Characterization of the Immune Basis for Reduced IFN-α in Serologically Active Clinically Quiescent SLE Patients

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Graduate Department of Immunology
University of Toronto

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Abstract

Serologically active clinically quiescent (SACQ) patients with systemic lupus erythematosus (SLE) are clinically quiescent despite the presence of autoantibodies. SACQ patients differ from serologically active clinically active (SACA) patients in terms of decreased levels of IFN-α and IFN signature. Given the role of IFN-α in SLE, we sought to determine the mechanisms underlying the lack of type I IFN elevation in SACQ patients. In assays containing peripheral blood mononuclear cells and nuclear extracts, SACQ serum induced decreased levels of IFN-α compared to SACA serum. Mixing experiments revealed that there are factors in SACQ serum that inhibit IFN-α production. The presence of anti-IFN-α antibodies was found in a small proportion of SLE patients and correlated with the ability to block IFN-α in-vitro but not with a decreased IFN signature in-vivo or clinical activity. These results suggest that SACQ patients have multiple mechanisms that lead to their decreased type I IFN production.
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<tbody>
<tr>
<td>AIAA</td>
<td>Anti-IFN-α antibody</td>
</tr>
<tr>
<td>ANA</td>
<td>Anti-nuclear antibody</td>
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<tr>
<td>BAFF</td>
<td>B cell activating factor</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domains</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double-stranded DNA</td>
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<tr>
<td>FcR</td>
<td>Fc receptor</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte–macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HC</td>
<td>Healthy control</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High-mobility group box 1 protein</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin receptor associated kinase</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>ISG</td>
<td>IFN-stimulated gene</td>
</tr>
<tr>
<td>ISGF3</td>
<td>IFN-stimulated gene factor 3</td>
</tr>
<tr>
<td>ISRE</td>
<td>IFN-stimulated response elements</td>
</tr>
<tr>
<td>JAK1</td>
<td>Janus kinase 1</td>
</tr>
<tr>
<td>MDA5</td>
<td>Melanoma differentiation-associated gene 5</td>
</tr>
<tr>
<td>mDC</td>
<td>Myeloid dendritic cell</td>
</tr>
<tr>
<td>MxA</td>
<td>Myxovirus resistance A</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>NE</td>
<td>Nuclear extract</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide oligomerization domain</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
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<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end products</td>
</tr>
<tr>
<td>RIG1</td>
<td>Retinoic acid inducible gene 1</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
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<tr>
<td>SACA</td>
<td>Serologically active clinically active</td>
</tr>
<tr>
<td>SACQ</td>
<td>Serologically active clinically quiescent</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
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<tr>
<td>S1P</td>
<td>Sphingosine-1-phosphate</td>
</tr>
<tr>
<td>SQCQ</td>
<td>Serologically quiescent clinically quiescent</td>
</tr>
<tr>
<td>SS</td>
<td>Sjögren's syndrome</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TIR</td>
<td>TLR-interleukin-1</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TRAF6</td>
<td>Tumor necrosis factor-receptor-associated factor 6</td>
</tr>
<tr>
<td>TYK2</td>
<td>Tyrosine kinase 2</td>
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1 Introduction

1.1 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by the production of autoantibodies against nuclear antigens. Autoantibody binding to nuclear antigens results in the formation of immune complexes that deposit in multiple organs, leading to inflammation and tissue damage. The worldwide prevalence of the disease ranges from 20 to 150 cases per 100,000 in the population (1). Women account for 70-90% of patients, with a peak age of onset between the late teens and early 40s (2). The course of the disease is highly variable with periods of remission and flare. Some patients experience a relatively mild disease, while others can have serious progression leading to significant and potentially life-threatening damage to organs, such as the kidneys, brain, heart and lungs. Due to its extensive clinical manifestations, 11 clinical and serological criteria have been established for the classification of SLE. According to the latest American College of Rheumatology (ACR) guidelines, 4 of these criteria must be fulfilled to make a definite diagnosis (2).

1.2 Etiology and Pathogenesis of SLE

Although the exact etiology of SLE remains unknown, it has been proposed that multiple genetic and environmental factors contribute to the development of the disease. These factors lead to a breach of self-tolerance resulting in a cascade of events that lead to clinical symptoms.

1.2.1 Genetic factors

The concordance rate for SLE is 24-35% in monozygotic twins and approximately 2% in dizygotic twins (3). The disease also demonstrates strong familial aggregation with a much higher frequency among first-degree relatives of patients, λs (sibling risk ratio) > 20 (4).
Although SLE can be associated with a single gene defect, such as a deficiency of complement components, C1q or C4, in most cases defects in multiple genetic loci are required. To date, genome-wide association studies (GWASs) have identified more than 30 loci linked to SLE susceptibility (5). These loci contain genes that mediate clearance of nucleic acids and immune complexes, antigen presentation, B-cell hyperactivity and T-cell dysregulation, as well as type I interferon signaling (5). However, it is still unclear how these genes interact with each other to contribute to the disease.

1.2.2 Environmental factors
There is strong evidence that environmental factors, such as ultraviolet light, demethylating drugs, and viruses contribute to SLE susceptibility. Exposure to sunlight induces apoptosis of keratinocytes in the skin, resulting in increased amounts of apoptotic material (1,6). Epstein-Barr virus may contribute to disease through molecular mimicry of the common SLE autoantigen Ro (1,7). Many drugs that inhibit DNA methylation including hydralazine and procainamide are known to induce lupus-like manifestations in healthy individuals (3).

1.2.3 Hormonal factors
Since the majority of SLE patients are female, it is possible that hormones play a role in SLE development. In murine models, administration of estrogen or prolactin promotes a lupus-like phenotype with an increase in mature high-affinity autoreactive B cells (8). Oral contraceptive use is associated with a slightly increased risk of developing SLE; however, it does not increase flares in women with stable disease (9). In addition, pregnancy can exacerbate disease in some cases. Although the timing of flares varies between studies, it appears that most flares occur during the second and third trimesters and the postpartum period (10,11). Since the levels of estrogen and progesterone are reduced during the second and third trimesters in SLE patients
compared to healthy women (12,13), and further decline postpartum, it is unclear whether rising levels of estrogen or progesterone are associated with an increased risk of flares during pregnancy.

1.2.4 Autoantibodies and tissue damage

One of the major immune defects in SLE patients is the production of anti-nuclear antibodies (ANAs). Although there is a wide spectrum of ANAs, the most characteristic are those against dsDNA. IgG, IgM, and to a lesser extent IgA anti-dsDNA antibodies are found in 60-80% of SLE patients, but in less than 0.5% of healthy individuals or patients with other autoimmune diseases (3). Some studies have reported that the levels of anti-dsDNA antibodies in serum correlate with disease activity (14–20). Anti-dsDNA antibodies are part of the normal repertoire of natural autoantibodies; however, most of these are low-affinity IgM antibodies that react weakly with self-antigens (21). Pathogenic anti-dsDNA antibodies in SLE patients are characterized by IgG subclasses with high-affinity for DNA and cross-reactive antigens (e.g. chromatin, nucleosomes or glomerular membrane) as well as complement-fixing ability (17). Although anti-dsDNA antibodies are the most extensively studied in SLE, other autoantibodies may also play a role in various clinical manifestations in SLE. Anti-Sm and anti-RNP autoantibodies are directed against small nuclear ribonucleoproteins (snRNPs) that contain RNAs U1-U6 and U1, respectively (22). They are increased in patients with CNS involvement and renal disease but are not always associated with disease activity (23). Anti-Ro and anti-La antibodies bind proteins of the Ro-RNP complex that associate with RNAs Y1-Y6 (24). The presence of anti-Ro antibodies confers an increased risk of fetal congenital heart block and cutaneous lupus in the offspring of SLE patients, while anti-La antibodies are more commonly found in patients with Sjögren's syndrome (SS) (3).
Most studies of autoantibody-mediated tissue damage in SLE patients have focused on the role of anti-dsDNA antibodies in patients with glomerulonephritis. It has been proposed that circulating immune complexes deposit in the glomerular basement membrane and cause an influx of inflammatory cells by activating the complement cascade (17,25). Alternatively, anti-dsDNA antibodies may bind to cognate antigens trapped inside the membrane or components of the basement membrane, such as nucleosomes, heparan sulfate or laminin that have shared epitopes or areas of similar charge, leading to a direct pathogenic effect on the renal glomerular basement membrane (26).

1.2.5 Defects in B and T cells associated with SLE

SLE is characterized by alterations in B cells, resulting in polyclonal B cell activation, increased numbers of antibody-producing cells, and production of autoantibodies that mediate tissue damage. Although study of animal models that completely lacked B cells revealed that the presence of B cells is critical to the development of SLE, this is not solely due to the capacity of B cells to differentiate to antibody-producing cells. In lupus-prone MRL/lpr mice that have been genetically manipulated so that their B cells are incapable of secreting antibodies, classic signs of autoimmunity still develop, including T cell activation, tissue damage and increased mortality (27,28).

A number of studies have identified abnormalities in B cell function, including abnormal B cell signaling, as evidenced by increased intracellular calcium flux and phosphorylation of cytosolic proteins. It has been shown that stimulation of B cells from SLE patients with BCR ligand leads to increased calcium responses and tyrosine phosphorylation of proteins compared to those from healthy individuals (29). There is some evidence that these changes in B cell signaling result from decreased expression of signaling molecules that negatively regulate BCR
signaling. B6 mice with a gene deletion of FcγRIIB, an inhibitory receptor in BCR signaling, have been shown to develop a lupus-like disease characterized by autoantibody production and severe glomerulonephritis (29). Notably, two polymorphisms of FcγRIIB resulting in decreased surface expression and impairment in lipid raft localization are found in a subset of SLE patients (30). Reduced expression of Lyn, an inhibitory molecule of BCR signaling is also associated with SLE (31). In addition to changes in B cell signaling, SLE patients have elevated levels of B cell activating factor (BAFF), which has been shown to rescue autoreactive B cells from peripheral deletion and promote their maturation into follicular and marginal zone B cells (32,33).

Recent work from our laboratory indicates that elevated levels of IFN-α may also contribute to the altered function of B cells observed in these patients, resulting in decreased apoptosis and increased proliferation of these cells (34). It is likely that these functional abnormalities promote the abnormal accumulation of activated B cells that is observed in lupus patients and murine models of this disease, which are proposed to promote activation of self-reactive T cells.

T cells are crucial in the disease pathogenesis as they provide necessary help to B cells for autoantibody production, as well as infiltrate target tissues causing inflammation and damage. Similar to B cells, abnormal signalling events partly contribute to an aberrant phenotype of T cells in SLE. For example, pre-aggregated lipid rafts observed in T cells may contribute to increased T cell activation and disease development, as disruption of these aggregates in lupus-prone MRL/lpr mice delays pathology (35,36). Furthermore, the TCR-CD3 complex is rewired in lupus T cells, with the CD3ζ replaced by the FcRγ chain, which recruits spleen tyrosine kinase (Syk) instead of ZAP70 (35). This FcRγ-Syk interaction leads to accelerated and heightened phosphorylation of signalling molecules and increased calcium flux as compared to the ζ chain-ZAP70 combination (35). SLE T cells also show increased and prolonged expression of CD40L upon activation, thereby leading to activation of autoreactive B cells and autoantibody
production via CD40-CD40L interaction (37). Aberrations in T cell signalling subsequently lead to defective gene transcription and cytokine production, as evidenced by a lack of IL-2 production, which results in the suppression of activation-induced cell death and thus increased survival of autoreactive T cells (35). There is also an increased proportion of IL-17 producing T cells in the peripheral blood of SLE patients (38). These cells are thought to home to the kidney in patients with nephritis, where they drive an inflammatory response (39).

1.2.6 Impaired clearance of apoptotic debris

Apoptosis is an important mechanism of cell death characterized by membrane blebbing, cell shrinkage, DNA fragmentation and formation of membrane-bound blebs (40). Autoantigens, including nucleosomes, Ro and La, that are commonly observed in SLE are exposed on the surface of these blebs (41–46). In healthy individuals, these blebs are efficiently cleared by macrophages without causing inflammation and tissue damage. It has been suggested that SLE patients have an increased rate of apoptosis and/or defective clearance of apoptotic cells, the latter leading to secondary necrosis where autoantigens are released and become immunogenic (47,48). Although limited data supports an increased rate of apoptosis in SLE in humans, an increasing body of evidence indicates that impaired clearance is an important cause of the increased load of apoptotic debris in SLE. *In vitro* studies have shown that monocytes from SLE patients have a decreased ability to engulf apoptotic debris compared to those from healthy individuals (40). Accumulated apoptotic debris has been found in the bone marrow of SLE patients and skin lesions of patients with cutaneous lupus (39). Deficiencies of complement components including C1q, C2 and C4 and reduced levels and activity of DNase I in serum, have also been found in some of SLE patients. Both conditions could lead to increased retention of apoptotic debris, and are associated with lupus-like autoimmunity in murine models (49,50).
1.3 Type I IFNs in SLE

1.3.1 Type I IFN signaling

Type I interferons (IFNs) are widely expressed cytokines that have potent antiviral and immunomodulatory properties. The type I IFN family consists of α subtypes (14 human and 11 mouse) and single β, ε, κ, τ, ζ and ω subtypes (51). All type I IFNs bind to the common heterodimeric type I IFN receptor (IFNAR). The receptor consists of two membrane spanning polypeptide chains, IFNAR1 and IFNAR2, which are constitutively associated with Janus kinase 1 (JAK1) and non-receptor tyrosine kinase 2 (TYK2) (51). The classical signaling pathway activated by type I IFNs is the JAK-STAT pathway (51,52). Activation of JAK1 and TYK2 results in the tyrosine phosphorylation and activation of several signal transducer and activator of transcription (STAT) family members (51,52). In most cells, these include STAT1, STAT2, STAT3 and STAT5, but in lymphocytes and endothelial cells, type I IFNs also induce activation of STAT4 and STAT6 (53). Activation of STAT1 and STAT2 leads to the recruitment of IFN regulatory factor (IRF) 9 and the formation of a STAT1–STAT2–IRF9 complex, which is known as the IFN-stimulated gene factor 3 (ISGF3) complex (51). This complex then migrates to the nucleus and binds to IFN-stimulated response elements (ISREs) in the promoters of IFN-stimulated genes (ISGs) to initiate gene transcription (51). While the JAK-STAT signaling cascade is the most extensively studied IFN-dependent pathway, it has been found that several other IFN-regulated signaling cascades are required for the generation of many of the responses to IFNs, including mitogen-activated protein kinase and phosphatidylinositol 3-kinase cascades (51). Some of these pathways operate independently of the JAK-STAT pathway, whereas others cooperate with STATs to optimize the transcriptional regulation of target genes.
1.3.2 Type I IFN induction pathways

Type I IFN production is normally induced by viruses, bacteria or microbial nucleic acids. Most types of cells can produce small amounts of type I IFN. However, the major type I IFN producer is the plasmacytoid dendritic cell (pDC). These cells constitute less than 1% of peripheral blood mononuclear cells (PBMCs) but produce large quantities of IFN-α (up to 10⁹ IFN-α molecules per cell within 24h) in response to a wide variety of exogenous and endogenous inducers (54–56). pDCs preferentially express TLR7 and 9 in their endosomal membranes and can therefore become activated by pathogens that invade the cell through receptor-mediated endocytosis (57). In addition, constitutive expression of IRF5 and 7 facilitates their capacity to produce IFN-α and other inflammatory cytokines (57).

The molecules that typically induce type I IFN secretion are sensed by pattern recognition receptors, such as Toll-like receptors (TLRs), retinoic acid inducible gene 1 (RIG 1)-like receptors and nucleotide oligomerization domain (NOD)-like receptors (58). TLRs involved in the production of type I IFN in pDCs include endosomal TLR7 and 9, which recognize ssRNA and unmethylated CpG-DNA, respectively (54,59). Ligand binding leads to TLR dimerization and conformational changes in both the receptor ectodomains and the cytoplasmic TLR-interleukin-1 (TIR) domains. The TIR domains then bind the adaptor protein MyD88, which associates with a complex consisting of tumor necrosis factor receptor-associated factor 6 (TRAF6) and interleukin receptor-associated kinase (IRAK) 1 and 4 (54,59). These factors lead to phosphorylation of IRF3, 5 and 7, translocation to the nucleus, and transcription of type I IFN genes (54,59). In contrast to pDCs, the majority of other cell types express cytoplasmic RNA helicases including RIG-1 and melanoma differentiation-associated gene 5 (MDA5) (54). These bind through caspase recruitment domains (CARD) to mitochondrial IFN-β stimulator 1 (IPS-1) also known as mitochondrial antiviral-signaling (MAVS), which initiates signaling cascades that
lead to IRF3, IRF7 and NF-KB activation resulting in IFN-α production (60).

1.3.3 Type I IFNs in SLE patients
Early observations that cancer and hepatitis patients develop a lupus-like syndrome after treatment with IFN-α provided the first evidence that type I IFNs have a central role in the pathogenesis of SLE (61,62). Increased serum levels of IFN-α were described in some SLE patients, with the levels correlating with disease activity and severity (63–66). For the majority of SLE patients whose serum levels of IFN-α are undetectable, there is increased expression of IFN-stimulated genes (ISGs) in the peripheral blood (the so-called IFN signature), indicating exposure to type I IFN (63,67–72). In cross-sectional studies, the level of ISG expression correlated with disease activity; however, in longitudinal studies, ISG expression did not fluctuate with disease activity, remaining stable over time despite changes in disease activity (73,74). Pediatric patients, who usually have more major organ involvement compared to adult patients, almost invariably display an IFN signature at early disease stages, which suggests that activation of the type I IFN system may be especially important in the initiation of the disease process (68). In addition, increased IFN-inducible chemokines in the serum of SLE patients are associated with active disease (75). Thus, the IFN signature in SLE patients is a prominent and reproducible marker for disease activity. Given that the majority of the IFN signature is inhibited by anti-IFN-α antibodies but not by antibodies to IFN-β or IFN-γ, IFN-α appears to be the major type I IFN implicated in SLE pathogenesis (76–78).

1.3.4 Plasmacytoid dendritic cells in SLE
It has been reported that patients with SLE have a reduced number of pDCs in their blood (79–81). Upon stimulation with TLR ligands, the pDCs from SLE patients have a normal capacity to secrete IFN-α, suggesting that the function of pDCs remains intact (78,79). In addition to IFN-α
production, pDCs have immunomodulatory functions, some of which may be abnormal in SLE patients. In fact, pDCs from SLE patients have a reduced capacity to induce regulatory T cells, leading to increased number of IL-17-producing Th17 cells, which may contribute to tissue inflammation and damage (81-83). While the number of circulating pDCs is decreased, there is increased accumulation of pDCs in tissues, particularly the skin, lymph nodes, and kidneys of SLE patients, where they are activated and synthesize IFN-α (84–87).

1.3.5 Inducers of type I IFN in SLE

There are several explanations for increased IFN-α production in SLE patients, including susceptibility genes linked to both the production and response to IFN-α (54). Notably, in human lupus, endogenous materials, particularly self-nucleic acids and immune complexes can stimulate pDCs and in some cases, other cell types to produce IFN-α by TLR-dependent pathways (54,89). Studies have shown that the immune complexes containing nucleic acids are internalized via the FcγRIIa receptor expressed on pDCs, resulting in localization to the endosome where they stimulate the relevant TLRs, leading to activation of transcription factors and IFN-α production (54,89). While TLRs mainly recognize nucleic acids of microbial origin, the uptake of immune complexes by FcγRIIa allows the trafficking of self-nucleic acids to TLR-containing endosomes (89). Moreover, binding of nucleic acids within nucleosomal or RNP particles or to autoantibodies prevents degradation by nucleases (89). In addition to the FcR-mediated pathway, several other molecules are involved in the IFN-α response to DNA-containing immune complexes. For example, interactions between high-mobility group box 1 protein (HMGB1) and receptor for advanced glycation end products (RAGE) can also mediate the TLR-9 activation triggered by DNA-containing immune complexes (90).
Endogenous stimuli that can induce IFN-α production by TLR-independent pathways have also been identified. These include cytosolic RNA sensed by the RIG-1 or MDA5 helicases, which are primarily expressed by cell types other than pDCs, such as myeloid DCs (mDCs), macrophages and granulocytes, resulting in much smaller quantities of IFN-α compared to pDCs (54,89,91). While most eukaryotic RNA species are known to lack 5’-triphosphate groups, which are required for recognition by RIG-1, the RNA transcripts in the nucleus and some RNA species in the cytosol of eukaryotic cells display 5’-triphosphates (89). Although there is limited evidence of IFN-α production via this pathway, it has been proposed that the uptake of apoptotic materials containing such RNA species by mDCs or macrophages may lead to RIG-1 engagement and type I IFN production. Intracellular administration of right-handed B-form dsDNA, the most common conformation of mammalian DNA, also triggers TLR9-independent production of type I IFN by mDCs, as demonstrated by Elkon and colleagues who reported increased gene expression of type I IFN in myeloid DCs from mice deficient in MyD88 and TLR9 (92,93). The specific sensors and adaptors recognizing cytosolic DNA remain to be elucidated.

The mechanisms leading to induction of IFN-α were initially described in late 1990s, when Ronnblom and colleagues found that the serum from SLE patients contain IFN-α inducing factors (IIFs) with the capacity to activate pDCs (94). These factors appeared to consist of immune complexes containing DNA and anti-DNA antibodies and could be mimicked by combining anti-dsDNA antibodies with plasmid pcDNA3 (95). Further studies revealed that cells transfected with TLR9 but not other TLRs responded to immune complexes in SLE serum, and blockade of FcγRIIa by neutralizing antibodies inhibited IFN-α production by pDCs (96). The immune complexes co-localized intracellularly with TLR9 and FcγRIIa, suggesting that DNA-containing immune complexes activate pDCs through cooperation of FcγRIIa and TLR9 (94).
Given the role of apoptosis in the generation of self-antigens in lupus, others demonstrated that apoptotic U937 cells combined with either SLE serum or purified SLE IgG induce IFN-α production by normal PBMCs (97). Notably, the IFN-α production correlated with the presence of antibodies against RNP but not anti-dsDNA antibodies, suggesting that similar to IIFs previously found in SLE serum, immune complexes containing RNA or RNA-binding proteins may have the potential to induce IFN-α in SLE (95). To assess the relative importance of DNA and RNA for IFN-α induction, subsequent studies have examined the effect of DNase or RNase on the ability of apoptotic materials to induce IFN-α when combined with SLE IgG. It was found that treatment with RNase completely abrogated IFN-α induction while treatment with DNase partially reduced IFN-α induction (98). In addition, IFN-α induction correlated with the presence of anti-snRNP antibodies but not anti-dsDNA antibodies (96). These observations suggest that immune complexes containing RNA rather than DNA may be associated with IFN-α production and IFN-α gene expression in SLE patients. In support of this concept, several studies have demonstrated the ability of immune complexes containing RNA components frequently targeted by lupus antibodies to stimulate IFN-α production (99–103). U-rich snRNA, especially U1, which are tightly bound to Sm and other snRNP components, were shown to stimulate IFN-α production when combined with anti-snRNP antibodies from SLE patients (103). It was also found that purified IgG containing anti-Ro antibodies induced high levels of IFN-α in PBMC cultures in the presence of supernatants from freeze-thawed cells or RNA particles (101,103). This response was strictly dependent on the RNA because treatment with RNase but not protease led to a complete loss of the IFN-α secretion (101). Both chloroquine and bafilomycin A1, which inhibit endosomal acidification and maturation, inhibited IFN-α production by U1 snRNP, implicating a role for TLRs, most likely TLR7 in the stimulation of IFN-α by RNA (100,101). The RNA-containing immune complexes appear to be associated with not only serum IFN-α
activity but also clinical manifestations, as they were found in the cerebrospinal fluid (CSF) of SLE patients with neuropsychiatric symptoms (104). These complexes were found to be capable of inducing the production of several chemokines reported to be elevated in the CSF of these patients (102).

1.3.6 Effects of type I IFN in SLE

Engagement of IFNAR by type I IFNs initiates several signal transduction pathways that lead to the expression of ISGs. The majority of ISGs are involved in inhibition of viral replication (53). For instance, activation of enzymes, such as myxovirus resistance A (MxA), 2′5′ oligoadenylate synthetase (OAS) and protein kinase 16 can inhibit viral transcription and translation and promote degradation of viral RNA (53).

In addition, type I IFNs have a wide range of effects on innate and adaptive immune cells, all of which can contribute to the development of SLE. In B cells, type I IFNs promote activation, isotype switching and differentiation into antibody-producing plasma cells, as well as expansion of B1 cells, the major producers of autoantibodies (105,106). In T cells, type I IFNs enhance T helper type I deviation, IFN-γ production and survival of CD4+ and CD8+ T cells (89).

Furthermore, type I IFNs inhibit B and T cell lymphopoiesis, resulting in increased homeostatic expansion of autoreactive cells (107). In DCs, type I IFNs promote mDC activation and upregulation of MHC and co-stimulatory molecules (CD40, CD80 and CD86), leading to self-antigen presentation to low-affinity autoreactive T cells (89). Type I IFNs also stimulate the secretion of the B cell survival factors BAFF and APRIL, which further promote the survival of autoreactive B cells (89,106). Moreover, type I IFNs provide a positive feedback mechanism in pDCs resulting in increased activation and upregulation of TLR7 and IRF7 (108).

Type I IFNs may also contribute to the retention of lymphocytes in lymphoid organs. The
level of sphingosine-1-phosphate (S1P) in lymphoid organs is relatively low compared to the lymphatic vessels, thereby forming an S1P gradient. S1P receptors on lymphocytes are responsive to this S1P gradient, promoting their egress from lymphoid organs (109). Type I IFNs induce a downregulation of this receptor, thereby causing lymphocyte retention in lymphoid organs (109). It is possible that this retention of cells leads to peripheral blood lymphopenia observed in SLE patients.

In summary, although many of the consequences of type I IFNs are beneficial in the context of viral infections, they can be detrimental in SLE-predisposed individuals by increasing the immune dysregulation.

1.4 Regulation of IFN-α and IFN-pathways in SLE

1.4.1 Role of C1q

One major protective role of the complement system is the clearance of apoptotic debris and immune complexes, which prevents the propagation of the autoimmune response. A deficiency in C1q, the first component of the classical complement pathway, is known as the strongest risk factor for the development of SLE. More than 90% of individuals with a genetic C1q deficiency develop the disease (110). A deficiency in C1r/C1s or C4 is also associated with SLE but at a lower frequency (111). Thus, the elevated risk of developing SLE in C1q deficiency compared to other complement deficiencies suggests that there must be other functions of C1q that are important in modulating the disease. In this context, C1q has been shown to regulate cytokine production from murine bone marrow-derived DCs following stimulation with TLR-4 or TLR-9 ligands (112). For example, C1q can inhibit IFN-α production by PBMCs in response to RNA-containing immune complexes, herpes simplex virus and CpG DNA, consistent with the previous finding that C1q-deficient patients have elevated serum levels of IFN-α (113,114). A similar
effect was seen for IL-6, IL-8 and TNF-α, suggesting that C1q has a general inhibitory effect on cytokine production without causing increased cell death (113). While the mechanism underlying the inhibition is still unclear, a study by Elkon et al. has proposed that it involves indirect interactions with CD14+ monocytes in PBMCs (112). When monocytes are absent, C1q enhances immune complex binding to pDCs and thereby increases IFN-α production (112). However, when monocytes are present, it preferentially promotes the uptake of immune complexes by these cells, leading to accumulation in early endosomes and decreased IFN-α production by pDCs (112). Serum and CSF from patients lacking C1q failed to suppress IFN-α production, resulting in a marked elevation of IFN-α and IP-10 in these fluids (112). All of these observations provide evidence for the potential regulatory function of C1q in SLE.

1.4.2 Role of IgG

Intravenous immunoglobulin (IVIG) has been used to treat several inflammatory and autoimmune diseases where type I IFN has been implicated in the pathogenesis (115,116). The main component of IVIG, IgG molecules has been shown to suppress inflammatory responses both in vitro and in vivo (115). This effect seems largely influenced by the amino acid sequence and glycosylation of IgG, as IgG that contains a glycan terminating in sialic acid has been shown to be more anti-inflammatory in certain mouse models (117,118). In a study by Elkon et al., it was found that the serum from neuropsychiatric SLE (NPSLE) patients has significantly decreased ability to induce IFN-α as compared to paired CSF, suggesting that the serum contains inhibitors that are not present in CSF (104). Mixing experiments revealed that the serum from healthy individuals, but not CSF, inhibits IFN-α stimulation by immune complexes containing autoantibodies from NPSLE CSF and exogenous nuclear antigens (103). Depletion of IgG from normal serum dramatically reduced this inhibition, confirming that at least one inhibitor in serum is IgG (103). Inhibition was restored by the addition of purified IgG; however, high
concentrations of IgG were required (103). In an attempt to identify the mechanisms of IFN-α inhibition by IgG and the importance of sialylation, subsequent studies have shown that IgG Fc fragments, but not F(ab)₂, inhibited IFN-α production induced by SLE immune complexes by competing for FcγRIIa on pDCs (119). However, in contrast to the studies demonstrating that the sialylated subset of IgG is critical for anti-inflammatory effects, the inhibition occurred independent of sialic acid (118).

1.4.3 Role of cytokines and inflammatory mediators

In addition to the inhibitory factors in serum, the regulation of IFN-α involves a complex network of interactions between various cell types and pro- and anti-inflammatory molecules. While previous reports have shown that C1q is required for the inhibition of IFN-α production through an indirect mechanism involving monocytes (112), others have found that even in the absence of C1q, monocytes have a profound inhibitory effect on IFN-α production by pDCs in PBMCs (120). In contrast, other cell types, including NK cells, have been shown to enhance IFN-α production (119). When the levels of TNF-α, IL-10, prostaglandin E₂ (PGE₂) and reactive oxygen species (ROS) were measured in different cell populations, it was found that monocytes are the major producers of PGE₂ and ROS in response to RNA-containing immune complexes (119). Further analysis revealed that PGE₂ as well as the cytokines TNF-α and IL-10 suppress IFN-α production in both PBMCs and purified pDCs (119). This effect was significantly counteracted by the ROS scavengers, serotonin and catalase in PBMCs but not monocyte-depleted PBMCs (119). Thus, in addition to C1q, several cytokines and mediators, which are largely produced by monocytes, have the capacity to regulate IFN-α response to immune complexes.
1.4.4 Role of anti-IFN-α antibodies

There is strong evidence in murine models that knockout of the type I IFN receptor in New Zealand Black lupus-prone mice ameliorates disease (121,122). In lupus, the role of IFN-α has been examined through administration or measurement of anti-IFN-α antibodies (AIAAs). *In vitro*, addition of neutralizing AIAAs inhibits the effects of serum IFN-α on various immune cells, including the maturation of DCs caused by SLE serum (123). Notably, a recent study by Behrens et al. found that a subset of SLE patients possesses endogenous AIAAs that are also able to neutralize IFN-α bioactivity (124). The majority of AIAA-positive patients were found to have significantly reduced levels of ISG expression and disease activity compared to AIAA-negative patients (123). However, others observed no correlation between the presence of AIAAs and disease activity, revealing some discrepancies with regard to the effect of AIAAs (125,126). Nevertheless, there is an increasing body of evidence that supports the role of AIAA in the therapy of SLE. Sifalimumab and Rontalizumab are human anti-IFN-α monoclonal antibodies that are currently being tested in phase II clinical trials (127–130). Both were shown to inhibit the ISG expression and disease flares in phase I clinical trials (126-129).

1.5 Serologically Active Clinically Quiescent (SACQ) SLE patients – a unique patient subset in prolonged clinical remission despite the presence of pathogenic autoantibodies

One of the challenges to treatment of SLE is the significant variability in clinical manifestations among patients and over time. Although there are currently no biomarkers that accurately predict disease flares, anti-dsDNA antibodies and hypocomplementemia have been strongly associated with disease activity in SLE in keeping with their role in disease pathogenesis (e.g. deposition of immune complexes and activation of complement with recruitment of pro-inflammatory mediators). Although many SLE patients demonstrate concordance in the levels of anti-dsDNA
antibodies and/or complement with disease activity (14–20), there is a subset of patients that maintain elevated levels of anti-dsDNA antibodies and/or hypocomplementemia for sustained periods of time in the absence of clinical activity (termed serologically active clinically quiescent or SACQ) (131,132). Since its first description by Gladman et al. in 1979, the existence of SACQ patients has attracted considerable attention (130, 131). It has been shown that SACQ patients represent up to 6% of the total SLE population followed prospectively at a large center (133). They differ from non-SACQ patients only in terms of a lower SLEDAI-2K score and lower frequency of use of steroids and immunosuppressives at presentation (132). Most of SACQ patients had active disease with similar frequencies of renal involvement, vasculitis and thromboembolism prior to the start of the SACQ period (132). Although SACQ patients are characterized by a sustained asymptomatic period, studies have found that 60% of SACQ patients become serologically active clinically active (SACA) after an average of 3 years (132). To ascertain whether there were predictors of this conversion, the levels of anti-dsDNA and serum complement were compared between patients who remained SACQ and those who became clinically active, with no difference being found (132). Some studies suggest that anti-chromatin antibodies correlate better with disease activity than anti-dsDNA antibodies and those with IgG isotype, especially complement-fixing IgG subclasses, are more pathogenic than IgM. However, a recent study by Steiman et al. observed no differences in the level of anti-chromatin IgG subclass or in the isotypes or ratio of IgG:IgM for either anti-dsDNA or anti-chromatin antibodies between patients who remained SACQ and who flared (134). These findings suggest that alternative immunologic mechanisms must be responsible for the clinical quiescence in SACQ patients.

1.5.1 Lack of IFN-α and IFN signature in SACQ patients

As an alternative explanation for the clinical quiescence in SACQ patients, our laboratory has
previously investigated the ability of SACQ patients to produce the proinflammatory cytokines that mediate inflammatory cell recruitment and tissue damage. Preliminary observations have shown that a number of cytokines including IFN-α, IL-6, IL-10, IP-10 and MCP-1 were significantly decreased in the plasma of SACQ compared to serologically active clinically active (SACA) patients, while the levels in SACQ and serologically quiescent clinically quiescent (SQCQ) patients were similar (135). Since IFN-α plays a central role in SLE pathogenesis, driving immune dysregulation of various immune cells and production of proinflammatory cytokines, such as IP-10 and MCP-1 (75), it was possible that the clinical quiescence in SACQ patients resulted from a lack of IFN-α production. Because of the difficulty in detection of IFN-α in serum, the IFN signature was measured, as previous work had suggested that the IFN signature correlated better with disease activity as well as the amount of IFN-α elaborated at the tissue level (66–73). Similar to the plasma levels of IFN-α, it was found that the sum of the expression levels of 5 ISGs, previously shown to be elevated in SLE (73), was significantly decreased in SACQ compared to SACA patients (manuscript submitted). Thus, despite the presence of autoantibody-containing immune complexes, which should be able to induce IFN-α and other proinflammatory cytokines through activation of pDCs and other cell populations, SACQ patients lack both an IFN signature and elevated levels of proinflammatory cytokines compared to SACA patients. This finding suggests that there are cellular or serologic factors in SACQ patients that inhibit the production of these factors.
1.6 Thesis hypothesis and objectives

SLE is a complex autoimmune disease in which multiple genetic and environmental factors lead to the breach of self-tolerance, resulting in the production of autoantibodies and formation of immune complexes that contribute to tissue inflammation and end-organ damage (3). Anti-dsDNA antibodies are highly specific for SLE and implicated in various aspects of disease pathogenesis including activation of complement as well as stimulation of pDCs and other cell types, resulting in production of IFN-α and proinflammatory cytokines. Although not as specific as anti-dsDNA antibodies, hypocomplementemia is frequently detected in SLE patients due to consumption by immune complexes (15,20). Despite the pathogenic role of anti-dsDNA and complement, a subset of SLE patients have acquired SACQ status after several years of disease activity, with some of them remaining SACQ for a prolonged period of time (131–133). Characterization of autoantibody specificity and isotype revealed no differences between SACQ and SACA patients (134). However, it has been shown that the plasma levels of IFN-α and proinflammatory cytokines as well as the IFN signature are significantly reduced in SACQ compared to SACA patients. Given the impact of IFN-α on immune cell activation and autoantibody production, we hypothesize that attenuated IFN-α production is the underlying mechanism for the clinical quiescence in SACQ patients and that these patients possess novel inhibitory mechanisms that lead to their lack of IFN-α production and decreased IFN signature.

We aim to test this hypothesis with the following objectives:

1. Determine whether SACQ and SACA patient serum differ in the ability to induce IFN-α and proinflammatory cytokine secretion.

2. Determine whether there are factors in SACQ and SACA patient serum that inhibit IFN-α and proinflammatory cytokine secretion.
2. Materials and Methods

2.1 Patient selection

A total of 24 SACQ, 28 SACA and 28 SQCQ patients attending the University of Toronto Lupus Clinic were included in the study. All patients fulfilled 4 or more of the revised 1997 ACR classification criteria for SLE. SACQ was defined as at least a 2-year period without clinical activity but with persistent serologic activity (SLE Disease Activity Index 2000 (SLEDAI-2K) score of 2 or 4, from positive anti-dsDNA antibody and/or hypocomplementemia only, at each clinic visit). These patients could be taking antimalarials, but those treated with corticosteroids or immunosuppressive medications were excluded. SACA was defined as clinical disease activity as determined by the clinical components of the SLEDAI-2K, which required corticosteroid or immunosuppressive treatment. SQCQ was defined as a SLEDAI-2K score of 0 (i.e. no clinical or serologic activity). Clinical and laboratory data were collected at each visit using a standardized protocol. Serum samples from all groups had been previously archived as part of longitudinal follow-up. Unselected patients with SLE were used to study the association between the IFN signature and anti-IFN-α antibodies and consisted of SLE patients with variable clinical and serologic activity. Healthy controls were recruited from hospital and laboratory staff members who had no family history of autoimmune disease. All subjects signed informed consent forms and patients signed a consent form allowing their anonymized clinical, serologic, and genetic data to be studied and reported.

2.2 Clinical serological testing

Anti-dsDNA antibodies were quantified by the Farr assay (normal range: 0-7 IU/ml). Serum complement C3 and C4 levels were measured by nephelometry (C3: normal range 0.9-1.8 gm/l, C4 normal range: 0.1-0.4 gm/l). Anti-Ro52, -Ro60, -La, -Sm and –RNP antibody levels were
measured by the FIDIS™ system based on the xMAP® technology at University of Calgary (normal range: 0-30 AU/ml).

2.3 PBMC isolation and culture

PBMCs were isolated from healthy donors using Ficoll-Paque density gradient centrifugation. Briefly, diluted blood was layered over Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ) and centrifuged at 400 x g for 30 minutes at room temperature. Cells at the interface were collected and washed in PBS. Red blood cells were depleted using lysis solution. The PBMCs were then seeded at 5 x 10^5 cells/well in U-bottom 96-well plates containing RPMI 1640 with 5% heat-inactivated fetal bovine serum (Invitrogen), 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen), 0.1 mM MEM nonessential amino acids (VWR International, Radnor, PA), 2 mM L-glutamine (Invitrogen) and 2-mercaptoethanol (Invitrogen). The cells were incubated with media alone (negative control), 0.5 μM CpG 2216 (positive control; Invitrogen) or a 1/20 dilution of SACQ, SAC, SQCQ or healthy control serum in the presence or absence of HeLa nuclear extract (NE) as a source of nuclear antigens. The cells used for IFN-α induction were primed with 500 U/ml universal type I IFN (recombinant IFN-α A/D; PBL Assay Science, Piscataway, NJ) and 2 ng/ml GM-CSF (R&D Systems, Minneapolis, MN) while those for proinflammatory cytokine measurement were left unprimed. To assess the presence of serum inhibitors, mixing experiments were performed with PBMCs incubated with 1/20 dilution of SACA serum alone or in the presence of 1/20 dilution of SACQ, SQCQ or healthy control serum. Supernatants were collected after 24 hours and stored at -20°C. For detection of anti-IFN-α antibodies, cells were seeded at 2.5 x 10^6 cells/ml in flat-bottom 24-well plates before stimulation with 2.5 μM CpG 2216. The supernatants were collected after 24 hours and incubated with 2 μg/ml anti-human IFN-α monoclonal antibody (positive control; PBL Assay Science) or 1/20 dilution of SACQ, SAC, SQCQ or healthy control serum. The supernatants
were collected after 2 hours and stored at -20°C. In each experiment, all serum samples were heat-inactivated (56°C for 30 minutes) prior to incubation with PBMCs or culture supernatants.

2.4 Preparation and analysis of nuclear extracts

HeLa cells were grown in DMEM (Princess Margaret Cancer Centre, Toronto, ON) with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin, 0.1 mM MEM nonessential amino acids, 2 mM L-glutamine and 2-mercaptoethanol. Nuclear extracts were isolated from HeLa cells as described previously (136,137). Briefly, cells were washed in PBS, followed by incubation in hypotonic buffer (0.01 M Tris, 0.01 M NaCl, 0.001 M MgCl₂, pH 7.4) for 10 minutes on ice. The cells were homogenized with a glass Dounce homogenizer and the nuclei were isolated by centrifugation at 3,300 x g for 15 minutes. Pelleted nuclei were resuspended in digestion buffer (Active Motif, Carlsbad, CA), and Enzymatic Shearing Cocktail (Active Motif) was added to digest the chromatin into smaller fragments. The extracts were obtained by centrifugation and equilibrated against PBS containing 20% glycerol. The protein concentration was determined by the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Briefly, the albumin standards and samples were added to a 96-well plate followed by the addition of the Working Reagent (50:1 mixture of Reagent A containing sodium carbonate, sodium bicarbonate, BCA and sodium tartrate and Reagent B containing cupric sulfate). The contents in the plate were mixed on a plate shaker for 30 seconds and incubated in the dark for 30 minutes. The absorbance was measured at 562 nm on a microplate reader.

2.5 Measurement of IFN signature

Total RNA was isolated from the peripheral blood of SQCQ, SACQ, SACA patients and healthy controls and reverse transcribed into cDNA. The expression levels of five ISGs (LY6E, OAS1, IFIT1, ISG15, and MX1) were determined by real-time polymerase chain reaction, normalized to
GAPDH and expressed as fold increase compared to those of healthy controls. For unselected SLE patients, a NanoString nCounter instrument (NanoString Technologies, Seattle, WA) was used to measure expression levels of EPST11, IFI44L, LY6E, OAS3, and RSAD2. For each patient, these values were summed to generate a composite IFN score.

2.6 Measurement of cytokines

The levels of IFN-α and proinflammatory cytokines in the supernatants were quantified by ELISA using commercially available kits as follows: IFN-α (Mabtech, Cincinnati, OH) and IP-10 (R&D Systems). Although a number of IFN-α ELISA kits are available, antibodies that detect IFN-α subtypes 8, 10, 14 and 17 were chosen because they do not cross-react with universal type I IFN used for priming PBMCs. 96-well plates were coated overnight with an anti-IFN-α or -IP-10 capture antibody at 4°C. After washing three times with PBS, the plates were blocked with PBS containing 1% BSA for 1 hour, washed three more times with wash buffer, and then incubated with standards or samples for 2 hours. After further washing, biotinylated detection antibody was added and incubated for 1 hour. The plates were washed again and incubated with streptavidin-alkaline phosphatase for 1 hour or streptavidin-horseradish-peroxidase for 20 minutes followed by the addition of substrate solution (pNPP or 1:1 mixture of H2O2 and Tetramethylbenzidine). The absorbance was measured at 405 nm or 450 nm using a microplate reader.

2.7 Detection of anti-IFN-α antibodies

Serum levels of IgG anti-IFN-α antibodies were measured by ELISA using an approach adapted from previous studies by Behrens et al (124). Briefly, 96 well plates were coated overnight with 0.5 μg/ml recombinant IFN-α1 (Prospec-Tany Technogene, East Brunswick, NJ) diluted in PBS at 4°C. The plates were washed with PBS and blocked with PBS containing 0.5% BSA for 1
hour at room temperature. After three washes with wash buffer, 1/50-diluted serum samples were added to the plates and incubated for 1 hour. The plates were washed again and incubated with 1/500-diluted alkaline phosphatase-conjugated goat anti-human IgG antibodies (Jackson Immunoresearch Labs, West Grove, PA) for 1 hour. After further washing, pNPP solution was added with incubation for 30 minutes. The absorbance was measured at 405 nm. To control for interplate and interassay variability, the same positive serum was included in each plate.

2.8 Statistical analysis

A Mann-Whitney non-parametric test was used for comparisons between two groups. Fisher’s exact test was used for comparisons of proportions between groups. Correlations between variables were assessed by Spearman’s rank correlation. A p value of <0.05 was considered significant. Statistical analysis was performed using Prism software (GraphPad, San Diego, CA).
3 Results

3.1 SACQ patients are demographically and serologically different from SACA patients

In this study, 24 SACQ, 28 SACA and 28 SQCQ patients were analyzed. Patient demographics, clinical manifestations and immunosuppressive drug use are shown in Table 1. The proportions of female patients were similar across all groups. The mean age of SACQ patients was significantly higher than that of SACA patients (44.5 ± 13.1 vs. 28.5 ± 13.5, p < 0.0001), while it was similar to that of SQCQ patients (44.5 ± 13.1 vs. 55.4 ± 12.3, p = 0.05). Both SACQ and SQCQ patients had longer disease duration as compared to SACA patients (19.0 ± 12.0, 24.3 ± 10.9 vs 7.7 ± 7.0, p < 0.0001). Similar proportions of SACQ and SACA patients were treated with antimalarials, but 82% of SACA patients were also receiving corticosteroids and 68% were taking immunosuppressive medications. The mean SLEDAI-2K of SACA patients was 16.6 ± 8.3, indicating that the majority of patients had serious organ involvement (>70% had active renal disease). SACQ patients had a mean SLEDAI-2K of 3.05 ± 1.17, from positive anti-dsDNA antibody and/or hypocomplementemia only.

As shown in Table 2, the levels of anti-dsDNA antibodies were significantly increased in SACA compared to SACQ patients (69.9 ± 39.2 vs. 23.1 ± 26.2, p < 0.0001). However, there was no difference in the proportion of anti-dsDNA positive patients (78.3% vs. 96.3%, p = 1.00). Anti-Sm and -RNP antibody levels were also significantly increased in SACA compared to SACQ patients (Sm: 57 ± 76.8 vs. 18 ± 50.2, p < 0.0001; RNP: 47 ± 37.1 vs. 14 ± 30.9, p = 0.003) with a significantly higher proportion of SACA patients with anti-RNP positivity (57.1% vs.12.5%, p = 0.02). Anti-Ro52, -Ro60 and -La antibody levels were similar between SACQ and SACA patients, again with similar proportions.
Table 1. Demographic and clinical characteristics of SACQ, SQCQ and SACA patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>SACQ (n=24)</th>
<th>SQCQ (n=28)</th>
<th>SACA (n=28)</th>
<th>p SACQ vs SQCQ</th>
<th>p SACQ vs SACA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>21 (87.5)</td>
<td>27 (96.4)</td>
<td>26 (92.9)</td>
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<td>1.00</td>
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<td>Age at study start, yrs</td>
<td>44.5 ± 13.1</td>
<td>55.4 ± 12.3</td>
<td>28.5 ± 13.5</td>
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<td>Disease duration at study start, yrs</td>
<td>19.0 ± 12.0</td>
<td>24.3 ± 10.9</td>
<td>7.7 ± 7.0</td>
<td>0.74</td>
<td>&lt;0.0001</td>
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<tr>
<td>SLEDAI-2K at study start</td>
<td>3.05 ± 1.17</td>
<td>0.0 ± 0.0</td>
<td>16.6 ± 8.3</td>
<td>&lt;0.0001</td>
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<td>Clinical features</td>
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<td>0 (0.0)</td>
<td>8 (28.6)</td>
<td>1.00</td>
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<td>Cutaneous</td>
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<td>0 (0.0)</td>
<td>11 (39.3)</td>
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<td>Vasculitis</td>
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<td>17 (60.7)</td>
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<td>1 (3.57)</td>
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<td>Steroid</td>
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<td>23 (82.1)</td>
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<td>Antimalarial</td>
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<td>Immunosuppressive</td>
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<td>19 (67.9)</td>
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</table>

Values are mean ± SD or n (%) unless otherwise stated. SLEDAI-2K, SLE Disease Activity Index 2000; CNS, central nervous system; SACA, serologically active clinically active; SACQ, serologically active clinically quiescent; SQCQ, serologically quiescent clinically quiescent.
Table 2. Serologic profiles of SACQ, SQCQ and SACA patients

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<tr>
<th></th>
<th>SACQ (n=24)</th>
<th>SQCQ (n=28)</th>
<th>SACA (n=28)</th>
<th>p SACQ vs SQCQ</th>
<th>p SACQ vs SACA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Presence of a positive test</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>α-dsDNA</td>
<td>(n=23) 18 (78.3)</td>
<td>0 (0.0)</td>
<td>(n=27) 26 (96.3)</td>
<td>&lt;0.0001</td>
<td>1.00</td>
</tr>
<tr>
<td>Low complement</td>
<td>(n=22) 14 (63.6)</td>
<td>0 (0.0)</td>
<td>(n=27) 23 (85.2)</td>
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<td>1.00</td>
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<tr>
<td>α-Sm</td>
<td>2 (8.33)</td>
<td>2 (7.14)</td>
<td>9 (32.1)</td>
<td>1.00</td>
<td>0.69</td>
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<tr>
<td>α-RNP</td>
<td>3 (12.5)</td>
<td>3 (10.7)</td>
<td>16 (57.1)</td>
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<td>0.02</td>
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<tr>
<td>α-Ro52</td>
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<td>5 (17.9)</td>
<td>9 (32.1)</td>
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<td>1.00</td>
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<tr>
<td>α-Ro60</td>
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<td>7 (25.0)</td>
<td>13 (46.4)</td>
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<tr>
<td>α-La</td>
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<td>1 (3.57)</td>
<td>4 (14.3)</td>
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<td>1.00</td>
</tr>
<tr>
<td><strong>Levels</strong></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>α-dsDNA</td>
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<td>3.43 ± 1.73</td>
<td>69.9 ± 39.2</td>
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<tr>
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<td>15 ± 24.2</td>
<td>1.00</td>
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</table>

Values are mean ± SD or n (%) unless otherwise stated. SACA, serologically active clinically active; SACQ, serologically active clinically quiescent; SQCQ, serologically quiescent clinically quiescent.
3.2 Optimization of an assay to examine the ability of SLE patient serum to induce IFN-α production by PBMCs *in vitro*

3.2.1 Priming enhances IFN-α production by PBMCs

Several studies with SLE serum have demonstrated that autoantibodies complexed with nuclear antigens stimulate the production of IFN-α and proinflammatory cytokines by PBMCs. To determine whether the differences in the levels of IFN-α and proinflammatory cytokines in SACQ and SACA patients arise from differences in the capacity of their immune complexes to induce these factors, we contrasted the ability of their serum to induce IFN-α and proinflammatory cytokine production by control PBMCs. Given the difficulty of measuring IFN-α by standard ELISA, we undertook several steps to optimize the IFN-α production induced by SLE patient serum. Previous studies by Ronnblom et al. have shown that co-stimulation with IFN-α and GM-CSF enhances IFN-α production by PBMCs in response to immune complexes (97,120,138). This phenomenon, termed priming, is thought to be due to increased expression of the transcription factors IRF-5 and IRF-7 and enhanced survival of the pDCs (139). We therefore examined the effect of IFN-α and GM-CSF priming on PBMCs following stimulation with immune complexes in SACA serum, to determine whether this would enhance our ability to detect IFN-α induced by SLE patient serum. As in a previous study, 500 U/ml universal type I IFN and 2 ng/ml GM-CSF were added to PBMCs co-incubated with various dilutions of SACA serum. Universal type I IFN is a hybrid IFN constructed from recombinant human IFN-α A and D. As shown in Figure 1, addition of universal type I IFN and GM-CSF alone did not lead to significant production of the subclasses of IFN-α detected by the ELISA, but led to a marked increase in the level of IFN-α induced by SACA 1162 serum (range: 6.3-9.2-fold). Although the level of IFN-α induced by SACA 1298 serum was considerably lower, suggesting a relative lack of immune complexes in this serum, it also showed a modest increase with priming. Thus, we
concluded that priming with IFN-α and GM-CSF could increase the levels of IFN-α induced by serum without increasing the background of the assay and consequently all PBMC cultures were primed with these cytokines to achieve optimal IFN-α production in future experiments.
Figure 1. Priming with IFN-α and GM-CSF enhances IFN-α production. Control PBMCs were incubated with SACA serum (1/20-1/2000 dilutions) in the presence or absence of universal type I IFN (500 U/ml) and GM-CSF (2 ng/ml). The levels of IFN-α in culture supernatants were measured after 24 hours by ELISA. Data shown are mean ± SD.
3.2.2 Complement inactivation leads to decreased inhibition of IFN-α production

It has been previously shown that the typical levels of C1q found in the serum can inhibit IFN-α production by pDCs in response to immune complexes through an indirect interaction with monocytes (113, 114). To determine whether serum complement affects our ability to detect differences in IFN-α production between SACQ and SACA patients, complement was inactivated using the same method as previously described (113). Eight SACA serum samples were heat-inactivated at 56°C for 30 minutes prior to incubation with PBMCs. As shown in Figure 2, heat-inactivation increased the level of IFN-α induced by SACA serum to a variable extent as compared to those without heat-inactivation (range: 0.7- to 24.7-fold). Of interest, SACA 44 and 1587 serum, which led to detectable levels of IFN-α in the absence of heat-inactivation, showed little or no increase following heat-inactivation, raising the possibility that they have low levels of complement. The remaining SACA serum resulted in increased levels of IFN-α, in most cases to levels above the limit of detection of the IFN-α ELISA, suggesting that complement potently inhibits IFN-α production. The extent of the increase varied between patients, suggesting that there are variable levels of complement in their serum or that additional mechanisms may modulate IFN-α production in some patients. These findings further confirm that complement plays a critical role in inhibition of IFN-α production induced by immune complexes. Consistent with previous studies, heat-inactivation not only reduced the inhibitory effect of complement, thereby enhancing our ability to detect IFN-α induced by SLE patient serum, but also eliminated complement as a variable in the differences in IFN-α production between patients. Thus, all serum samples were heat-inactivated in subsequent experiments.
Figure 2. Complement inactivation restores IFN-α production. Control PBMCs were incubated with SACA serum (1/20 dilution) with or without heat-inactivation. The levels of IFN-α in culture supernatants were quantified after 24 hours by ELISA. Data shown are mean ± SD. HI, heat-inactivation.
3.3 Decreased ability of serum from SACQ patients to induce IFN-α

As outlined previously, SACQ patients have a reduction in their IFN signature and IFN-α levels despite similar frequencies of autoantibodies and reduced complements to SACA patients (132,133, and submitted). However, there were differences in the levels of some of the autoantibodies, and thus one potential explanation for the difference in Type I IFN levels between these two patient subsets is that SACQ patients lack immune complexes that can drive IFN-α production. To assess this possibility, control PBMCs were incubated with SACQ, SACA, SQCQ or healthy control serum and the ability of immune complexes in the serum to stimulate IFN-α production was measured. As shown in Figure 3A, incubation of PBMCs with SACQ serum induced significantly decreased levels of IFN-α as compared to SACA serum (p = 0.0023), with the levels comparable to those induced by SQCQ and healthy control serum (p = 0.12; p = 0.19).

Since formation of immune complexes requires the presence of antigen, we next investigated whether the reduced levels of IFN-α induction seen with SACQ and SQCQ serum, as compared to SACA serum, were due to a lack of nuclear antigens. IFN-α production was assessed in the presence and absence of an exogenous source of nuclear antigens, HeLa nuclear extract. Although SLE is thought to be driven by antigens released from apoptotic cells, a large number of studies have used the HeLa nuclear extract with SLE serum to stimulate IFN-α production in vitro (104,120,136,137,140). This nuclear extract has been shown to contain key SLE antigens as well as low levels of RNases, proteases and other inhibitory factors, thus resulting in potent IFN-α production (137,141). In preliminary experiments, we assessed whether addition of HeLa nuclear extract could augment the IFN-α production induced by SACA serum. Control PBMCs were incubated with representative SACA sera alone or in the presence of HeLa
nuclear extract. At a serum dilution of 1/20, all of the SACA sera tested induced low levels of IFN-α in the absence of nuclear extract (Figure 3B). Following the addition of nuclear extract, the levels of IFN-α induced by SACA 1162 and 1633 serum increased in a dose-dependent manner reaching the highest level at concentrations of 40 and 60 μg/ml, respectively. This finding suggests that even in active SLE patients many of the anti-nuclear antibodies are not complexed with nuclear antigens. Since the differences observed for SACA 1162 and 1633 serum were most apparent with the use of 60 μg/ml of nuclear extract, this concentration was used to examine whether SACQ sera lack endogenous nuclear antigens in subsequent experiments.

As shown in Figure 3C, upon addition of nuclear extract, there were significant increases in the levels of IFN-α produced following stimulation with SACQ and SACA serum as compared to serum alone (p = 0.0043; p < 0.0001) while no difference was seen for SQCQ serum (p = 0.27). However, the levels of IFN-α induced by SACQ serum remained significantly decreased as compared to SACA serum (p = 0.0002).

Based on the ability of serum to induce IFN-α in the presence of nuclear extract, SACQ and SACA patients were clustered into two distinct subgroups: IFN<sup>low</sup> and IFN<sup>high</sup>. A threshold for IFN<sup>high</sup> was defined as greater than 2 SDs above the mean for serum from healthy controls. For the majority of SACA sera, addition of nuclear extract led to increased levels of IFN-α secretion upon co-incubation with control PBMCs, with 25/28 (89%) being IFN<sup>high</sup>, indicating that the autoantibodies in these patients are capable of inducing IFN-α when complexed with nuclear antigens (Figure 3D). In contrast, for the majority of SACQ sera (14/22) their ability to induce IFN-α secretion remained low even after addition of nuclear extract, and similar findings were observed of SQCQ patients.
To determine whether the inability of these SACQ sera to induce IFN-α production by PBMCs was due to a lack of high titer autoantibodies, we contrasted the serologic profile in IFN_{high} and IFN_{low} SACQ patient sera. There was a trend to decreased levels of autoantibodies in the IFN_{low} compared to IFN_{high} group but only anti-RNP antibodies achieved statistical significance when corrections were made for multiple comparisons (Figure 4; p = 0.044). This suggests that differences in the amount or specificity of immune complexes derived from autoantibodies especially anti-RNP antibodies may account for some of the differences in IFN-α production between SACQ and SACA patients. Consistent with this possibility, the level of C3 was significantly increased in the IFN_{low} compared to IFN_{high} group, indicating the impaired ability of autoantibodies to form immune complexes in vivo. However, a subset of IFN_{low} SACQ patients showed elevated levels of autoantibodies, in some cases to levels comparable to the IFN_{high} group, raising the possibility that there may be other mechanisms that lead to decreased IFN-α production in these patients.

To determine the type of autoantibodies that are associated with high levels of IFN-α production in vitro for the various SLE patient subsets, we contrasted the complement and autoantibody levels in IFN_{high} and IFN_{low} patient groups. There was a trend to increased levels of autoantibodies in IFN_{high} as compared to IFN_{low} patients for all three SLE patient groups, with the exception of anti-dsDNA antibodies for SQCQ patients, which by definition were absent in this patient subset (Figure 4). These differences achieved statistical significance for anti-RNP antibodies in SACQ patients. Consistent with a relative lack of immune complexes in vivo in SQCQ patients, the levels of C3 were higher in these patients that those in SACQ and SACA patients, and similar in both IFN_{high} and IFN_{low} groups. In both SACQ and SACA patient subsets, there was a trend to decreased levels of complement in the IFN_{high} group, which was significant for the SACQ subset, suggesting that there is a correlation between the ability of the
autoantibodies to form immune complexes and activate complement in vivo with their capacity to bind to nuclear antigens and induce IFN-α in vitro.

Notably, within the SACQ subset there is a group of patients that has high levels of autoantibodies, particularly of anti-Ro and anti-dsDNA specificities, but that has an impaired ability to induce IFN-α production even in the presence of exogenous nuclear antigens. For this subset of patients, alternative immune mechanisms, such as serum inhibitors may be responsible for the lack of IFN signature and pro-inflammatory factors, a possibility that will be explored later in my thesis.
Figure 3. SACQ serum has a decreased ability to induce IFN-α as compared to SACA serum. Control PBMCs were incubated with (A) SACQ, SACA, SQCQ or healthy control serum alone (1/20 dilution) (B) SACA serum alone or with various concentrations of HeLa nuclear extract (C) SACQ, SACA, SQCQ or healthy control serum with nuclear extract (60 μg/ml). (D) Changes in IFN-α production following addition of nuclear extract. The threshold for IFN<sup>high</sup> is shown as the dotted horizontal line. The levels of IFN-α in supernatants were measured after 24 hours by ELISA. Results are expressed as log of percentage of CpG control. Each symbol represents the results of an individual patient. Horizontal lines represent the mean of each group tested. Statistical differences between groups were determined by the Mann-Whitney non-parametric test. *p<0.05, **p<0.01, ***p<0.0001. NE: nuclear extract; HC: healthy control; n.s.: not significant.
Figure 4. Comparison of autoantibody and complement levels between IFN$_{\text{low}}$ and IFN$_{\text{high}}$ patients following addition of nuclear extract. Results are shown independently for each of the three patient subsets. Each symbol represents the results of an individual patient. Horizontal lines represent the mean of each group tested. Statistical differences between groups were determined by the Mann-Whitney non-parametric test with a Bonferroni’s multiple comparison test. *p<0.05. HC: healthy control; n.s.: not significant.
3.4 IFN-α induction does not correlate with IFN signature

To determine whether the ability of the serum to induce IFN-α in vitro correlated with the IFN signature in vivo, the levels of five ISGs (LY6E, OAS1, IFIT1, ISG15 and MX1) were measured and summed to generate a composite IFN score, that has been previously reported to be elevated in SLE (63,67,73). As seen in Figure 5A and B, there was no correlation between the levels of IFN-α induced by serum in the presence or absence of exogenous nuclear extract and IFN score for either SACQ or SACA patients (serum alone: p = 0.44, r = 0.20; p = 0.96, r = -0.009; serum with nuclear extract: p = 0.30, r = 0.25; p = 0.03, r = 0.88). Nor were significant differences in the IFN score observed when patients were stratified into IFN<sub>low</sub> and IFN<sub>high</sub> groups (data not shown). These findings suggest that the autoantibodies and/or immune complexes contained within the peripheral blood are not the primary drivers of the IFN signature in vivo. Given the accumulation of pDCs in the tissues of SLE patients, it is possible that the IFN signature is driven by IFN-α produced in response to local tissue immune complexes rather than those found in the peripheral blood (54). Alternatively, the IFN signature might reflect the balance between immune mechanisms that drive IFN-α production, such as nuclear antigen-containing immune complexes, and a number of regulatory mechanisms, as outlined in the introduction to the thesis.
Figure 5. Lack of association between IFN scores and IFN-α induction \textit{in vitro}.

Comparison of IFN scores and IFN-α induction by (A) serum alone or (B) serum in the presence of HeLa nuclear extract using Spearman’s correlation. The IFN-α levels are expressed as log of percentage of CpG control. The IFN score was calculated by summing the expression levels of 5 ISGs (LY6E, OAS1, IFIT1, ISG15 and MX1), as outlined in the Methods. Each symbol represents the result for an individual patient.
3.5 SACQ patients have serum inhibitors of IFN-α production

Since serum from healthy donors is known to contain several inhibitors that suppress IFN-α production stimulated by immune complexes, it is possible that the differences in IFN-α induction observed between SACQ and SACA patients result from differences in the presence of serum inhibitors. To assess this possibility, we performed a pilot experiment in which we measured IFN-α production by PBMCs that were stimulated with SACA serum in the presence or absence of SACQ, SQCQ and healthy control serum, as potential inhibitors. To determine whether any potential inhibition was antibody-mediated, we depleted IgG in SACQ, SQCQ and healthy control serum using protein G sepharose and compared the effects of complete serum with IgG-depleted serum (104,119). In these pilot experiments, we used SACA serum with two different specificities to assess whether the nature of inhibition differed depending on the type of immune complexes present. As shown in Figure 6A, the addition of SACQ serum to a SACA serum with anti-dsDNA antibodies alone (SACA 1) dramatically decreased the levels of IFN-α induced by SACA serum, and similar results were seen for SQCQ and healthy control serum. This inhibitory effect was minimally reversed by IgG depletion. These findings suggest that there are non-IgG serum inhibitors in SACQ, SQCQ and healthy control that block the ability of DNA-associated immune complexes to induce secretion of IFN-α by control PBMCs. In contrast, for another SACA serum containing both anti-dsDNA and anti-Ro antibodies (SACA 2), only one SACQ serum decreased the levels of IFN-α detected following PBMC stimulation, while the remaining SACQ, SQCQ or healthy control serum had little or no effect (Figure 6B). The inhibitory effect of this SACQ serum was completely abrogated by IgG depletion, suggesting that it is antibody-mediated. These findings suggest that there may be multiple serum inhibitors of IFN-α secretion in SACQ sera, and that the mechanisms may differ depending on whether the complexes contain DNA or RNA-associated complexes.
Figure 6. Selective SACQ serum samples mediate inhibition of IFN-α induced by SACA serum. Control PBMCs were incubated with a mixture of SACA serum and SACQ, SQCQ or healthy control serum (1/20 dilution each) in the presence of HeLa nuclear extract (60 μg/ml). SACQ, SQCQ and healthy control serum were left untreated or IgG-depleted prior to incubation. (A) IFN-α induced by DNA-associated complexes was generally inhibited by SACQ, SQCQ and healthy control serum and the inhibition was not mediated by IgG. (B) IFN-α induced by RNA-associated complexes was inhibited by one of SACQ serum and the inhibition was dependent on IgG. SACA 1, anti-dsDNA positive; SACA 2, anti-dsDNA and -Ro positive. The levels of IFN-α in supernatants were measured after 24 hours by ELISA. Data shown are mean ± SD. HC, healthy control; NE, nuclear extract.
3.6 Serum from SACQ patients can induce proinflammatory cytokines

To investigate whether the inhibition by SACQ serum played a role in the lack of production of other proinflammatory cytokines in these patients, control PBMCs were incubated with SACQ, SACA, SQCQ or healthy control serum in the presence or absence of nuclear extract. In this experiment, PBMCs were unprimed to avoid production of cytokines that occurred by priming alone (data not shown). As shown in Figure 7A, the addition of SQCQ, SACQ and SACA serum all resulted in significantly increased levels of IP-10 as compared to healthy control serum (healthy control vs. SQCQ p = 0.002; healthy control vs. SACQ p = 0.001; healthy control vs. SACA p<0.0001), with no differences between groups. This suggests that SQCQ and SACQ serum have immune complexes that can stimulate IP-10 production to levels comparable to SACA serum and that proinflammatory cytokine production may not be regulated similarly to IFN-α by serum factors. However, following the addition of nuclear extract, the level of IP-10 induced by SACA serum was significantly increased as compared to either SACQ or SQCQ serum (p = 0.002; p<0.0001), while no difference was seen between SQCQ and SACQ serum (Figure 7B). Since SACA serum has been shown to induce IFN-α to much higher levels than SACQ serum in the presence of nuclear extract (mean difference: SACA 29.4; SACQ 10.1), and IFN-α induces secretion of IP-10, we questioned whether the increase in IP-10 observed for SACA, as compared to SACQ, serum, was due to the increased levels of IFN-α induced by SACA serum. To block the effect of IFN-α, an anti-human IFN-α antibody was added to the PBMC culture along with SQCQ, SACQ or SACA serum and nuclear extract. For either SACQ or SQCQ serum, there was no difference in IP-10 with or without anti-IFN-α antibodies, suggesting that IFN-α levels may be too low to augment IP-10 production (Figure 7C). Alternatively, much of IP-10 may be driven by other cytokines, such as IFN-γ. In contrast, there
was a 1.6-fold decrease in the levels of IP-10 induced by SACA serum in the presence of anti-IFN-α antibodies, suggesting that some of the enhanced IP-10 secretion seen for SACA serum may arise from IFN-α-induced secretion of IP-10.
Figure 7. SACQ serum has immune complexes that can induce proinflammatory cytokines. Control PBMCs were incubated with (A) SACQ, SACA, SQCQ or healthy control serum alone (1/20 dilution) or (B) serum in the presence of HeLa nuclear extract (60 μg/ml) (C). The enhanced IP-10 production seen in SACA patients is partially mediated by IFN-α. Anti-IFN-α antibody (2 μg/ml) was added to PBMCs co-incubated with SACQ, SQCQ or healthy control serum (1/20 dilution) and HeLa nuclear extract (60 μg/ml). The levels of IP-10 in the supernatants were measured after 24 hours by ELISA. Results are expressed as log of percentage of CpG control. Each symbol represents the results of an individual patient. Horizontal lines represent the mean of each group tested. Statistical differences between groups were determined by the Mann-Whitney non-parametric test. *p<0.05, **p<0.01, ***p<0.0001. HC: healthy control; NE, nuclear extract; n.s.: not significant.
3.7 Serum from SACQ patients can block detection of IFN-α by ELISA

Given that the inhibition of IFN-α production/detection by SACQ serum in vitro appeared to be mediated at least in part by IgG, we explored whether this activity was attributable to the presence of anti-IFN-α antibodies in SACQ serum. To determine the presence of anti-IFN-α antibodies, SACQ, SACA, SQCQ and healthy control sera were incubated with the supernatants of PBMC cultures that had been stimulated with CpG 2216, as a source of IFN-α, and then examined for their ability to block detection of IFN-α by ELISA. Following incubation of supernatant with SACQ 987 serum, that we had previously shown to have an IgG inhibitor (see Figure 6), there was a 9-fold decrease in the level of IFN-α detected, consistent with the possibility that this serum has IgG anti-IFN-α antibodies (Figure 8A). However, assessment of all patient sera revealed only 5 samples that demonstrated marked inhibition of IFN-α detection (SACQ 3/24; SQCQ 1/28; SACA 1/28), and there were no differences in mean levels of IFN-α detected in each group (mean IFN-α detected as a percent of control without added serum: SACQ 87.5; SACA 96.4; SQCQ 96.4; Figure 8B), suggesting that the anti-IFN-α antibodies do not play a major role in SACQ patients.
Figure 8. Detection of IFN-α in culture supernatants following incubation with SACQ, SACA, SQCQ or healthy control serum. Control PBMCs were incubated with media alone or media containing CpG 2216 (2.5 μM). The supernatants were collected and incubated with 1/20 dilution of SACQ, SACA, SQCQ or healthy control serum prior to measurement by ELISA. Anti-IFN-α antibody (2 μg/ml) was used as a positive control. (A) Bar graph of representative results showing a marked decrease in IFN-α levels following incubation with SACQ 987 serum. (B) Scatter plot showing IFN-α levels following incubation with all serum samples from each group. Results are expressed as percentage of IFN-α detected in the supernatants without serum. Each symbol represents the results of an individual patient. Horizontal lines represent the mean of each group tested. Statistical differences between groups were determined by the Mann-Whitney non-parametric test. HC: healthy control; n.s.: not significant
3.8 Anti-IFN-α antibodies are associated with decreased IFN-α induction

Previous studies have used several methods to detect anti-IFN-α antibodies in SLE serum (124–126). While some of our patients were shown to block detection of IFN-α by ELISA, raising the possibility that they have anti-IFN-α antibodies, the observation that addition of exogenous anti-IFN-α antibodies at concentrations <10 μg/ml failed to block detection suggested that this approach was relatively insensitive. Alternatively, the epitope recognized by the anti-IFN-α antibodies in the ELISA could differ from that of some of the endogenous anti-IFN-α antibodies, limiting the ability of this blocking assay to detect some of the clinically relevant anti-IFN-α antibodies present in patients. Therefore, to more directly measure the levels of anti-IFN-α antibodies in the serum of SLE patients, we utilized a previously published ELISA in which recombinant IFN-α1 was used as a substrate. In this assay, the majority of the serum samples did not exhibit signals above background, with only 7 out of 80 demonstrating elevated levels (Figure 9). As shown in this figure, there was a trend to a higher proportion of patients with anti-IFN-α antibodies in SACQ as compared to the SACA or SQCQ subsets, although these differences were not significant (% positive: SACQ 12.5; SQCQ 7.14; SACA 7.14). These results are consistent with the results for inhibition of IFN-α detection, confirming that anti-IFN-α antibodies are not the major type of inhibitor in SACQ patients.

Since anti-IFN-α antibody therapy has been shown to effectively neutralize IFN-α in some studies (123,128,130,142), we determined whether the anti-IFN-α antibody (AIAA) status was associated with differences in measures of IFN-α induction *in vitro* and the IFN signature *in vivo*. There was a trend to reduced levels of IFN-α induced by either serum alone or by a combination of serum and nuclear extract in PBMC co-cultures and lower IFN scores in the AIAA-positive group as compared to the AIAA-negative group (Figure 10A-C), but this did not achieve statistical significance. Further analysis revealed that serum from all AIAA-positive
patients demonstrated IFN-α induction below that of healthy controls, with the majority (5/7) remaining as IFN$^{\text{low}}$ even after addition of nuclear extract (Figure 10A and B and Table 3). In contrast, the IFN scores varied with the AIAA levels, with a large discordance observed for SACA 1316 (Table 3). Taken together, the data suggest that the presence of AIAAs is associated with low IFN-α induction but is not always associated with a low IFN signature. While the antibodies may play a critical role in AIAA-positive patients, other mechanisms must be responsible for the lack of IFN-α induction in AIAA-negative SACQ patients.
Figure 9. Lack of endogenous anti-IFN-α antibodies in SACQ, SACA, SQCQ and healthy control serum. Scatter plots showing IgG anti-IFN-α antibody levels as measured by ELISA. Each symbol represents the results of an individual patient. Horizontal lines represent the mean of each group tested. Statistical differences between groups were determined by the Mann-Whitney non-parametric test. HC: healthy control; n.s.: not significant.
Figure 10. Comparison of IFN-α induction and IFN scores between AIAA-positive and AIAA-negative patients. SACQ, SACA and SQCQ patients were stratified into AIAA-positive and AIAA-negative groups based on signals in the ELISA. Levels of IFN-α induced by (A) serum alone (B) serum with HeLa nuclear extract, expressed as percentage of CpG control. The threshold for IFN^{high} is shown as the dotted horizontal line. c) IFN scores, quantified as described in the Methods. Each symbol represents the results of an individual patient. Horizontal lines represent the mean of each group tested. Statistical differences between groups were determined by the Mann-Whitney non-parametric test. AIAA: anti-IFN-α antibody; HC: healthy control; n.s.: not significant.
Table 3. Presence of anti-IFN-α antibodies is associated with low IFN-α induction but a variable IFN signature.

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<td>0.40</td>
<td>0.163</td>
<td>1.08</td>
<td>10.1</td>
</tr>
<tr>
<td>SQCQ 289</td>
<td>0.055</td>
<td>0.00</td>
<td>0.00</td>
<td>5.40</td>
</tr>
<tr>
<td>SQCQ 514</td>
<td>0.099</td>
<td>0.206</td>
<td>1.88</td>
<td>57.1</td>
</tr>
<tr>
<td>SACA 1316</td>
<td>&gt;3</td>
<td>0.00</td>
<td>0.305</td>
<td>126.6</td>
</tr>
<tr>
<td>SACA 140</td>
<td>0.153</td>
<td>0.557</td>
<td>19.9</td>
<td>4.11</td>
</tr>
</tbody>
</table>

<sup>a</sup> Expressed as % of CpG control
<sup>b</sup> Sum of the expression levels of 5 ISGs.
3.9 Anti-IFN-α antibodies are associated with a variable IFN signature.

Our study of AIAAs in SACQ, SQCQ, and SACA patients suggested that the presence of AIAAs was not necessarily associated with normalization of the IFN signature. To further explore this possibility, we measured the AIAA levels in an independent cohort of 121 unselected SLE patients for whom IFN scores were available. Approximately 25% of patients were positive for AIAAs, with the majority of those patients that were positive having low levels of AIAAs (Figure 11A). Similar to the trends observed for SACQ and SACA patients, there was no correlation between AIAA levels and IFN scores (Spearman’s r = -0.026, p= 0.86; Figure 11B). While the presence of AIAA may be associated with decreased ability to induce IFN-α in vitro, the majority of AIAA-positive patients have elevated IFN signatures, which as shown in Figure 11C often markedly exceeded the levels seen in healthy controls. These findings suggest that endogenous AIAAs may be insufficient to neutralize IFN-α in vivo. Alternatively, other types I IFNs, for example IFN-β, may be involved in the expression of IFN signature in these patients.
Figure 11. Lack of association between anti-IFN-α antibodies and IFN signature in SLE patients. (A) Scatter plot showing IgG anti-IFN-α antibodies levels as measured by ELISA (n = 121). Each symbol represents the results of an individual patient. Horizontal lines represent the mean of each group tested. (B) The levels of IgG anti-IFN-α antibodies were compared to IFN scores using Spearman’s correlation (n = 49). (C) IFN scores obtained for healthy controls and SLE patients that were positive for anti-IFN-α antibodies. The dotted line shows the mean (+2 SD) IFN score for healthy controls (n = 23). AIAA: anti-IFN-α antibody; HC: healthy control; n.s.: not significant.
4.0 Anti-IFN-α antibodies are not associated with lower disease activity.

To determine the impact of AIAAs on disease activity, we compared various clinical features between AIAA-positive and -negative patients. As shown in Table 4, AIAA-positive patients had a significantly lower mean disease duration at the time of blood collection compared to AIAA-negative patients, raising the possibility that the occurrence of antibodies occurs early in the disease course and gradually normalizes over time. The mean SLEDAI-2K scores for AIAA-positive and-negative patients were 8.28 and 6.41, respectively with no difference between groups, indicating that the majority of patients were active regardless of AIAA status. The frequencies of clinical manifestations, including rash, arthritis, vasculitis, renal and CNS diseases appeared to be similar between groups, suggesting a lack of association between the presence of AIAAs and specific clinical manifestations. Similarly, no difference in the frequency of antimalarial or steroid use was observed between groups. Taken together, the data suggest that the presence of endogenous AIAAs in SLE patients is not associated with modulation of overall disease activity or particular organ involvement.
Table 4. Demographic and clinical characteristics of AIAA-positive and AIAA-negative SLE patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>AIAA-positive (n=21)</th>
<th>AIAA-negative (n=49)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>21 (87.5)</td>
<td>27 (96.4)</td>
<td>1.00</td>
</tr>
<tr>
<td>Age at study start, yrs</td>
<td>30.4 ± 11.9</td>
<td>37.4 ± 13.3</td>
<td>0.28</td>
</tr>
<tr>
<td>Disease duration at study start, yrs</td>
<td>6.37 ± 4.29</td>
<td>13.2 ± 8.58</td>
<td>0.01</td>
</tr>
<tr>
<td>SLEDAI-2K at study start</td>
<td>8.28 ± 5.14</td>
<td>6.41 ± 6.39</td>
<td>1.00</td>
</tr>
<tr>
<td>Clinical features</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arthritis</td>
<td>2 (9.52)</td>
<td>4 (8.16)</td>
<td>1.00</td>
</tr>
<tr>
<td>Cutaneous</td>
<td>2 (9.52)</td>
<td>6 (12.2)</td>
<td>1.00</td>
</tr>
<tr>
<td>Vasculitis</td>
<td>2 (9.52)</td>
<td>4 (8.16)</td>
<td>1.00</td>
</tr>
<tr>
<td>Renal</td>
<td>9 (42.9)</td>
<td>13 (26.5)</td>
<td>1.00</td>
</tr>
<tr>
<td>CNS</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1.00</td>
</tr>
<tr>
<td>Medications</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steroid</td>
<td>14 (66.7)</td>
<td>35 (71.4)</td>
<td>1.00</td>
</tr>
<tr>
<td>Antimalarial</td>
<td>18 (85.7)</td>
<td>36 (73.5)</td>
<td>1.00</td>
</tr>
<tr>
<td>Immunosuppressive</td>
<td>0 (0.0)</td>
<td>1 (2.04)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Values are mean ± SD or n (%) unless otherwise stated. SLEDAI-2K, SLE Disease Activity Index 2000; CNS, central nervous system.
4 Discussion and future directions

Previous studies from our laboratory have highlighted the lack of IFN-α and IFN signature in SACQ patients. Here we demonstrate that the serum from SACQ patients has a significantly reduced ability to induce IFN-α production by PBMCs compared to the serum from SACA patients and that the mechanisms underlying the differences involve, at least in a subset of patients, the presence of inhibitors in SACQ serum.

It is now well established that the immune complexes between autoantibodies and nuclear antigens stimulate IFN-α production by pDCs in an FcR-dependent manner (78,96,98,103). Given that the majority of SACQ patients have elevated levels of autoantibodies (Table 2), they should have immune complexes that can stimulate IFN-α production. However, incubation of control PBMCs with SACQ serum resulted in decreased levels of IFN-α as compared to SACA serum. Characterization of patients as IFN\text{low} and IFN\text{high} based on IFN-α induction revealed a higher proportion of SACA patients in the IFN\text{high} group compared to SACQ patients. Upon addition of nuclear extract, the majority of SACA patients induced higher levels of IFN-α, in most cases to levels corresponding to IFN\text{high} patients. In contrast, 22 out of 24 SACQ patients were classified as IFN\text{low} with 8 of these patients showing a marked increase in IFN-α levels with nuclear extract. This suggests that IFN-α induction in these patients may be low due to a lack of endogenous nuclear antigens. Examination of SACQ patients that remained IFN\text{low} despite the presence of exogenous nuclear antigens showed no significant difference in the levels of anti-dsDNA, -Ro, -La or –Sm antibodies compared to those in the IFN\text{high} group. In contrast, the difference in anti-RNP antibodies suggests that there may be an association between the levels of these autoantibodies and IFN-α production observed in IFN\text{high} SACQ patients. Nevertheless, the presence of elevated autoantibodies in a subset of IFN\text{low} SACQ patients indicates that other immune mechanisms may be involved in modulating IFN-α production in these patients.
There are two mechanisms that regulate the availability of nuclear antigens in SLE patients: rate of apoptosis and clearance of apoptotic debris (40,47,143,144). It is well known that lymphocytes from SLE patients undergo an increased rate of apoptosis compared to those from healthy controls and other autoimmune disorders and that the rate of apoptosis correlates with disease activity (42). Thus, the rate of apoptosis of lymphocytes from SACQ patients may be lower than apoptosis of lymphocytes from SACA patients, leading to decreased levels of antigens available for immune complex formation. This may be the case for SACQ patients whose IFN-α induction increased in the presence of nuclear extract. For the remaining SACQ patients, IFN-α induction did not increase to the levels seen with SACA serum, suggesting that this phenomenon is not simply a result of lack of antigens due to defects in apoptosis.

There are several molecules that mediate clearance of apoptotic debris, including C1q and DNase I. It has been shown that the reduced activity of any of them in mice or humans arising from gene mutations or increased consumption by autoantibodies is strongly associated with active disease (145–150). In addition to activating complement pathway, C1q contributes through unknown mechanisms to inhibition of IFN-α production by pDCs in response to SLE immune complexes (113,114). Although there could be differences in the level and/or activity of C1q between IFN_{\text{low}}/SACQ and SACA patients, it is important to note that all serum samples were complement-inactivated prior to incubation with PBMCs. This argues that other factors are present in SACQ serum, but does not exclude the possibility that C1q is involved.

DNase I is a common endonuclease found in serum that facilitates degradation of chromatin during apoptosis (47). It has been implicated in SLE pathogenesis owing to the observation that serum levels of DNase are decreased in patients with the disease (151). In addition, diminished transcription and expression of renal DNase I were observed in lupus-prone
mice (152). These defects in DNase 1 may allow more immune complexes to persist and further promote disease progression. A recent study by Leffler et al. found that patients with lupus nephritis have impaired ability to degrade neutrophil extracellular traps (NETs), a source of autoantigens in SLE that contains chromatin and neutrophil proteins (153–156). Serum from most of these patients efficiently degraded NETs in remission, suggesting that NET degradation varies with disease activity (153). Another study found that the impaired degradation was linked to the presence of DNase I inhibitors (e.g. G-actin) or antibodies against NETs that protect NETs from the nuclease (157). Although the precise role of DNase I was not explored in this study, it is possible that IFN<sup>low</sup> SACQ patients have increased DNase I activity as compared to SACA patients, leading to decreased immune complex formation and decreased production of IFN-α and ultimately downstream gene expression in the IFN pathway. Because of the difficulty of measuring DNase I levels in serum, further studies will measure the ability of patient serum to degrade nuclear antigens, such as NETs using an approach adapted from previous studies. Based on the differences in the amount of degradation observed between SACQ and SACA patients, the presence of DNase I inhibitors or anti-NET antibodies will be examined to identify the mechanisms underlying these differences between the two groups.

Alternatively, the differences in IFN-α induction between SACQ and SACA patients may result from the differences in the ability of their autoantibodies to either bind nuclear antigens or activate pDCs. In fact, studies have found that one serum inhibitor that downregulates IFN-α production by pDCs is IgG (104,119). Depending on the amino acid sequence or glycosylation, IgG molecules can exert a strong anti-inflammatory effect in certain mouse and human models of autoimmune disease (118). In regards to its effect on IFN-α, it has been shown that it directly impedes immune complex binding to FcγRIIa on pDCs (119). However, this mechanism of IgG inhibition has been questioned as FcγRIIa has low affinity for monomeric IgG (103). The
dilution of SACQ serum (1/20) used in our assay was likely too low to achieve inhibition by competitive binding to the FcR, as high concentrations of IgG are required to attenuate IFN-α production by this mechanism (103). However, it is still possible that a distinct subgroup of IgG inhibits IFN-α through mechanisms other than competition resulting in the decreased IFN-α induction by SACQ serum.

To determine whether SACQ patients have serum inhibitors of IFN-α production, we performed mixing experiments. The addition of SACQ serum to SACA serum at equal dilutions (1/40) reduced the levels of IFN-α measured by ELISA far in excess of the dilution factor. The mechanism of inhibition appeared to vary with the autoantibody profile of SACA serum, indicating the presence of multiple inhibitors in SACQ serum. Following incubation with SACA serum containing anti-dsDNA antibodies, similar inhibition was observed for SACQ, SQCQ and healthy control serum, suggesting that IFN-α induction by DNA-containing complexes may be inhibited by mechanisms found in both SLE and healthy individuals. The fact that the inhibition was not reversed by IgG depletion raises the possibility that nuclease activity plays a role in the clearance of DNA-containing immune complexes. In contrast, only SACQ serum caused inhibition following incubation with SACA serum containing anti-Ro and -dsDNA antibodies, which was completely abrogated by IgG depletion. It is possible that at least one factor that inhibits IFN-α induction by Ro or other RNA-containing immune complexes is IgG. Taken together, these findings indicate that the serum from SACQ patients contains potent inhibitors of IFN-α production, some of which are lacking or decreased in SACA, SQCQ and healthy individuals. To further support this concept, future studies will investigate the inhibitory effect of all SACQ, SQCQ and healthy control serum on IFN-α induced by multiple SACA serum with different autoantibody specificities.
In addition to IFN-α, many proinflammatory cytokines and chemokines, such as IL-6, IL-10, IP-10 and MCP-1 were detected at lower levels in SACQ serum as compared to SACA serum. Although the exact mechanism responsible for stimulation of these cytokines has not been identified, there is abundant evidence to suggest that nucleic acid-containing immune complexes can be taken up by B cells, mDCs or monocytes through the BCR or FCR (57). Binding of nucleic acids to TLR-7 or TLR-9 then leads to activation of NFkB or MAPK signaling cascades, resulting in the production of proinflammatory cytokines as well as B cell survival factors that mediate inflammation in SLE (57). We found that SACQ serum contains immune complexes that can drive cytokine production, in particular IP-10, which is augmented by addition of nuclear antigens. While this finding appears to be incompatible with the possibility that nucleases may inhibit IFN-α production by degrading nuclear antigens in immune complexes, it is possible that production of these cytokines is less sensitive to the effects of nucleases. Alternatively, there may be other non-Ig inhibitors in normal serum that specifically inhibit cytokine production by pDCs, the major producers of IFN-α in our assay.

While IP-10 and MCP-1 were previously shown to be induced by type I IFNs (104), the increase in IP-10 production following stimulation with SACQ serum suggests that it may be driven by direct activation of immune cells by immune complexes; however, the exact pathway leading to this event is not fully understood. The ability of a neutralizing anti-IFN-α antibody to inhibit some of IP-10 production in response to SACA serum but not SQCQ or SACQ serum suggests that immune complexes and IFN-α induced by SACA serum work in concert to augment IP-10 production. Exploring the mechanisms that drive cytokine production in SACQ patients is an area of future interest and will help elucidate the pathways that lead to decreased levels of these cytokines in SACQ patients.
The elevated expression of ISGs is characteristic of many SLE patients and correlates with disease activity (66–73). The mechanism of induction is still unclear but it is likely through activation of IFNAR by IFN-α. This is supported by the observations that IFN-α is elevated in SLE patients and the gene expression induced by IFN-α correlates strongly with the expression pattern seen in SLE patients (72). In this study, we have shown that SACQ serum has a reduced ability to induce IFN-α as compared to SACA serum, possibly mediated by the presence of serum inhibitors, such as IgG and nucleases. Although these results are compatible with the preliminary observations demonstrating reduced levels of plasma IFN-α and IFN signature in SACQ patients, we found no significant correlation between the IFN signature and IFN-α induction in vitro for either SACQ or SACA patients. Given the increased numbers of pDCs detected in the skin, lymph nodes and renal tissues of SLE patients, it has been suggested that the IFN signature is derived from IFN-α produced at the tissues rather than the peripheral blood (84,85,87). The lack of correlation between IFN signature and IFN-α induced by serum factors therefore raises the possibility that alternative mechanisms are involved in the regulation of IFN-α production at the tissue level. Since the IFN signature was obtained from PBMCs that had been exposed to IFN-α produced by pDCs in patients whereas IFN-α measured in this study was produced by control PBMCs, there could also be differences in the ability of control and lupus, or alternatively, tissue and blood pDCs to respond to immune complexes as well as interactions with serum inhibitors. In addition, as outlined in the introduction to the thesis, there are a number of other cellular populations that regulate production of IFN-α by pDCs and it is possible that differences in these populations play a significant role in the modulation of IFN signature in vivo.

The IgG-mediated inhibition of IFN-α production in response to RNA-containing immune complexes was likely facilitated by the presence of AIAAs. Previous studies have found that a substantial proportion of SLE patients (~25%) exhibit endogenous AIAAs in serum, which
effectively neutralizes IFN-α \textit{in vitro} (124). More specifically, Ching et al. reported that SACQ patients have a significantly higher frequency of AIAAs compared to SLE patients with various disease manifestations, although the sample size was small (4/11 AIAA-positive) (158).

Regardless, these results suggested that AIAAs could be another important mechanism that modulates IFN-α production and the IFN signature as well as disease activity in SACQ patients.

In this study, we used two methods to detect AIAAs in patient serum. The first method examined the functional capacity of AIAAs to bind IFN-α by measuring the level of IFN-α induced by CpG following incubation with patient serum. IFN-α complexed with AIAAs would prevent binding of ELISA antibodies and subsequently inhibit detection by ELISA. Using this method, we found that a total of 3 SACQ, 1 SQCQ and 1 SACA serum dramatically reduced the level of IFN-α detected by ELISA. These included the SACQ serum that previously inhibited IFN-α production, which was completely abrogated by IgG depletion, indicating a potential role for AIAAs. Although there was a slightly higher frequency of these antibodies in SACQ patients, the differences between groups were not significant. While these findings indicate that the majority of SACQ patients do not have AIAAs, several limitations need to be addressed. There is a possibility that serum AIAAs and ELISA antibodies recognize different epitopes on IFN-α, thus the ability of serum to block detection by ELISA may not accurately reflect the level of AIAAs. Furthermore, the incubation of IFN-α with a relatively high dose of exogenous anti-human IFN-α antibodies did not block detection, further questioning the sensitivity and specificity of the assay.

Thus, as an alternative strategy to measure the AIAAs in serum, we developed an ELISA specific for AIAAs and despite the issues encountered in our first assay, we found a significant correlation between the two results. With the exception of two SACQ patients, the same SACQ, SACA and SQCQ patients were positive for AIAAs. Given the low frequency of AIAAs in our patient cohort, our findings suggest that AIAAs are rare in SLE patients, probably due to the
importance of IFN-α in anti-viral response (51). Notably, this frequency of positive antibodies is considerably lower than previous estimates of ~25%. This discrepancy could be attributable to the differences in the methods used to detect AIAAs. For our analysis, an ELISA was used to detect AIAAs; however, it has been previously noted that ELISAs are less sensitive than other assays, resulting in the identification of only 10% of AIAA-positive patients compared to 25% from using an SPR-based assay (124). Thus, modifications of the ELISA may be necessary to increase its sensitivity for all types of AIAAs including those with low-affinity. More importantly, our findings indicate that AIAAs are not the major inhibitory factor in SACQ serum that leads to the lack of IFN-α production and disease activity.

Analysis of IFN-α induction and IFN scores for patients stratified by AIAA status revealed no differences between the two groups. Although the majority of SACQ and SACA patients who exhibited low levels of IFN-α induction (“IFN\text{\textsuperscript{low}}”) and the IFN signature were AIAA-negative, those who were AIAA-positive were all within the IFN\text{\textsuperscript{low}} group with the values ranging from 0.0 to 1.28% of CpG. These findings are consistent with a previous study by Behren et al. demonstrating the ability of AIAA-positive serum to neutralize IFN-α \textit{in vitro} and further indicate that neutralizing AIAAs could be one of the mechanisms by which SACQ patients attenuate their IFN-α production (123). In contrast to these \textit{in vitro} results, IFN scores reflecting \textit{in vivo} IFN-α production varied between patients with one SACA patient, SACA 1316, showing high levels of ISGs despite high levels of AIAAs. Since these AIAAs can effectively neutralize IFN-α \textit{in vitro}, as evidenced by low levels of IFN-α induction seen for AIAA-positive patients, the question arises as to what is inducing the IFN signature in this patient \textit{in vivo}. Although it has been shown that antibodies to IFN-α but not antibodies to IFN-β or IFN-θ markedly inhibit the ISG expression induced by SLE serum (76,78), it is possible in some patients this is not the case. To explore this possibility, we measured the levels of AIAAs using...
the same method in a large cohort of unselected SLE patients with active disease that was previously recruited by our center and compared this with their IFN signature. In agreement with previous studies, a considerably higher percentage of patients had AIAAs (~24.8%). Similar to the observations seen with SACA 1316, there was no significant correlation between the levels of AIAAs and IFN scores. This finding further suggests that other type I IFNs may be involved in type I IFN activity in some of SLE patients. In support of this concept, studies have reported that antibodies against IFN-β or IFN-θ led to a modest, though not significant, inhibition of ISG expression (159). Furthermore, increased expression of IFN-β and IFN-ω has been found in lupus blood compared to healthy donors (160). Thus, although ISG expression may be primarily induced by IFN-α, it is possible that other isoforms contribute to some of this increased expression. Alternatively, the levels of endogenous AIAAs may be insufficient to inhibit the IFN-α response in vivo. Given that our ELISA detects AIAAs specific for IFN-α1, the IFN pathway in these patients could be driven by other IFN-α subtypes. Future studies will look into characterization of AIAAs by affinity and neutralizing effect in vitro and measurement of IFN-β or IFN-θ in patient serum to confirm their role in ISG expression.

In addition to IFN scores, many studies have investigated an association between the presence of AIAAs and clinical disease activity (123-125). Given the lack of association between changes in IFN signature and changes in disease activity, we asked whether the presence of AIAAs could have an impact on clinical activity without causing a change in IFN scores. However, there was no difference in overall disease activity or the frequency of different types of organ involvement between AIAA-positive and –negative patients, suggesting that AIAAs do not play a significant role in modulating disease activity. This finding is in agreement with a previous report by Slavikova et al. indicating a lack of correlation between AIAAs, as measured by ELISA, and lower disease activity (126). Given that most of these patients were clinically
active, similar to the results seen with IFN scores, it is possible that their IFN-α levels may be present at levels higher than AIAA levels, resulting in not only elevated IFN scores but also sustained immune activation and clinical signs of the disease. This conclusion is further supported by the observation that for all patients with ELISA signals higher than 2, their SLEDAI score was 0. Alternatively, other type I IFNs may be involved in IFN-α-mediated or other immunological pathways that lead to clinical disease in patients.

5 Conclusions

Autoantibodies in the serum from SACQ patients have an impaired ability to induce IFN-α compared to those in the serum from SACA patients. These differences were, in part, explained by an inhibitory effect of serum factors, such as IgG. Our results suggest that at least one subfraction of IgG is AIAAs. Patients with AIAAs have evidence of lower levels of IFN-α induction in vitro, but variable IFN signatures and disease activity. Given the proposed role of IFN-α in SLE, therapeutics targeting type I IFN pathway are being developed. The lack of association between AIAAs and IFN signature and disease activity implicates other type I IFNs in the disease process and further questions the efficacy of monoclonal anti-IFN-α antibodies in the treatment of the disease. Furthermore, the low prevalence of AIAAs in SACQ patients indicates that other inhibitory factors are present. Characterization of inhibitory factors in SACQ serum may provide insight into the mechanisms that modulate disease activity in response to autoantibody production and may ultimately lead to the elucidation of better treatment strategies and biomarkers of disease activity.
References


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