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Identity and Activation of the Natural Interferon-α Producing Cells

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SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

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Abstract

This thesis focuses on the identity and activation of the human natural interferon-α (IFN-α) producing cells (NIPC), infrequent but highly efficient producers of IFN-α/β among peripheral blood mononuclear cells (PBMC) upon exposure to most types of virus. The capacity of NIPC to become activated by bacteria and immuno-stimulatory DNA (isDNA) is studied, as is the involvement of NIPC and IFN-α in the autoimmune disease systemic lupus erythematosus (SLE). It was found that also the bacteria *E. coli* and *Staphylococcus aureus* Cowan I (SAC) induced IFN-α production in PBMC, and that at least SAC activated NIPC. This effect of SAC appeared to require bacterial surface proteins, such as protein A, on intact bacteria. The SAC also inhibited the IFN-α production induced by herpes simplex virus (HSV) in NIPC. The phenotype of HSV-induced NIPC determined by flow cytometry (FCM) resembled that of immature dendritic cells (DC), as did the morphology and antigen presenting ability of NIPC purified by fluorescence-activated cell sorting. The number of functional NIPC was greatly reduced at the blood level in SLE patients. The NIPC was partially reconstituted by exposure *in vitro* to the costimulatory cytokines IFN-α2b, IFN-γ and GM-CSF. An ongoing production of IFN-α is commonly found in SLE patients. Interestingly, several SLE sera were found to induce IFN-α production in normal PBMC *in vitro*, indicating presence of a circulating IFN-α inducing factor (IIF) in the disease. The low levels of NIPC in SLE could therefore be due to their depletion by activation, as well as deficient costimulatory cytokines. The IIF was frequently found in SLE serum, especially in serum from patients with active disease and measurable serum IFN-α levels. The activity of IIF on normal PBMC was markedly increased by IFN-α2b and GM-CSF. The SLE-IIF was shown by means of FCM to specifically trigger NIPC. The SLE-IIF had a molecular weight of 300-1000 kD and appeared to consist of immunoglobulin G (IgG) and DNA, possibly as small immune complexes. Further analysis of SLE-IIF revealed that the essential IgG component was anti-dsDNA antibodies, and that the DNA could well be isDNA with unmethylated CpG motifs. The SLE-IIF may be a pathogenic factor in SLE by causing production of IFN-α, which then promotes development of autoimmunity. The combination of anti-DNA antibodies and the isDNA containing plasmid pcDNA3 was shown to mimic the SLE-IIF with respect to specificity for NIPC. They were also both influenced in a similar manner by cytokines, being stimulated by IFN-α, IFN-β and GM-CSF, but strongly inhibited by IL-10. The results of the present thesis have further clarified the composition and function of the normal IFN-α/β system and its role in the development of SLE.

Keywords: type I interferon, dendritic cells, systemic lupus erythematosus, interferon inducer, immunostimulatory DNA, autoimmunity, herpes simplex virus, Sendai virus.

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To the memory of Karl Svensson

Abstract

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Contents

Appendix

Papers I-V

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. **Svensson, H.**, Cederblad, B., Lindahl, M. and Alm, G.V. Stimulation of natural interferon-α/β-producing cells by *Staphylococcus aureus*. *J. Interferon Cytokine Res. 16*: 7-16, 1996.

II. **Svensson, H.**, Johannisson, A., Nikkilä, T., Alm, G.V. and Cederblad B. The cell surface phenotype of human natural interferon-α producing cells as determined by flow cytometry. *Scand. J. Immunol. 44*: 164-72, 1996.

III. Cederblad, B., Blomberg, S., **Vallin, H.**, Perers, A., Alm, G.V. and Rönnblom, L. Patients with systemic lupus erythematosus have reduced numbers of circulating natural interferon-α producing cells. *J. Autoimmun. 11*: 465-70, 1998.

IV. **Vallin, H.**, Blomberg, S., Alm, G.V., Cederblad, B. and Rönnblom, L. Patients with systemic lupus erythematosus (SLE) have a circulating inducer of interferon-alpha (IFN-α) production acting on leucocytes resembling immature dendritic cells. *Clin. Exp. Immunol. 115*: 196-202, 1999.

V. **Vallin, H.**, Perers, A., Alm, G.V. and Rönnblom, L. Anti-dsDNA antibodies and immunostimulatory plasmid DNA in combination mimic the endogenous IFN-α inducer in systemic lupus erythematosus that activates natural IFN-α producing cells. *Submitted for publication*.

Abbreviations

AF-1	activation function 1
CD	cluster of differentiation
CDK	cyclin-dependent kinase
DELFIA	dissociation-enhanced lanthanide fluoroimmunoassay
DC	dendritic cells
DNA	deoxyribonucleic acid
ds	double-stranded
E. coli	Escherichia coli
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FCM	flow cytometry
GAS	gamma activation site
GM-CSF	granulocyte-macrophage colony-stimulating factor
Hk	heat killed
HLA	human leukocyte antigen
HSV	herpes simplex virus type 1
IFN	interferon
IFNAR	interferon-α/β receptor
IFNGR	interferon-γ receptor
IIF	interferon-α inducing factor
Ig	immunoglobulin
IL	interleukin
IPC	interferon-α/β producing cells
IRF	interferon regulatory factor
is	immunostimulatory
ISGF3	interferon-stimulated gene factor 3
ISRE	interferon-stimulated response element
JAK	janus tyrosine kinases
mAb	monoclonal antibody
MHC	major histocompatibility complex
NF-κB	nuclear factor kappa B
NIPC	natural interferon-α/β producing cells
NK	natural killer
NRE	negative regulatory element
PBMC	peripheral blood mononuclear cells
PKR	dsRNA-dependent protein kinase
poly I:C	polyinosinic-polycytidilic acid
PRD	positive regulatory domain
RNA	ribonucleic acid
SAC	Staphylococcus aureus Cowan I
SLE	systemic lupus erythematosus
SpA	Staphylococcal protein A
ss	single-stranded
STAT	signal transducer and activator of transcription
Str	streptomycin inhibited
SV	Sendai virus
Th	T helper cells
TNF	tumor necrosis factor
U	units
UV	ultra violet
VRE	virus responsive element

Introduction

Discovery and definition of interferon

Interferon (IFN) was first described in 1957 by Isaacs and Lindenmann (Isaacs & Lindenmann, 1957). They found transient production of a new soluble factor when heat-inactivated influenza A virus was incubated with chick chorio-allantoic membranes. Since the factor was able to interfere with the growth of live virus in fresh pieces of chick chorio-allantoic membrane it was called interferon. Soon they found that IFN, induced by heat-inactivated influenza A virus, was active against other types of live virus, such as Sendai, Newcastle disease and vaccinia virus (Isaacs *et al.*, 1957), and that the IFN had a remarkable pH stability (Lindenmann *et al.*, 1957).

When IFN first was discovered it was thought to be one single compound, but during the following years it became obvious that it was composed of several different molecules. Since different kinds of inducers caused production of IFNs with disparate stability at low pH, the IFN was divided into two groups: type I IFN insensitive to low pH, and type II IFN sensitive. It is now known that there are differences between the groups also at the molecular level. The IFN system has reviewed (De Maeyer & De Maeyer-Guignard, 1988; De Maeyer & De-Maeyer-Guignard, 1998; Meager, 1998).

In 1980 IFN was defined as follows "To qualify as an interferon a factor must be a protein which exerts virus non-specific, antiviral activity at least in homologous cells through cellular metabolic processes involving synthesis of both RNA and protein" (Stewart, 1980).

IFN genes and proteins

The IFN genes of several species have been cloned (Weissmann & Weber, 1986), but only findings in man will be summarized below.

Type I IFN

The type I IFN constitute a multigene family, further divided into the three subfamilies IFN-α, IFN-β and IFN-ω, all lacking introns and located on the short arm of chromosome 9, at band 9p21 (Allen & Diaz, 1996; De Maeyer & De Maeyer-Guignard, 1988; Diaz, 1995; Diaz *et al.*, 1996; Meager, 1998; Weissmann & Weber, 1986). In man there are 14 closely related nonallelic IFN-α genes, including one pseudogene. They code for proteins, several of which are glycosylated, consisting of 165 to 167 amino acids. The homology within the IFN-α subfamily is high, 80-100% in the coding region. The IFN-β is a single copy gene encoding a glycosylated protein of 166 amino acids, showing 30% homology at the amino acid level to IFN-α. The IFN-ω consists of one functional gene coding for a glycosylated protein of 172 amino acids, as well as six pseudogenes. There is about 70% sequence homology with IFN-α genes.

Type II IFN

The type II IFN has only one member, IFN-γ, a single copy gene with three introns, located on chromosome 12 and coding for a glycoprotein (De Maeyer & De Maeyer-Guignard, 1988; Meager, 1998; Weissmann & Weber, 1986). The IFN-γ gene shows no significant homology with type I IFN genes. The IFN-γ has antiviral activity as the type I IFNs, but is produced in other cells (activated T and NK cells) and acts on another receptor than the type I IFNs.

Regulation of IFN-α/β production

The IFN-α and -β genes are transiently expressed after stimulation with the appropriate inducers. They are strictly regulated by different mechanisms, but some transcription factors are common for both gene types (Doly *et al.*, 1998; Hiscott *et al.*, 1995; Pitha & Au, 1995).

The human IFN-α genes have a virus responsive element (VRE) in their promoter regions, and several transcription factors with binding affinity for VRE have been identified. The interferon regulatory factor-1 (IRF-1) has been shown to increase the IFN-α gene expression. Furthermore, the induction-specific complex AF-1 is probably modified by virus stimulation, and is suggested to be required together with IRF-1 for efficient IFN-α gene expression (Pitha & Au, 1995). A suppressing factor, IRF-2, has the same binding site as IRF-1, and can thereby inhibit the positive effects of the latter. The production of both IRF-1 and IRF-2 is induced by viruses as well as by IFN (Harada *et al.*, 1989).

The IFN-β gene is regulated through four positive (PRDI to PRDIV) and one negative (NRE) overlapping domains within the promoter region. The regulatory domains interact with several transcription factors required for efficient virus stimulated IFN-β gene expression, such as IRF-1, NF-κB and ATF-2 (Hiscott *et al.*, 1995).

Another more important activator of type I IFN gene expression is the IFN-stimulated gene factor 3 (ISGF3), a transcription factor complex activated by IFN-α/β, which have been shown to bind to the VRE and PRDI with higher affinity than IRF-1 and -2 (Yoneyama *et al.*, 1996). Recent investigations have revealed another transcription factor, IRF-3, that is involved in the regulation of type I IFN genes (Hiscott *et al.*, 1999; Schafer *et al.*, 1998).

Inducers of IFN-α/β production

Virus is the classical inducer of IFN-α/β production, but also bacteria, parasites as well as some synthetic compounds are relatively potent inducers (De Maeyer & De Maeyer-Guignard, 1988; Ho, 1984).

Almost all viruses are able to induce production of IFN-α/β. In the present thesis two different enveloped viruses were used, that is herpes simplex virus type I

(HSV) and Sendai virus (SV). The HSV contains linear double stranded DNA, whereas SV contains a negative stranded RNA genome. Little is known about the mechanism whereby viruses induce IFN-α/β production. Double-stranded RNA has been suggested to be an important intermediate, at least in the inductions by RNA viruses (Long & Burke, 1971; Marcus & Sekellick, 1977). Synthetic dsRNA, such as polyinosinic-polycytidylic acid (poly I:C), has been reported to be potent inducers of IFN-α/β production (Field *et al.*, 1967; Lampson *et al.*, 1967). However, viral replication seems not to be necessary for the induction, since UV-irradiated viruses (Clavell & Bratt, 1971; Ito *et al.*, 1978) and glutaraldehyde-fixed virus infected cell lines (Lebon *et al.*, 1982; Lebon *et al.*, 1980) are efficient inducers. Furthermore, the existence of a cell-membrane-bound receptor required for viral induction of IFN-α/β production in leukocytes has been proposed (Lebon, 1985). Several studies also indicate that viral glycoproteins can induce IFN-α/β production (Ankel *et al.*, 1994; Ankel *et al.*, 1996; Ankel *et al.*, 1998; Baudoux *et al.*, 1998a; Baudoux *et al.*, 1998b; Charley & Laude, 1988). Viral proteins are therefore important in the induction of IFN-α/β, but the further requirement of RNA or DNA cannot be excluded.

Several bacteria and parasites have been recognized as potent inducers of IFN (Ho, 1984; Niesel & Klimpel, 1992; Rönnblom *et al.*, 1983a; Rönnblom *et al.*, 1983b). Regarding the induction mechanism, it has been demonstrated that DNA from bacteria can induce IFN-α/β production (Yamamoto *et al.*, 1992b). Immunostimulatory DNA (isDNA) sequences, containing palindromes with unmethylated CpG, seem to be responsible for the induction (Klinman *et al.*, 1997; Pisetsky, 1996; Roman *et al.*, 1997; Sato *et al.*, 1996; Sonehara *et al.*, 1996; Sun *et al.*, 1998; Yamamoto *et al.*, 1992a). Such unmethylated DNA with iterative sequences of the general form 5'-pu-pu-CpG-pyr-pyr-3' are present in bacterial and plasmid (prokaryotic) DNA, but are suppressed and methylated in eukaryotic DNA (Bird, 1986; Sved & Bird, 1990). This could explain why eukaryotic DNA has been reported to be without immunostimulatory effects (Sun *et al.*, 1997; Yamamoto *et al.*, 1992a).

Bacterial isDNA was originally identified by its tumoricidal effects and NK cell activation (Shimada *et al.*, 1985; Yamamoto *et al.*, 1992a), but has also been shown to activate B cells and immunoglobulin production (Messina *et al.*, 1991). The isDNA can also activate macrophages and immature dendritic cells to express MHC class II and costimulatory molecules and to produce several cytokines such as IFN-α/β, IFN-γ, IL-1, IL-6, IL-12 and TNF-α (Lipford *et al.*, 1997; Sparwasser *et al.*, 1997a; Sparwasser *et al.*, 1997b; Stacey *et al.*, 1996). It is likely that the immunostimulatory effects of isDNA are largely mediated by these cytokines. It has been suggested that a pattern recognition system identifies isDNA motifs, and mediates the further activation of various genes, such as the cytokine genes. Recent results indicate that isDNA is taken up by cells by endocytosis and endosomal maturation, and this process is coupled to signaling via the stress kinase pathway, where p38 kinase activation is

important (Häcker *et al.*, 1998). This in turn appears to activate the transcription factor AP-1 and conceivably other transcription factors involved for instance in the regulation of type I IFN genes.

The involvement of such isDNA in the pathogenesis of the autoimmune disease systemic lupus erythematosus (SLE) has also been suggested (Krieg, 1995; Sato *et al.*, 1999).

The IFN-α/β producing cells

Many cell types can produce IFN-α/β upon stimulation by the inducers described above. Cells such as fibroblasts produce mainly IFN-β, while leukocytes produce predominantly IFN-α (De Maeyer & De Maeyer-Guignard, 1988). Isolated human peripheral blood mononuclear cells (PBMC) have been extensively examined and B cells, NK cells, monocytes, dendritic cells and null cells have been suggested to be the main producer of IFN-α/β. As reviewed by Fitzgerald-Bocarsly (1993), two main populations of IFN-α/β producing cells among human PBMC are now recognized, i.e. monocytes and the so-called natural IFN-α/β producing cells (NIPC).

The monocytes produce IFN-α/β upon stimulation with RNA viruses, such as SV and influenza virus (Roberts *et al.*, 1979; Saksela *et al.*, 1984; Sandberg *et al.*, 1990; Stanwick *et al.*, 1981; Yamaguchi *et al.*, 1977; Åkerlund *et al.*, 1996). However, most virus types and other IFN-α/β inducers cannot activate monocytes, but are able to stimulate the NIPC (see below).

The NIPC are responsible for the IFN-α/β production when human PBMC are stimulated *in vitro* with HSV. They are infrequent in blood (about 1 per 1000 PBMC), but highly efficient IFN-α producers, which can produce 1-2 antiviral units (U) IFN per cell (Cederblad & Alm, 1990; Gobl *et al.*, 1988; Rönnblom *et al.*, 1988; Sandberg *et al.*, 1990). In contrast, SV-stimulated monocytes produce ten times less IFN-α per cell, but are at least ten times as frequent (Gobl *et al.*, 1988). Cells resembling NIPC can be induced by many other viruses than HSV (Feldman *et al.*, 1994; Fitzgerald-Bocarsly, 1993), several types of bacteria (Funa *et al.*, 1985; Rönnblom *et al.*, 1983a) and parasites (Rönnblom *et al.*, 1983b). However, the exact phenotype of the actual IFN-α/β producing cells stimulated by these inducers remains to be determined.

The HSV-induced NIPC are nonadherent, non-phagocytic cells, expressing MHC class II, CD4 and the thrombospondin receptor CD36. They lack markers characteristic for T, B and NK cells as well as monocytes, that is CD2, CD3, CD7, CD8, CD10, CD11b, CD11c, CD13, CD14, CD15, CD16, CD19, CD33, CD34, CD38, CD56 and CD57 (Bandyopadhyay *et al.*, 1986; Fitzgerald-Bocarsly *et al.*, 1988; Perussia *et al.*, 1985; Sandberg *et al.*, 1991; Sandberg *et al.*, 1989; Sandberg *et al.*, 1990). Because of this phenotype the NIPC were suggested to be related to dendritic cells (DC) (Feldman & Fitzgerald-Bocarsly,

1990; Ferbas *et al.*, 1994; Ghanekar *et al.*, 1996; Perussia *et al.*, 1985). However, phenotypic and functional differences between NIPC and DC have also been reported (Chehimi *et al.*, 1989). In Paper II in the present thesis the phenotype of NIPC and their relationship to DC are further explored.

A few studies have been carried out regarding the identity and activation of the IFN-α/β producing cells (IPC) *in vivo*. Type I IFN was detected in serum of both pigs (Artursson *et al.*, 1995) and mice (Eloranta *et al.*, 1996; Riffault *et al.*, 1996), following intravenous or subcutaneous injection of UV-inactivated virus, while the IPC were found in the spleen or the draining lymph node. The IPC in spleens of newborn piglets, challenged with a non-infectious coronavirus, lacked markers specific for T cells and B cells. Furthermore, two-thirds of the IPC expressed MHC class II, whereas one-third expressed a marker of macrophages and granulocytes (Riffault *et al.*, 1997). The IPC in the spleen of mice injected with UV-HSV were localized in the marginal zones and lacked MHC class II and markers typical for T and B cells, red pulp macrophages and DC. A majority of the IPC expressed a marker for metallophilic macrophages while a few expressed a marker for marginal zone macrophages (Eloranta & Alm, 1999). Consequently, the phenotype of the IPC *in vivo*, especially in mice, does not agree with that of the NIPC in human peripheral blood. The reason for this discrepancy must be elucidated.

Effects of IFN-α/β

Activation of gene expression by IFN

The type I IFN use the same receptor (IFNAR) (Merlin *et al.*, 1985), and by binding to it, they activate the JAK-STAT signal transduction pathway (reviewed in Uzé *et al.*, 1995). Binding of IFN-α/β activates IFNAR associated Janus kinases JAK1 and Tyk2, which phosphorylate the STAT proteins STAT1 and STAT2. The latter assemble with the p48 protein and translocate to the nucleus as ISGF3, which activates transcription of the IFN inducible genes by binding to the IFN-α/β stimulated response element (ISRE), located in their promoter region (Levy, 1995; Lewerenz *et al.*, 1998; Meager, 1998). The proteins encoded by these genes are responsible for many of the effects of IFN. Several genes have been identified, and the encoded proteins have important functions (see below), while the function of others remain to be clarified. In contrast, the IFN-γ uses a distinct receptor (IFNGR) resulting in formation of activated STAT1 dimers that translocate and bind to the gamma activation site (GAS) in promoter regions in certain genes that subsequently are activated (Levy, 1995).

Antiviral effects

The antiviral activity of the IFN is mediated by IFN-induced proteins that can affect various steps of the viral multiplication, for example viral penetration,

uncoating of virions, transcription, translation and assembly of progeny virus (Staeheli, 1990). An example is the 2'-5'A system, which is induced by both type I and II IFN and shows specificity for at least some picornaviruses (Sen & Ransohoff, 1993). In the 2'-5'A system the enzyme 2'-5'oligoadenylate synthetase is induced by IFN and activated by dsRNA, resulting in synthesis of 2'-5'-linked oligoadenylates that in turn activate latent RNase L. The RNase L inhibits viral replication by degradation of viral and cellular RNA. The dsRNA-dependent protein kinase (PKR) is also induced by both type I and II IFN. The PKR is activated by dsRNA and inhibits the multiplication of many types of virus by inhibiting the initiation of protein synthesis (Williams, 1995). The human MxA protein, induced preferentially by IFN-α/β, is able to inhibit replication of a variety of RNA viruses, such as influenza virus, measles, vesicular stomatitis virus and Semliki Forest virus (Landis *et al.*, 1998; Pavlovic *et al.*, 1993; Pavlovic & Staeheli, 1991). Interestingly, most laboratory mice lack functional Mx genes, in contrast to wild mice.

Cellular effects

The type I IFN have, in addition to the antiviral activity, several other effects on cells (De Maeyer & De Maeyer-Guignard, 1988) that may grouped into cellular effects and immunological effects. This section briefly describes the cellular effects, which may be subgrouped into antiproliferative, cytotoxic and differentiation effects. This is a large and complicated field, at least in part due to the fact that most cells have IFNAR and that the stage of differentiation of a particular cell can determine the type I IFN effects.

Type I IFN can efficiently inhibit cell growth, by way of for instance inhibition of G1 cyclin-dependent kinase (CDK), or via c-myc, pRb and other factors involved in regulation of the cell cycle (Grandér *et al.*, 1997; Kimchi, 1992; Sangfelt, 1998). Type I IFN may also be directly cytotoxic to cells by means of induction of apoptosis (Sangfelt, 1998; Sangfelt *et al.*, 1997). Furthermore, type I IFN can induce apoptosis specifically in virally infected cells and this effect may involve the 2'-5'A system (Castelli *et al.*, 1997; Diaz-Guerra *et al.*, 1997; Tanaka *et al.*, 1998). Therefore some of the antiviral effects of type I IFN may be mediated by such apoptosis. Finally, IFN can cause differentiation of cells (De Maeyer & De Maeyer-Guignard, 1988), for instance chronic lymphocytic lymphoma cells into plasmacytoid cells (Östlund *et al.*, 1986).

Immunological effects

The type I IFN have many regulatory effects of the immune system (Belardelli, 1995) by which they may enhance their antiviral action (Gresser, 1997). For example, type I IFN can increase the number of Fc receptors on macrophages and enhance their phagocytic activity. The type I IFN also increase the expression of MHC class I and β_2-microglobulin on cells, which facilitates recognition and killing of virus infected cells by cytotoxic lymphocytes. Type I

IFN have also stimulatory effects on NK cells and can regulate the traffic of leukocytes (Biron, 1997; Biron, 1998).

Furthermore, IFN-α has a regulatory role in the development the Th1 type of immunity, which is characterized by T cell production of IFN-γ, IL-2 and TNF-β, activated macrophages, delayed-type hypersensitivity reactions and formation of IgG2a antibodies (Belardelli, 1995; Gresser, 1997). In contrast, Th2 immunity is characterized of production of IL-4, IL-5, IL-10 and IL-13, as well as eosinophils, mast cells and IgE antibodies. It has been shown in mice that IFN-α promotes antibody responses dominated by the IgG2a isotype and suppresses IgE secretion and splenic IL-4 mRNA levels (Finkelman *et al.*, 1991). In man, IFN-α can inhibit the IL-5 production by CD4 positive T cells (Nakajima *et al.*, 1994; Schandene *et al.*, 1996).

Species differences have been reported regarding the regulation of Th1/Th2 pathways. During early Th2 development, the IL-12 pathway in T cells is extinguished by loss of a subunit of the IL-12 receptor. The IL-12 receptor is maintained in an upregulated and functional state on activated Th cells by IFN-γ in mice (Szabo *et al.*, 1997), but by IFN-α/β (not IFN-γ) in man (Rogge *et al.*, 1997). Furthermore, IFN-α cannot alone promote development of the Th1 type of immunity in mice, but requires synergy with IFN-γ and IL-12 (Wenner *et al.*, 1996). In contrast, a recent study shows that IFN-α, but not IFN-γ, can drive human T cells to Th1 development (Rogge *et al.*, 1998). The reason may be that in man IFN-α/β can activate STAT4, necessary for Th1 development.

It is also interesting that type I IFN produced during viral infections promote proliferation of bystander memory cytotoxic T cells and thereby may help maintain immunological memory (Gray, 1996; Tough *et al.*, 1996; Tough & Sprent, 1998). Furthermore, isDNA with unmethylated CpG motifs stimulates T cells by inducing antigen presenting cells to produce type I IFN, which in turn acts on T cells via the IFNAR (Sun *et al.*, 1998). Recently, it has been described that type I IFN can keep activated T cells alive, and thus may prevent the antigen-induced death of activated T cells during infections (Marrack *et al.*, 1999), further suggesting a central role of the type I IFN system in the regulation of the immune response.

Effects of cytokines on the IFN-α/β production

It is a well known that low doses of IFN-α/β can enhance the production of IFN-α/β induced by viruses and bacteria. The phenomenon is referred to as priming (De Maeyer & De Maeyer-Guignard, 1988; Rosztóczy, 1988; Stewart, 1979), and can in some cases be essential for the IFN-α/β expression (Erlandsson *et al.*, 1998). Furthermore, primed cells are able to produce IFN-α/β more quickly than non-primed cell (Stewart, 1979). The mechanism of priming is not yet completely understood, but since priming with IFN-α/β enhances the production of other cytokines besides IFN-α/β, such as IL-6 and TNF-α, the priming effect

has been suggested to be on an early step in the signal transduction common for the viral induction of several cytokines (Rosztóczy & Pitha, 1993). Type I IFN causes rapid and efficient expression and activation of STAT1, STAT2 and p48 proteins in human PBMC and macrophages (Lehtonen *et al.*, 1997). These factors form the ISGF3 complex which stimulates expression of IFN-a/β genes by binding to their promoters (see above). However, other mechanisms may operate since at least the transcription factors IRF-1, IRF-3, and NF-κB have been shown to be involved in the regulation of type I IFN genes (Hiscott *et al.*, 1995; Hiscott *et al.*, 1999; Pitha & Au, 1995).

Other cytokines than type I IFN can affect the IFN-α/β production. For example, GM-CSF, IL-3 and IFN-γ enhance the IFN-α production induced by HSV-infected WISH cells in human PBMC, whereas IL-1, IL-2, IL-4, IL-6 and TNF-α have no effect (Cederblad & Alm, 1991). In contrast, IL-4 decreases the production of IFN-α and -β by human monocytes stimulated with SV (Gobl & Alm, 1992). Furthermore, IL-10 strongly inhibits the IFN-α production induced by several viruses, including HSV and SV (Payvandi *et al.*, 1998).

Biological relevance of the IFN-α/β system

The biological relevance of IFN-α/β in resistance against viruses has been demonstrated in mice. In several experiments it was shown that injections of anti-IFN-α/β antibodies in mice dramatically decrease resistance to various viruses, such as HSV and vesicular stomatitis virus, and also several oncogenic viruses (Gresser, 1984; Gresser, 1997). In addition, mice with deletions of IFN-α/β receptor genes or genes involved in the intracellular pathway of IFN signal transduction show a highly increased susceptibility to various virus infections (Durbin *et al.*, 1996; Fiette *et al.*, 1995; Meraz *et al.*, 1996; Müller *et al.*, 1994; Van den Broek *et al.*, 1995). Some caution is however prudent in the interpretation of data derived from studies of the type I IFN system in mice, because the common laboratory strains have inactivated Mx genes and their Th1 cells do not respond to type I IFN in the same way as their human counterpart (see above).

Experimental studies of the relevance of the type I IFN system in man are limited to studies of the correlation between infections and type I IFN system function. The PBMC from infection-prone children have a markedly decreased IFN production induced by SAC (Bondestam *et al.*, 1985). The IFN induced by SAC was found to be acid labile IFN-α and IFN-γ, and produced by null cells (Funa *et al.*, 1985). As described in Paper I, the SAC actually stimulates NIPC. Therefore, it cannot be excluded that defects in components of the type I IFN system, such as NIPC, can cause increased susceptibility to infections also in man.

The fact that some viruses have developed means to subvert the IFN-α/β system attests to its biological relevance (Gresser, 1997). For example, vaccinia

virus and other orthopoxviruses encode a soluble type I IFN receptor with high affinity for human type I IFN (Symons *et al.*, 1995), whereas hepatitis B virus and type 5 adenovirus have gene products that inhibit the IFN gene expression (Kerr & Stark, 1992). Furthermore, Epstein-Barr virus encodes viral IL-10 which blocks production of several cytokines (Seow, 1998), and IL-10 has been shown to strongly inhibit the IFN-α production in human NIPC (Payvandi *et al.*, 1998). As a final example, HSV inhibits the antiviral activity of IFN by inhibiting one of the enzymes in the 2'-5'A system, namely the RNase L (Sen & Ransohoff, 1993).

Clinical use of IFN-α/β

The inhibitory actions of IFN-α/β on viral infections and on some tumors both *in vivo* and *in vitro* in experimental systems (see above) have motivated production IFN-α and IFN-β on a large scale for clinical studies in man. Today, IFN-α treatment is used in malignant diseases such as hairy cell leukemia, chronic myelogenous leukemia, low-grade non-Hodgkin lymphoma, cutaneous T cell lymphoma, carcinoid tumors, renal cell carcinomas, multiple myelomas, melanomas and renal cell carcinomas. The effects are significant but usually not dramatic. The relevant mechanism of action on the tumors remain to be determined, and could be due to any of the cellular and immunomodulatory effects described before in this Introduction. Also viral diseases have been treated, and at present the main use of IFN-α is in chronic active hepatitis caused by hepatitis B and C viruses, with quite good results (Gutterman, 1994) (Strander, 1986; Stuart-Harris & Penny, 1997). A third major type of clinical indication is in multiple sclerosis, where IFN-β reduces the frequency and severity of exacerbations and the progression of disability (Jacobs *et al.*, 1996; The IFNB multiple sclerosis study group, 1995; The PRISMS study group, 1998).

When patients are treated with IFN-α, several serious side effects have been observed (Strander, 1997). These include acute symptoms resembling an influenza infection, and also development of autoantibodies and even autoimmune diseases (see the section on SLE below).

Systemic lupus erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a multisystem inflammatory autoimmune disease characterized by production of autoantibodies against up to 50 different autoantigens and is regarded as the prototype for organ non-specific autoimmune disorders (Wallace & Hahn, 1997). The SLE has been extensively studied, a wealth of facts are available and it is therefore not possible to describe this disease at any length. The clinical manifestations are indeed quite complex. Cytotoxic autoantibodies and immune complexes (Foster *et al.*, 1993; von Mühlen & Tan, 1995) cause inflammation and damage of various organ systems, such as skin and mucous membranes, joints, kidneys, brain (neuropsychiatric manifestations), serous membranes, lung, heart and

sometimes gastrointestinal tract. Per definition, at least two organ systems are involved in the disease, but usually multiple organs are afflicted over time. Constitutional complaints such as malaise, marked fatique, weight loss and fever are important. Morbidity and mortality increase significantly when vital organs such as kidneys are involved, but may also be caused by the therapeutic drugs.

In 1985 the prevalence of SLE in southern Sweden was reported to be 39 cases per 100 000 individuals (Nived *et al.*, 1985), but higher figures have been reported in other ethnic groups and geographic areas (Fessel, 1974). Furthermore, the incidence of SLE in Minnesota, USA has for unknown reason(s) nearly tripled over the four past decades (Uramoto *et al.*, 1999). Predominantly females during their reproductive years are afflicted. All this, especially the considerable mortality and morbidity as well as economic costs to our society (direct and indirect), make SLE an important disease to study.

The autoantibody production in SLE is antigen driven with production of high affinity IgG antibodies via somatic mutation. A vast literature exists with regard to different autoantibodies and their specificity in SLE (Wallace & Hahn, 1997). Anti-dsDNA antibodies are highly specific for SLE and reflect disease activity but other autoantibodies with specificity for e.g. histone, P, Ku, SS-A and SS-B, DNase I, Sm, RNP, nucleosomes and cell-surface antigens (including phospholipids) may also be involved in the disease process. A favored hypothesis is that DNA/protein complexes (e.g. nucleosomes) can initiate the autoimmune response (Koutouzov *et al.*, 1996; Mohan *et al.*, 1993).

The etiology and pathogenesis of SLE is largely unknown, but many factors of importance have been reported (Wallace & Hahn, 1997). For instance, many genes are associated with increased risk for SLE, such as complement, TNF-α and MHC genes. Mapping disease associated genes is one way of understanding the cause of the disease. A not widely appreciated potential causative factor in SLE is the type I IFN system. In an early phase of the disease, a majority of SLE patients have detectable levels of IFN-α in serum (Kim *et al.*, 1987; Klippel *et al.*, 1985; Shou-Nee *et al.*, 1987; Strannegård *et al.*, 1982; von Wussow *et al.*, 1988; Ytterberg & Schnitzer, 1982), and almost all patients have increased levels of the IFN-α/β inducible Mx protein in peripheral blood leukocytes, indicating endogenous IFN production (von Wussow *et al.*, 1996; von Wussow *et al.*, 1989). The serum IFN-α levels correlate to disease activity and IFN-α can also be detected in cerebrospinal fluid of SLE patients with brain involvement (Lebon *et al.*, 1983; Shiozawa *et al.*, 1992).

The main reason for linking IFN-α to development of autoimmunity such as SLE is that IFN-α treated patients with malignant and infectious disorders show increased frequencies of both autoantibodies and autoimmune diseases (Burman *et al.*, 1985; Fentiman *et al.*, 1988; Fritzler, 1994; Rönnblom *et al.*,

1990; Rönnblom *et al.*, 1991). Clinical overt autoimmune disease develops in particular in individuals with pre-existing autoantibodies or subclinical autoimmunity (Rönnblom *et al.*, 1991). Furthermore, a case of SLE-like disease developing during IFN-α treatment was initially reported (Rönnblom *et al.*, 1990), and further cases have subsequently been published (Flores *et al.*, 1994; Mehta *et al.*, 1992; Sanchez Roman *et al.*, 1994; Schilling *et al.*, 1991; Tolaymat *et al.*, 1992; Wandl *et al.*, 1992). Interestingly, SLE associated with IFN-α therapy is reversed when the therapy is discontinued. While overt SLE-like syndromes during IFN-α therapy are infrequent, antibodies against dsDNA develop in 8% of the patients (Kälkner *et al.*, 1998), and such antibodies are considered specific for SLE (Austin *et al.*, 1983). It is also relevant that the lupus in (NZB/W)F1 mice is accelerated by administration of IFN-α (Adam *et al.*, 1980). In another murine autoimmune disease, insulitis with beta-cell destruction and diabetes in BB rats, islet cell expression of IFN-α genes precedes lymphocyte infiltration. A causative role for IFN-α in this animal model is suggested by the fact that antibodies against IFN-α inhibit development of the diabetes (Huang *et al.*, 1994; Huang *et al.*, 1995; Stewart *et al.*, 1993).

In all, these results indicate a connection between the type I IFN system and autoimmunity, hypothetically because IFN-α prevents maintenance of tolerance to self antigens. The mechanisms of action of the IFN-α remains to be determined, but several of the immunological effects described in a preceding section (Effects of IFN-α/β: Immunological effects) could be involved. Other mechanisms may however certainly be involved. For instance, mitogen-activated normal lymphocytes exposed to IFN-α produce antibodies with a lupus idiotype, including anti-dsDNA antibodies (Schattner *et al.*, 1986). Because IFN-α can inhibit apoptosis in B lymphocytes (Jewell *et al.*, 1994; Strasser *et al.*, 1991) and prolong survival of activated T cells, autoreactive cells may not be normally deleted in the presence of an activated type I IFN system. This hypothesis is supported by findings that mice and men with deficient apoptosis develop an autoimmune disease resembling SLE (Rieux-Laucat *et al.*, 1995). Increased levels of anti-apoptotic Bcl-2 have been reported in SLE lymphocytes (Gatenby & Irvine, 1994). Type I IFN can however cause apoptosis in other cell types (see Effects of IFN-α/β: Cellular effects) which could generate more autoantigens (e.g. nucleosomes) and isDNA (see Papers IV and V), stimulating autoimmunity. Interesting defects of immune complex clearance mechanisms, including nucleosome receptor dysfunction, have been found in SLE patients and may be relevant in the disease process (Bennett *et al.*, 1987; Bennett *et al.*, 1986; Wallace & Hahn, 1997).

Aims of the present study

The general aim of this thesis was to study the identity and activation of the natural IFN-α producing cells (NIPC), including their involvement in SLE. The more specific aims were:

1. To determine to what extent production of IFN-α, -β and -γ are induced in PBMC by the gram-positive bacteria *Staphylococcus aureus* Cowan I (SAC) and the gram-negative *Escherichia coli* (*E. coli*). Furthermore, it was asked whether the NIPC were the actual producers of IFN-α/β. Finally, attempts were made to gain some insight into the mechanisms whereby SAC induces production of IFN-α.

2. To establish and use a flow cytometric method, involving the simultaneous detection of cell surface antigens and intracellular IFN-α, to determine the phenotype of the HSV-induced NIPC. Furthermore, the morphology and antigen presenting ability of purified NIPC were investigated. Especially the relationship between NIPC and dendritic cells (DC) was explored.

3. To look for any abnormal function of the type I IFN system at the blood level in SLE. This was done by studying the *in vitro* ability of PBMC to produce IFN-α spontaneously, as well as after stimulation of NIPC by HSV and of monocytes by SV. A reduced HSV-induced IFN-α production by NIPC was seen, and possible reasons for this were sought. In particular, it was asked whether NIPC function was restored by costimulatory cytokines and whether there were inhibitory (or stimulatory) factors in SLE serum.

4. To determine whether the activity and occurrence of the IFN-α inducing factor (IIF) in SLE serum, discovered in the previous work, correlated to disease activity and serum levels of IFN-α. It was also asked if the SLE-IIF activity was dependent on the costimulatory cytokines IFN-α2b and GM-CSF, and if NIPC were responsible for the IFN-α production. Finally, it was investigated whether the SLE-IIF had a defined molecular weight and whether it contained immunoglobulin or nucleic acid.

5. To more extensively characterize the SLE-IIF, especially to determine whether it consisted of complexes between anti-dsDNA antibodies and isDNA. It was found that anti-DNA antibodies and isDNA (plasmid pcDNA3) induced IFN-α production. It was therefore further asked whether this potential SLE-IIF model also involved activation of NIPC. Finally, the ability of several cytokines to either stimulate or inhibit SLE-IIF and the combination of anti-DNA antibodies and isDNA was determined.

Results and Discussion

Stimulation of NIPC by *Staphylococcus aureus* (Paper I)

Most studies of type I IFN production have focused on stimulation by viruses, but also bacteria can induce production of IFN in various cell types *in vitro* and *in vivo*. Both *E. coli* and SAC have been shown to induce production of IFN by PBMC *in vitro* (Rönnblom *et al.*, 1983a). In previous attempts to characterize the IFN response to SAC, bioassays were used to measure the IFN activity. These studies showed that acid labile IFN-α was produced, and that the IFN activity was neutralized by antibodies to both IFN-α and -γ (Funa *et al.*, 1985; Gore *et al.*, 1983; Rönnblom *et al.*, 1983a) Furthermore, these early studies suggested that SAC induces IFN-α production in null lymphocytes (Funa *et al.*, 1985).

In the present study, we used immunoassays in addition to bioassays in order to determine to what extent actually IFN-α, -β and -γ were produced by normal PBMC exposed to SAC or *E. coli in vitro*. The bioassays were used with and without neutralizing antisera to IFN-α, -β or -γ. For detection of IFN-β and IFN-γ classical enzyme-linked immunosorbent assays (ELISA) were used, while a more sensitive dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) (Artursson *et al.*, 1995; Hemmilä *et al.*, 1984) was established for measurement of IFN-α.

When PBMC prepared from normal blood donors were cultured with SAC or *E. coli* for 24 h, IFN-α and -γ, and much lower levels of IFN-β were produced. The concentrations of IFN determined by bioassay were usually about 10-fold higher than by immunoassays. The discrepancies between bioassay and immunoassay results are most likely caused by potentiation of the biological activity by synergy between IFN-α/β, IFN-γ (Czarniecki *et al.*, 1984) and other cytokines such as TNF-α (Feduchi *et al.*, 1989; Wong & Goeddel, 1986). Such synergies may also explain the previous observations of SAC-induced acid labile IFN-α, as well as IFN with properties of both IFN-α and -γ (Funa *et al.*, 1985; Gore *et al.*, 1983; Rönnblom *et al.*, 1983a).

Subsequently, the study focused on the IFN-α inducing capacity of SAC. We found that the kinetics of the IFN-α responses induced by SAC resembled that induced by HSV-infected WISH cells, most IFN-α being produced within 20 h after addition of the inducer to PBMC *in vitro*. When the IPC frequencies were determined by *in situ* hybridization, immunohistochemistry and ELIspot assay, SAC-IPC showed very strong hybridization signals, intense immunohistochemical staining and produced large plaques in the ELIspot assay. The frequency of SAC-IPC was about 1 per 1000 PBMC and the amount of IFN-α produced per cell was approximately 1 U. The cell surface phenotype of SAC-IPC was studied by immunohistochemistry, where the cells were

double stained for intracellular IFN-α and cell surface antigens. It was found that SAC-IPC expressed CD4 and HLA-DR, but not the T cell marker CD3, the B cell marker CD19 or the monocyte marker CD14. The phenotype of SAC-IPC therefore resembled that previously described for NIPC, as did the low frequency and impressive IFN-α producing capacity (Fitzgerald-Bocarsly, 1993).

It was further found that heat-killed SAC (Hk-SAC) induced IFN-α production in PBMC more efficient than Streptomycin-inhibited SAC (Str-SAC). The Str-SAC, but not Hk-SAC, caused a significant decrease in the IFN-α response induced by HSV-infected WISH cells. In addition, supernatants from heat treated SAC reduced the IFN-α response of PBMC to both virus and bacteria. These results indicate the existence of cell wall components that inhibit the IFN-α response and are removed from SAC by heat treatment. In contrast, both Str-SAC and Hk-SAC were equally inhibitory to the SV-induced IFN-α production, suggesting that the heat-sensitive components in contrast are not essential for inhibition of the IFN-α production in monocytes. This inhibition may be caused by phagocytosis of particles, possibly also bacteria, by monocytes (Sandberg *et al.*, 1990).

Streptomycin-inhibited SAC were much more potent as IFN-α inducer when harvested during exponential growth compared to stationary phase, whereas equally good IFN-α production was obtained with Hk-SAC regardless of growth phase. These results suggest that changes in bacterial properties during their growth, such as expression of surface structures, influence their ability to stimulate or inhibit IFN-α responses in NIPC.

Regarding the mechanism whereby SAC trigger NIPC to produce IFN-α, the results of this study suggest that interactions with bacterial surface proteins, such as staphylococcal protein A (SpA), is important. Pronase treatment was shown to destroy most of the IFN-α inducing capacity of SAC, and mutants lacking SpA induced less IFN-α than corresponding parent strains. However, pure SpA did not induce IFN-α. By mechanical disruption of SAC, the IFN-α inducing ability was lost, indicating that the interferogenic structures must be presented on a particulate phase, that is on the bacteria. The results of this study do not exclude the possibility that the actual IFN-α inducer in SAC is bacterial isDNA, needing one or more bacterial surface proteins to penetrate the cells.

The ability of SAC to either stimulate or inhibit production of IFN-α/β by NIPC may be biologically relevant for several reasons. The NIPC appeared similar to if not identical to immature dendritic cells (see Paper II). It is therefore possible that the bacteria can affect both production of immunoregulatory cytokines such as IFN-α/β and antigen presentation, and thereby have a positive or negative impact on the resistance to the bacterial infection.

Phenotype, morphology and antigen presenting ability of NIPC, and relationship of NIPC to dendritic cells (Paper II)

The actual cellular identity of NIPC is not clear. Due to the lack of lineage specific markers and the expression of CD4 and MHC class II antigens they have been suggested to be related to dendritic cells (DC) (Feldman & Fitzgerald-Bocarsly, 1990; Ferbas *et al.*, 1994; Perussia *et al.*, 1985; Sandberg *et al.*, 1990). However, also discrepancies between NIPC and DC have been described (Chehimi *et al.*, 1989). Therefore we further investigated the cell surface phenotype of NIPC, as well as their ultrastructure and ability to present antigens to autologous T cells. The properties of NIPC were compared to previously published data on DC.

Previous investigations on the phenotype of NIPC are reviewed by Fitzgerald-Bocarsly (1993). Many of these studies were done by Sandberg *et al.*, using different combinations of immunohistochemical staining, in situ RNA-RNA hybridization and cell sorting by flow cytometry (FCM) (Sandberg *et al.*, 1991; Sandberg *et al.*, 1989; Sandberg *et al.*, 1990). We used an immunohistochemical double staining technique to determine the phenotype of SAC-IPC in the previous work (Paper I). All these methods are time consuming, and therefore a more efficient method was developed to allow more comprehensive phenotyping of NIPC. The method involved the simultaneous flow cytometric analysis of the expression of cell surface antigens and intracellular IFN-α by HSV-induced NIPC. In brief, normal PBMC were stimulated to produce IFN-α by UV-inactivated HSV for 5 h and then fixed in paraformaldehyde. The fixed cells were sequentially stained by mAbs reactive with various cell surface antigens and FITC-labelled polyclonal anti-mouse Ig antibodies. The cells were then permeabilized by Tween 20 treatment, stained for intracellular IFN-α by a biotinylated anti-IFN-α mAb and phycoerythrin-labelled streptavidin, and finally analyzed by FCM.

The results showed that NIPC stained for intracellular IFN-α formed a population of brightly fluorescent cells. These cells had distinct light scatter characteristics and were located between the populations of lymphocytes and monocytes. The frequency of NIPC determined by this technique correlated well with frequency estimates by the ELIspot assay, known to detect essentially all HSV-induced IFN-α secreting cells (Cederblad & Alm, 1990). The NIPC were confirmed to lack specific markers of T cells (CD3), B cells (CD19), NK cells (CD16) and monocytes (CD11b, CD14 and CD64), and to express CD4, the thrombospondin receptor CD36 and MHC class II. Furthermore, they expressed CD44 and CD45RB, and like DC they expressed CD40, CD83 and FcεRI (Jaffe, 1993; Romani *et al.*, 1994; Zhou & Tedder, 1995). However, the NIPC were found to lack markers previously identified on more mature DC, such as CD5 (O'Doherty *et al.*, 1994; Wood *et al.*, 1991), the integrin CD11c (Freudenthal & Steinman, 1990; O'Doherty *et al.*, 1994) and the costimulatory B7 molecules CD80 and CD86 (Hart *et al.*, 1993; Thomas *et al.*, 1993a).

Consequently their phenotype differ from that of typical mature DC. Instead, we found that NIPC expressed markers known to be expressed on immature DC, that is CD72 (O´Doherty *et al.*, 1993), low levels of the myeloid markers CD13 and CD33 (O´Doherty *et al.*, 1994; Thomas & Lipsky, 1994) and higher levels of CD45RA than of CD45R0 (Jaffe, 1993; O´Doherty *et al.*, 1994; O´Doherty *et al.*, 1993; Romani *et al.*, 1994; Steinman, 1991).

To determine the ultrastructure of NIPC, IFN-α containing cells among HSV-stimulated PBMC were purified by the use of fluorescence-activated cell sorting (FACS), and processed for electron microscopy. The examination of the ultrastructure revealed irregularly shaped nuclei, many mitochondria and vesicles, and a somewhat rough cell membrane. However, the cells lacked the projections or veils characteristic of cultured mature DC (Romani *et al.*, 1994; Van Voorhis *et al.*, 1983). Thus, the morphology of NIPC appears to be similar to that reported for immature DC (O´Doherty *et al.*, 1994; O´Doherty *et al.*, 1993; Thomas *et al.*, 1993b; Thomas & Lipsky, 1994).

Sorted NIPC were also examined for their ability to present antigen to autologous T cells. We showed that HSV-induced NIPC caused proliferation of T cells from HSV-immune donors. However, NIPC were relatively poor stimulators compared to monocytes. In fact, immature DC are reported to be inferior to mature DC in stimulating T cells (O´Doherty *et al.*, 1994; Thomas & Lipsky, 1994).

The results of the present investigation show that NIPC in many respects resemble immature DC, but clearly differ from mature DC. However we can still not exclude that they constitute a unique population with type I IFN production as the main function.

Type I IFN production by PBMC in systemic lupus erythematosus (Paper III)

Patients with systemic lupus erythematosus (SLE) often have signs of an activated IFN type I system with measurable levels of IFN-α in serum, that could be of pathogenic significance (see Introduction). The continual production of IFN-α in SLE raises questions regarding the identity of IFN-α producing cells and the mechanisms by which they are activated. In the present study we examined the type I IFN system at the blood level in SLE patients for any abnormality. In particular we examined the IFN-α production induced by HSV in NIPC or by SV in monocytes. We also measured serum levels of IFN-α and spontaneous production of type I IFN by PBMC.

It was found that seven of 12 SLE patients, but none of the controls, had measurable levels of IFN-α in serum. Still, no spontaneous IFN-α production by their PBMC in cultures could be detected by immunoassay or ELIspot. However, we found that PBMC of SLE patients (SLE-PBMC) have a

decreased production of IFN-α *in vitro* compared to control PBMC. The IFN-α response induced by HSV in NIPC, as determined by immunoassay, was much more impaired than that induced by SV in monocytes, indicating that NIPC in some way are involved in the disease. The frequency of NIPC among SLE-PBMC, as determined by ELIspot, was reduced 70-fold compared to control PBMC, but the few remaining NIPC produced normal amounts of IFN-α (about 1 U/cell). Furthermore, the frequency of NIPC increased 10-fold in SLE-PBMC, but not in control PBMC, when the cultures were costimulated with IFN-α, IFN-γ and GM-CSF. We therefore conclude that patients with SLE have a reduced number of functional NIPC in blood, which in part may be caused by a reduced production of costimulatory cytokines. Our results also may explain previous discrepant results regarding the ability of PBMC from SLE patients to produce IFN-α *in vitro* (Alarcón-Segovia *et al.*, 1974; Neighbour & Grayzel 1981; Strannegård *et al.*, 1982), because especially NIPC were deficient in our study and at least some of the previous investigations measured IFN-α production by monocytes.

Besides a deficiency in costimulatory cytokines, the low IFN-α production in SLE-PBMC could be due to the presence of inhibitory factors, including autoantibodies to cellular antigens or to cytokines such as IFN-α. No anti-IFN-α antibodies were however found in the SLE sera. Furthermore, SLE sera added to cultures of normal PBMC did not inhibit the IFN-α production stimulated by HSV. The only inhibitor found was anti-HSV antibodies, but they appeared to be equally inhibitory in both SLE and control sera. It has actually been shown before that antibodies to HSV can inhibit the IFN-α production induced by this virus (Lebon, 1985).

While we failed to identify any inhibitor in SLE sera, we found that four of 11 SLE sera, but none of the control sera, actually induced production of IFN-α in normal PBMC. This is interesting because it indicates the presence of an IFN-α inducing factor in SLE sera, which may explain the continuous production of IFN-α in SLE. This endogenous IFN-α inducing factor (IIF) was further examined in Paper IV.

The findings that many SLE patients have detectable levels of circulating IFN-α, reduced numbers of NIPC and the lack of spontaneous production of IFN-α by SLE-PBMC, indicate that the IFN-α producing cells may be elsewhere. In fact, Blomberg *et al.* (to be published) have demonstrated IPC in skin biopsies of SLE patients. Furthermore, IPC are found mainly in lymphoid organs in mice and pigs after viral injections (Artursson *et al.*, 1995; Eloranta *et al.*, 1996; Riffault *et al.*, 1996). Therefore it seems reasonable that the low number of NIPC among PBMC in SLE is due to increased recruitment to tissues, where the IFN-α is produced.

The endogenous IFN-α inducing factor in SLE (Paper IV)

An activated type I IFN system has previously been demonstrated in SLE patients (Kim *et al.*, 1987; Klippel *et al.*, 1985; Preble *et al.*, 1983; Strannegård *et al.*, 1982; von Wussow *et al.*, 1988; von Wussow *et al.*, 1989; Ytterberg & Schnitzer, 1982). In Paper III we confirmed these results, and our results further indicated the presence of an endogenous IFN-α inducing factor in sera from patients with SLE (SLE-IIF). The SLE-IIF may be responsible for the activation of the type I IFN system in SLE. In Paper IV we further studied this SLE-IIF.

We found that sera from three to seven of 34 SLE patients, but none of 18 control sera, induced production of IFN-α in cultures of normal PBMC. When the PBMC cultures were supplemented with the cytokines IFN-α2b and GM-CSF, all sera induced IFN-α. However, the IFN-α levels induced by 14 to 21 of the 34 SLE sera were significantly higher than those induced by the control sera. In fact, sera from some SLE patients had a very high IFN-α inducing capacity, similar to that of HSV. The proportion of patients with IFN-α inducing sera and the IFN-α inducing capacity of the sera were significantly higher in SLE patients with active disease than in patients with inactive disease. This difference became less pronounced in presence of costimulatory GM-CSF and IFN-α2b. Patients with active SLE also more often had significant IFN-α levels in serum. Furthermore, sera from patients with IFN-α in serum more frequently and efficiently induced IFN-α production in normal PBMC.

The IFN-α producing cells induced by SLE serum in PBMC cultures had a lower frequency than the NIPC induced by HSV, as shown by detection of IFN-α mRNA containing cells by *in situ* hybridization. However, they showed the same high IFN-α production per cell as typical NIPC. Their phenotype, as determined by FCM, was also clearly similar to that previously (Paper II) shown for NIPC. Thus, they expressed CD4, CD36, CD40, CD45RA, CD83 and HLA-DR, but not CD80, CD86 and a variety of markers for the T, B, NK and monocyte lineages. Accordingly, SLE-IIF appears to specifically activate NIPC.

An initial characterization of the SLE-IIF revealed a molecular weight of 300-1000 kD, as determined by ultrafiltration. Furthermore, it was retained by a protein G column indicating an IgG content. Eluted IgG was inactive, but was partially reconstituted when recombined with the column effluent. This suggested that SLE-IIF besides IgG contained some further component(s). One essential such component appeared to be DNA, because the nuclease Benzonase and DNase I, but not RNase A or T1, largely destroyed the IFN-α inducing activity. The results therefore suggest that SLE-IIF consists of antigen-antibody complexes, containing DNA. The specificity of the antibodies and the nature of the DNA are further characterized and discussed in Paper V.

The main finding in the present paper was therefore the demonstration of an IFN-α inducing factor in SLE serum that could well explain the correlation between serum IFN-α levels and disease activity (Kim *et al.*, 1987; Strannegård *et al.*, 1982; Ytterberg & Schnitzer, 1982). The type I IFN have many immunoregulatory functions that may inhibit self tolerance and promote autoimmunity, and IFN-α has been shown to cause autoimmune diseases in both man and mice (see Introduction). It is therefore suggested that the SLE-IIF by inducing IFN-α production in NIPC could be involved in the pathogenesis of SLE (see General discussion).

Further characterization of the SLE-IIF (Paper V)

The results described in Paper IV suggested that the SLE-IIF contained IgG and DNA as essential components. We therefore asked if SLE-IIF consisted of complexes of anti-DNA antibodies and isDNA.

Using a selected panel of sera from SLE patients and control individuals, we found that the SLE-IIF activity showed positive correlation with serum levels of anti-dsDNA antibodies. We further separated SLE serum on a dsDNA cellulose column. All SLE-IIF activity was retained by the column and eluted material induced IFN-α production in normal PBMC cultures when they were costimulated with IFN-α2b. The IFN-α inducing activity of the eluate, containing mainly anti-DNA antibodies and possibly some residual DNA, was strongly enhanced by addition of the plasmid pcDNA3. This plasmid contains isDNA sequences and can induce IFN-α production when transfected into PBMC (unpublished results), but not without transfection. The importance of anti-DNA antibodies in the IFN-α induction was further demonstrated by showing that a human anti-ss/dsDNA mAb also could convert pcDNA3 into a potent IFN-α inducer, especially in presence of costimulatory IFN-α2b. These results suggest that anti-DNA antibodies can transfect IFN-α inducing DNA into cells. In fact, certain anti-DNA antibodies have been shown to penetrate cells (Alarcón-Segovia *et al.*, 1996; Golan *et al.*, 1993; Vlahakos *et al.*, 1992), and also internalize larger proteins (Weisbart *et al.*, 1998) and nucleosomes (Koutouzov *et al.*, 1996).

We also examined to what extent isDNA in the form of pcDNA3 enhanced the IFN-α inducing capacity of a panel of IgG preparations from SLE and control sera. The IgG fractions were derived from sera treated with DNase I to destroy the SLE-IIF activity. Addition of pcDNA3 increased the capacity of IgG fractions to induce IFN-α production in normal PBMC *in vitro*, especially if the IgG fractions contained anti-dsDNA antibodies. Similar results were obtained when pcDNA3 was added to cultures with the corresponding unseparated sera. In addition, the activity of pcDNA3 was destroyed by methylation of the cytosines in its CpG dinucleotides. These results support the view that isDNA is part of the SLE-IIF. We have so far failed to directly isolate and identify the DNA with IFN-α inducing ability in sera from SLE patients (unpublished

results). Immunostimulatory DNA sequences have however been identified in serum of SLE patients by molecular cloning (Miyata *et al.*, 1996; Sano *et al.*, 1989; Sato *et al.*, 1999), but such isDNA in the biologically active unmethylated form was not directly isolated.

It was also found that the combination of SLE-IgG and pcDNA3 induced IFN-α production in cells that had the same low frequency among PBMC as NIPC, and the same light scatter characteristics by means of FCM analysis. The combination of anti-DNA antibodies and isDNA therefore constitutes a convenient tool to clarify how SLE-IIF activates NIPC to produce IFN-α, but may also have more general application in studies of IFN-α/β induction mechanisms.

The results in Paper IV and in the present study indicated that the cytokines IFN-α2b and GM-CSF in many cases strongly stimulated the production of IFN-α induced by SLE-IIF or its likely components. It was of interest to further define cytokines that could regulate the IFN-α production. We chose to study the cytokines IFN-α2b, IFN-β, IFN-γ, GM-CSF, IL-4, IL-10, IL-12, IL-13 and IL-18, mainly representing the Th1 and Th2 cytokine profiles. We found that type I IFNs and GM-CSF strongly and in an additive way enhanced the IFN-α production induced in PBMC *in vitro* by SLE serum, while mainly type I IFNs enhanced the induction by SLE-IgG combined with pcDNA3. In contrast, the effects of both inducers were inhibited by IL-10, in accordance with other results showing that IL-10 can inhibit virus-induced IFN-α production in human PBMC (Payvandi *et al.*, 1998). The results therefore show that cytokines can markedly both enhance and inhibit the IFN-α production by NIPC caused by inducers like SLE-IIF *in vitro*. It is suggested that regulatory cytokines could have similar effects also *in vivo*, and be important in SLE.

General discussion

The NIPC appear indeed to be very special cells. We have concluded that they resemble immature DC, at least with respect to antigenic phenotype and morphology (Paper II). Surprisingly, we found (unpublished results) that the NIPC did not carry an antigen present on DC progenitors and recognized by the mAb M-DC8 (Schäkel *et al.*, 1998). There may however exist other types of immature DC. For instance, humans have at least two different DC precursors, the myeloid pDC1 and the lymphoid pDC2 (Bottomly, 1999; Olweus *et al.*, 1997; Rissoan *et al.*, 1999; Sallusto & Lanzavecchia, 1994), which should be investigated for their relation to the NIPC. It certainly cannot be completely excluded, as mentioned in Paper II, that NIPC represent a unique cell population.

The NIPC are infrequent in blood, but they have an extremely high IFN-α producing capacity. Logically, the immune system should utilize such NIPC *in vivo* to produce IFN-α in tissues and organs to protect them against a viral infection. In line with this, results from experimental work in pigs and mice indicate that actually most virus-induced IFN-α is produced by relatively infrequent cells, predominantly localized in the lymphoid organs (Artursson *et al.*, 1995; Eloranta *et al.*, 1996; Riffault *et al.*, 1997; Riffault *et al.*, 1996). The NIPC in peripheral blood are perhaps recruited to such sites, and this could explain the much decreased NIPC numbers in blood of SLE patients (Paper III). However, the identity of the IPC in tissues certainly requires further consideration, since they were suggested to mainly correspond to marginal metallophilic macrophages in murine spleens (Eloranta & Alm, 1999).

An interesting property of the NIPC is their ability to become activated to produce IFN-α by a variety of agents, such as bacteria, many types of virus, SLE-IIF and antibody-plasmid complexes (see Papers I, IV, V). This raises questions regarding the nature of the IFN inducer and the mechanisms whereby IFN inducers interact with NIPC and trigger the IFN-α gene expression. Uptake of an IFN inducer appears to be mediated by binding to receptor-like structures on the NIPC, such as the mannose receptor (Milone & Fitzgerald-Bocarsly, 1998) or CD4 (Capobianchi *et al.*, 1992), followed by internalization via endocytosis. It has been claimed that viral proteins themselves, presumably by binding to NIPC, could induce IFN-α production (see Introduction). This does not explain the findings in Paper I, indicating that protein structures on bacteria were necessary but not sufficient to induce IFN-α, and that the whole bacteria were required. Furthermore, the results in Papers IV and V indicate that a protein was necessary for IFN-α induction, that is anti-DNA antibodies, but a DNA component was required in addition. In both these cases it can be argued that the protein served to internalize the actual inducer. The latter probably consists of DNA with isDNA motifs containing unmethylated CpG (see Introduction and Paper V). Perhaps also RNA can form such

immunostimulatory motifs. The mechanism whereby isDNA motifs trigger IFN-α gene expression is not known, but could be elucidated using well defined and small isDNA IFN-α inducers. Such isDNA might for instance bind and activate enzymes or transcription factors involved in type I IFN gene regulation. In summary, the minimal IFN-α/β inducer consists of two components. One component is necessary for penetrating cells and may vary depending on the inducer. The other component consists of isDNA or molecules that mimic isDNA and is the actual activator of type I gene expression.

An IPC must be able to efficiently internalize the type I IFN inducer, and must also possess the required enzymes (e.g. kinases) and transcription factors that activate type I IFN gene expression. The NIPC may be richly endowed in these respects. Since these cells appear related to DC they may have a very active endocytotic mechanism and unique ability to internalize (Cella *et al.*, 1997; Lanzavecchia, 1996; Milone & Fitzgerald-Bocarsly, 1998). The fact that the IFN-α production by the NIPC in many cases is very much dependent on the costimulatory cytokines type I IFN and GM-CSF (Papers I, III-V) may indicate that the cells must upregulate production of for instance essential transcription factors such as STAT 1, STAT 2 and p48 upon cytokine exposure. In fact, it was shown before that the expression of IFN-α/β mRNA in NIPC was very much dependent on new protein synthesis (Cederblad *et al.*, 1991).

The main findings in Papers III-V were the demonstration of an endogenous IFN-α inducer in blood of SLE patients, termed SLE-IIF, and that this inducer consisted of anti-dsDNA antibodies and DNA, most likely of the isDNA variety. These new findings were supplemented by other relevant findings, for instance that costimulatory cytokines markedly enhanced the activity of SLE-IIF on normal PBMC *in vitro* and that the IFN-α producers were NIPC. All these findings are highly interesting, especially against the background that SLE patients have an ongoing production of IFN-α that correlates with disease activity, and that IFN-α therapy can cause autoimmune diseases, including frequent development of dsDNA antibodies and occasionally SLE-like disease (see Introduction). It may be relevant that a striking concentration of the type I IFN production to lymphoid organs has been shown (see above) and that T and B lymphocytes therefore are exposed to high IFN concentrations, in the case of SLE for prolonged periods, while encountering autoantigens.

We have formulated a hypothesis based on our own results and the findings of others, concerning the role of the type I IFN in the etiology and pathogenesis of SLE (see Introduction and in particular Papers III-V for references). In individuals with the genotype predisposing for SLE, there is an initial period of production of IFN-α and perhaps other immunostimulatory cytokines, and increased apoptosis. This may occur during a viral infection. Because of the action of these cytokines, especially IFN-α, products of apoptotic cells cause autoimmunization. The autoantibodies are directed against major products of

apoptosis such as nucleic acids and associated proteins, including nucleosomes. Once anti-dsDNA antibodies develop they form IFN-α inducing complexes by combining with isDNA. We suggest that this isDNA is derived from apoptotic cells, rather than of microbial origin. The IL-10 produced in SLE patients may inhibit the production of IFN-α, obviously not with complete efficacy. Instead, the IL-10 may assist IFN-α in driving the disease by means of its ability to cause apoptosis in T cells (Georgescu *et al.*, 1997) and stimulate B lymphocytes and Th2 lymphocytes (Llorente *et al.*, 1997; Llorente *et al.*, 1994; Llorente *et al.*, 1995). Consequently, a *circulus vitiosus* is envisaged where the autoimmune response is maintained largely because of continual formation and action of the endogenous IFN-α inducer. The generation of its components, that is anti-DNA autoantibodies and isDNA, but also costimulatory cytokines (e.g. IFN-α and GM-CSF), may determine disease activity. Thus, disease activity is reduced when any of these components are reduced and increased when these components increase. Obviously, the current hypothesis is not complete and may certainly be incorrect in some respects. However, it defines several problems that can be approached experimentally.

General summary

The work in the present thesis mainly concerned the identity of the natural IFN-α producing cells (NIPC), as well as the capacity of NIPC to become activated not only by virus, but also by bacteria and by isDNA in the form of a plasmid linked to anti-DNA antibodies. Furthermore, the autoimmune disease SLE was studied, with the aim to determine the reason for the activated IFN-α production in SLE and to understand its role in the pathogenesis of the disease. The following results were obtained:

1. Human PBMC produced high levels of antiviral activity, as determined by bioassay, when stimulated *in vitro* by the bacteria *Staphylococcus aureus* Cowan I (SAC) and *E. coli*. Specific immunoassays demonstrated the presence of both IFN-α and -γ, and for SAC also low levels of IFN-β. The frequencies of SAC-induced IFN-α producing cells (IPC) were up to 1-2 per 1000 PBMC. These IPCs expressed the HLA-DR and CD4 antigens, but not CD3, CD14 or CD19, thus resembling NIPC. The SAC was a more efficient IFN-α inducer when heat killed than when growth inhibited by streptomycin. Especially streptomycin-treated SAC also inhibited the IFN-α production induced by virus. Both pronase treatment and mechanical disruption of SAC cells abolished their capacity to induce IFN-α production. Staphylococcal strains lacking or expressing low levels of protein A (SpA) showed a decreased ability to induce IFN-α production. However, purified SpA did not itself induce IFN-α. Possibly, SpA together with other bacterial surface proteins are important for the capacity of SAC to induce IFN-α production in NIPC. The ability of bacteria to stimulate or inhibit NIPC may be of biological significance in the early immune response.

2. A flow cytometry (FCM) method was developed to simultaneously determine expression of cell surface antigens and intracellular IFN-α in the HSV-stimulated NIPC. The NIPC were infrequent, <3 per 1000 PBMC, and displayed a homogeneous and relatively typical light scatter. They were confirmed to lack leukocyte lineage specific markers, and to express CD4, CD36 and HLA-DR. Furthermore, the NIPC expressed high levels of CD44, CD45RA and CD45RB, and lower levels of CD40, CD45R0, CD72 and CD83. The expression of CD13, CD33 and FceRI were weak but significant, while no CD5, CD11b, CD16, CD64, CD80 or CD86 were detected. FACS-purified HSV-activated NIPC had irregular shaped nuclei, many mitochondria and vesicles, and rugged cell membranes without veils. Such FACS-purified NIPC were able to stimulate proliferation of autologous T-cells from HSV immune donors. Altogether, the results show that NIPC in many respects resemble immature DC, but clearly differ from typical mature DC. Because immature DC are not well defined as yet, it still cannot be excluded that NIPC constitute a unique population of efficient IFN-α producing cells.

3. The *in vitro* IFN-α producing ability of PBMC of SLE patients was studied. It was found that IFN-α production induced by HSV in the NIPC was strongly reduced, while that induced by Sendai virus (SV) in monocytes was less affected. At the cell level, the frequency of HSV-activated NIPC was reduced 70-fold, but residual NIPC produced normal amounts of IFN-α (1-2 U/cell). The NIPC frequency increased 10-fold in SLE-PBMC, but not in control PBMC, when PBMC were costimulated by the combination of IFN-α, -γ and GM-CSF. No spontaneous IFN-α production by PBMC was detected in SLE patients. Furthermore, no SLE serum factor inhibiting IFN-α production was found. However, the very interesting discovery was made that the sera of four out of 11 SLE patients, but none of the sera from healthy control individuals, induced IFN-α production in normal PBMC. This pointed to the existence of an IFN-α inducer in SLE blood. It was proposed that NIPC in SLE blood is reduced in number both because of lack of costimulatory cytokines and because they are recruited to tissues and activated by an endogenous IFN-α inducer.

4. The finding that some SLE sera induced IFN-α production in normal PBMC *in vitro* motivated a more extensive search for an endogenous IFN-α inducing factor (IIF) in SLE patients. It was found that SLE sera frequently contained such IIF activity, especially when the PBMC were costimulated with the cytokines IFN-α2b and GM-CSF. The IIF activity in SLE sera was sometimes as high as that of a virus and was present especially in patients with active disease and with measurable IFN-α levels in serum. The IFN-α producing cells induced by SLE-IIF displayed the same phenotype as the NIPC stimulated by HSV, as determined by FCM. The frequency of IFN-α mRNA containing cells induced by SLE-IIF, as determined by *in situ* hybridization, was lower than that of HSV-induced NIPC, but they had the same high mRNA content. The IIF had an apparent molecular weight of 300 -1000 kD and appeared to consist of at least immunoglobulin (adhering to protein G columns) and DNA (destroyed by DNase), possibly being immune complexes. This new endogenous IFN-α inducer is of great interest, because it may have a pathogenic role in SLE.

5. The IIF in blood of SLE patients was further characterized. The SLE-IIF activity correlated to serum levels of anti-dsDNA antibodies, and such antibodies were found to be an essential component of SLE-IIF. Purified anti-dsDNA antibodies or IgG from SLE patients caused only a weak IFN-α production in cultures of normal PBMCs *in vitro*, and only in the presence of costimulatory IFN-α2b. However, they converted the plasmid pcDNA3 into an efficient IFN-α inducer for PBMC, as did a human anti-ss/dsDNA mAb. Unmethylated plasmid CpG DNA motifs were essential in the IFN-α induction by the plasmid. Like SLE-IIF, the combination of SLE-IgG and pcDNA3 induced IFN-α production specifically in the NIPC. The combination of anti-DNA antibodies and plasmid isDNA therefore appears

to constitute a minimal model for SLE-IIF, which will be of value in further studies. It was furthermore found that the IFN-α production induced by SLE-IIF was greatly increased by type I IFN (IFN-α2b and IFN-β), and by GM-CSF, and these two types of cytokines had at least additive effects. Only type I IFN could stimulate IFN-α production by IgG-plasmid complexes. In contrast, the cytokine IL-10 markedly inhibited the IFN-α production, while several other cytokines were essentially without effects. The action of SLE-IIF is therefore regulated by cytokines in a pronounced and interesting manner.

The results of the present thesis have led to a more clear picture of the composition and function of the type I IFN system and its role in the etiology and pathogenesis of SLE.

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