Mechanisms of Interferon-α Induction in Systemic Lupus Erythematosus

BY

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Abstract
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Patients with systemic lupus erythematosus (SLE) have an activated type I interferon (IFN) system with an ongoing IFN-α synthesis. This may be caused by circulating immune complexes, consisting of anti-DNA antibodies (Abs) and DNA, with IFN-α inducing capacity. Produced IFN-α may be crucial in the pathogenesis, because this cytokine can break tolerance and promote autoimmunity.

In the present thesis, possible mechanisms of the IFN-α production in SLE were studied. To investigate whether IFN-α inducing material could be derived from apoptotic cells, IgG from SLE patients (SLE-IgG) were combined with apoptotic cells. This combination induced high IFN-α production in normal peripheral blood mononuclear cells (PBMC). The IFN-α induction was associated to presence of anti-RNP Abs, but not to anti-dsDNA Abs, indicating that two inducers could be active in SLE, one containing DNA and the other RNA.

Apoptotic cells and SLE-IgG exclusively activated the natural interferon producing cells (NIPC) and the IFN-α response was enhanced by type I IFN and inhibited by IL-10 and TNF-α. The IFN-α induction was dependent on FcγRII, because blocking this receptor reduced IFN-α production and NIPC were found to express FcγRIIa.

To further elucidate the role of different autoantibodies in the IFN-α induction, sera from patients with Sjögren´s syndrome (SS), containing autoantibodies to RNA binding proteins (SSA, SSB, RNP and/or Sm) were investigated. The combination of SS or SLE sera and apoptotic or necrotic cell material induced high IFN-α production in PBMC. RNA, but not DNA, was required for IFN-α induction, indicating that RNA and Abs to RNA-binding proteins form potent IFN-α inducing complexes.

The findings in this thesis can explain central mechanisms for the activation of NIPC in SLE, and perhaps also other autoimmune diseases. This activation is mediated by interferogenic immune complexes, and modulating the NIPC activation may be a novel therapeutic approach in SLE.

Keywords: Systemic lupus erythematosus, interferon inducer, apoptosis, autoantibodies, natural interferon-alpha producing cell, FcgammaRII, Sjögren´s syndrome

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PAPERS INCLUDED IN THE THESIS

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

I   Ullvi Båve, Gunnar V. Alm, Lars Rönnblom. 2000.
           The combination of apoptotic U937 cells and lupus IgG is a potent IFN-α inducer. *The Journal of Immunology* 165: 3519-3526

II   Ullvi Båve, Helena Vallin, Gunnar V. Alm, Lars Rönnblom. 2001.
           Activation of natural interferon-α producing cells by apoptotic U937 cells combined with lupus IgG and its regulation by cytokines. *Journal of Autoimmunity* 17: 71-80

III   Ullvi Båve, Mattias Magnusson, Maija-Leena Eloranta, Anders Perers, Gunnar V. Alm, Lars Rönnblom.
           FcγRIIa is expressed on natural IFN-α producing cells (plasmacytoid dendritic cells) and is required for the IFN-α production induced by apoptotic cells combined with lupus IgG. *Submitted*.

IV   Ullvi Båve, Gunnel Nordmark, Tanja Lövgren, Johan Rönnelid, Maija-Leena Eloranta, Gunnar V. Alm, Lars Rönnblom.
           Anti-SSA, -SSB, and -RNP autoantibodies in Sjögren´s syndrome and systemic lupus erythematosus in combination with apoptotic or necrotic cell material are potent IFN-α inducers. *Manuscript*.

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Contents

Abbreviations

Ab	antibody
ACR	American College of Rheumatology
ANA	anti-nuclear Ab
Ag	antigen
APC	antigen presenting cell
BDCA	blood dendritic cell Ag
BCR	B cell receptor
C	Complement
CD	cluster of differentiation
CpG	cytosine-phosphate-guanine
CRP	C-reactive protein
DC	dendritic cell
DsDNA	double stranded deoxyribonucleic acid
FcR	Fc receptor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HSV	herpes simplex virus
IC	immune complex
IDDM	insulin-dependent diabetes mellitus
IFN	interferon
IFNAR	interferon-α/β receptor
IIF	IFN-α inducing factor
Ig	immunoglobulin
IL	interleukin
IRF	interferon regulatory factor
LE	lupus erythematosus
MAb	monoclonal Ab
MDC	myeloid DC
MHC	major histocompatibility complex
NFκB	nuclear factor kappa B
NIPC	natural IFN-α producing cell
ODN	oligodeoxynucleotides
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells

PDC	plasmacytoid dendritic cell
RNA	ribonucleic acid
RNP	ribonucleoprotein
RT-PCR	reverse transcriptase polymerase chain reaction
SAP	serum amyloid protein
SLE	systemic lupus erythematosus
SLEDAI	SLE disease activity index
Sm	Smith Ag
SSA/Ro	Sjögren syndrome-A/Ro
SSB/La	Sjögren syndrome-B/La
SV	Sendai virus
Th1	T helper cell type 1
Th2	T helper cell type 2
TGF	transforming growth factor
TLR	toll like receptor
TNF	tumor necrosis factor
U	units
UV	ultraviolet

INTRODUCTION

Autoimmune diseases affect approximately 5% of the human population, and women are disproportionately often affected. These diseases can be described as a state where the immune system reacts to the host´s own tissues (self). The prognosis of many of these diseases have improved markedly since the introduction of different medications such as glucocorticoids, but still cause morbidity and increased mortality. It is therefore of major importance to elucidate the events leading to autoimmunity with the ultimate goal of finding specific pharmacological intervention.

Systemic Lupus Erythematosus (SLE) is often described as the classical systemic autoimmune disease due to its wide spectrum of clinical and immunological abnormalities (1). Findings from studies of SLE could therefore be important for the general understanding of autoimmunity and have implications also for other autoimmune diseases. The complexity of autoimmunity is obvious, considering the large variation of factors contributing to its genesis as well as the multitude of clinical manifestations observed. These various parts can be described by the metaphor of a mosaic pattern where the pieces consist of genetics, immune abnormalities as well as hormonal and environmental factors (2). This is further illustrated by patients with autoimmune diseases that have an increased risk for developing also other autoimmune diseases or conditions, as well as by patients with overlapping syndromes. The precise pathways from autoimmune associated genes to an overt clinical disease are largely unknown. In this thesis, possible mechanisms involved in the pathogenesis of SLE and to some extent also Sjögren´s syndrome have been studied.

The history of SLE

Systemic lupus erythematosus (SLE) is not a novel disease, and it has been suggested that cutaneous lesions were described already by Hippocrates

under the term *herpes esthiomenos* (3). Lupus, wolf in Latin, a term first associated to disease in the 10th century, presumably to describe skin lesions reminding of a wolf´s bite (1). Due to the large variety of disease manifestations and to the common misinterpretation of lupus as being a variant of tuberculosis, it was not until 1872 that the physician Moriz Kaposi described lupus erythematosus (LE) as an entity of skin lesions with occasional systemic symptoms. In 1906, the Wasserman test for syphilis became widely used and soon false positive tests for syphilis were noted in patients with LE, a sign still included in the ACR criteria for SLE today (1, 4). The LE cell was discovered in 1948 and was a breakthrough that contributed to the understanding of the pathogenesis of disseminated LE (5). Today the LE cells have been found to contain apoptotic bodies (6), which connects history to the present thesis.

Definition and epidemiology of SLE

SLE, also termed lupus, is a chronic autoimmune disease with tissue damage caused by autoantibodies and immune complexes. The disease has a wide spectrum of clinical manifestations that include non-erosive arthritis and skin lesions, but also inflammation in internal organs that cause nephritis, pleuritis, pericarditis and nervous system involvement. General symptoms such as malaise, fever and fatigue are also common in SLE patients. Most patients have antinuclear antibodies (ANA) and during active disease, leukopenia and/or complement consumption are frequently seen. The disease is also varyingly active, with periods of exacerbations that are followed by remissions (1).

For more mild SLE, without threatening organ involvement, salicylate, non-steroid anti-inflammatory drugs (NSAID) and glucocorticoids are used for therapy. Antimalarials are also frequently used in SLE, both for treatment of several manifestations and to prevent relapses. To more severely ill patients, high-dose glucocorticoids and immunosuppressive agents are used, including azathioprine, methotrexate and cyclophosphamide (1).

Due to the fact that SLE patients can have many different clinical manifestations, rheumatologists have developed a set of criteria to facilitate collection of data for clinical trials and to compare different patient populations. In 1982, the American College of Rheumatology (ACR) revised the preliminary criteria from 1971 for the classification of SLE (Table I) (4). A person is considered to have SLE if at least four of the 11 criteria are present which gives a high sensitivity and specificity for presence of SLE (4, 7). Of all individual parameters, anti-dsDNA antibodies were found to be the best discriminator. It is important to make the distinction that these criteria

are not intended to be used for diagnosis, because more than 50% of the patients did not fulfill the ACR criteria at a given time point, although all did with time (8, 9).

Predominantly women (9:1, female:male ratio) during their child bearing years are afflicted by SLE and the incidence has been estimated to be 4.8/100 000 and the prevalence to be 68/100 000 inhabitants in southern Sweden (10). In the US, the overall prevalence has been estimated to be between 14.6 to 50.8 cases per 100 000 persons (11, 12). An increase in the incidence has been reported, perhaps due to increased use of oral contraceptives, estrogen replacement therapy, increased exposure to UV-light due to depletion of the ozone layer, or to improved recognition of mild disease (13).

Despite a much better prognosis today in comparison to the 1950s, with a 10-year survival of 50% and 80% respectively, the survival of SLE patients is still markedly decreased in comparison to the normal population (1, 14). The main causes of death of SLE patients include infections, end-stage organ failure and most importantly today cardiovascular disease (10, 14).

Table I. The 1982 ACR classification criteria for systemic lupus erythematosus.

Criterion:	Definition:
1. Malar rash	Fixed malar erythema over the malar eminence, flat or raised
2. Discoid rash	Raised erythematous patches, in older lesions scarring may occur
3. Photosensitivity	Skin rash as an unusual reaction after exposure to sun
4. Oral ulcers	Oral or nasopharyngeal lesions observed by physician
5. Arthritis	Non-erosive arthritis characterized by swelling, tenderness or effusion, in at least two peripheral joints
6. Serositis	Pleuritis or pericarditis
7. Renal disorder	Persistent proteinuria (> 0.5 g/d or >3+) or cellular casts
8. Neurologic disorder	Seizures or psychosis, in the absence of other causes
9. Hematologic disorders	Hemolytic anaemia, leukopenia, lymphopenia or thrombocytopenia
10. Immunologic disorder*	Positive LE cell preparation or anti-dsDNA, anti-Sm, or false positive test for syphilis
11. ANA	Abnormal titre of antinuclear antibodies

*In 1997, an update of these criteria was published, where two changes were proposed; deletion of positive LE cell preparation, and addition of positive antiphospholipid antibodies (7).

Disease activity indices

The activity of the disease varies over time for most SLE patients. When monitoring SLE patients, it is essential to quantify such variations, and for this reason several scoring systems have been constructed to assess disease activity. One frequently used scoring system in clinical studies is the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score which is calculated by summing weights of 24 different signs and symptoms (15). More severe manifestations of the disease, that can be life threatening, such as involvement of the central nervous system and vascular manifestations, are given higher weights. The descriptors are seizure, psychosis, organic brain syndrome, visual disturbance, cranial nerve disorder, lupus headache, cerebral vascular accident or vasculitis (weight 8/each), arthritis, myositis, urinary casts, hematuria, proteinuria or pyuria

(weight 4/each), rash, alopecia, mucosal ulcers, pleurisy, pericarditis, low complement or increased DNA binding (weight 2/each), fever, thrombocytopenia or leukopenia (weight 1/each).

Etiology of SLE

Genetics

SLE is a genetic complex disorder where the disease predisposing alleles frequently are normal variants, but where an unfortunate combination of these increases susceptibility to SLE and eventually results in disease, depending on penetrance as well as environmental factors. Relatives to patients with SLE have increased levels of autoantibodies, such as ANA, and are also more commonly affected by SLE (16). Interestingly, other autoimmune disorders are more frequent among both SLE patients and their relatives, illustrating that certain genes predispose to autoimmunity in general (17, 18).

Genes associated with the development of SLE code mostly for functions in the immune system including immune reactivity, immune regulation and scavenging functions. Allelic polymorphisms are identified in the following genes in SLE patients: MHC class I and II, complement components such as C2, C4, C1q, cytokine genes such as the TNF-α gene, as well as its receptor, and IL-10, FcγR genes, and PARP, a protein involved in DNA repair and apoptosis (19, 20).

An exception to the picture of SLE as a multigenetic disorder is the homozygous deficiency in the gene coding for the complement component C1q, where over 90% of the carriers develop SLE (21). This genotype is very rare in the population (approximately 40 persons have been described in the world) and can only account for unique cases of SLE. In addition, individuals with defects in the pro-apoptotic gene Fas may also develop a lupus like syndrome (22).

Environmental factors

Several factors in the environment can contribute to onset and relapses of SLE. Mainly chemical and physical factors have been studied in SLE, whereas social and behavioral aspects have been less investigated.

Exposure to UV radiation from the sun is an established environmental risk factor in SLE. More than 50% of the SLE patients are photosensitive, and in these individuals UV-light exposure can lead not only to dermal lesions, but also to systemic manifestations (23). UV-light has several effects on cells and among these, induction of apoptosis may be of relevance in

SLE, considering that many autoantibodies in SLE are directed to antigen generated during apoptosis (24).

Drugs such as chlorpromazine, hydralazine, procainamide, sulfasalazine and isoniazide can induce a reversible SLE syndrome, termed drug-induced lupus (DIL) (25). The mechanisms of DIL are unknown, but the action for some of these drugs, i.e. procainamide and hydralazine is the ability to demethylate DNA and thereby increase the risk of developing autoreactive T-cells (26). Treatment with the cytokine IFN-α of patients with malignant diseases or chronic hepatitis C virus infections can induce SLE, which will be further discussed below. Recently, treatment of RA patients with TNF-α inhibitors has shown side effects such as development of anti-dsDNA antibodies and in rare cases SLE (27).

Infections have been noted to occur before onset or flares of SLE (28-30). Especially viral infections have been proposed to trigger the autoimmune process, and increased frequencies of ANA and anti-RNP Abs have been reported during influenza virus and cytomegalovirus (CMV) infections (31, 32). Several case reports also describe the onset of SLE after or during a viral infection (28, 33). A history of varicella-zoster infection was noted to be a risk factor for developing SLE (34). Other viruses with association to lupus are EBV, endogenous retroviruses, and HTLV-1 (1). However, no clear association between SLE and a single viral agent has been found, but the fact that most viruses cause IFN-α production may instead be relevant for the triggering of SLE. This possibility is supported by the finding that lupus prone mice, infected with lymphocytic choriomeningitis virus, develop nephritis to a lesser extent when treated with antibodies that neutralize type I IFN (35).

Other risk factors associated to SLE are use of tobacco and intake of alf-alfa sprouts. Predisposing factors such as sun-sensitive skin, hypertension and drug allergy also increase the risk of developing SLE (1, 36). In contrast, use of alcohol was negatively associated to SLE (36).

Hormonal aspects

Female gender is a clear risk factor for development of lupus, which most frequently occurs during the childbearing years. Elevated levels of estriol and progesterone, as well as rapid changes in hormonal concentrations in pregnancy, have been associated to flares of the disease (37).

Furthermore, in women with lupus, several abnormalities in the metabolism of sex hormones have been noted, i.e. increased levels of 16-hydroxyestrone and estriol in serum, as well as decreased levels of androgens (1, 38). Women taking estrogen replacement therapy had an increased risk of developing lupus, especially when treatment duration was two years or more (39).

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The pathogenesis of SLE

Immune cells in the pathology of lupus

Several immune abnormalities have been described in SLE patients. Typical findings are T-cell activation, autoantibody production by B cells and immune complex formation. The cellular abnormalities in lupus are numerous and closely connected to the antigens and autoantibodies forming immune complexes (IC) that provide inflammatory stimuli to e.g. dendritic cells (DC), monocytes and T- and B cells. In this thesis, some of the immune disturbances described in lupus are summarized.

In SLE patients, T cells are reduced in the circulation, which can be seen as a lymphocytopenia. Despite the low number, an increased T cell activation has been demonstrated and an increased MHC II expression (40). Because T cells regulate B cell function and thus the production of autoantibodies, T cell abnormalities are central in the disease process (41). This has further been studied in IFN-γ transgenic mice developing a form of murine lupus, in which a depletion of α/β T cells resulted in reduced autoantibody production and an abolishment of kidney disease (42). Furthermore, treatment with anti-CD4 Abs can diminish autoantibody production and ameliorate disease (43).

B cells also play an important role in SLE, which is indicated by B cell hyperactivity, with production of a large number of different autoantibodies. The B cells are clonally expanded, producing antibodies that demonstrate somatic mutation, affinity maturation and IgM to IgG switching, which indicates an antigen-driven immune response (44). Although many studies of B-cell function have been performed, especially in murine lupus, the exact mechanism(s) causing the B-cell hyperactivity and Ab production in SLE patients is not known. Interestingly, inhibition of secretion of Ig did not ameliorate the disease in autoimmune prone mice (45). This indicates that other factors than autoantibodies are also of importance for autoimmune disease.

Novel research points out the dendritic cell (DC) as a pivotal cell in the autoimmune process. The DC are professional antigen presenting cells (APC) and constitute the only APC capable of activating naïve T-cells (46). In the absence of pro-inflammatory cytokines, immature DC migrate to lymphoid organs where they induce T cell tolerance (47). There are several different types of DC such as myeloid DC and plasmacytoid DC, both originating from haematopoetic stem cells (48, 49). The myeloid DC, also termed type 1 DC (DC1), comprise three main types, i.e. the Langerhans DC, interstitial DC and monocyte-derived DC. While Langerhans DC and interstitial DC can develop directly from haematopoetic stem cells, the monocyte-derived DC develop from monocytes stimulated by the cytokine

combinations IL-4 and GM-CSF or IL-3 and IFN-α. These DC express myeloid markers, including CD11c. When matured by stimuli, such as TNF-α, they efficiently stimulate development of T helper (Th) 1 cells (49). The plasmacytoid DC, also known as type 2 DC or natural IFN producing cell (NIPC), are described in the section below dealing with the IFN system.

Several changes in the DC populations have been described in SLE patients. The number of both myeloid and plasmacytoid DC in circulation are reduced in SLE patients, and the number of myeloid DC correlate to disease activity (50, 51). Increased numbers of monocyte-derived DC have been detected in blood of SLE patients and may be due to the ongoing production of IFN-α in SLE, because IFN-α is known to promote maturation of monocyte-derived DC (52).

Monocytes also demonstrate other abnormalities in SLE patients, such as decreased phagocytic ability, decreased production of IL-1 and increased apoptosis (53-55), which could contribute to increased susceptibility to infections. The defects in uptake of foreign antigens and immune complexes are interesting in the context of autoimmune responses, as will be further discussed below.

Defects in tolerance

Antigen recognition by T-cells can result in either cell activation followed by differentiation or in tolerance. Tolerance is a term used to describe the ability of the immune system to accept and not react to self-antigens, and implies an active process, not a passive lack of responsiveness (56). It is normally induced both in the thymus and in the periphery. In SLE patients this capacity of discriminating self from non-self is altered towards responsiveness also to self (56). The reason for this defect is unknown, especially in human SLE, but in lupus prone mice defects in peripheral tolerance of T and B lymphocytes has been found, leading to increased autoantibody production (57). Although the presence of autoreactive T cells, with a high affinity for self-antigens, suggests that negative selection in the thymus could be defective, central tolerance has been reported to remain intact in murine lupus (58).

The autoreactive T and B cells that do not undergo anergy or apoptosis when reacting to self, could be activated due to the elevated concentrations of apoptotic cell material in a pro-inflammatory environment (59). The apoptotic antigens, such as RNP, Sm and SSA/SSB that have been detected in apoptotic blebs (24, 60), can be processed by APCs and presented to autoreactive T helper cells that in turn stimulate B cell autoantibody production (61).

Autoantigens and autoantibodies

More than 50 different autoantibodies have been identified in sera from SLE patients. At least 98% of SLE patients have antibodies binding to antigens located in the cell nucleus, antinuclear antibodies (ANA) (62). The ANA are usually detected by immunofluorescence microscopy (IF) of Hep-2 cells incubated with serum, and different staining patterns are obtained depending on the predominant specificity of the autoantibodies. Typical patterns of lupus patients are the homogenous pattern that indicate presence of anti-dsDNA antibodies, whereas the speckled pattern indicate presence of antibodies binding to non-histone structures, including RNP, Sm, SSA/Ro or SSB/La. In the rare cases of ANA-negative SLE patients, it is of diagnostic value to study the autoantibody pattern by IF or by sensitive ELISA techniques that could be positive despite negative ANA (63). IgG antibodies to dsDNA are typical for SLE and their presence correlates to several changes in the SLE pathology such as deposition of IC at the basement membranes in glomerulonephritis. Presence of anti-Sm Abs is a diagnostic sign of SLE (see table I), and is closely associated with presence of anti-RNP antibodies. Similarly, presence of anti-SSB/La antibodies is associated with presence of anti-SSA/Ro antibodies. The SSA antibodies occur most commonly in patients with primary Sjögren´s syndrome (SS). These autoantibodies are associated with several clinical manifestations such as hypergammaglobulinemia, purpura, leukopenia and lymphopenia, but also to congenital heart block and subacute cutaneous lupus (64).

Autoantibodies in SLE or other autoimmune diseases may form immune complexes, or can directly bind to tissues and cause inflammation. Certain autoantibodies may interfere with normal cellular functions and in this way cause specific clinical manifestations, such as the thrombosis caused by antiphospholipid antibodies (65).

Immune complexes and clearance

Immune complexes (IC) consist of an antigen bound to an antibody, or of larger complexes of several antibodies and antigens. The IC can exist in the circulation, but are rapidly removed by phagocytosis in healthy individuals. The clearance of IC is deficient in SLE patients and there are several explanations for this. A deficient mononuclear phagocyte system is one of the main factors contributing to this decreased clearance. The complement system and FcγR function have been pointed out as important factors for clearance of immune complexes (21, 66), which is discussed in more detail below. The pentraxins, especially C-reactive protein (CRP) and serum amyloid protein (SAP), are also involved in the opsonization and can thereby facilitate phagocytosis of IC, but also of apoptotic cells (67).

There are several ways by which IC contribute to the central autoimmune process of SLE. Recently, Rifkin et al (68) reported that certain immune complexes can efficiently activate autoreactive B-cells. These complexes are especially efficient when the autoantigens were SLE-associated, i.e. nuclear or cytoplasmic autoantigens (68). Furthermore, these complexes were shown to simultaneously activate both BCR and a member of the MyD88-dependent receptor Toll-like receptor (TLR) family, probably TLR9 (69). IC can also have many other effects mediated via activation of different Fc receptors (70), e.g. induction of the cytokine IL-10 (71). In addition, SLE patients have circulating IC that can induce IFN-α production, which will be further discussed in the section Results and Discussion.

The complement system

The complement system consists of a number of proteins with three main biologic activities; opsonization of for instance immune complexes or bacteria, lysis of target cells and chemotaxis of leukocytes (72). Solubilization and clearance of immune complexes and removal of apoptotic cells, as well as viral neutralization are important functions that are mediated by the early complement factors. The complement system is of importance in the pathogenesis of SLE, as demonstrated by the fact that genetic defects in early complement components considerably increase the risk of developing SLE (21).

During disease flares SLE patients can develop acquired hypo-complementemia (73). The reasons for this include consumption of complement components by binding to IC or directly to antigens. The early complement components are required for the uptake of apoptotic cells, and a lack of C1q can therefore lead to prolonged exposure of autoantigens to the immune system and potentially promote an autoimmune response (74).

Fcgamma Receptors (FcγR)

Receptors for IgG, termed FcγR due to their binding of the constant region of the IgG (Fc), are important for clearance of IC, but also in regulating immune responses. Their main functions include phagocytosis, antibody-dependent cell-mediated cytotoxicity, and their ability to mediate release of cytokines and proteases upon stimulation (75). The FcγR are divided into three classes, FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), all binding to IgG, but with different affinities; FcγRI binds with high affinity to monomeric IgG, whereas FcγRII and FcγRIII bind to IgG in the form of IC with low affinity (76). The FcγR are expressed on different immune cells such as DC, B cells, macrophages, Langerhans cells, neutrophils and eosinophils (76).

The FcγR possess intracellular motifs regulating the response of a cell, termed immunoreceptor tyrosine-based activation or inhibitory motif (ITAM and ITIM), respectively. Of the three FcγRII isoforms, i.e. FcγRII a, b and c, FcγRIIa and c contain ITAM, whereas FcγRIIb contain ITIM. The ITIM mediates downregulation of immune responses by aggregated antibodies or IC. The ITAM mediates activation of several processes in cells, including phagocytosis and production of cytokines (75). However, in some cases the ITAM-containing FcγRIIa can also induce negative signals when the extracellular domain is clustered (77, 78). The balance of these positive and negative signals is crucial for the final response mediated by an activated cell.

In SLE patients, functional polymorphisms of FcγRIIa and FcγRIIIa have been described to be related to pathologic changes. Certain genotypes of FcγRIIIa were found to be associated to disease manifestations, such as hematologic cytopenias and arthritis, whereas genotypes of FcγRIIa affected the immune complex clearance (66). Notably, in other studies, SLE patients with nephritis were found to be homozygous for the FcγRIIa alleles R131/R131 or heterozygous for R131/H131, whereas the H131/H131 genotype was associated with protection against nephritis (75). Similar results have been observed for patients with FcγRIIIa polymorphisms, and in both cases the homozygous, but not the heterozygous, individuals have a decreased clearance (75).

The decreased clearance due to FcγR polymorphisms also affects the uptake of immune complexes containing apoptotic cells, or clearance of apoptotic cells bound to pentraxins. The main receptor binding to CRP is FcγRIIa, indicating that also nucleosomes bound to CRP could be affected by polymorphisms of FcγRIIa (79). While pentraxins have been suggested to bind to FcγRs, existence of specific receptors for pentraxins cannot be excluded (67).

Apoptosis

Apoptosis (falling in Greek, as in falling leaves from a tree) is an active and controlled process of cell death, also referred to as programmed cell death. It is an evolutionary conserved process with condensation of the cytoplasm and the chromatin, cell membrane blebbing with expression of pro-phagocytic signals leading to rapid ingestion by phagocytes without inflammation (80, 81). When apoptotic material is ingested, the phagocytes produce cytokines such TGF-β that inhibit inflammation (82). Apoptotic signaling is mediated via a number of different receptors, including the well-known transmembrane protein termed Fas/APO-1/CD95, which belongs to the TNF

family of receptors. After Fas ligation, a proteolytic cascade leads to activation of a family of cystein proteases termed caspases. Apoptosis is regulated by a number of genes, and mutations in these genes can contribute to the development of different pathologic effects, including autoimmune, neurodegenerative and malignant diseases (83).

Apoptosis contrasts to necrosis, which typically is described as a degenerative process induced by cell damage and loss of cell membrane integrity. This leads to release of intracellular contents such as proteases and proinflammatory signals (81). Although apoptosis and necrosis principally are regarded as two different events, necrosis can follow apoptosis. This can appear when cells receiving apoptotic stimuli are short of energy, or are exposed to intense apoptotic/necrotic stimuli (84). Such secondary apoptosis also appear when apoptotic cells are not removed by phagocytosis (85). Necrosis and apoptosis can be induced by the same signals and are often found simultaneously in the same diseased tissue (84).

The removal of dying cells is suggested to be most relevant biological difference between apoptosis and necrosis. Apoptotic cells express surface components such as phosphatidylserine that is recognized by phagocytes, whereas necrotic cells do not (86). Instead, necrotic cells induce an inflammatory response and are removed only after lysis of the cell membrane (84). The products generated during necrosis are proinflammatory cytokines and an induction of differentiation of DC may occur (81, 87).

In certain situations, apoptotic cells can induce an autoimmune response, e.g. when the phagocytic system is overloaded and secondary necrosis is generated (81). Autoantigens from apoptotic or secondary necrotic cells can therefore potentially activate the immune system (24, 88). In addition, apoptotic cells can trigger an immune response in an IFN-α rich environment or in the presence of heat-shock proteins (87).

Apoptosis in lupus
The decreased clearance of apoptotic material in SLE patients is thought to be the actual reason for the observed increase in the number of cells undergoing cell death, and an impaired phagocytic uptake of apoptotic cells have been demonstrated in macrophages (89). However, an increased rate of apoptosis has also been reported in lymphocytes from SLE patients (90, 91). An increased number of apoptotic cells have also been suggested to break tolerance (92). The apoptotic blebs and bodies that appear when cells undergo apoptosis contain known autoantigens, such as SSA/SSB and U1-RNP (24). Consequently, autoantibodies from SLE patients can bind to antigens released during apoptosis (93).

In addition, SLE patients may have an increased expression of the proto-oncogene bcl-2 in lymphocytes that may protect these cells from apoptosis (94). This could explain the longevity of autoimmune T and B cells.

The importance of normal apoptosis is illustrated by the fact that individuals with mutation in the CD95/Fas gene and impaired apoptosis often develop lupus-like disease (also referred to as the Canale-Smith syndrome), although four ACR criteria for SLE are rarely fulfilled (22). Similarly, in the MRL-lpr/lpr murine model of lupus a mutation in the CD95/Fas molecule has been found (92).

Thus, numerous abnormalities concerning apoptosis are found in lupus patients that may contribute to the pathogenesis of SLE.

Cytokines

Cytokines are proteins or glycoproteins that are produced as a consequence of cell activation and they are of major importance for the development and regulation of the immune response. Thus, cytokines affect most cells in the immune system, and are involved in autoimmune reactions.

The paradigm of T helper (Th) 1/Th2 cells describe two important outcomes of the activation of Th0 cells by DC. When DC produce cytokines, such as Il-12 and IFN-α, Th1 cells develop (95). In the absence of these cytokines, Th2 cells will differentiate. Th1 cells are characterized by the production of a spectrum of cytokines, including IL-2, IL-3, IFN-γ, TNF-β. In contrast, Th2 cells are characterized by production of a different set of cytokines including IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. As a consequence of their cytokine profiles, Th1 cells promote macrophage functions, development of cytotoxic T cells and B cells production of opsonizing and complement binding antibodies, i.e. IgG1 and IgG3. Th2 cells promote B cell proliferation and differentiation to IgE, IgM and IgG4 producing cells (96). Under certain conditions regulatory T cells, develop, termed Tr or Th3, that may produce the inhibitory cytokines TGF-β and IL-10 (97).

Depending on which cytokines predominate in a disease, the diseases can be classified as Th1 or Th2 dependent. Most autoimmune diseases, such as rheumatoid arthritis, are mainly Th1 driven, whereas allergic reactions are derived from an imbalance towards Th2 cytokines. SLE has often been described as a Th2 driven disease, but this view seems to be obsolete because many novel studies indicate that also Th1 cytokines are crucial, especially in the beginning of the disease (98). Thus, a mixed Th1/Th2 balance is characteristic of SLE, where the Th1 cytokines are suggested to have an initiating role (99).

Cytokines in lupus

Numerous cytokines have been reported to be increased in serum of SLE patients. The first cytokine described to be elevated in SLE was IFN-α (100, 101), which is discussed in depth below. Other cytokines that are altered (mostly increased) include IL-2, IL-6, IL-10, IL-12, IL-15, IL-18, IFN-γ, TNF-α, GM-CSF and TGF-β (102, 103). The reasons for the altered levels of these cytokines in SLE patients are unclear, but several cytokines can contribute to the autoimmune response whereas others may act as negative feedback signals. Herein, only some especially relevant cytokines for this thesis will be discussed in more detail.

TNF-α can be increased in SLE sera and this cytokine display both activating and inhibitory functions in the immune system. For instance, TNF-α is known to cause maturation of DC (104), activate macrophages and endothelial cells, but can also act anti-inflammatory perhaps due to its pro-apoptotic effects (56).

Both IL-6 and IL-10 are elevated in SLE sera (105) and have the ability to promote antibody production. Especially IL-10 has been closely studied and serum levels of IL-10 have been found to correlate to SLEDAI, presence of anti-dsDNA antibodies as well as to low levels of C3 (103, 105). The role of IL-10 in the pathogenesis of SLE has mainly been attributed to the proliferative effect of IL-10 on B-cells, which are known to be hyperactive in SLE (see above). However, the effects of IL-10 are complex in SLE, because this cytokine deactivates DC and promote apoptosis in SLE lymphocytes and is a potent inhibitor of several cytokines such as IL-1, TNF-α and IL-6 (102). This is further demonstrated by the finding that lupus prone mice made deficient in IL-10 developed much more severe lupus than their IL-10 intact littermate controls (106).

IFN-α in lupus

The frequently elevated levels of IFN-α observed in sera of SLE patients are found to correlate to disease activity and severity (101, 107, 108). In addition, the IFN-α levels correlate to signs of immune activation measured as IC levels, complement consumption and levels of anti-dsDNA Abs (107, 108). The MxA protein that is induced by IFN-α is elevated in >90% of the SLE patients, indicating a general IFN-α activation in most SLE patients (109). These findings were confirmed in March 2003 when two different groups reported that SLE patients have increased levels of expression of IFN inducible genes, using the microarray technique (110, 111).

The increased levels of IFN-α in serum of SLE patients can be explained by the presence of endogenous IFN-α inducers. In fact, an IFN inducing factor (SLE-IIF) was frequently observed in sera of SLE patients and was associated to disease activity (51, 112). The SLE-IIF activated natural

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interferon producing cells/plasmacytoid dendritic cells (NIPC/PDC) exclusively and consisted of anti-DNA antibodies and DNA, possibly in complex (112, 113). This observation is one of the key findings that constitute the background to the present thesis.

A causative role for IFN-α in the pathogenesis of SLE is further supported by the finding that IFN-α therapy, given to patients without known autoimmune disorders, may develop ANA, anti-dsDNA antibodies, and occasionally also SLE (114-116). Interestingly, recent findings demonstrate that both NZB and lpr lupus prone mice lacking the IFN-α/β receptor (IFNAR) developed a marked amelioration of the disease (117, 118).

In conclusion, all these observations strongly suggest that the type I IFN system is essential in the pathogenesis of SLE (119, 120).

The interferon system

In 1957 Isaac and Lindenmann discovered interferon (IFN), the first cytokine to be described, and characterized it as a soluble factor interfering (hence the term interferon) with the effects of influenza A virus infection of chick chorio-allantoic membranes (121). Subsequent studies revealed that several IFNs exist. These IFNs, together with inducers of the IFN-production, the IFN producing cells as well as target cells for the IFN and their functions constituting the type I IFN system.

There are two types of IFN, type I include IFN-α, β, ω, τ, κ and ε, and type II, only IFN-γ. In humans, the type I IFN system consists of 13 IFN-α genes, one IFN-β gene and one IFN-ω gene, all without introns situated on chromosome 9, whereas IFN-γ is encoded by one gene with three introns on chromosome 12 (122).

The IFN-α, -β and -ω bind to the same receptor IFNAR, with its two subunits IFNAR-1 and IFNAR-2, whereas IFN-γ binds to a different receptor IFNGR. The signals produced by binding to the IFNAR are mediated by the JAK-STAT signaling pathway through phosphorylation and subsequent translocation of the STAT 1 and STAT 2 that together with IRF-9 constitute a complex (ISGF-3), which is subsequently translocated into the nucleus where it binds to the IFN-α/β inducible genes (122, 123). In humans (but not in mice), also STAT 4 is activated, resulting in differentiating of Th0 to Th1 cells. In this thesis solely the type I IFN system is studied and the Type II IFN will therefore not be further described here.

The natural interferon producing cells
The term natural interferon producing cells (NIPC) was first established by Rönnblom et al in 1983, demonstrating a very infrequent cell population

with a high IFN-α producing capacity (124, 125). Several cells can produce type I IFN, including monocytes, transformed B cells and fibroblasts, in response to certain viral inducers, but not in as high quantity per cell as the NIPC (126). Thus, although the NIPC are much less frequent than monocytes they produce much more IFN-α per cell (1-2 U/cell) than do the monocytes (0.1 U/cell) (127). In recent years, a tremendous progress in the characterization of the phenotype and function of the NIPC has been made.

In 1997, a cell population termed plasmacytoid moncytes was described that was localized to the T cell rich areas of lymphoid tissues (128). These cells undergo apoptosis in culture unless they are exposed to CD40L and IL-3 that cause these cells to mature into DC (128). Another term for these cells is the precursor of type 2 dendritic cells (pDC2), indicating its ability to modulate the immune system into a Th2 response (129). The term plasmacytoid dendritic cell (PDC) is now more often used for these cells, which have the phenotype as NIPC and was shown to have the same capability to produce high amounts of IFN-α in response to viral stimuli (130). The origin of NIPC/PDC remains controversial because both myeloid and lymphoid markers have been observed, and recently Comeau et al (131) hypothesized that they could convert from lymphoid to myeloid cell type. The NIPC/PDC are not only found in peripheral blood, but are also found in the bone marrow, lymph nodes, spleen, tonsillar tissue and in the thymus of healthy individuals (126).

The NIPC/PDC lack cell surface markers typical for T cells, B cells, monocytes or NK cells. They do not express CD11b or CD11c, markers of monocytes or mature DC, respectively, or the costimulatory molecules CD80 and CD86. They do express the markers CD45RA, CD45RB, CD45R0, CD83 and HLA-DR, CD4, CD36, CD44, CD72, and IL-3R (CD123) (126, 132).

Previously, it has been difficult to purify the NIPC/PDC, due to the lack of specific markers. However, Dzionek et al recently identified two cell surface molecules termed BDCA-2 and BDCA-4 that are selectively expressed on NIPC/PDC (133). Ligation of BDCA-2, a type II C-type lectin, reduces the IFN-α production markedly upon stimuli, indicating that this receptor could be of importance for the induction of IFN-α (134). This inhibiting action of BDCA-2 could switch the NIPC/PDC to produce IL-12, and thereby perhaps further promote a Th1 response (135).

Furthermore, the NIPC/PDC regulate the immune response by linking the innate response to the cell mediated adaptive immune response directly. When the NIPC/PDC have produced the IFN-α, which is an important part of the innate immune system, they loose this capacity and may begin to act as APC. In this more mature state they can induce Th1, Th2 or regulatory T cell responses, depending on the stimuli received (129, 136, 137). Although

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Th2 responses can be readily induced in vitro, Th1 responses occur when IFN-α and IL-12 are produced, for instance at viral infections (138).

Inducers of type I IFN

Many viruses, including UV-inactivated and therefore non-replicative viruses, as well as the synthetic dsRNA poly:IC can induce IFN-α production in NIPC/PDC. The IFNs are also important in the innate response to parasites and bacteria, and bacteria such as S aureus and Mycobacterium tuberculosis have been reported to induce of IFN-α production in NIPC/PDC (126, 132, 139). Furthermore, IFN-α can also be produced in response to bacterial DNA containing unmethylated CpG motifs and to the drug imiquimod that has antitumor and antiviral effects (126, 140, 141).

The requirements for the structure of the IFN-α inducers are not fully comprehended. Several studies concerning the nature of the interferogenic nucleic acids show that unmethylated DNA induces more IFN-α, that CpG motifs are required and that certain oligodeoxynucleotides (ODN) can induce high levels of IFN-α due to specific bases at the endings of the sequence, i.e. poly G tails (142). However, this may be true only for ODN with poly G tails that induce IFN-α production in the absence of lipofectin and for double stranded DNA. Single stranded DNA does not always require such unmethylated CpG motifs (143).

Glycoproteins have an important role in the IFN-α induction, probably in the interaction of the inducer and the cell surface receptors such as C-type lectins. This has been studied by mutated pathogens where glycoproteins altered at a single site loose their IFN-α inducing properties (144). Thus, the uptake of inducers seems to be important for induction, which is further demonstrated by the abolished or reduced IFN-α production caused by inhibitors of endocytosis (144). The uptake of the actual inducer can in some cases be facilitated by lipofectin or IgG antibodies (143, 145). Moreover, inhibitors of endosomal acidification, such as chloroquine, inhibit the IFN-α production (126) and is a common treatment of SLE.

Receptors involved in the IFN-α induction

Toll like receptors (TLR) recognize conserved pathogen-associated molecular patterns (PAMPs) that are crucial for the initiation of the innate immune system. The TLRs are transmembrane proteins expressed on APCs, and upon interaction with PAMPs, cytokines are produced. Among the TLR family members (TLR1-10), TLR9 and TLR7 are expressed on human PDC and are required for IFN-α production stimulated by oligodeoxynucleotide (ODN) rich in CpG-DNA or the synthetic compound imiquimod respectively (146, 147). The other important subset of DC, myeloid DC (MDC) express TLR7 and TLR4. When TLR7 is stimulated in MDC, they produce IL-12

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whereas PDC produce IFN-α (147). This indicates that stimulation of TLR7 promotes the development of a Th1 response.

In some cases, the induction of PDC requires further stimuli than that mediated through TLRs alone. The IFN-α and IL-12 production in PDC induced by CpG (through TLR9) was for instance synergistically increased by ligation of CD40 on NIPC/PDC by CD40L (146).

Other receptors on NIPC/PDC are members of the chemokine receptor family, including CXCR3, whose ligand IP-10 is upregulated in SLE patients and could promote migration of NIPC/PDC to peripheral tissues (136). Two molecules in the mannose receptor family are also upregulated on NIPC/PDC, the mannose receptor and the DC-SIGN, both C-type lectins (126).

Priming

An observation made concerning the induction of IFN-α production is that addition of IFN-α or -β before or at the same time as the addition of inducers can increase the production of IFN-α. The IFN-α production can be further increased also by other cytokines such as IFN-γ, IL-3 or GM-CSF or inhibited by IL-10 or IL-4 (148, 149). This stimulatory effect is known as priming (150). Priming is actually required for IFN-α responses in some situations (151). The exact mechanisms are not completely known, but one major reason is the induction of type I IFN transcription factors required for induction of IFN-α genes, such as IRF 7, whose expression is stimulated by type I IFN (152, 153).

The functions of the type I IFN system

One main function of IFNs is their inhibition of viral replication. This is effectuated by inhibition of viral translation through two pathways; the 2-5A pathway and the protein kinase pathway (122). A number of proteins with anti-viral effects are induced by IFN-α. These include the Mx proteins that interfere more specifically with replication of influenza virus (122).

The type I IFN also affects several cellular functions and can for instance induce apoptosis in virally infected cells, and inhibit cell proliferation (122, 154).

In addition to the role in the early defence against viral infections, the type I IFN has many immunomodulatory effects. These include enhancement of the NK cell and macrophage activity, increased expression of MHC class I molecules, and promotion of T cell and DC differentiation (155). IFN-α/β are also essential mediators of apoptosis, but require the mediators PKR and 2-5A-dependent RNAse L (122). A Th1 response is commonly seen in autoimmune disorders and this can be enhanced by IFN-α (156). The inhibitory effects of the type I IFN on Th2 clones also indirectly

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promote a Th1 response (157). Furthermore, it has recently been shown that type I IFN can keep activated T cells alive, and protect B cells from apoptosis (158, 159), which in certain situations could interfere with the deletion of autoreactive lymphocytes. IFN-α can also promote Ig class switching, leading to increased production of high affinity Abs (160).

IFN-α in autoimmunity

The actions of type I IFN described above could contribute to an autoimmune process in several ways. It is therefore not surprising that a variety of autoimmune diseases have been reported during IFN-α therapy (116, 161). Besides SLE, autoimmune diseases induced by IFN-α therapy include thyroiditis, Raynaud´s syndrome, autoimmune hemolytic anemia, rheumatoid arthritis, insulin dependent diabetes mellitus (IDDM), polymyositis and pernicious anemia (114, 116, 161, 162). Moreover, SLE, IDDM, psoriasis, Crohn´s disease, celiac disease and dermatomyositis have all been found to have increased expression of IFN-α (162). With the exception of SLE, IDDM is probably most thoroughly studied, and IFN-α has been detected both in the pancreas and sera of patients with IDDM (163, 164). Also, in psoriasis, both cells producing IFN-α and the IFN-α inducible MxA protein is found in skin (165) and in dermatomyositis IFN inducible genes were elevated (166).

Taken together, these results suggest an important role for the type I IFN system as a trigger of many autoimmune disorders, whereas the genetic predisposition of a person will determine which disease or syndrome that will result.

Sjögren´s syndrome

Sjögrens´s syndrome (SS) was described in 1933 by the Swedish ophtalmologist Henrik Sjögren as an inflammatory disease mainly affecting salivary and lacrimal glands, leading to dryness of the eyes and mouth. It mainly affects women (9:1) with prevalence figures up to approx 2-4%, depending on classification criteria used (167). The disease can occur solely and is then referred to as primary SS, but SS can also occur secondarily to other rheumatologic diseases such as SLE or RA. Herein, only primary SS will be dealt with. There are several sets of classification criteria for SS, of which the most commonly used are the European, the San Diego, and the Copenhagen criteria. The San Diego criteria (used in paper IV in this thesis), include the following four objective criteria; 1) Keraconjunctivitis sicca verified by Shirmer´s test and by rose bengal or fluorescein dye staining. 2) Xerostomia verified by clinical examination and decreased salivary flow rate

on testing 3) Minor salivary gland biopsy with lymphocyte infiltrates with ≥2 foci/4mm^2 4) Evidence of a systemic autoimmune process, manifested by presence of autoantibodies, i.e. positive rheumatoid factor, or positive ANA or positive SSA/SSB antibodies (168). Definitive SS fulfill four criteria and possible SS three criteria.

Although SS and SLE are two distinct diseases, many SS and SLE patients have several signs and symptoms in common. These include presence of ANA, antibodies to SSA and/or SSB, increased cytokine levels, and involvement of the joints, skin and lung tissue. Concerning the pathogenesis, both diseases are characterized by the production of autoantibodies and immune complex formation may occur. However, only SLE patients usually have high levels of circulating IC. In SS patients, B cells producing anti-SSA and anti-SSB antibodies have been detected in salivary glands, indicating autoantibodies present at the site of inflammation (169). These antibodies are directed to antigens in apoptotic blebs and apoptosis have been reported to take place in salivary glands in SS patients (24, 170). Therefore, an important part of the pathogenesis in SS could include inflammation caused by immune complexes formed in tissues by apoptotic cell antigens and autoantibodies. This inflammation could cause tissue damage and dysfunction of the glands due to a number of factors including antibodies to muscarin receptors and produced cytokines (171). However, the role of type I IFN system in SS has not been investigated, but an intriguing observation is that SS has been reported to develop during IFN-α treatments (172).

PRESENT INVESTIGATIONS

AIMS

General aim

The general aim of the studies was to investigate the mechanisms leading to the increased IFN-α production observed in SLE patients. The background for this, is that the SLE-IIF previously described consists of anti-DNA antibodies (Abs) and DNA (112, 113), which could be generated from apoptotic cells that are increased in SLE patients (90). To assess this we used an experimental model depicted in Figure 1.

Specific aims

Paper I

- To investigate whether apoptotic cells in the presence of autoantibodies from SLE patients (SLE-IgG) can act as an IFN-α inducer in normal PBMC.
- To clarify whether anti-DNA Abs, or Abs of other specificities are involved in the IFN-α production triggered by apoptotic cells.

Paper II

- To characterize the IFN-α producing cell when PBMC are stimulated with apoptotic U937 cells and SLE-IgG.
- To test the effects of several cytokines, suggested to be important in the SLE disease process, on the IFN-α production in this in vitro model.

Paper III

- To identify the role of the FcγR in the IFN-α production induced by apoptotic cells and SLE-IgG.
- To characterize the presence of FcγRII on the NIPC/PDC.

Paper IV

- To investigate whether the autoantibodies present in SS could induce IFN-α in the presence of apoptotic or necrotic cell material.
- To study the relationship of the specificities of the autoantibodies of SS, SLE and RA patients to the IFN-α inducing ability.
- To evaluate the association of IFN-α inducing ability in sera from SS patients and the presence of clinical manifestations.

1. Apoptosis is induced in e.g. U937 cells by UV-irradiation.

UV

4 hours

U937 cells

Apoptotic U937 cells
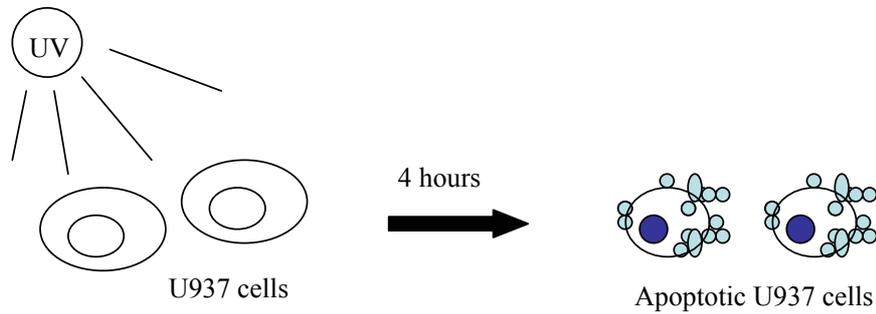
2. After culture for 4 hours, IgG from SLE patients is added to the apoptotic cells.

SLE -IgG   +   Apoptotic U937 cells

Combination of
apoptotic U937 cells
And SLE-IgG
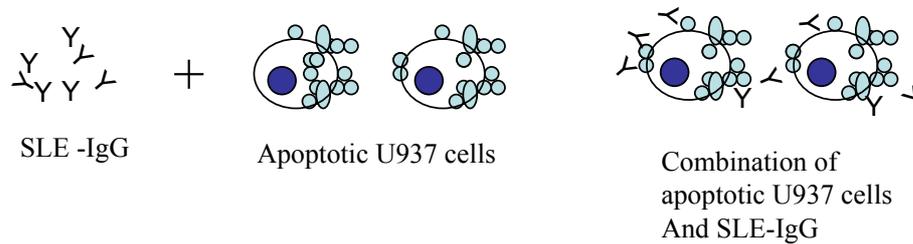
3. Subsequently, IFN-α2b-stimulated peripheral mononuclear cells (PBMC) from
a healthy blood donor are added and cultured for 24 h.

+

Combination of
apoptotic U937 cells
and SLE-IgG
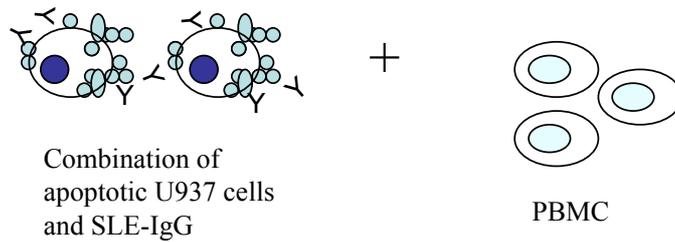
PBMC

4. Finally, the IFN-α produced by PBMC is quantified using an immunoassay.

24 hours

PBMC cultured with the combination of
SLE-IgG and apoptotic U937 cells
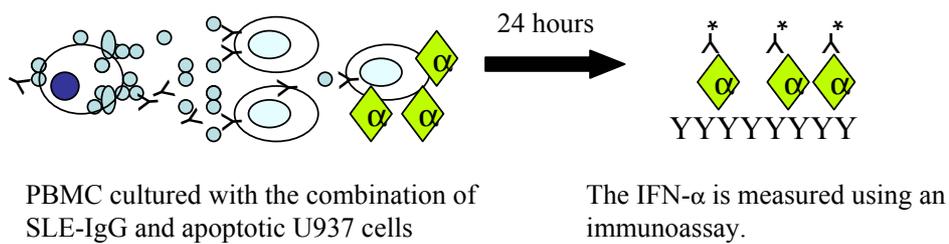
The IFN-α is measured using an
immunoassay.

Figure 1. Experimental in vitro model of IFN-α production by peripheral blood
mononuclear cells (PBMC) induced by apoptotic U937 cells in combination with IgG
from SLE patients.

RESULTS AND DISCUSSION

The combination of lupus IgG and apoptotic cells is a potent IFN-α inducer (Paper I).

The background to this study was the findings that SLE patients have a circulating IFN inducer (SLE-IIF) consisting of DNA and anti-DNA Abs. Because SLE patients have an increased number of apoptotic cells and a decreased clearance of such material, and because apoptotic blebs can contain autoantigens, we hypothesized that the DNA in the SLE-IIF could originate from apoptotic cells (89, 90).

To examine this we constructed an in vitro model using the human monocytic cell line U937 in which apoptosis was induced, and sera or IgG from SLE patients and cultured these components with IFN-α2b costimulated PBMC from a normal blood donor (Figure 1). Initially, we added apoptotic U937 cells to the induction cultures with SLE sera, which increased the levels of IFN-α produced (Table I, paper I). We then asked whether the component in sera required for IFN-α production could be IgG, since previous studies had shown that IgG was required for sera to induce IFN-α production (112). Therefore, we first treated sera with DNAse followed by purification on a protein G column. The DNAse treatment destroyed the endogenous DNA, to avoid IFN-α production due to SLE-IIF. The eluate containing IgG was essential to the IFN-α production induced in combination with apoptotic U937 cells, whereas the effluent free from IgG was unable to induce IFN-α in this model.

To examine the different effects of inducers of apoptosis, etoposide and anti-Fas Abs were used in addition to UV irradiation. All three preparations of apoptotic U937 cells together with SLE-IgG, but not normal IgG, could induce IFN-α in PBMC (Table II, paper I). We further examined whether other cell lines (MonoMac 6, H9, Jurkat and U266), when made apoptotic by UV irradiation and cultured together with SLE-IgG, could induce IFN-α production in PBMC. All cell lines treated with UV irradiation induced IFN-production when combined with SLE-IgG (Table III, paper I).

To verify the importance of apoptosis in the IFN-α induction, an inhibition of apoptosis by the irreversible caspase inhibitor zVADfmk was performed. The rate of apoptosis in the U937 cells decreased in cultures to which zVADfmk was added, resulting in diminished IFN-α production (Figures 2 and 3, paper I).

These results show that apoptotic material, given certain conditions such as presence of autoantibodies, may trigger the production of IFN-α Interestingly, apoptotic cells without autoantibodies induce production of anti-inflammatory cytokines such as IL-10 and TGF-β (173, 174).

Since the SLE-IIF consists of DNA and anti-dsDNA Abs (113) we hypothesized that the specificity of the Abs required for the IFN-α production induced by apoptotic cells could be directed to dsDNA. Therefore, IgG from two groups of patients, one with and one without Abs against dsDNA, was used together with apoptotic U937 cells in PBMC cultures. Together with UV-irradiated U937 cells, all SLE-IgG containing anti-dsDNA Abs, but also the IgG from a patient without anti-dsDNA Abs induced high levels of IFN-α (Table IV, paper I). An association was also noted between IFN-α inducing ability and disease activity, but this association was not confirmed using SLE sera.

Purification of SLE-IgG using dsDNA and ssDNA columns alone or in sequence confirmed that anti-dsDNA Abs were not a prerequisite to IFN-α induction by apoptotic cells. In an attempt to further elucidate the specificity of the Abs involved, sera from 22 SLE patients were examined with regard to IFN-α inducing ability alone, or in combination with apoptotic U937 cells. This was related to serum content of different autoantibodies. This analysis showed that the Abs required for apoptotic cells to induce IFN-α may be of anti-RNP specificity and is therefore different from that of SLE-IIF.

In conclusion, it was shown that apoptotic cells have the ability to promote a pro-inflammatory response under certain conditions, i.e. in presence of certain antibodies. The anti-DNA antibodies were not an essential part of the combination of SLE-IgG and apoptotic cells for the IFN-α production, instead anti-RNP antibodies were of importance for IFN-α production in this experimental model. This indicates that the IFN-α inducing component of the U937 cells could consist of RNA/protein complexes, released from apoptotic cells (24). This is interesting in light of the fact that viruses are the classical IFN-α inducers and dsRNA is considered to be responsible for their induction. The origin of the DNA in SLE-IIF could not be defined in this study. Possibly, the DNA could still be hidden within apoptotic cells, and not exposed to the IgG used. A support for this is new findings from our laboratory showing that the IFN-α production induced by SLE-IgG and material released from apoptotic cells is abolished with RNAse and decreased with DNAse treatment (Lövgren, to be

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published). Consequently, these results suggest that more than one IFN-α inducer could be active in SLE patients, perhaps operating in different tissue compartments.

Thus, all components present in SLE patients, i.e. IFN-α production, presence of autoantibodies and increased levels of apoptosis are found in this in vitro model. Therefore it is possible that this model reflects the in vivo situation in SLE patients. In papers II and III, the cellular mechanisms for the IFN-α induction were further studied using this experimental model.

The natural IFN-α producing cells are activated by apoptotic cells in combination with SLE-IgG and regulated by cytokines (Paper II).

In order to characterize the IPC in the experimental model described in paper I, PBMC induced by SLE-IgG and apoptotic U937 cells were stained for intracellular IFN-α production and analyzed by flow cytometry (FCM). A brightly fluorescent cell population stained for intracellular IFN-α was detected, located between monocytes and lymphocytes (Figure 1, paper II). This was a very infrequent cell population, 2.3 ± 0.2 cells/10^4 PBMC, similar to the frequency induced by HSV, 7.5 ± 0.7 cells/10^4 PBMC. The phenotype of these IPC was identical to that induced by HSV, expressed high levels of IL-3R, HLA-DR, CD36, CD45RA and CD83 (>80%), were clearly positive for CD40 and CD68 (>50%), and had lower levels of CD4 (44%). The cells lacked typical markers for monocytes (CD14), T-cells (CD3) and B-cells (CD19), as well as CD11b, CD11c, CD32, CD86 and IL-10R (Table I, paper II).

The phenotype and frequency of these cells are identical to the previously described NIPC (132) with the same phenotype as immature dendritic cells, more specifically that of the PDC (128, 175). These cells are typically found close to the high endothelial venules of lymphoid organs, where they could be rescued from apoptosis by signals from T cells, indicating important immunological functions (129). The discrepancy between the numbers of NIPC induced by SLE-IgG and apoptotic cells and those induced by HSV could be due to potency of the stimuli, or may be due to activation of different subtypes among the NIPC. More recent studies have shown that when defining NIPC by presence of the antigens BDCA-2 or BDCA-4, it is clear that not the whole population positive for these antigens produce IFN-α (176). This could be due to differences in the properties of the inducer, i.e. induction of IFN-α by SLE-IIF, but not HSV, is dependent on FcγRII (145), paper III).

Since IFN-α production can be enhanced or inhibited by different cytokines, we asked how some cytokines known to be produced in SLE, that is IL-6, IL-10, IFN-γ, IFN-α, TNF-α and TGF-β (103), affected the IFN-α production. Furthermore, GM-CSF and IFN-β were used because they increase the IFN-α production in PBMC induced by HSV (113, 148).

In this study, the type I IFNs, IFN-α or -β increased the IFN-α production severalfold, as also demonstrated in paper I. Both IL-10 and TNF-α had inhibitory effects in a dose dependent way (Figure 3, paper II), of which IL-10 completely inhibited the IFN-α production. In contrast, the cytokines IFN-γ, IL-6, TGF-β and GM-CSF had no clear effects (Figure 2, paper II).

It has previously been reported that apoptotic cells can produce IL-10 (174, 177) and induce IL-10 production in PBMC (173). In addition, immune complexes may induce IL-10 production (71, 178). Furthermore, IL-10 has been reported to inhibit the IFN-α production induced by HSV (149). Therefore we hypothesized that IL-10 could be produced, and possibly decrease the IFN-α production, in this experimental model. However, no IL-10 (<4 pg/ml) was detected in the supernatants of PBMC stimulated by UV-irradiated U937 cells and SLE-IgG. Addition of anti-IL-10 Abs did not elevate the IFN-α production, but inhibited the effect of exogenously added IL-10 (Figure 4, paper II).

Patients with SLE have an increased production of IL-10 (179) and a positive correlation between IL-10 and IFN-α has also been reported in such patients (108). We have previously suggested that the increased IL-10 production in SLE patients could be due to a negative feedback mechanism, inhibiting the IFN-α production (113). The hypothesis is in accordance with the findings in this study, showing a strong inhibition of the IFN-α production by IL-10. Further support for this is that PDC have been shown to induce Th2 subsets of cytokines, including IL-10 which kill PDC (129).

In summary, apoptotic U937 cells and SLE-IgG induce IFN-α production in NIPC/PDC. This IFN-α production was markedly enhanced by type I IFN. This priming effect could be important in both the onset and relapses of the disease. The IFN-α production caused, for example, by viral infections may prime natural IPC to respond to complexes of IgG and material from apoptotic cells and initiate a sustained synthesis of IFN-α, which could promote autoimmunity.

The FcγRIIa is expressed on NIPC/PDC and is crucial in the IFN-α production induced by apoptotic cells and lupus IgG (Paper III).

The aim of this study was to examine the role of the Fc portion of the SLE-IgG and different FcRs on the IFN-α production induced by apoptotic U937 cells and SLE-IgG. Initially, we cleaved SLE-IgG to Fab and F(ab′)$_2$ fragments, and demonstrated that only intact SLE-IgG was able to stimulate production of IFN-α. This finding suggests that the Fc part of the antibodies may be necessary for the induction of IFN-α in presence of apoptotic U937 cells (Figure 1, paper III).

The fact that the Fc part of the SLE-IgG was essential, indicated that FcγR was expressed on NIPC/PDC and was important for IFN-α production in this experimental system. To evaluate this, normal IgG and heat-aggregated IgG, known to block FcγR, were added to cultures with apoptotic material and SLE-IgG. Indeed, normal IgG as well as IgG aggregated by heat treatment (aggregated IgG) could inhibit the IFN-α production in a dose-dependent manner, but a 100-fold stronger inhibition was observed with aggregated IgG (Figure 2, paper III).

To study the inhibition more specifically, different antibodies to FcγR were used. The IFN-α response induced by apoptotic cells and SLE-IgG was inhibited by antibodies to FcγRII, but not to FcγRI or FcγRIII. Both the anti-FcγRII Ab IV.3, directed to the activatory forms FcγRIIa and c, and the anti-FcγRII Ab AT10 directed to all three forms, including the inhibitory FcγRIIb, inhibited the IFN-α production induced by apoptotic cells and SLE-IgG. In contrast, the effect of anti-FcγRII antibodies on the IFN-α production induced by HSV differed. Thus, when the antibody IV.3 was added to HSV-induced PBMC, the IFN-α production was not altered, but presence of AT10 inhibited the IFN-α production in a dose dependent manner (Figure 3, paper III). One explanation to these observations is that the inhibitory FcγRIIb mediated this inhibition. We therefore continued the investigation by further determining the presence of this receptor on NIPC by two different methods depicted below. This was also motivated by the fact that previous studies actually failed to detect expression of FcγRII on NIPC/PDC (132, 133).

Initially we examined whether FcγRII did exist on NIPC/PDC by flow cytometry. PBMC were stained with antibodies to the NIPC/PDC marker BDCA-2 and with an antibody detecting all three forms of FcγRII (FLI8.26). We also stained PBMC induced by HSV for FcγRII and intracellular IFN-α. By both stainings we demonstrated a population of the NIPC/PDC that clearly expresses FcγRII on their surface, although at low levels (Figure 4, paper III).

Because of the different actions of IV.3 and AT10 on the IFN-α induction by HSV, we decided to further determine the expression of each of the three forms of FcγRII (a,b,c) on NIPC/PDC. First, a purification of the NIPC/PDC was carried out by magnetic BDCA-4 cell sorting and subsequently the NIPC/PDC were sorted by FACS using BDCA-2 mAb labeling. Second, a PCR using primers to detect each of the three forms of FcγRII (a, b and c) was performed on the purified NIPC/PDC. The PCR revealed that only mRNA for FcγRIIa was expressed by NIPC/PDC (Figure 5, paper III). This was unexpected because ligation of FcgRIIa by the AT10 then appears to result in a general inhibitory effect on inducers such as HSV, that do not use FcR.

Conceivably, as some reports indicate, the FcγRIIa could act inhibitory. This inhibition may act through the regulator Src homology 2 domain-containing inositol 5-phosphatase (SHIP), which when activated by phosphorylation downregulates the activatory functions of the FcγRII (77, 78). This occurs as a consequence of clustering of the FcγRIIa on the surface, and results in down-regulation of the transcription factor NF-κB, which induces production inflammatory cytokines (77). The suggestion that the SHIP acts as a regulator to prevent hyperinflammation could be in accordance with the findings in this study.

Furthermore, the results in this study indicate an essential role for the FcγRIIa in the stimulation of NIPC/PDC by of interferogenic IC containing nucleic acid released from apoptotic cells. The FcγRIIa may act in concert with TLR, receptors of importance for the IFN-α induction of NIPC/PDC induced by CpG-DNA (TLR9) or by imiquimod (TLR7). Further investigations are necessary to clarify this issue.

Sera from primary Sjögren´s patients induce IFN-α when cultured with apoptotic or necrotic cell material (Paper IV).

The results in paper I indicated that antibodies to RNP could be important for the IFN-α production induced by apoptotic cells and SLE-IgG, and recent findings in our laboratory show that the production of IFN-α induced by apoptotic cells and SLE-IgG is almost abolished when the apoptotic material is treated with RNAse (180, paper I), Lövgren et al, submitted). Because patients with SS often have antibodies to the RNA-binding SSA or SSB antigens, we found it important to investigate whether sera from SS patients also could induce IFN-α production by PBMC, when cultured together with apoptotic or necrotic U937 cell material. We found that the SS sera were at

least as effecient as SLE sera in inducing IFN-α production in PBMC. In contrast, RA sera and sera from normal subjects did not induce any IFN-α production, except one RA serum with SSA antibodies (Figure II, paper IV). The IFN-α production was usually higher when induction was made with necrotic material, and titrations of sera were characterized by steep slopes in the dose-response curves (Figure 1, paper IV). Furthermore, purified IgG from an SS serum in combination with material released by apoptotic or necrotic cells also induced IFN-α production, albeit at lower levels than those produced by serum (Figure 2, paper IV).

In contrast to the strong inducing ability of low serum concentrations, the levels of IFN-α detected in serum of SS patients were remarkably low or absent (Figure 5, paper IV).

These results suggest that dead cell material also in SS could be pathogenic in the presence of certain autoantibodies and in an environment of IFN-α. This is interesting in the light of the findings that increased apoptotic material is found in epithelial cells in the salivary gland of SS patients, whereas the infiltrating cells demonstrate a lower degree of apoptosis, thus sustaining an autoimmune process (170).

To analyze the content of the SS sera responsible for the IFN-α production, an association study of IFN-α inducing capacity and presence of autoantibodies with different specificities was performed. This investigation showed that occurrence of SSA autoantibodies in sera was strongly associated to IFN-α inducing ability, both for SS and SLE sera (Table I, paper IV). A weaker association was noted also for anti-SSB Abs, which was significant when necrotic material and SS sera were used. Trends could be seen for occurrence of anti-RNP antibodies in SLE sera and IFN-α inducing ability with apoptotic material. Unfortunately, few sera were positive for antibodies to RNP and Sm, which made it difficult to evaluate the importance of these Abs. Therefore we grouped the sera containing Abs with specificities to RNA-binding proteins, i.e. SSA, SSB, RNP and/or Sm and compared these sera with sera without such Abs. Here, the association was clear between the presence of any of these antibodies and IFN-α inducing ability (Figure 4, Paper IV). Taken together, these results strengthen the hypothesis that the actual IFN-α inducing material in these complexes is RNA.

It is known that presence of SSA and/or SSB antibodies are associated to clinical features such as photosensitive skin rash, interstitial pneumonitis, and neonatal lupus syndrome (17). We therefore wanted to investigate the possibility that some clinical manifestations could be related to the IFN-α inducing ability of sera. Because there is no disease activity index for SS patients, the extraglandular manifestations were chosen as a marker for more severe disease. We noted associations between IFN-α production induced by

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the combination of apoptotic cell material and dermatologic, pulmonary and hematologic involvement (Table II, paper IV). Interestingly, no association of clinical manifestations and IFN-α inducing ability with necrotic cell material was found. Although the number of patients in each group of clinical manifestations was low, making comparisons difficult, the more frequent association to induction with antibodies combined with apoptotic cells could be due to the possibility that apoptosis could be a more frequent event in vivo than necrosis.

Furthermore, the strongest association was noted between IFN-α inducing capacity of sera and plasma IgG levels. This could be due to a generally increased production of IgG, or presence of IFN-α inducing complexes activating B-cells, that has been demonstrated by Leadbetter et al (69).

In conclusion, these results demonstrate a novel view of the pathogenesis of SS, where dead cell material can, in presence of antibodies directed to RNA-binding proteins, induce high levels of IFN-α, which has strong immunomodulatory effects and could drive the immune response towards a pro-inflammatory state. It is therefore important to further investigate the activity of the type I IFN system in SS patients.

Conclusions

Paper I:
- Apoptotic cells in combination with SLE-IgG were able to induce IFN-α production in normal PBMC.
- The ability of SLE sera to induce IFN-α production together with apoptotic cells was associated with occurrence of anti-RNP Abs, but not with that of anti-DNA Abs.

Paper II:
- The IFN-α producing cell stimulated by apoptotic cells and IgG from SLE patients had the phenotype of NIPC/PDC.
- The type I IFN, IFN-α and IFN-β had stimulatory effects, while IL-10, and to a lesser extent TNF-α, had inhibitory effects on the IFN-α production by PBMC induced by apoptotic U937 cells and SLE-IgG.

Paper III:
- The IFN-α production induced by apoptotic U937 cells and SLE-IgG requires FcγRII.
- The FcγRII is expressed on the surface of NIPC, and the form FcγRIIa, but not b or c, was detected by PCR in the population of highly purified NIPC.

Paper IV:
- Sera from SS patients in combination with apoptotic or necrotic cell material can induce high levels of IFN-α by PBMC.
- The IFN-α inducing ability of sera was strongly associated with presence of autoantibodies binding to RNA-binding proteins in sera of both SS and SLE patients
- Clinical manifestations in SS patients i.e. dermatologic, pulmonary and hematologic involvement were associated with IFN-α inducing ability when induction was made with apoptotic cells combined with patients sera.

GENERAL DISCUSSION

SLE patients have an activated type I IFN system and serum IFN-α levels correlate to disease activity and severity (101, 108). Several important observations in recent years corroborate the pivotal role of this system in the etiopathogenesis of SLE. Among these are the finding of SLE-IIF, the observation that such IC activate NIPC/PDC and the observation of activated NIPC/PDC in SLE tissues (181, 182). Consequently, a possible explanation for the ongoing IFN-α production in SLE patients has been found.

In this thesis the mechanisms of the IFN-α induction in SLE was studied in more detail. One key finding was that apoptotic and necrotic cell material can induce IFN-α production in the presence of autoantibodies. This may be highly relevant, considering the fact that SLE patients have reduced clearance of apoptotic cell material. Consequently, endogenous interferogenic inducers, generated from dying cells, are available in large amounts in vivo. The results may also explain disease flares in SLE patients caused by sun exposure, because UV-light induces apoptosis in keratinocytes and thereby interferogenic cell material. Patients with SS clearly have apoptosis in the epithelial cells of salivary glands (170) and local production of autoantibodies (169). Therefore, all components necessary for formation of IFN-α inducing IC are present also in this disease, as suggested by our results. However, the role of the type I IFN system in SS is largely unknown, but there are a few case reports describing that the disease can be initiated by IFN-α treatment (172). This indicates that the described mechanisms for IFN-α induction in SLE can be operative also in other autoimmune diseases.

The view that NIPC/PDC are central in the pathogenesis of SLE was strengthened by the finding that only these cells were stimulated by the combination of SLE autoantibodies and apoptotic cells. The IFN-α production could be markedly enhanced by IFN-α or IFN-β, in many cases from none to considerable amounts of IFN-α. In vivo, IFN-α production caused by viral infections could therefore prime the immune system to be further activated by endogenous IFN-α inducers, i.e. IC consisting of autoantibodies and nucleic acids. Case reports have also described a connection between infections and SLE onset or disease flares (29, 30). In contrast, IL-10 and to some extent also TNF-α down-regulated the IFN-α

response. This is interesting because both these cytokines could be elevated in SLE patients and have been suggested to be involved in the pathogenesis of SLE (102, 103). The inhibitory effects of IL-10 on the IFN-α production could represent a negative feedback signal in SLE. This conjecture is supported by the finding that IL-10 gene deficient lupus prone mice develop a more severe disease (106). However, the exact role of IL-10 in human SLE is at the moment unclear (102). TNF-α may have several effects in SLE, one being protective due to the decreased IFN-α production (paper II) and another being deleterious and linked to increased atherosclerosis. The latter may be caused by the proinflammatory actions of TNF-α, whereas the suppression of the IFN-α may downregulate the autoimmune process. The downregulating actions are compatible with the observations that RA patients receiving anti-TNF-α treatment may develop SLE and that TNF-α administration ameliorates nephritis in lupus prone mice (183, 184).

The NIPC/PDC were found to express the receptor FcγRIIa, mediating both positive and negative signals in the IFN-α induction, depending on the nature of the IC. The FcγRIIa was required for the IFN-α production induced by the combination of apoptotic cells and SLE-IgG. Interestingly, elevated levels of FcγRIIa mRNA expression have been detected in SLE patients (110), possibly indicating increased levels of FcγRIIa on the surface of NIPC/PDC that can interact with interferogenic IC. Furthermore, a genetic association exists between certain alleles of FcγRIIa and SLE (20, 66). Thus, the expression and function of this receptor appears important in the pathogenesis of SLE, and this may at least partially be linked to the role in the IFN-α production.

Obviously, there are many parts of the autoimmune process in SLE that can be caused or facilitated by type I IFN. In Figure 2, a hypothesis is outlined to explain the role of IFN-α in the etiopathogenesis of SLE. Apoptotic and/or necrotic cell material can be generated as a direct effect of e.g. sun exposure, as described above. During infections, pathogens can induce apoptosis per se, and the IFN-α, can also be pro-apoptotic (122, 185). Certain viruses, e.g. influenza virus, can trigger the production of autoantibodies with specificities for nuclear antigens (32, 186). This will lead to the formation of IC with IFN-α inducing properties. The produced IFN-α causes monocytes and NIPC/PDC to mature to professional APC. These cells can then present the autoantigens to autoreactive T cells (also activated by IFN-α). The latter cells subsequently activate B cells to produce autoantibodies that will form more interferogenic IC. Such IC may also directly activate B cells (69) Thus, once tolerance to the autoantigens is broken, more endogenous complexes of nucleic acids and autoantibodies can
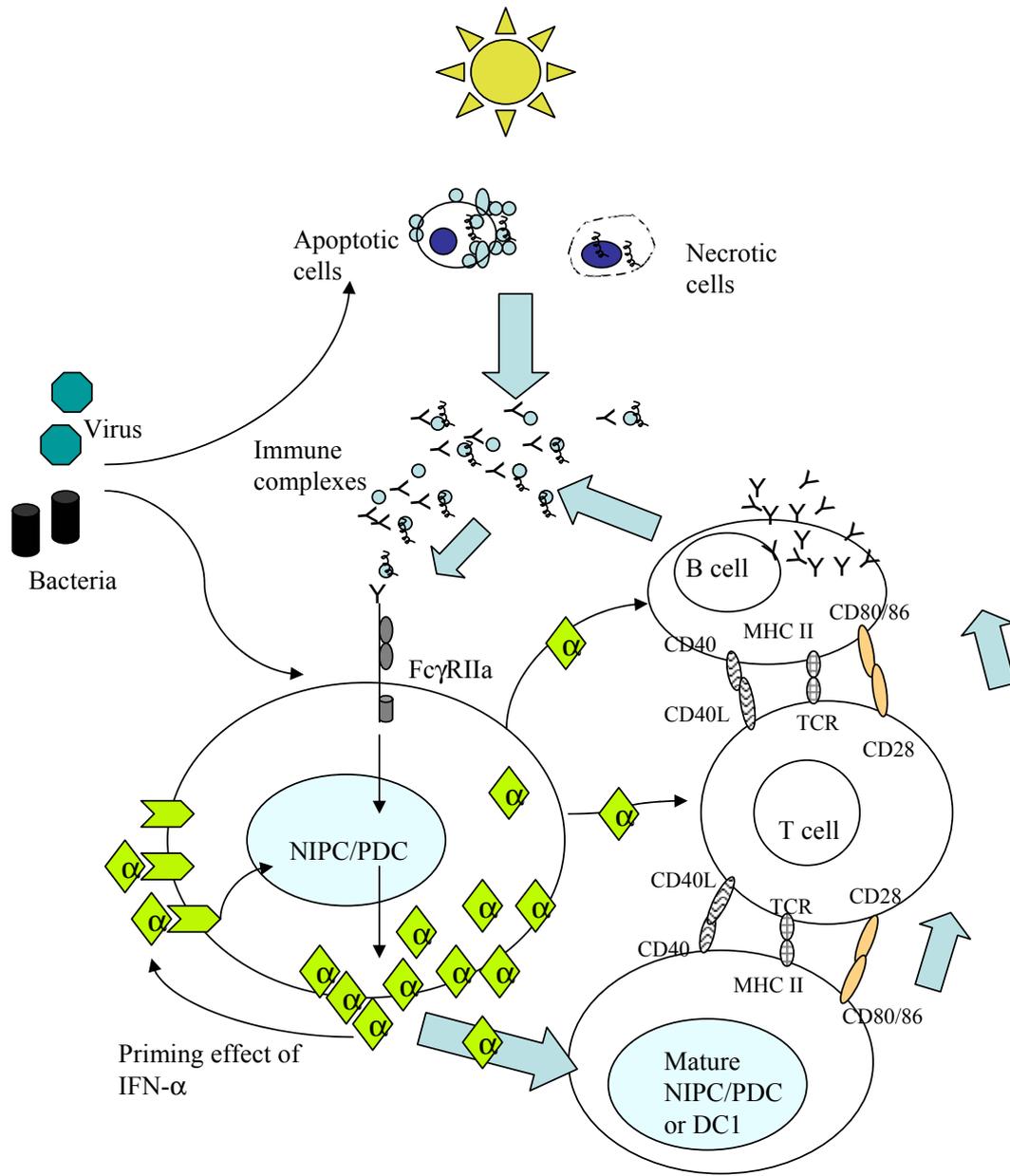
Figure 2. The hypothesis for the role of NIPC/PDC in the etiopathogenesis of SLE.

be formed and continue to drive the autoimmune response by a process resembling a vicious circle (119, 120).

The results in the present thesis, as well as other studies, show that the type I IFN system could be important in several other autoimmune disorders beside SLE (116, 161, 162). The reason for this is that the type I IFN system bridges the innate and adaptive immune system and is probably pivotal in breaking tolerance. The genetic predisposition in an individual will determine which specific autoimmune disease that will appear during sustained IFN-α production. In addition, environmental factors will influence the final disease outcome. However, more research is required to define the role of the type I IFN system in each autoimmune disease. One objective is of course to obtain specific therapies targeting this system. One therapeutic target could be the FcγRIIa, because specific blocking of this receptor inhibits the IC induced IFN-α production. An advantage with this therapeutic strategy would be that the virally induced IFN-α production is maintained. The described hypothesis in Figure 2 suggests several other approaches to modulate the activated type I IFN system in SLE. Whether inhibition of this system actually is of therapeutic value remains to be determined in future clinical trials.

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