Autoantibodies and the Type I Interferon System in the Etiopathogenesis of Systemic Lupus Erythematosus

BY

STINA BLOMBERG
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Abstract

In sera remitted for anti-nuclear antibody (ANA) analysis, the supplement of a sensitive anti-SSA/Ro ELISA to the conventional ANA screening by immunofluorescence (IF) revealed that one fourth of the individuals with IF-ANA negative, but SSA/Ro ELISA positive sera, had systemic lupus erythematosus (SLE) or cutaneous LE. Consequently, adding a sensitive anti-SSA/Ro ELISA to the ANA screening is valuable for the serological detection of ANA negative SLE/LE patients.

SLE patients often have measurable interferon-alpha (IFN-α) levels in serum, and IFN-α treatment of patients with non-autoimmune diseases can induce SLE. Thus, the type I IFN system seems to be important in SLE and was therefore investigated. Initially, a decreased IFN-α producing capacity, due to a 70-fold reduction in the number of circulating natural IFN-α producing cells (NIPC), was noted in peripheral blood mononuclear cells (PBMC) from SLE patients. SLE-sera contained an endogenous IFN-α inducing factor (SLE-IIIF), consisting of IgG and DNA in the form of small immune complexes (300-1000 kD). The SLE-IIIF selectively activated NIPC and was more common in sera from patients with active disease compared to individuals with inactive disease. IFN-α producing cells could be detected by immunohistochemistry in both lesional and unaffected skin from SLE patients, and IFN-α gene transcription could be verified by in situ hybridisation in some of the skin biopsies. A reduced number of NIPC, detected by expression of the blood dendritic cell antigen (BDCA)-2, was noted among SLE-PBMC. The IFN-α production triggered by SLE-IIIF in SLE-PBMC was inhibited by monoclonal antibodies (mAbs) to BDCA-2 and markedly decreased by anti-BDCA-4 mAbs.

The observations in the present thesis may explain the ongoing IFN-α production in SLE patients, indicate an important role for the activated type IFN system in the pathogenesis, and suggest that direct targeting of SLE-NIPC may constitute a new therapeutic principle in SLE.

Keywords: Systemic lupus erythematosus, type I interferon, interferon inducer, dendritic cell, natural interferon-alpha producing cell, immune complex, SSA/Ro, dsDNA, BDCA

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Till Simon, Nils och Hedvig
This thesis is based on the following papers, which are referred to in the text by their Roman numerals:


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>ACLE</td>
<td>acute cutaneous lupus erythematosus</td>
</tr>
<tr>
<td>ACR</td>
<td>American College of Rheumatology</td>
</tr>
<tr>
<td>ANA</td>
<td>anti-nuclear Ab</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>BDCA</td>
<td>blood dendritic cell Ag</td>
</tr>
<tr>
<td>C</td>
<td>complement</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CpG</td>
<td>cytosine-phosphate-guanine dinucleotides</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded DNA</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc receptor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
</tr>
<tr>
<td>IC</td>
<td>immune complex</td>
</tr>
<tr>
<td>ID</td>
<td>immuno diffusion</td>
</tr>
<tr>
<td>IDDM</td>
<td>insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>IF</td>
<td>immunofluorescence</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IFNAR</td>
<td>IFN-α/β receptor</td>
</tr>
<tr>
<td>IIF</td>
<td>IFN-α inducing factor</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IPC</td>
<td>IFN-α producing cell</td>
</tr>
<tr>
<td>IRF</td>
<td>interferon regulatory factor</td>
</tr>
<tr>
<td>is</td>
<td>immunostimulatory</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LE</td>
<td>lupus erythematosus</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal Ab</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NIPC</td>
<td>natural IFN-α producing cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PDC</td>
<td>plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>polyinosinic-polycytidylic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNP</td>
<td>ribonucleo protein</td>
</tr>
<tr>
<td>SCLE</td>
<td>subacute cutaneous lupus erythematosus</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>SLE disease activity index</td>
</tr>
<tr>
<td>Sm Ag</td>
<td>Smith Ag</td>
</tr>
<tr>
<td>SSA/Ro</td>
<td>Sjögren syndrome-A/Ro</td>
</tr>
<tr>
<td>SSB/La</td>
<td>Sjögren syndrome-B/La</td>
</tr>
<tr>
<td>SV</td>
<td>Sendai virus</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper cell type 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper cell type 2</td>
</tr>
<tr>
<td>TLR</td>
<td>toll like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>WB</td>
<td>western blot</td>
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</table>
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INTRODUCTION

Autoimmunity is defined as a condition when an individual’s immune system begins reacting against its own tissues. Autoimmune diseases can be either systemic, involving multiple organs, or organ-specific. One important autoimmune disease is systemic lupus erythematosus (SLE), a multisystemic inflammatory disorder characterized by production of antibodies (Abs) to many different autoantigens (autoAgs). Disturbances in most parts of the immune system have been described in SLE, which has a chronic and relapsing course. This disease serves as a prototype for systemic autoimmune diseases and therefore, a better knowledge of the pathogenesis in SLE may give us a greater understanding of autoimmune mechanisms in general.

History of SLE

The term lupus, latin for wolf, appeared as early as in the 10th century and refers to the medical description of cutaneous lesions reminiscent of a wolf’s bite 1. Until the 16th century, lupus was mainly a term associated with ulcerative lesions on the legs. It was then used preferably as a description of facial lesions, although the cause of the lesions could be multiple and were not easily distinguished from e.g. tuberculosis. In the middle of the 19th century, the term “lupus erythemateux” was introduced by Cazenave to describe butterfly-like facial lesions, preferentially occurring in outdoor workers 2,3. Soon thereafter, Kaposi described systemic symptoms in association with a disseminated form of cutaneous lupus lesions, and he also noted that this disease was more frequent in women 4. At the turn of the century, a more definite recognition of SLE arrived with the description of multiple extracutaneous manifestations associated with lupus lesions, such as arthritis, nephritis and endocarditis. In the beginning of the 20th century, the first immunological manifestation in SLE was described in the form of false positive results for syphilis by the Wassermann test in these patients. The next important observation was the demonstration of the LE-cell phenomenon by Hargraves 1948 5. This discovery gave rise to a more extensive search for immunological abnormalities possibly involved in the
pathogenesis of SLE. In the 1950s, antinuclear Abs (ANA) were detected by immunofluorescence in SLE patients, soon followed by the detection of more specific autoAbs and deposition of immunoglobulins (Ig) in the skin. Today more than 50 different autoAbs and several cellular immunological dysfunctions, have been described in SLE patients.

Clinical manifestations and diagnosis of SLE
The broad spectrum of signs and symptoms in SLE patients reflects the systemic and multifaceted nature of this disease. Common symptoms during an SLE disease flare are arthralgia, myalgia, fever, fatigue and weight loss. Organ specific manifestations include arthritis, skin changes, pleuritis/pericarditis, nephritis, engagement of the central nervous system and adenopathy. Typical laboratory findings that can occur in SLE patients are presence of ANA, anti-dsDNA Abs, low complement, anemia, leukopenia, proteinuria and thrombocytopenia. Long term complications in SLE patients are for example osteoporosis and cardiovascular disease, the latter associated with dyslipoproteinemia described in these patients.

Approximately 2% of SLE patients are negative for IF-ANA test, and these patients are considered to have ANA negative SLE. A higher incidence of leukopenia, photosensitivity and antiphospholipid syndrome are reported in these patients.

Cutaneous manifestations
Skin lesions can be seen in up to 90% of SLE patients, and in approximately one fourth of the individuals skin disease is the presenting SLE symptom. Four out of the eleven SLE criteria encompass cutaneous symptoms (malar rash, discoid rash, photosensitivity and oral ulcers), reflecting the prominent occurrence of skin manifestations in SLE.

A classification system of the different lupus skin lesions was created by Gilliam in 1982. In this system, the lesions are divided to be either LE-specific or LE-non-specific. The LE-specific lesions are further subdivided in acute cutaneous LE (ACLE), subacute cutaneous LE (SCLE) and chronic cutaneous LE (CCLE). ACLE is represented by the typical malar rash, SCLE shows strong association with anti-SSA/Ro Abs and CCLE is dominated by discoid LE. The LE-non-specific lesions occur in up to 75% of all SLE patients and the most common lesions are alopecia and cutaneous vascular disease, such as vasculitis and Raynaud's phenomenon.
The lupus band test detects a band of Ig and complement components at the dermal-epidermal junction by immunofluorescence (IF) microscopy. However, a positive lupus band staining is not specific for lupus, but in contrast to isolated cutaneous lupus disease, it is often seen in unaffected skin from SLE patients. Thus, this test can sometimes be used to discriminate between isolated cutaneous LE and SLE.

**SLE diagnosis**

In the every day clinical practice, the combination of typical SLE manifestations in two out of seven organs, and the presence of ANA is sufficient for an SLE diagnosis. However, for clinical research purposes, when data from SLE patients in one study are compared with results obtained from other studies, a more strict definition of the disease is necessary. Therefore, the American rheumatology association (ARA), at present called American college of rheumatology (ACR), created classification criteria for SLE in 1971, later revised by Tan in 1982. Hereby, the specificity of the diagnosis in a particular patient using the ACR criteria is high, although mild and early cases of SLE are not always detected. To achieve an SLE diagnosis, at least four out of the eleven ACR criteria have to be fulfilled some time during the disease course (Table 1).
Table 1. ACR classification criteria for SLE:

<table>
<thead>
<tr>
<th>No.</th>
<th>Criteria</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Malar rash</td>
<td>Fixed erythema, flat or raised, over the malar eminences</td>
</tr>
<tr>
<td>2</td>
<td>Discoid rash</td>
<td>Erythematous raised patches</td>
</tr>
<tr>
<td>3</td>
<td>Photosensitivity</td>
<td>Skin rash as a result of unusual reaction to sunlight</td>
</tr>
<tr>
<td>4</td>
<td>Oral ulcers</td>
<td>Oral or nasopharyngeal ulceration</td>
</tr>
<tr>
<td>5</td>
<td>Arthritis</td>
<td>Nonerosive arthritis involving 2 or more peripheral joints</td>
</tr>
<tr>
<td>6</td>
<td>Serositis</td>
<td>Pleuritis or pericarditis</td>
</tr>
<tr>
<td>7</td>
<td>Renal disorder</td>
<td>Persistent proteinuria greater than 0.5 grams per day or cellular casts</td>
</tr>
<tr>
<td>8</td>
<td>Neurologic disorder</td>
<td>Seizures or psychosis</td>
</tr>
<tr>
<td>9</td>
<td>Hematologic disorder</td>
<td>Hemolytic anemia, leukopenia, lymphopenia or thrombocytopenia</td>
</tr>
<tr>
<td>10</td>
<td>Immunologic disorder*</td>
<td>Positive LE cell preparation, anti-dsDNA Ab, anti-Sm Ab or false positive serologic test for syphilis</td>
</tr>
<tr>
<td>11</td>
<td>ANA</td>
<td>An abnormal titre of antinuclear Ab</td>
</tr>
</tbody>
</table>

*The presence of anticardiolipin Abs or lupus anticoagulant was added in 1997.

SLE activity indices

Several disease activity indices have been developed to measure the SLE disease activity in a single patient. The three most commonly used indices are SLE Disease Activity Index (SLEDAI), the British Isles Lupus Assessment Group scale (BILAG) and Systemic Lupus Activity Measure (SLAM). These indices differ from each other, but are all found to be valid measurements of SLE disease activity in both adults and children. The SLEDAI, was developed by Bombardier et al, and is based on clinical and laboratory findings related to SLE during the past ten days. A total of 24 parameters are evaluated (Table 2).
Table 2. SLE related manifestations, each giving the indicated SLEDAI score

<table>
<thead>
<tr>
<th>Manifestation</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seizure, psychosis, organic brain syndrome, visual disturbance, cranial nerve disorder, lupus headache, cerebrovascular accident or vasculitis</td>
<td>8</td>
</tr>
<tr>
<td>Arthritis, myositis, urinary casts, hematuria, proteinuria or pyuria</td>
<td>4</td>
</tr>
<tr>
<td>New rash, alopecia, mucosal ulcers, pleurisy, pericarditis, low complement or increased DNA binding</td>
<td>2</td>
</tr>
<tr>
<td>Fever, trombocytopenia or leukopenia</td>
<td>1</td>
</tr>
</tbody>
</table>

A modified SLEDAI (mSLEDAI), which does not include dsDNA binding Abs and complement levels, is the activity index referred to in the investigations presented in this thesis.

The definition of an SLE flare is controversial. It has been proposed that a change in SLEDAI score, or decision of the physician to increase the treatment can be used to define disease flares. Recently, Gladman and colleagues suggested that a flare can be defined as an increase in the SLEDAI score of > 3 29.

Epidemiology and prognosis of SLE

SLE has an incidence and prevalence of 4.8 and 68 per 100 000 inhabitants respectively, with an approximately 7:1 female gender bias 30,31. Both incidence and prevalence vary worldwide, and a 2- to 5-fold higher SLE incidence is reported in non-caucasians 31.

In the last 50 years the prognosis in terms of survival rates has improved from a 5-year survival rate of 50% in the 1950s to over 90% in the 1990s 30,32. The more frequent detection of milder cases of SLE by the use of ANA assays and the introduction of more efficient therapy may have contributed to this improvement. Corn stones in the medical treatment of SLE patients are glucocorticoids, anti-malarials and, when indicated, immunosuppressive drugs.

The mortality among SLE patients is related to active disease, infections and, especially later in the disease course, cardiovascular morbidity 14,33,34.
Etiology of SLE

The etiopathogenic factors involved in SLE disease onset and development are considered to be multiple, where a genetic predisposition interplays with environmental factors.

Genetic factors

The familial prevalence of SLE is estimated to be 10%, and siblings to SLE patients have a 20-40-fold increased risk to develop SLE compared with the general population. There is evidence for an association between SLE disease and the MHC class II and III regions on chromosome 6. Furthermore, there is a strong association between autoAb production, such as anti-SSA/Ro Ab and anti-SSB/La Ab, and the MHC class II subsets HLA-DR2 and DR3. Associations are also seen between SLE and complement deficiencies (C1q, C2 and C4), FcγRIIA polymorphisms, IL-10 promoter polymorphism, and most recently programmed cell death 1 gene (PDCD1) polymorphism.

Environmental and hormonal factors

In monozygotic twin studies the disease concordance is not complete, although 10-fold increased compared with dizygotic twins. This strongly suggests that other factors than hereditary susceptibility must have a role in the initiation of SLE, and several environmental factors contributing to the SLE disease process have been identified.

UV light

Nearly three quarters of the SLE patients report photosensitivity, and ultraviolet (UV) radiation induce skin lesions in these patients. Systemic symptoms can also be induced by sun exposure, although the exact relationship between UV exposure and disease activity is difficult to demonstrate, because of a lag period between the UV exposure and the disease flare. UVA and UVB were found to induce necrosis or apoptosis, respectively, in keratinocytes. SLE related autoAg such as SSA/Ro, SSB/La and calreticulin cluster in apoptotic blebs at the cell surface, and may serve as B cell autoAg. In this way the immune system may be exposed to autoAg, that can lead to an autoimmune response.
Drugs
A number of drugs have been reported to induce SLE or a lupus-like reaction, termed drug-induced lupus (DIL). The latter is characterized by a reversible development of ANA and at least one clinical feature of lupus, and full recovery after discontinuing treatment. The high-risk drugs reported to induce DIL are procainamide and hydralazine, followed by quinidine, chlorpromazine, sulfasalazine and penicillamine.

Autoimmune manifestations, including SLE, have also been reported in patients after immunotherapy with cytokines and anti-cytokines. Treatment with interferon-alpha (IFN-α) of patients with non-autoimmune diseases, induces development of both autoAbs and autoimmune diseases. Consequently, during long term IFN-α treatment of patients with carcinoid tumors, 22% developed ANA, 8% anti-dsDNA autoAbs and 0.7% developed SLE. This issue will be further discussed below. The recently introduced TNF-α blocking agents, used in rheumatoid arthritis and inflammatory bowel disease, have also been reported to induce autoAb production, such as anti-dsDNA Abs, and a lupus like syndrome.

Infectious agents
Both viruses and bacteria have been suspected to be involved in the initiation of SLE. Among viruses, especially Epstein-Barr virus, cytomegalovirus and retrovirus may be linked to the development of SLE. Elevated Ab titers to several viruses have been reported in SLE patients, and both onset and exacerbations of the disease are reported at concurrent infections. Possible mechanisms for triggering of SLE by microorganisms, or their components, are for example polyclonal B cell activation in predisposed individuals, cross reactivity with host Ags through molecular mimicry or exposure of cryptic epitopes. Relevant in the context of the present thesis, is the fact that viruses as well as certain bacteria are potent IFN-α inducers.

Hormones
The fact that the incidence of SLE has a strong female-to-male preponderance during reproductive years, with diminishing ratio during childhood and menopause, indicates hormonal influences on onset and development of SLE. This is further underlined by the fact that female SLE patients may show increased lupus symptoms in the premenstrual period and more disease flares during pregnancy.
Other environmental risk factors
A number of additional risk factors for SLE have been described. Smoking, a history of hypertension, drug allergy, sun-reactive skin type and intake of alfalfa seeds are all reported to increase the risk of developing SLE \(^{62,63}\). In contrast, alcohol consumption is reported inversely associated to development of SLE, and may thus have a protective effect \(^{63}\).

The immune system and SLE
SLE is characterized by alterations in many different parts of the immune system, but most patients share some central characteristics; a lack of tolerance and the production of pathogenic autoAbs with immune complex formation. Some of the most important immunological findings seen in SLE are summarized below.

Cellular abnormalities

T cells and B cells
Nearly half of all SLE patients have decreased levels of circulating leukocytes, manifested as granulocytopenia and/or lymphocytopenia. The lymphocytopenia is primarily due to a decrease in T cells, and both the CD4\(^+\) and CD8\(^+\) subsets seem to be affected \(^{64}\). However, the T cells in SLE patients show signs of an in vivo activation, and autoreactive T cells are considered to have an important role in the disease process \(^{65,66}\).

The hallmark of the B cell population in SLE is hyperactivity, with increased proliferation rates, elevated numbers of long-lived autoreactive B cells and differentiation to plasma cells. This results in the production of autoAbs and polyclonal hypergammaglobulinemia \(^{67,68}\). T cells may contribute to this Ab production, as well as the cytokine milieu and functional abnormalities, such as an increased expression of the costimulatory molecule CD40L \(^{69-71}\).

Dendritic cells (DC), monocytes/macrophages and natural killer (NK) cells
Relatively little has been known about the DC population in SLE patients, but a recent study demonstrated a reduced number of circulating DC and an impaired T cell-stimulatory capacity by such DC \(^{72}\).
In contrast, the number of monocytes among peripheral blood mononuclear cells (PBMC) from SLE patients is often increased, with an enhanced rate of spontaneous apoptosis \(^{73}\). Interestingly, these monocytes may be induced to differentiation into DC by the presence of IFN-α in SLE serum \(^{74,75}\). Such DC can function as Ag-presenting cells (APC) that may explain the T cell activation seen in SLE.

Decreased levels and activity of NK-cells are reported in active SLE patients, as well as reduced Ab-dependent cellular cytotoxicity in vitro \(^{64}\).

Autoantibodies

Auto Abs can be seen in healthy individuals, although these natural autoAbs are usually of a low affinity IgM isotype. They do not undergo isotype switching or affinity maturation and do not cause autoimmune disease or tissue damage \(^{67}\). Furthermore, in a normal healthy population, approximately 3% of the individuals are ANA positive by IF, detecting only those of IgG isotype. Higher ANA frequencies are seen in several autoimmune diseases, chronic infections and drug-induced conditions, and reach a frequency of 98% in SLE patients. The autoAbs in SLE are preferably of the IgG1 or IgG3 isotype, and are of high affinity \(^{76}\).

The autoAb production in SLE is thought to be of pathogenic relevance, mainly by the formation of immune complexes (IC), with deposition in target tissues such as glomeruli, heart, skin and vessels. The deposited IC then participate in inflammatory processes involving complement activation, eventually causing tissue damage. In addition, IC may have other properties, such as activation of autoAb production \(^{77}\). Auto Abs can also interfere with normal cellular functions and thereby contribute to disease manifestations \(^{78}\).

Autoantibody specificities

More than 50 different autoAbs specificities have been described in SLE, and these Abs are typically directed to nuclear Ags such as DNA, RNA, chromatin, and different ribonucleoprotein complexes \(^{79}\). The screening method to detect ANA is usually performed by IF on Hep-2 cells, a human cell line derived from a laryngeal cell carcinoma. Certain autoAgs are not expressed in the nucleus and consequently, SLE patients with autoAbs to these Ag could be ANA negative. In this thesis anti-dsDNA Abs and anti-SSA/Ro Abs have been especially investigated, and are therefore discussed in more detail.
Anti-dsDNA
The presence of Abs directed to dsDNA is a relatively specific feature in SLE. In the majority of patients with SLE, anti-dsDNA Abs can be detected in serum at some time during the disease course. Furthermore, high titers of anti-dsDNA Ab often indicate disease activity and are associated with an increased risk for nephritis \(^{80,81}\). Not all anti-dsDNA Ab are associated to in vivo disease activity, or tissue damage in experimental systems \(^{82}\). The cause of this heterogeneity among anti-dsDNA Abs with regard to pathogenic potential is unclear, but the avidity of a certain anti-dsDNA Ab may at least partly play a role. Possible target Ags for anti-dsDNA Abs are the nucleosomes, containing double stranded DNA, that are released from cells undergoing apoptosis. However, The nature of the DNA itself can also contribute to the immune response, by acting as an adjuvant, as seen with CpG rich DNA. \(^{83,84}\). Some anti-dsDNA Abs were early on shown to penetrate cells and induce apoptosis \(^{85}\). By this mechanism, anti-dsDNA Abs could supply the immune system with autoAgs for autoreactive B cells.

Anti-SSA/Ro
Anti-SSA/Ro Abs are found in one third to nearly half of all SLE patients, partly depending on the assay used. In contrast to anti-dsDNA Abs, the occurrence of anti-SSA/Ro is not specific for SLE, and can be found in higher frequencies among patients with Sjögren’s syndrome. Anti-SSA/Ro Abs are closely associated with certain clinical features in SLE, such as SCLE, ANA-negative SLE, photosensitivity, interstitial pneumonitis, thrombocytopenia, complement 2 (C2) deficiency and nephritis \(^{86}\). In addition, neonatal lupus dermatitis and congenital heart block can be seen in the offspring to healthy women with circulating anti-SSA/Ro Abs \(^{87,88}\).

The Ags for anti-SSA/Ro Abs comprise a family of proteins binding to human cytoplasmic (hY) RNAs. The major antigenic component in the SSA/Ro complex is a 60 kD peptide \(^{89,90}\). In addition, a 52 kD polypeptide has been demonstrated as an antigenic subunit \(^{91}\). The SSB/La Ag is also associated with the hY RNAs, and anti-SSB/La Ab positive SLE patients almost always have concomitant anti-SSA/Ro Ab.

Methods for anti-SSA/Ro detection
The SSA/Ro Ag is easily denatured, and the occurrence of anti-SSA/Ro Ab in a serum sample can therefore be overlooked in the IF-ANA screening. More robust methods for the detection of anti-SSA/Ro Abs are therefore needed, and traditionally double immunodiffusion (ID) has been used as golden standard \(^{92}\). The ID is relatively specific, but has the disadvantage of being time consuming and thus expensive. An immunoblotting method, such
as Western blot (WB), has the disadvantage of using denatured Ags, but is instead sensitive. The enzyme linked immunosorbent assays (ELISA) provides a high degree of sensitivity in Ab detection, but artefactual binding by contaminants and degradation products is more common in this assay, thus decreasing the specificity \(^{93,94}\). To overcome the disadvantages of denaturation of SSA/Ro Ags, cell lines hyperexpressing the 60 kD Ag can be used for ANA screening by IF, such as Hep-2000 cells \(^{95,96}\).

**Apoptosis**

Apoptosis is a continuously occurring programmed cell death, followed by rapid clearance of the apoptotic cell material by complement binding and activation of the mononuclear phagocyte system \(^{97}\). In a healthy individual this provides a subtle control of the lifespan of cells in the body, most importantly involving cells of the immune system. In SLE patients, the apoptotic mechanisms are disturbed, with an increased rate of lymphocyte apoptosis as well as increased numbers of apoptotic cells. The latter observation could partially reflect the impaired clearance of apoptotic cells by macrophages in SLE \(^{98-100}\).

**Immune complexes (IC), clearance and the complement system**

SLE patients have high levels of circulating IC, and a deficient clearance of IC is typical for this disease \(^{101}\). The impaired IC clearance correlates to nephritis and is likely to contribute to IC deposits in tissues \(^{102}\).

The complement system consists of several plasmaproteins cooperating in cascades, and with an important role in the defence against microorganisms and clearance of IC. Genetic defects in the early classical pathway, involving complement components C1, C4 and C2, are associated with SLE and a lupus-like disease. Acquired complement abnormalities are a frequent finding in SLE patients, and serum levels of C1q, C4, C2 and C3 correlate with disease activity \(^{103,104}\).

**Cytokines**

Cytokines constitute a large group of proteins and are produced by activated cells. They act mainly in an autocrine or a paracrine fashion, exhibit effects on cells at low concentrations and contribute to cell growth, differentiation
and activation or inhibition. Several cell types including T cells, monocytes/macrophages, B cells, epithelial cells and fibroblasts can produce cytokines. The effects of cytokines are pleiotropic, and thus, depending on surrounding cytokine milieu and target cell, a single cytokine can both promote and inhibit an immune response.

Depending on the effect of the immune response, some cytokines may be referred to as Th1 or Th2 associated cytokines. The Th1 cytokines, for example IL-2, IL-12, IFN-γ and IFN-α, favour a T cell-mediated immune response, whereas the Th2 cytokines, for example IL-4, IL-5 and IL-10, promote Ab production. However, this separation of cytokines is somewhat artificial, as for example IFN-γ activates macrophage cell killing but also promote Ig secretion and class switch. However, the Th1/Th2 subsets of T cells should be considered as a broad outline of Th function.

There exist an abundant literature concerning the possible role of different cytokines in the pathogenesis of human SLE. Contradictory results have however, been obtained in investigations studying serum levels, intracellular mRNA or protein content or cytokine production in cell cultures. One explanation for this could be that serum cytokine levels probably reflect cytokine production in tissues, such as lymphoid organs, that cannot always be compared to analyses of cytokine secretion by PBMC in cell cultures.

Among the cytokines, IFN-γ, IL-2, IL-6, IL-10, IL-15, IL-16, IL-18 and TNF-α have all been reported elevated in serum from SLE patients. In many cases, the role of these cytokines in the disease process is controversial, and both positive and negative effects of the same cytokine can be expected. For example, IL-10 may promote autoAb production by B cells, but can also deactivate DC and inhibit IFN-α production.

Furthermore, the proinflammatory cytokine TNF-α are elevated in SLE patients, but blockade of this cytokine in patients with rheumatoid arthritis and inflammatory bowel disease can, as discussed earlier, induce an SLE syndrome.

**Interferon-α**

The first cytokine reported to be increased in sera from SLE patients was IFN, subsequently characterised as IFN-α, and this observation has later been confirmed in a number of reports. In many of the SLE patients the raised serum IFN-α levels are as high as in patients with viral infections. In fact, signs and symptoms in SLE may resemble those in viral infections or during IFN-α therapy, such as fever, fatigue, myalgia, arthralgia, and leukopenia. Consequently, some SLE symptoms may actually be caused by IFN-α in these patients. IFN-α has also been reported in cerebrospinal fluid from SLE patients with psychosis. The increased IFN-α synthesis in SLE
patients have been confirmed by the report that most of these individuals express the IFN-\( \alpha \) induced protein MxA in leukocytes and skin biopsies \(^{123,124}\).

The observation that IFN-\( \alpha \) treatment can induce development of ANA, anti-dsDNA Abs, and also an overt SLE syndrome, more directly suggests a causative role for IFN-\( \alpha \) in the pathogenesis of SLE \(^{48-50,125}\). Interestingly, several other autoimmune disorders may also develop during IFN-\( \alpha \) therapy, such as thyreoiditis, pernicious anemia, autoimmune hepatitis, insulin-dependent diabetes mellitus (IDDM), rheumatoid arthritis, polymyositis and vasculitis \(^{47,126}\). Furthermore, increased serum levels of IFN-\( \alpha \) have been noted in patients with IDDM, and IFN-\( \alpha \) is expressed in pancreatic beta cells from these patients \(^{31,127,128}\). An increased expression of IFN-\( \alpha \) inducible genes in muscle biopsies from patients with juvenile dermatomyositis was also recently reported \(^{129}\). Taken together, these findings suggest a role for IFN-\( \alpha \), not only in the pathogenesis of SLE, but also in breaking tolerance and induction of autoimmunity in general.

The observations, that SLE patients have an ongoing IFN-\( \alpha \) production, and that SLE can be induced by IFN-\( \alpha \) treatment, constitute the background to the present thesis and were the actual reasons for the investigations performed in paper II-V.

The interferon system

In 1957, Isaacs and Lindenmann for the first time described a soluble substance that could interfere with virus replication and protect cells from virus infection. Because of this interference, the proteins found were called interferons (IFN) \(^{130}\). Subsequent studies revealed that IFNs inhibited cell proliferation but also had many immunoregulatory effects. Today, the term “the IFN system” is used to denote the different genes encoding the IFNs, encoded proteins, the IFN producing cells, the IFN-\( \alpha/\beta \) receptor (IFNAR) and target cells affected by IFNs.

Interferons, receptors and genes

There are two types of IFNs, type I and type II IFN, divided due to dissimilarities at the molecular level and the binding to separate receptors \(^{131}\). All type I IFNs share a common receptor, IFNAR, consisting of two subunits
Type I interferon effects

The binding of type I IFN to IFNAR is the beginning of a cellular signal cascade. Activation of Janus tyrosine kinase (Jak)-1 and Tyrosine kinase (Tyk)-2 by IFNAR ligation, subsequently leads to phosphorylation and dimerisation of signal transducer and activator of transcription (STAT) -1 and STAT-2 proteins. These proteins together with interferon regulatory factor (IRF) -9 form a complex called IFN-stimulated gene factor 3 (ISGF-3) that translocate to the nucleus and activate numerous IFN-inducible genes.

The proteins encoded by the IFN-inducible genes are the final effector molecules, which exert the IFN-α-induced antiviral actions on cells. This is done by direct interference in the viral replication process, including effects on viral penetration, transcription and translation. Representatives of the IFN-α induced proteins are the double stranded RNA dependent protein kinase (PKR), the 2’5’oligoadenylate synthetase (OAS), the Rnase L, and Mx proteins. In addition, IFN-α exerts effects on many other cellular mechanisms. It has an impact on the cell cycle regulation, leading to inhibited cell proliferation. IFN-α can also exert cytotoxic actions by inducing apoptosis in virally infected cells.

IFN-α also has a number of immunomodulatory properties. Thus, IFN-α enhances the expression of MHC class I molecules, promotes NK-cell-mediated cytotoxicity, upregulates FcRs on macrophages and enhances their phagocytic capacity.

Several IFN-α effects more directly involve the adaptive immune response. Consequently, IFN-α could stimulate maturation of immature myeloid dendritic cells, promote DC migration into lymph nodes and increase the DC capacity of Ag presentation to T cells, as well as the subsequent T cell activation. It also stimulates T cell differentiation,
inhibits apoptosis of activated T cells, enhances proliferation of memory cytotoxic T cells and augments a Th1 response. With regard to B cells, IFN-α promotes their survival and activation, by for example enhancing B cell responses to B cell receptor (BCR) ligation. IFN-α activated DC can induce CD40-independent Ig class switching through upregulating B lymphocyte stimulator protein (BLys) and a proliferation-inducing ligand (APRIL). However, the human in vivo relevance of these in vitro observations remain to be established.

The natural IFN-α producing cell

Almost all viruses have the capability of inducing IFN-α production and, depending on virus, a wide variety of cells can produce type I IFN, such as monocytes and fibroblasts. However, these cells are relatively poor IFN-α producers in contrast to the major IFN-α producer per cell, the natural interferon producing cell (NIPC). These cells respond to a wide variety of agents, are infrequent (1/1000 PBMC) but highly efficient, producing up to 3-10 pg or 1-2 U IFN-α/cell, for example in response to herpes simplex virus (HSV). These cells were early on characterised as not T, B, NK cells or monocytes, and were therefore designated NIPC. When investigating the phenotype of these cells, they were found to express MHC class II, CD4, and CD36 CD40, CD83, CD45RA, but lacked CD11b, CD11c and the costimulatory molecules CD80 and CD86, thus resembling immature dendritic cells.

The term ‘plasmacytoid’ is descriptive and refers to the discovery by pathologists in the 1950s of a T cell/monocyte that resembled plasma cells, but lacked B cell markers. Several years later this cell was recognized in the T cell rich area in the lymph nodes. It was then found to be CD123 bright with capability to differentiate into a mature dendritic cell (DC) in presence of IL-3 and CD40L. Parallel to this, the uniform origin of DC was questioned and two distinct DC subsets were described in peripheral blood, one lymphoid and one myeloid. The plasmacytoid dendritic cells (PDC) were first suggested to belong to the myeloid lineage and were called plasmacytoid monocytes. In 1999 the PDC was found to produce IFN-α in response to HSV, and the NIPC was hereafter considered contained within the PDC population. Thus, they are lineage-negative but MHC class II+, CD11c-, CD123+ NIPC/PDC, presumably with a lymphoid origin, although a myeloid subpopulation within the NIPC/PDC population can not be excluded.
Two novel cell markers for NIPC/PDC, blood dendritic cell Ag (BDCA)-2 and BDCA-4, were recently described by Dzionek et al. The BDCA-2 is present on lineage negative CD11c-, CD123+ cells, but not on the myeloid CD11c+ DC. After culture with IL-3, BDCA-2 is down regulated after 48h. In contrast, BDCA-4 is upregulated on NIPC/PDC after culture, and also appear on CD11c+ DC after a few hours in cell cultures, thus being a less specific NIPC/PDC marker. The detection of these Ags by monoclonal Abs (mAbs) facilitates the study of the NIPC/PDC population, which in this way can be more easily recognized and also purified.

Further studies of the BDCA-2 revealed that this molecule shows homologies with type II C-type lectins, and had qualities of a signalling receptor that activating protein-tyrosine kinases. Ligation of BDCA-2 on NIPC/PDC from healthy individuals by mAbs rapidly internalised the Ag, and a role in Ag targeting and presentation was proposed. Interestingly, ligation of BDCA-2 on such NIPC/PDC also potently inhibited their IFN-α production. A recent report proposes that BDCA-2 ligation promotes production of IL-12 in favour of the inhibited IFN-α production.

The BDCA-4 molecule is identified as the neuronal receptor neuropilin-1, which participates in the contact between naïve T cells and DC via homophilic interaction. However, the exact role of BDCA-4 on NIPC/PDC remains to be determined. Other receptors expressed by NIPC/PDC are the mannose receptor, DC-SIGN and the Toll-like receptors (TLR), for example TLR7 and TLR9. The NIPC/PDC also express certain chemokine receptors, such as CXCR3, CXCR4 and CCR5. The ligand for CXCR3 is IP-10, that is elevated in SLE patients and could contribute to migration of these cells.

**IFN-α-inducing agents and IFN-α induction in NIPC/PDC**

Enveloped viruses are among the most potent IFN-α inducing agents. One such virus, the DNA virus HSV, is one of the most studied IFN-α inducing viruses, triggering specifically the NIPC/PDC. Several other agents are capable of IFN-α induction in NIPC/PDC, such as certain bacteria, parasites, CpG DNA and the low-molecular-weight molecule imiquimod.

Induction of IFN-α production in NIPC/PDC possibly involves several of the NIPC/PDC receptors described above, although the exact role of these receptors is at the moment unclear. One possible mechanism involved in the induction of IFN-α in NIPC/PDC is endocytosis, because blocking of endosomal acidification, by for example chloroquine, inhibits IFN-α production in NIPC/PDC. The endosome located Toll-like receptor
TLR9, is critical for the induction of IFN-α in NIPC/PDC by immunostimulatory CpG-DNA, further supporting an endocytosis mediated induction \(^{177, 178}\). TLR7 is required for the induction of IFN-α in NIPC/PDC by imiquimod, but the natural ligand to this receptor is unknown \(^{179}\). Other receptors expressed on NIPC/PDC that could play a role in IFN-α induction are Fc receptors (FcR), especially FcγRII (CD32), apparently of importance in the induction of IFN-α by the combination of poliovirus and Abs, (Båve et al, manuscript submitted) \(^{180}\).

Depending on stimulation, NIPC/PDC can produce other cytokines beside IFN-α. Viral triggering can induce the production of small amounts of IFN-β, IFN-ω TNF-α, IL-6 as well as the chemokines MIP-1α and IP-10 \(^{175, 181}\). CpG DNA and CD40 ligation can induce NIPC/PDC to produce IL-12 \(^{177, 182}\).

**Regulation of IFN-α production in NIPC/PDC**

Induction of IFN-α and -β is generally regulated by members in the IRF family \(^{183}\). A recent report suggests that NIPC/PDC express all of the IRFs constitutively, although IRF-4 and IRF-7 are expressed in high levels \(^{175}\). Certain cytokines can enhance the IFN-α production, such as IFN-α itself, IFN-β, IL-3, GM-CSF and finally also IFN-γ \(^{184, 185}\). This phenomenon is referred to as priming, and at least for the priming effect of IFN-α it may be explained by the upregulation of IRF-7 in NIPC/PDC \(^{186}\). Among cytokines, IL-10 is the most potent inhibitor of IFN-α production, but TNF-α can also downregulate the IFN-α response \(^{117, 118}\).

**Immune regulation by NIPC/PDC**

The NIPC/PDC that produce IFN-α are immature cells, not expressing the costimulatory molecules CD80/CD86 and therefore, they are poor Ag-presenting cells. Upon maturation, for instance by exposure to CpG-DNA, CD40L or IL-3, they acquire CD80/CD86 and can stimulate T cells \(^{177}\). A Th1 T cell profile is seen after stimulation of NIPC/PDC with CpG ODN alone or in combination with CD40 L \(^{177, 187}\) as well as after viral stimulation and CD40 ligation \(^{188}\). A Th2 T cell profile is described when NIPC/PDC are cultured with the survival and differentiation promoting cytokine IL-3 alone or with CD40L \(^{162, 187}\). In addition, regulatory CD8+ T cells are reported to be induced by CD40L activated NIPC/PDC \(^{189}\). This differential ability of mature PDC to stimulate Th1 or Th2 appears to correlate to their production of IFN-α/IL-12 (Th1) or not (Th2).
PRESENT INVESTIGATIONS

AIMS
The general aim of this thesis was to study the clinical and etiopathogenic aspects of SLE with special reference to the role of autoAbs and the type I IFN system. The more specific aims were:

I To investigate the diagnostic relevance of performing an anti-SSA/Ro Ab ELISA on ANA negative sera remitted for IF-ANA screening in a routine clinical setting.

II To investigate the number and function of circulating NIPC in SLE patients. This was done by studying their in vitro capacity to produce IFN-α both spontaneously and after virus stimulation, with and without costimulatory cytokines. It was also asked if there exist factors present in SLE serum that could interfere with the IFN-α production.

III To characterize the IFN-α inducing factor in SLE serum (SLE-IIF) discovered in the previous work, to correlate the occurrence of SLE-IIF to SLE disease activity and serum IFN-α and to clarify the identity of the IFN-α producing cell triggered by this factor. It was also asked, if the IFN-α production triggered by SLE-IIF was dependent on the costimulatory cytokines IFN-α2b and GM-CSF.

IV To study if IFN-α producing cells could be detected in apparently normal and lesional skin biopies from patients with SLE.

V To determine the IFN-α inducing effect of SLE-IIF on PBMC from SLE patients and to determine the absolute and relative numbers of circulating NIPC/PDC in SLE patients by the surface markers BDCA-2 and -4. Further, to investigate if BDCA-2 or -4 are potentially therapeutic targets in SLE, by blocking the SLE-IIF induced IFN-α production with anti-BDCA-2 and -4 monoclonal Abs in PBMC from SLE patients.
RESULTS AND DISCUSSION

Lupus among patients remitted for an IF-ANA screening, with ANA-negative and SSA/Ro-positive sera (paper I)

When signs and symptoms in a certain patient suggest an autoimmune rheumatic disorder, an ANA screening on Hep-2 cells by IF is often performed. Most antinuclear auto Abs can in this way be detected. However, Abs directed to SSA/Ro Ag can be missed because this Ag is present in low amounts in the intact cell and is often destroyed by fixation. Therefore, we investigated the diagnostic value of adding a sensitive anti-SSA/Ro Ab determination by ELISA to the conventional ANA screening. The clinical picture in the ANA negative patients with positive outcome in the ELISA was then evaluated.

A total of 4025 sera remitted to the Department of clinical immunology at Uppsala University Hospital for routine IF-ANA screening during one year were analysed. Anti-SSA/Ro Abs were found by ELISA in 285 (7%) remitted sera, compared with less than 1% found in healthy individuals. Approximately one fourth of the anti-SSA/Ro Ab positive sera (n=75), derived from 64 patients, were negative by the IF-ANA on Hep-2 cells.

In order to evaluate the SSA/Ro ELISA, positive samples in this assay were investigated with three other SSA/Ro assays; ID, IF using Hep-2000 cells transfected to hyperexpress the 60 kD SSA/Ro Ag, and WB detecting both the 60 and the 52 kD SSA/Ro Ag subunits. In 86% (55/64) of the patient sera the positive outcome in the ELISA could be confirmed by at least one of the other three assays. WB was the most sensitive assay as it detected 81% of ELISA-positive samples. In approximately half of the patient samples, anti-SSA/Ro Abs were detected by ID and/or Hep-2000.

When the charts from the 64 ANA negative but SSA/Ro positive patients were reviewed a total of 12 patients were found to fulfil the ACR criteria for SLE. Another five patients turned out to have cutaneous LE. Thus, in this
patient population, 26% of the ELISA positive and ANA negative individuals turned out to suffer from cutaneous LE and SLE.

The relatively high number of SLE patients among the ANA negative and SSA/Ro positive individuals is interesting, and in the same year Reichlin could demonstrate that all ANA negative SLE patients had detectable anti-SSA/Ro Abs in an ELISA assay \(^{190}\). Taken together, these data suggest that a sensitive anti-SSA/Ro Ab ELISA could be used as a supplement to IF-ANA, when looking for auto Abs in patients with suspect SLE.

**IFN-α producing cells in PBMC from patients with SLE (paper II)**

Patients with SLE have signs of an ongoing IFN-α production, with measurable serum levels of IFN-α and increased expression of the IFN-α induced protein MxA in blood and skin. The reason to activation of the type I IFN system in SLE patients was completely unknown when this study commenced. We therefore decided to investigate the type I IFN system in these patients in more detail.

In a first set of experiments, we could confirm that a majority of the SLE patients had measurable levels of IFN-α in serum, reflecting increased IFN-α production in vivo. In contrast, this was not detected in any of the control sera. In the next step, we examined if SLE-PBMC produced IFN-α spontaneously in vitro, but no such IFN-α production could be detected. In order to further elucidate the induced IFN-α producing capacity in SLE-PBMC, these cells were stimulated by HSV and Sendai virus (SV). The IFN-α production by SLE-PBMC was markedly decreased compared with PBMC from healthy controls (control-PBMC). This decrease in IFN-α production, determined by an immunoassay, was more impaired in HSV stimulated PBMC cultures (> 95%) compared with SV induced cultures (~70%). This latter virus is known to trigger IFN-α induction by monocytes and not in NIPC/PDC. These results indicated a defect preferentially in the NIPC population in SLE, at least at the blood level.

In order to clarify if the decreased IFN-α production among SLE-PBMC was due to a lack of circulating IPC, the frequency of IPC among PBMC was determined by an ELIspot assay \(^9\). The amount of IFN-α produced per IPC was calculated by dividing the IFN-α concentration in culture supernatants by the numbers of IPC detected. These experiments showed that SLE-PBMC had a 70-fold reduction of the IPC frequency, compared with control-PBMC, but had a mean IFN-α producing capacity comparable with normal NIPC, e.g. about 1-2 U IFN-α/cell.
We then asked whether the frequency of NIPC could be increased by costimulatory cytokines and consequently, the cytokines IFN-α, IFN-γ and GM-CSF were added to the HSV-induced cultures. Priming with these cytokines increased the frequency of NIPC ten-fold in SLE-PBMC. This finding indicates a recruitment of NIPC by the costimulatory cytokines in the SLE-PBMC cultures. Thus, the low number of circulating NIPC in SLE patients could reflect lack of a sufficient amount of costimulatory cytokines in vivo for these cells.

Another explanation to the reduced NIPC frequency in circulation of SLE patients could be the presence of inhibitory serum factors. No anti-IFN-α Abs could be detected in SLE sera. However, Abs directed to HSV, which are known to inhibit HSV induced IFN-α production \(^{176}\), were present in both SLE and control sera. These Abs indeed inhibited IFN-α production, although at equal levels in SLE-PBMC and control-PBMC.

The observation that SLE patients have decreased numbers of functionally normal NIPC, suggests that these cells have been recruited into tissues. This is an interesting possibility, as IPC have been detected in for example lymph nodes and spleen after experimental viral induction in mice \(^{192,193}\). The hypothesis that IPC in SLE patients are localized in tissues was directly investigated in paper IV.

Perhaps the most interesting finding in this study was the observation that four out of eleven SLE sera actually induced IFN-α production in normal PBMC, when added to otherwise unstimulated cultures of PBMC from healthy controls. Because SLE patients have an ongoing IFN-α production, the existence of an endogenous IFN-α inducing factor could explain the increased serum IFN-α levels in these individuals and be of pathogenic relevance. This IFN-α inducing factor was therefore examined in paper III.

The circulating endogenous IFN-α inducer in SLE (paper III)

Normally, IFN-α gene expression is triggered by microorganisms or their constituents, such as viruses or bacterial CpG DNA \(^{175}\). Because several different microorganisms have been implicated in the etiopathogenesis of SLE \(^{194}\), it was of great interest to clarify the nature of the endogenous IFN-α inducing factor in SLE sera (SLE-IIF) found in paper II. We also wanted to examine the frequency of SLE patients demonstrating presence of SLE-IIF in blood, and the target cell triggered to IFN-α production by this factor.

Serum from three to seven (depending on the PBMC donor) out of 34 SLE patients induced IFN-α production in normal PBMC cell cultures. No
IFN-α inducing capacity was seen in the sera from any of the 18 healthy controls. When the PBMC cultures were supplemented by the costimulatory cytokines IFN-α2b and GM-CSF, all sera had an IFN-α inducing capacity. Depending on the PBMC donor stimulated, 14 to 21 of the 34 SLE sera induced IFN-α in significantly higher levels than the sera from healthy controls. Sera from normal individuals induced IFN-α production in the range of 1-25 U/ml while as much as 1000 U/ml was observed in cultures induced by SLE sera. In fact, these latter IFN-α levels are in the same magnitude as those observed after viral triggering. The patients with active disease, determined as mSLEDAI score ≥2, had higher IFN-α content in serum, had more often SLE-IIF and also showed a more pronounced IFN-α inducing capacity in cultures without costimulatory cytokines, compared to patients with inactive disease.

The IFN-α production in PBMC induced by SLE serum was also investigated by in situ hybridisation for detection of IFN-α at the mRNA level. As compared to HSV-induced NIPC, SLE sera induced a lower frequency of IFN-α producing cells, but showed a similar heavy labelling intensity, reflecting the massive IFN-α production per cell.

The phenotype of the IFN-α producing cells induced by SLE-IIF was analysed by flow cytometry, and a high expression of CD36, CD45RA, CD83 and HLA-DR and lower levels of CD4 and CD40 were noted. In addition, markers for the T, B, NK cells and monocytes as well as CD80 and CD86 were not expressed. Thus, the phenotype was identical to that of the HSV-induced NIPC. The IFN-α inducing factor in serum from SLE patients, designated SLE-IIF, thus specifically activates NIPC.

In order to characterise the SLE-IIF, ultrafiltration of SLE sera was performed and this revealed that SLE-IIF had a molecular weight between 300-1000 kD. Further characterisation by passage of SLE serum over a protein G sepharose column eliminated the IFN-α inducing capacity of the sera, indicating that SLE-IIF contained IgG. Neither the effluent nor the eluate had any IFN-α inducing effects on PBMC. However, when combining the effluent and eluate, the IFN-α inducing capacity was partly restored, indicating that SLE-IIF could consist of small IC. By treatment of SLE serum with different nucleases this component appeared to contain DNA, since benzonase and DNase I eliminated the IFN-α inducing ability. In contrast, RNase A or T1 did not affect IFN-α induction. Consequently, SLE-IIF probably consists of Ag-Ab complexes containing DNA.

SLE patients have a deficient clearance of IC with increased levels of circulating IC. The traditional view is that these IC contribute to SLE pathogenesis when deposited in tissue, causing inflammation and organ destruction such as nephritis. The main discovery in this study was that IC from SLE patients elicit IFN-α production by NIPC, and suggests an
additional role for IC in SLE pathogenesis. This hypothesis is supported by the fact that IFN-α can contribute to loss of tolerance and autoAb production, as seen during long-term treatment with IFN-α in patients with malignant and infectious disorders. This issue will be further elaborated on in the general discussion.

IFN-α producing cells in skin from SLE patients (paper IV)

The elevated IFN-α levels seen in serum from patients with SLE, together with the decreased frequency of NIPC at the blood level, indicate that the actual IFN-α production is confined to cells localized in tissues. An organ relatively frequently affected in SLE is the skin, and increased expression of the IFN-α induced MxA protein has earlier been reported in skin biopsies from SLE patients \(^{124}\). We therefore decided to investigate if actively IPC could be detected in skin biopsies from SLE patients.

Eleven SLE patients, visiting either the department of rheumatology or dermatology at Uppsala university hospital, who presented a typical cutaneous lupus rash were consecutively selected for this study. Punch biopsies, 3 mm diameter, were taken from lesions and unaffected skin from patients and from six healthy controls.

The IFN-α containing cells were determined by immunohistochemistry (IH) by intracellular staining with a polyclonal anti-IFN-α Ab. All SLE patients had IFN-α containing cells in their lesional skin biopsies. IFN-α positive cells were also detected in 10 out of 11 biopsies obtained from unaffected SLE-skin, but were not as numerous as in lesional skin. In all biopsies from SLE patients the IPC detected were evenly distributed between the papillary and reticular dermis. An ongoing IFN-α gene transcription could be verified in a majority of the SLE patients by in situ hybridisation (ISH) with oligonucleotide probes for IFN-α mRNA, both in lesional and non-lesional biopsies. Serum IFN-α levels were found in six of the SLE patients. Four of these patients had detectable IFN-α mRNA containing cells in the skin.

We did not perform any phenotypical characterisation of the IPC in this study, but Farkas et al have demonstrated PDC (CD11c-, CD123+) in skin biopsies from SLE patients \(^{195}\). In the latter study no IFN-α staining was performed, but an increased expression of MxA protein was detected. Taken together, these two studies strongly support the hypothesis put forward in paper II, that the reduced number of circulating NIPC in SLE patients can be due to migration of these cells into skin. Because NIPC are triggered to IFN-
α production by special interferogenic IC, it is tempting to speculate that the large amount of IC, known to be deposited in both lesional and apparently normal skin of SLE patients, act as the IFN-α inducing factor for dermally located NIPC 196. An intriguing observation is the fact that SLE patients also have activated IPC in non-lesional skin, reflecting a more general activation of these cells in the disease. Recently, the importance of NIPC in inflamed skin has been emphasized by the finding of PDC in different inflammatory skin diseases, such as psoriasis and contact dermatitis 197.

An important tissue for immune activation is the lymphoid organs and recently, we could also demonstrate IFN-α producing cells in a lymph node from an SLE patient 198. In fact, the expression of the chemokine receptor CXCR3 and CXCR4 on NIPC/PDC 199 together with the observation that SLE patients have elevated levels of the CXCR3-ligand IP-10 175,200 further support an active recruitment of these cells into tissues. Recent reports further show that SDF-1 acting on CXCR4 is an important recruiter of NIPC/PDC to both tumors and lymph nodes 201,202. Relevant in this context is the fact that IFN-α can induce IP-10 production, and it is therefore possible that NIPC/PDC themselves attract additional NIPC/PDC. Such a mechanism will enhance an immune response, but can also be deleterious if leading to loss of tolerance and a self-perpetuating immune reaction.

Expression of BDCA-2 and -4 on NIPC/PDC in SLE patients and inhibitory effects of anti-BDCA mAbs on the IFN-α production induced by SLE-IIF (paper V)

Taken together, the studies in paper II-IV indicate an important role of NIPC/PDC in the pathogenesis of SLE. However, in the earlier studies, virus was the IFN-α inducing agent used when SLE-PBMC were investigated. We now asked if SLE-IIF could induce SLE-PBMC to IFN-α production. We also wanted to determine the expression of the recently described BDCA-2 and -4 markers on SLE-NIPC/PDC, and if all, or only a subpopulation of the BDCA-2 and -4 expressing NIPC/PDC, actually produced IFN-α after induction. In addition, the recently described inhibitory action of anti-BDCA-2 Abs on the IFN-α production in NIPC/PDC from normal PBMC donors, also presented an ability to modulate IFN-α production in SLE patients 167. We therefore asked if the inhibitory action of these Abs was applicable on the IFN-α production induced by SLE-IIF in PBMC from SLE patients.
First we established that SLE-IIF induced IFN-α production in SLE-PBMC, although not as efficiently as in PBMC from healthy controls. This decreased capability of SLE-IIF to induce IFN-α production in SLE-PBMC was in line with our previous finding that SLE-PBMC have a reduced capacity to produce IFN-α in vitro (paper II). In that study, a reduced number of NIPC among SLE-PBMC was demonstrated by the detection of IPC after HSV-induction. A reduced number of IPC, detected by ELISPOT, can not exclude a reduced function within the NIPC/PDC population as an explanation to the impaired IFN-α production in SLE-PBMC. We therefore investigated the numbers of circulating NIPC/PDC by their expression of the BDCA-2/-4 markers, and found that the proportion of BDCA-2/-4 expressing cells was significantly reduced among SLE-PBMC compared to control-PBMC. Thus, not only are the number of functional NIPC decreased in SLE patients, but also the circulating NIPC/PDC population, as detected by anti-BDCA-2/-4 Abs, is reduced in these patients. This finding is important, because conflicting data has been reported concerning CD11c-DC in SLE patients detected by different surface markers. One study reports an almost normal CD11c- DC subset, whereas another study show decreased levels of CD123+, HLA-DR+ CD11c- cells in pediatric SLE patients. In addition, we found that another subset of DC, expressing BDCA-3, was decreased in SLE-PBMC. These cells are known to express CD11c but not CD1c or CD123, and have been reported to be reduced in the blood of SLE patients. Thus, both the DC populations detected by BDCA-2/-4 and BDCA-3 markers are decreased in the circulation of SLE patients.

By double staining PBMC with anti-IFN-α Abs and anti-BDCA-2 Abs after HSV-stimulation, we investigated the IFN-α producing capacity of BDCA-2 expressing cells in both SLE-PBMC and control-PBMC. A lower frequency of IFN-α producers in SLE-PBMC was seen compared with normal PBMC, as well as a lower mean fluorescence intensity (MFI) of the BDCA-2 staining. Only a minority of all cells in the BDCA-2 cell population was actual IPC, but the proportion of IPC varied widely in control-PBMC. In SLE-PBMC, this proportion could not be estimated due to the low BDCA-2 MFI. The frequency of BDCA-2 and -4 expressing PBMC before culture was also correlated to the amount of IFN-α produced in the cultures upon stimulation by HSV. There was a correlation between these parameters only among SLE patients. This observation, together with the decreased BDCA-2 MFI after culture in SLE patients, is interesting and we suggest that it may reflect an in vivo influence on NIPC/PDC in SLE, perhaps caused by cytokines with priming properties. Such priming would result in more prompt IFN-α production upon stimuli and more rapid differentiation of cells with a concomitant downregulation of BDCA-2 expression.
Coincubation with anti-BDCA-2 Abs in control- and SLE-PBMC cultures induced by SLE-IIF almost completely inhibited IFN-α production (median 98 and 97% respectively). However, a partial inhibitory effect was also seen with anti-BDCA-4 Abs (median 70 and 57% respectively). Only anti-BDCA-2 Abs had an inhibitory effect on the IFN-α production induced by HSV. Therefore, the anti-BDCA-2 Ab is a potent inhibitor of IFN-α production by PBMC from SLE patients stimulated not only by virus, but also, even more efficiently, after induction by the endogenous IFN-α inducer SLE-IIF. Thus, these results support the view that anti-BDCA-2 Ab in a humanized form could be a potential therapeutic agent in SLE patients. In addition, anti-BDCA-4 Abs preferentially, but incompletely, decrease the IFN-α production stimulated by SLE-IIF, but leaves the IFN-α production triggered by HSV unaffected. This could be beneficial in a therapeutic situation, because the endogenously derived IFN-α in SLE patients in this way may be decreased without disturbing the protective IFN-α to viral infections. Because NIPC appears central in the pathogenesis of SLE, further evaluation of these Abs as possible therapeutic agents acting on NIPC seems reasonable.
GENERAL DISCUSSION

The main findings in this thesis were i) that SLE patients have an endogenous IFN-α inducer (SLE-IIF) in sera consisting of Abs in complex with DNA, ii) that SLE-IIF selectively activates the NIPC/PDC, iii) that these cells are reduced in circulation but are localized to tissues in SLE patients where they produce IFN-α, iii) that SLE-NIPC/PDC express BDCA-2/-4 and that ligation of these molecules with mAbs inhibits the action of SLE-IIF. In addition, adding a sensitive anti-SSA/Ro ELISA to standard IF-ANA screening was shown to be of benefit in order to detect ANA negative SLE patients. Interestingly, recent observations in our laboratory suggest that anti-SSA/Ro Abs may be important in the activation of NIPC/PDC (Båve et al, to be published). Thus, these Abs could be of relevance in the understanding of the activated type I IFN system in SLE patients, and this will be further discussed below.

SLE is characterised by the production of auto Abs, typically directed to nucleic acid and associated proteins, IC formation and tissue inflammation. Anti-dsDNA Abs is associated with disease activity \(^{80}\) and in this thesis an association was also found between the occurrence of SLE-IIF and active SLE. Further studies by Vallin et al have revealed that SLE-IIF consists of anti-dsDNA Abs and DNA, where the latter could be mimicked by hypomethylated plasmid DNA \(^{203}\). This indicates that immune stimulatory (is) DNA sequences, consisting of unmethylated CpG motifs, may be of importance in triggering the IFN-α production in SLE. In fact, the presence of such isDNA in SLE sera has been identified by molecular cloning \(^{204}\), but when using oligodeoxyribonucleotides (ODN) originally cloned from SLE sera, the unmethylated CpG sequences were not necessary for IFN-α induction \(^{205}\). This suggests that interferogenic DNA motifs can occur in eukaryotic cells. The DNA component in SLE-IIF could therefore be derived from apoptotic cells, and recently Båve et al showed that the combination of apoptotic cells and SLE autoAbs can induce IFN-α production by NIPC/PDC \(^{206}\). The increased apoptosis and decreased clearance of apoptotic cell material in SLE patients could therefore contribute to the SLE disease process in several ways. First, by supplying interferogenic nucleic acid for NIPC/PDC activation and second, by being Ags for autoreactive T and B
cells. The NIPC/PDC may execute both these functions; IFN-α production as an immature cell and Ag presentation after maturation. Necrotic cell material can also induce IFN-α production in combination with SLE autoAbs in vitro, shown in our laboratory by Lövgren et al (manuscript, submitted). The IFN-α inducing capacity of these IC is sensitive to RNase treatment, indicating that also RNA in complexes can act as an IFN-α inducer. This suggests that lupus-associated RNA-containing Ag, such as SSA, SSB and RNP, also can possess interferogenic properties after IC formation. This opens the possibility that for example anti-SSA/Ro Abs may be of importance in IFN-α induction as well, but this remains to be established.

Although reduced in circulation, the residual NIPC/PDC among PBMC are functionally normal, each cell synthesising large amounts of IFN-α when activated. The observation that NIPC/PDC in SLE patients can be induced to IFN-α production by SLE-IIF, strengthens the conjecture that these IC are relevant IFN-α inducers in vivo. Several studies support the presence of activated NIPC/PDC in peripheral tissues, including the present investigation 195,197. Apparently, these cells have the capacity to migrate to sites of inflammation, as they are found not only in SLE skin lesions but also in contact dermatitis and in plaques from patients with psoriasis 197,207. The NIPC/PDC may therefore have a role in inflammatory reactions in general, and this is also supported by the presence of several TLRs on these cells. However, this issue definitively needs more studies. The observation that IPC could be detected in a lymph node from an SLE patient, supports the central role for the NIPC/PDC in the initiation and development of the autoimmune response in this disease 198. Here, activated NIPC/PDC can stimulate T and B cells, increase the APC function of DC 187 as well as induce differentiation of monocytes to APC 74,75. An ongoing IFN-α production by these NIPC/PDC will cause a continuous immune stimulation that will lead to loss of tolerance and promote autoimmunity. Such a mechanism for the progression from self tolerance to autoimmune disease is supported by the fact that patients receiving continuous IFN-α administration occasionally develop ANA, anti-dsDNA Abs and SLE 47-49.

Based on the findings in the present thesis, and other observations, a hypothesis has been formulated concerning the role of the type I IFN system in SLE 208,209 (Figure 1).
Figure 1. Mechanisms for activation of the type I interferon system in SLE
Initially, IFN-α and perhaps other cytokines are produced as a consequence of for example a viral infection. The produced IFN-α or the microorganism can increase apoptosis and necrosis. A reduced clearance of apoptotic cell material seen in SLE will increase the availability of autoAg consisting of nucleic acids and different cellular proteins. By the described actions of IFN-α on various cells in the immune system, tolerance can be broken and autoAbs produced. An increased frequency of autoAbs, including ANA, has also been noted during viral infections \(^{57,58}\). In SLE patients, endogenous IFN-α inducers can be generated from autoAbs and DNA/RNA that form interferogenic IC. They can now replace the exogenous IFN-α inducer. Once formed, these endogenous IFN-α inducers will cause an ongoing IFN-α production that sustains the autoimmune process by promoting autoAb production and formation of more IC acting as SLE-IIF.

Several factors of suspected or known importance for the etiopathogenesis of SLE can influence this IFN-α driven autoimmune process. For example, the UV-light induced photosensitivity reactions, common among SLE patients, may generate Ag for SLE-IIF, because UV light can induce apoptosis and target autoantigen in apoptotic blebs on the cell surface \(^{43}\). Viral infections with concomitant IFN-α production may, during periods of SLE remissions, boost this system by priming and recruitment of NIPC/PDC and cause a disease flare \(^{54,59}\). Certain drugs, such as procainamide, can demethylate DNA and increase the amount of isDNA, and in this way contributes to formation of interferogenic DNA motifs \(^{210,211}\).

The cytokines IL-10 and TNF-α have both been reported to be elevated in serum from SLE patients \(^{110,111}\). These cytokines downregulate the IFN-α production by NIPC/PDC and thus IL-10 and TNF-α may act as negative feedback signals in the type I IFN system \(^{117,118}\). A lack of such a negative feedback circuit, could contribute to the development of ANA and also SLE reported in RA patients during anti-TNF-α treatment \(^{51-53}\).

This hypothesis may not be correct in every detail, but our model is indirectly supported by two related findings. Serum IFN-α levels correlate with SLE disease activity and severity \(^{312}\), and these patients have an increased expression of IFN inducible genes, detected by global gene expression profiling of PBMC \(^{213,214}\). The model of SLE pathogenesis, outlined above, suggests several new therapeutic targets. It remains to be determined whether inhibition of the type I IFN system actually will ameliorate the SLE disease in patients. However, at least two observations support the contention that such a strategy could be successful. First, chloroquine, that blocks endosomal acidification and thereby inhibits IFN-α production in NIPC/PDC, is an effective drug in SLE patients \(^{176}\). Second, in two recent studies, lupus prone B6/lpr and NZB mice with null mutations for
the IFNAR showed a dramatic decrease in lupus manifestations, compared with wild type mice. Relevant in this context is our observation that SLE-NIPC/PDC express BDCA-2 and -4 and that ligation of these molecules markedly reduced SLE-IIF induced IFN-α production. Consequently, administration of humanised anti-BDCA-2 or -4 Abs to SLE patients could be an attractive therapeutic alternative. Another option in SLE treatment is of course administration of soluble IFNAR or anti-IFN-α Abs that could neutralise the hyperinterferonemia seen in these patients.

This thesis has dealt with the etiopathogenic role of the type I IFN system in SLE. However, it is important to keep in mind, that this system also may have a crucial role in autoimmune diseases in general. This is illustrated by the fact that increased expression of IFN-α genes have been reported in such divergent diseases such as polymyositis and type I diabetes, and the observation that during IFN-α treatment most types of autoimmune disorders can develop. The genetic susceptibility of a certain individual will determine the autoimmune disease that may evolve during prolonged exposure of the immune system to IFN-α. It is therefore of great importance to further explore the role of the type I IFN system in autoimmune diseases and to define the essential regulatory mechanisms involved.
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