid units) were detected by ELISA. Anti-Sm, anti-Ro/SSA, anti-La/SSB, anti-dsDNA antibodies, and rheumatoid factor were not detected. Levels of C3 and C4 were 110 and 18 arbitrary units, respectively. The subject was treated successfully with nonsteroidal anti inflammatory agents.

Subject UB2, the 14-year-old half-brother of subject UB1, had the same chromosomal, developmental, and physical abnormalities as those described above, with joint pain and mild sicca symptoms (Figure 5-2A). He was observed to have facial erythema, Raynaud's phenomenon, joint tenderness without swelling, and mild joint deformities. Hand radiographs showed narrowing of the carpal joint spaces and diffuse bony demineralization. The subject had a history of grand mal seizures since age 3 years that were treated with phenytoin for 8 years. At the time of evaluation, phenytoin had been replaced with divalproex sodium and oxcarbazepine and, 1 year later, with oxcarbazepine and levetiracetam because of poorly controlled seizures. His serum was anti-Ro positive (by ELISA) and negative for anti-Sm, anti-RNP, anti-La, anti-dsDNA, rheumatoid factor, and anticardioplin antibodies. The levels of C3 and C4 were 99 and 31 arbitrary units, respectively. There was no change in his ANA status after phenytoin was changed to divalproex/oxcarbazepine or when levetiracetam was substituted for divalproex. The patient achieved relief of symptoms following treatment with artificial tears and nonsteroidal antiinflammatory agents.
The karyotype of the mother of these 2 patients (subject B1 in Figure 5-1) was 46,XX,t(9;21)(q22.31;q21.2), consistent with a balanced translocation involving the long arm of Chromosome 9 and the long arm of Chromosome 21 (breakpoints 9q22.31 and 21q21.2) (Figure 2B). The subject was healthy, without evidence of autoimmunity. In addition to her 2 sons (subjects UB1 and UB2), the mother had a male child with a balanced translocation at 9;21 (subject B2 in Figure 5-1), a male child who died neonatally, and 2 miscarriages (solid circles in Figure 1). One of the mother's siblings also had a balanced translocation (subject B3 in Figure 5-1). Similar to subject B1, subjects B2 and B3 had no developmental abnormalities, seizures, or autoimmunity. There was no other family history of autoimmunity.

**Immunologic Findings**

ANA patterns were speckled and nuclear (in subject UB1) and cytoplasmic (in subject UB2) (Figure 5-3A). Sera from their healthy mother (subject B1) (Figure 5-3A) and from subjects B2 and B3 (results not shown) did not contain ANAs. Analysis of serum autoantibodies by radioimmunoprecipitation confirmed the presence of serum anti-RNP and anti-Ro 60 autoantibodies in subjects UB1 and UB2, respectively (Figure 5-3B). Anti-Ro 60 antibodies remained detectable in the half-brother (subject UB2) after valproic acid was discontinued (results not shown). In contrast, sera from healthy relatives B1, B2, and B3 showed an autoantibody pattern similar to that in nonautoimmune controls (Figure 5-3B, right). Anti-Ro 52 autoantibodies were undetectable in the sera from subjects UB1, UB2, B1, B2, and B3 (results not shown).

Because subjects UB1 and UB2 were both receiving anticonvulsant medications, some of which have been associated with a low risk of drug-induced lupus syndrome (40), their sera were tested for autoantibodies characteristic of the drug-induced lupus syndrome (anti-ssDNA and antichromatin). Tests of the sera of subjects UB1 and UB2 as well as the sera of family members
B1, B2, and B3 all yielded negative results (not shown) for these autoantibodies associated with drug-induced lupus.

The absence of autoantibodies that are usually found in drug-induced lupus and the presence of autoantibodies that are not associated with drug-induced lupus (anti-RNP and anti-Ro 60) along with the continued production of anti-Ro 60 in subject UB2 after anticonvulsant therapy was changed led us to investigate other potential explanations for the development of lupus-like disease and serologic changes in both of the subjects with an unbalanced translocation compared with the absence of these features in their relatives with a balanced translocation. In view of recent evidence implicating IFN in the pathogenesis of SLE (139, 194, 237), it was notable that the IFN cluster is located on the short arm of Chromosome 9 (9p21) (Figure 5-2) (187, 232).

Karyotype analysis indicated that subjects UB1 and UB2 were trisomic for the IFN cluster (Figure 5-2A). Coincidentally, genes for the 2 chains of the type I IFN receptor (IFNAR1 and IFNAR2) as well as the IFN-inducible genes Mx1 and Mx2 are located on Chromosome 21, but these were not involved in the translocation (i.e., subjects UB1 and UB2 had 2 copies of each).

We therefore investigated IFNα4 and IFNβ expression in PBMCs from subjects UB1 and UB2 and their healthy mother (subject B1) in comparison with that in the PBMCs from 10 SLE patients and 10 normal healthy controls (Figures 5-4A and B). In comparison with controls, IFNα4 and IFNβ expression was increased in the PBMCs from subjects UB1 and UB2 and was increased in those from the patients with SLE (Figures 5-4A and B). Expression of IFNα4 and IFN3 in subjects UB1 and UB2 was increased 7-8-fold over the mean value in healthy controls. In contrast, the mother (subject B1) expressed IFNα4 and IFNβ messenger RNA (mRNA) at normal levels. Not only was type I IFN mRNA expression increased in the PBMCs from subjects
UB1 and UB2, but IFN protein expression was increased as well, since the expression of Mx1, a gene induced specifically by IFN (176), also was increased in the PBMCs from subjects UB1 and UB2 and in those from the SLE patients, as compared with those from healthy controls and subject B1 (Figure 5-4C).

Expression of the β-chain of IFNAR2 is down-regulated upon binding of IFNα/β (238). The levels of IFNAR2 in the PBMCs from subjects UB1, UB2, and the SLE patients were somewhat decreased compared with those in healthy controls and subject B1 (results not shown). Comparison of subjects UB1, UB2, and B1 with SLE patients and healthy controls did not reveal significant differences in the levels of IL-6 (Figure 5-4D) or TNFα (results not shown).

Subject UB2 exhibited persistently high levels of IFN, since blood samples obtained 10 months apart showed similar IFNα4, IFNβ, and Mx1 expression (Figure 5-5A). Circulating plasmacytoid dendritic cells, the major IFN-producing cell type, were examined in subjects UB1 and UB2 by flow cytometry (Figure 5-5B). It is known that SLE patients have low numbers of circulating plasmacytoid dendritic cells compared with healthy controls (150, 194), probably reflecting activation and migration to secondary lymphoid tissues (190). Subjects UB1 and UB2 had plasmacytoid dendritic cell counts that were lower than those in healthy controls (n = 10) and comparable with those in SLE patients (n = 10). Unexpectedly, subject B1 also had low numbers of circulating plasmacytoid dendritic cells (Figure 5-5B).

**Discussion**

We describe herein a pedigree with a chromosomal translocation involving Chromosomes 9 and 21. Three individuals with a balanced translocation (subjects B1, B2, and B3) had no signs of autoimmunity. Two other family members (subjects UB1 and UB2) had chromosome aberrations due to a 1:3 segregation (1 of the 4 translocated chromosomes segregated to one pole, while 3 segregated to the other pole), leading to trisomy of chromosome 9p and the proximal arm
of 9q as well as monosomy of the proximal arm of 21q. Remarkably, both patients with trisomy of 9p presented with lupus-like autoimmunity and abnormalities in the regulation of type I IFN. We hypothesize that their autoimmunity may have been related to having 3 copies of the type I IFN cluster located on the p arm of chromosome 9 (187, 232).

It is unlikely that the presence of lupus-like disease in 2 family members with an unbalanced translocation and its absence in 3 relatives with a balanced translocation was coincidental. Since subjects UB1 and UB2 had different fathers, a genetic predisposition to autoimmunity, if present, would most likely have been derived from the mother. However, neither the mother nor any maternal relatives had evidence of autoimmunity. Although their mother had low numbers of circulating plasmacytoid dendritic cells, as has been seen in patients with SLE (150, 197), unlike subjects UB1 and UB2, the mother did not express high levels of IFNα4 or IFNβ mRNA (Figures 5-4 A and B), nor was there increased type I IFN signaling, as indicated by the low Mx1 expression (Figure 5-4C). Thus, although it is possible that subject B1 had an abnormality in regulating plasmacytoid dendritic cells, she appeared to have normal regulation of type I IFN production. The most likely interpretation is that the autoimmune phenotype exhibited by subjects UB1 and UB2 was related to the presence of 3 copies of the type I IFN gene cluster.

A previous report of lupus-like disease in a patient with the karyotype 46,XX,-6,+der(6),+t(6,9)pat, which resulted in a partial trisomy of Chromosome 9 (239, 240), further supports the idea that the lupus-like disease was related to a chromosomal abnormality. Similar to subjects UB1 and UB2, this patient with a translocation 6;9 had seizures. Although her lupus-like disease was attributed to anticonvulsant therapy with sodium valproate (239), this would be a rare occurrence, and definitive evidence supporting the diagnosis of drug-induced
lupus was not presented. It is unlikely that subjects UB1 and UB2 had drug-induced lupus.

Subject UB1 was taking phenytoin, which is associated with a low risk of drug-induced lupus (40), whereas subject UB2 initially received phenytoin, but it was replaced with sodium valproate/oxcarbazepine and subsequently with levetiracetam. Sodium valproate is associated only anecdotally with drug-induced lupus (239, 241-243), and the anti-Ro 60 autoantibodies persisted after sodium valproate was replaced by oxcarbazepine and levetiracetam, neither of which is associated with drug-induced lupus (244). Since the lupus-like syndrome and autoantibody production did not abate when the anticonvulsant regimen was altered, and since the autoantibodies classically associated with drug-induced lupus (anti-ssDNA and antichromatin) (40) were not detected, whereas subjects UB1 and UB2 both produced autoantibodies (anti-RNP, anti-Ro 60) not associated with drug-induced lupus, we conclude that subjects UB1 and UB2 did not have drug-induced lupus.

Because Chromosome 9p was involved in the translocation (187, 232) and the patients with trisomy of the type I IFN cluster had higher levels of IFN4α, IFNβ, and Mx1 than did their mother or healthy controls (Figure 5-4), we suggest that increased gene dosage may have promoted lupus-like disease by enhancing IFN production. It is often assumed that genes present in 3 copies in trisomic individuals will be expressed at a 1.5-fold higher level relative to euploid, a prediction that has been formally tested in the case of chromosome 21 (245). The mean overexpression of all aneuploid genes on chromosome 21 was very close to the predicted 1.5-fold increase in a mouse Down syndrome model. However, only 37% of the genes were expressed at the theoretical value of 1.5-fold, whereas 45% were significantly underexpressed. Interestingly, 18% were overexpressed (levels significantly higher than 1.5-fold). Our data (Figures 5-4-5) are consistent with the possibility that the type I IFNs (on Chromosome 9) may
be among the genes overexpressed in trisomic individuals. It is possible that this is related to the positive feedback regulation of type I IFN production, i.e., interaction of secreted type I IFN with the type I IFN receptor stimulates the production of additional type I IFN (246). Another issue is the fact that alterations in gene expression measured at the transcript level may not always be accurately reflected at the protein level (247). However, the strikingly high Mx1 expression (induced by IFN protein binding to the IFN receptor) strongly suggests that in this case, increased transcript level was closely tied to increased protein level.

Subjects UB1 and UB2 may exemplify individuals having a genetic defect that affects the regulation of type I IFN production and promotes susceptibility to SLE. The data are consistent with the possibility that other genetic polymorphisms, point mutations, or chromosomal abnormalities affecting type I IFN production may be associated with lupus (31). Some patients treated with recombinant IFNa develop ANAs, SLE, or autoimmune thyroiditis (139, 194), and disease is accelerated in lupus-prone mice treated with IFNa (237). However, increased type I IFN also is associated with idiopathic SLE (141, 142, 183, 184, 186). Thus, the high levels of IFN exhibited by subjects UB1 and UB2 could be either the cause of their lupus or secondary to lupus. Although it is impossible to completely exclude the possibility that the increased IFN expression was secondary to lupus, the fact that disease was restricted only to family members with a partial trisomy of 9p supports a causal role.

In summary, disordered regulation of type I IFN was found in association with a chromosomal translocation resulting in trisomy of the type I IFN cluster. Individuals with trisomy of 9p developed a lupus-like syndrome, whereas their relatives with the balanced translocation showed no evidence of autoimmunity. The data support the idea that abnormal regulation of type I IFN production is involved in the pathogenesis of SLE (160).
Figure 5-1. Pedigree. Diagram of the kindred, indicating individuals with an unbalanced translocation 9;21 resulting in trisomy of chromosome 9p (subjects UB1 and UB2) and with a balanced translocation 9;21 (subjects B1, B2, and B3). The maternal grandmother of subjects UB1 and UB2 was also known to have a balanced translocation (not shown). Miscarriages are indicated as solid circles. The proband (index case) is indicated with an arrow.
Figure 5-2. Karyotype analysis of subject UB2 and subject B1. A) The karyotype of subject UB2 was determined by GTG-banding (similar to that of subject UB1 [not shown]). Right, diagram of 9;21 and the derivative chromosomes 9 and 21 from subjects UB1 and UB2. Chromosome 9 is shown in dark hatching and chromosome 21 in light hatching. Chromosome bands 9p21 (type I interferon [IFN-I] gene cluster), 21q22.11 (IFN-I receptors 1 and 2), and 21q22.3 (Mx1, Mx2) are shown as dark bands on their respective chromosomes. B) The karyotype of subject B1 (the mother of subjects UB1 and UB2) was determined by GTG-banding. Right, diagram of 9;21 and the derivative chromosomes from subjects B1, B2, and B3.
Figure 5-3. Autoantibody testing. A) Fluorescence antinuclear antibody test. HeLa cells were stained with sera from subjects UB1, UB2, and B1 (1:160) followed by Alexa-conjugated goat anti-human IgG and 4',6-diamidino-2-phenylindole staining as described in patients and methods. Subject UB1, nucleoplasmic immunofluorescence pattern. Subject UB2, cytoplasmic pattern. Subject B1, no difference from control normal human serum (not shown). B) Immunoprecipitation. Serum samples from subjects UB1, UB2, and B1, B2, and B3 were used to immunoprecipitate proteins from 35S-methionine/cysteine-labeled K562 cell extract followed by analysis on 12.5% sodium dodecyl sulfate-polyacrylamide gels. Radiolabeled proteins were detected by fluorography. Sm = anti-Sm reference serum; Ro/La = anti-Ro/SSA/anti-La/SSB reference serum; Ro = anti-Ro/SSA reference serum; N = normal human serum. Positions of the U1 small ribonucleoprotein antigens A, B'/B, C, D1/D2/D3, E, F, and G as well as the 200K (doublet) U5 small ribonucleoprotein antigens are indicated. Anti-Sm antibodies immunoprecipitate A-G plus 200K, whereas anti-RNP antibodies immunoprecipitate only A-G. Positions of the Ro60 and La/SSB antigens are also indicated.
Figure 5-4. Type I interferon (IFN) expression. Peripheral blood mononuclear cells (PBMCs) were isolated as described in patients and methods. Expression of IFNα (A), IFNβ (B), the IFN-inducible gene Mx1 (C), and interleukin-6 (IL-6) (D) was analyzed by real-time polymerase chain reaction. Total RNA was isolated from PBMCs (subjects UB1, UB2, and B1) and from patients with systemic lupus erythematosus (SLE) and 10 healthy controls (NHC).
Figure 5-5. Regulation of interferon (IFN) production. A) Gene expression pattern in subject UB2 remains similar over time. RNA was isolated from peripheral blood mononuclear cells (subject UB2) on 2 separate occasions 10 months apart, and Mx1, IFNβ, IFNα4, IFNAR2, interleukin-6 (IL-6), and tumor necrosis factor α (TNFα) gene expression was measured by real-time polymerase chain reaction. B) Low numbers of circulating plasmacytoid dendritic cells (PDC). Numbers of circulating plasmacytoid dendritic cells (Lin-,HLA-DR+,CD123+,CD11c-) in peripheral blood of subjects UB1, UB2, and B1 and from 10 patients with systemic lupus erythematosus (SLE) and 10 healthy controls (NHC) were enumerated by flow cytometry. Bars show the median, boxes indicate the 25th and 75th percentiles, and bars outside the boxes indicate the 10th and 90th percentiles.
CHAPTER 6
CONCLUSION AND FUTURE DIRECTIONS

The main goal of this research was to study the role of IFN-I and IFN-I producing cells in the pathogenesis of SLE. IFN-I levels were assessed using real-time PCR to measure IFN-I inducible gene expression. IFN-I producing cells were characterized in the peripheral blood of lupus patients and finally, individuals with a trisomy of chromosome 9p (location of the Type I interferon gene cluster) provided a natural model to study the effects of IFN-I over-production in the pathogenesis of lupus.

We found that IFN-I is up-regulated in patients with SLE in comparison with healthy controls and patients with other autoimmune disease. Abnormally high IFN-I inducible gene expression (Mx1) was seen in about 30% of lupus patients. The high IFN-I levels were not associated with age, race or gender. Common treatments of SLE (low-does corticosteroids, antimalarials) did not have significant effects on IFN-I levels although high does of corticosteroids (e.g. 1 gram of methylprednisolone IV daily for 3 days) did reduce IFN-I expression dramatically. High IFN-I levels in SLE were associated with the production of certain autoantibodies, such as ANA, anti-dsDNA, anti-Sm/RNP antibodies and was negatively associated with antiphospholipid antibodies. High IFN-I levels were also associated with active disease. The numbers of PDCs, the most efficient IFN producing cells, as well as MDCs were significantly decreased in lupus circulation. We further demonstrated that low PDC/MDC counts were correlated with production of anti-Sm/RNP antibodies and negatively associated with antiphospholipid antibodies. Low PDC/MDC counts were seen in patients with renal involvement as well as more extensive disease.

Secondly, we found increased numbers of circulating CD14+CD71+ monocytes in SLE compared with healthy controls. The numbers of these cells were negatively correlated with PDC
numbers and positively with IFN-I levels in lupus patients. These cells produced significantly higher IFN-I in compared with CD14^+CD71^- or CD14^- cells. We also found increased TLR7 expression on lupus monocytes. Stimulation with the TLR7 ligand loxoribine induced IFN-I as well as IL-6 production by lupus monocytes. Stimulation of LPS (TLR4 ligand) also stimulated IFN-I by SLE monocytes, but less efficiently than loxoribine. We also found that the numbers of CD14^+CD71^+ monocytes were higher in patients with antibodies against U1 small nuclear ribonucleoproteins (snRNPs), suggesting that endogenous RNA ligands for TLR7 might play a role in the pathogenesis of the “interferon signature” in SLE patients.

Finally we reported a pedigree with a chromosomal translocation involving chromosomes 9 and 21. Three individuals with a balanced translocation had no signs of autoimmunity. Two other family members had chromosome aberrations due to a 1:3 segregation resulting in trisomy of the proximal arm of 9q including the IFN-I cluster which is located on 9p22 as well as monosomy of the proximal arm of 21q. Both patients with trisomy of 9p presented with lupus-like autoimmunity and production of ANA as well as anti Sm/RNP or anti-Ro60 antibodies. Both patients had higher IFN-I levels than the normal family members with balanced translocation or normal controls. IFN-I levels were comparable to those found in idiopathic lupus. Interestingly both patients had low dendritic cell numbers, similar to SLE patients, and this was also seen in their mother’s peripheral blood (balanced translocation 9:21 without autoimmunity).

Type I interferon is important cytokine in both innate and adaptive immunity. In addition to its antiviral effects, IFN-I also induces MHC class II expression on antigen presenting cells, promotes dendritic cell (DC) maturation and survival and the survival of activated T cells and antigen-activated B cells. The high IFN-I levels as well as low dendritic cell count might be explained by the maturation of DCs. It has been proposed that upon maturation, PDCs upregulate
expression of chemokine receptor CCR7 and become responsive to chemokines such as SLC and ELC, resulting in migration out of the circulation and into secondary lymphoid tissues. Further studies of the distribution as well as maturation of dendritic cells, monocytes, and macrophages in skin, kidney and other lymphoid tissues will be helpful to understand the fate of IFN-I producing cells in SLE. Gene expression assays and immunostaining of IFN-I in these tissues could be used to define the origin of IFN-I directly.

A monocyte phagocytosis defect affecting the clearance of dying cells has been reported in about half of lupus patients. In the TMPD-lupus model, immature interferon producing monocytes also are poorly phagocytic. We hypothesized that similar cells in the peripheral blood of SLE patients might be responsible for the observed monocyte phagocytosis defect. To test this hypothesis, phagocytosis assays will be carried out in the future using purified CD14⁺CD71⁺ monocytes and CD14⁺CD71⁻ monocytes.

Genomic variability exists in different scales, including SNP, small insertion and deletions, and large scale chromosome structure variations. Here we reported patients with unbalanced translocation between Chromosome 9 and 21 developed a lupus like disease, suggesting gene dosage effect of IFN cluster was responsible for the increased risk of SLE. With recent development of gene array technology, several studies have revealed the existence of copy number variants of small DNA regions (248, 249). This phenomenon exists abundantly not only under pathogenic conditions but also in normal individuals. To test the hypothesis that the elevated production of IFN-I in subgroup of SLE patients might due to copy number variants of IFN cluster, microarray experiments of regions related to IFN cluster will be performed.

In conclusion, IFN-I produced by a novel population of immature CD71⁺ monocytes appear to be a significant factor in the pathogeneisis of SLE.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Haoyang Zhuang was born in Tianjin, China. She received her bachelor’s degree in microbiology from Nankai University, Tianjin, China in 1998. She then worked as a sales manager in the Department of Import and Export at Shenzhen Agriculture Product Co., Ltd, Guangdong, China. In 2000, she started work as a research assistant in the Institute of Microbiology, Chinese Academy of Science, Beijing, China, under the supervision of Dr. Yanhe Ma. She was admitted to the Interdisciplinary Program in Biomedical Sciences at the University of Florida in 2002. She did her dissertation research under the supervision of Dr. Westley H. Reeves in the Division of Rheumatology and Clinical Immunology and was awarded the Doctor of Philosophy in 2008.