Reactivity of Human and Porcine Natural Interferon-α Producing Cells to Immunostimulatory DNA

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Abstract

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The interferon-α (IFN-α) inducing capacity of various forms of immunostimulatory DNA and the identity of the IFN-α producing cells (IPC) were studied in human and porcine leukocytes. The DNA vaccine vector pcDNA3 induced production of IFN-α in porcine peripheral blood mononuclear cells (PBMC), but only if used with the transfecting agent lipofectin. Unmethylated CpG dinucleotides in the plasmid were necessary for induction of IFN-α, but pcDNA3 retained this ability after mutation of the CpG-motifs (5'AACGTT 3') in the ampicillin resistance gene. Lipofection and presence of an unmethylated CpG were also prerequisites for the ability of the double stranded (ds) phosphodiester oligodeoxyribonucleotide (ODN) H (5' TTTTCAATTCGAAGATGAAT 3') to activate production of IFN-α in human and porcine PBMC. Human, but not porcine, PBMC could still produce high levels of IFN-α in response to certain single stranded (ss) ODNs, devoid of unmethylated CpG dinucleotides. This indicates that there are species differences in the recognition of immunostimulatory DNA and that eukaryotic DNA sometimes can be interferogenic. Certain CpG-containing ODNs with flanking poly-G sequences were very potent inducers of IFN-α production in the absence of lipofectin, both as phosphorothioate/ phosphodiester chimeric ODNs or as phosphodiester ODNs. Addition of poly-G sequences to the phosphodiester ODN H clearly enhanced its activity, but did not replace the need for lipofectin. The natural IFN-α producing cells (NIPC), also termed plasmacytoid dendritic cells (PDC), were the only cells among human or porcine PBMC that produced IFN-α in response to immunostimulatory DNA.

The human NIPC/PDC also produce IFN-α in response to apoptotic cells in combination with autoantibodies from patients with systemic lupus erythematosus (SLE). This activation was dependent on Fcγ-receptor type II (FcγRII), and the NIPC/PDC were shown to express FcγRIIa, but not the FcγRIIb/c isoforms. The FcγRIIa may also be inhibitory, because aggregated IgG that binds FcγR had a general inhibitory effect on IFN-α production induced by immunostimulatory DNA or herpes simplex virus.

Elucidation of the mechanisms whereby NIPC/PDC are activated may result in more efficient vaccine adjuvants and also provide new targets aiming at inhibition of the pathologic activation of NIPC/PDC in autoimmune diseases.

Key words: CpG-DNA, interferon inducer, dendritic cells, natural interferon-α producing cells, type I IFN, FcγRII.

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Contents

Appendix

Papers I-IV

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

I. Magnusson, M., Johansson, E., Berg, M., Eloranta, M-L., Fuxler, L. & Fossum, C. 2001. The plasmid pcDNA3 differentially induces production of interferon-α and interleukin-6 in cultures of porcine leukocytes. *Veterinary Immunology and Immunopathology 78,* 45-56.

II. Magnusson, M., Magnusson, S., Vallin, H., Rönnblom, L. & Alm, G. V. 2001. Importance of CpG dinucleotides in activation of natural interferon-α producing cells by a lupus-related oligodeoxynucleotide. *Scandinavian Journal of Immunology 54,* 543-550.

III. Domeika, K., Magnusson, M. Eloranta, M-L., Fuxler, L., Alm, G. V. & Fossum, C. Characteristics of oligodeoxyribonucleotides that induce interferon-α (IFN-α) in the pig and the phenotype of the IFN-α producing cells. Manuscript submitted for publication.

IV Båve, U., Magnusson, M., Eloranta, M-L., Perers, A., Alm, G. V. & Rönnblom, L. 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. *The Journal of Immunology.* In press.

Abbreviations

ADV	Aujeszky's disease virus
APC	antigen presenting cell
BDCA	blood dendritic cell antigen
CD	cluster of differentiation
CpG-DNA	DNA containing unmethylated CpG dinucleotides
CpG-ODN	ODN containing unmethylated CpG dinucleotides
DC	dendritic cell
DNA	deoxyribonucleic acid
ds	double stranded
FcR	Fc-receptor
Fc□R	Fc□-receptor
HSV	herpes simplex virus
IC	immune complex
IFN	interferon
IFNAR	type I IFN receptor
IL	interleukin
IRF	IFN regulatory factor
IS-DNA	immunostimulatory DNA
ISG	IFN stimulated gene
ISGF	IFN stimulated gene factor
ISRE	IFN stimulated response element
ISS	immunostimulatory sequence
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
LPS	lipopolysaccharide
MHC	major histocompatibility complex
Mx	myxovirus resistance
NK-cells	natural killer cells
OAS	2', 5' oligoadenylate synthetase
ODN	oligodeoxyribonucleotide
PBMC	peripheral blood mononuclear cells
PAMP	pathogen associated molecular pattern
PDC	plasmacytoid DC
PKR	dsRNA-dependent protein kinase
PRD	positively regulatory domain
PRR	pattern recognition receptor
RNA	ribonucleic acid
SLE	systemic lupus erythematosus
SLE-IIF	IFN-□ inducing factor in SLE
ss	single stranded
Stat	signal tranducer and activator of transcription
SV	Sendai virus
Tc cell	cytotoxic T cell
Th cell	helper T cell
TNF	tumor necrosis factor

Introduction

General overview of the topic

Deoxyribonucleic acid (DNA), especially DNA from microorganisms, has the ability to activate the mammalian immune system. The ability to recognize DNA from pathogens has been interpreted as an innate ability of our immune system to combat infections. One of the most rapidly activated defence mechanisms upon infection is the activation of production of type I interferon (IFN), a cytokine that inhibits viral replication and regulates ensuing immune responses. The early activation of type I IFN is part of the innate immune system (see below) and is crucial to effectively combat invading microorganisms, but a sustained activation of the innate immune system may also initiate and maintain an attack on the body itself.

The innate immunity is the first line of defence against invading microorganisms and it is activated irrespectively of whether the pathogen has been encountered before or not. The adaptive immune response acts via molecules (antibodies and T-cell receptors) that specifically interact with structures of the invading microorganisms, and the pathogen is therefore more efficiently eliminated. This adaptive immunity to microorganisms is often long lived, forming the basis of an immunological memory. Upon re-encountering of the pathogen, the adaptive immune response is therefore more rapidly activated and the clearance of the pathogen is more effective, preventing or mitigating the disease. The ability to elicit a specific immunological memory is an important property of vaccines against microorganisms.

The innate immune system roughly includes the skin, mucosal surfaces, phagocytes, natural killer (NK) cells, antigen presenting cells (APC), complement factors, chemokines, cytokines and acute phase proteins. Together, they restrain the spread of invading pathogens by a number of mechanisms, including ingestion or lysis of pathogens or direct inhibition of their replication. The type I IFN participates in these events by binding to the type I IFN receptor, which rapidly activates transcription factors that induce transcription of a number of molecules with direct antiviral effects. The production of type I IFN can be induced by bacteria, virus and molecules derived from them, e.g. lipopolysaccharide (LPS), glycoproteins, double stranded (ds) ribonucleic acid (RNA) and certain so called immunostimulatory DNA (IS-DNA) sequences. These type I IFN inducers activate transcription factors that then translocate to the nucleus and bind to promoters of type I IFN genes, thereby activating their transcription. Most cell types have the ability to produce type I IFN, but a distinct cell type termed natural IFN producing cell (NIPC) or plasmacytoid dendritic cell (PDC) has the ability to rapidly produce very high levels of type I IFN. The type I IFN and other cytokines produced by NIPC/PDC and other cells of the innate immune system, such as macrophages and dendritic cells (DC), can stimulate the development of efficient adaptive immunity in many ways. Therefore, the cytokines and the cells that produce them can be thought of as the link between innate and adaptive immunity. The present thesis focuses on the type I IFN inducing capacity of molecules

containing immunostimulatory nucleic acids derived from microorganisms (exogenous) or eukaryotic host cells (endogenous), and the cells that produce type I IFN in humans and pigs.

The type I IFN system

Type I IFN genes and proteins

The type I IFNs are immunoregulatory proteins that are unique among cytokines in that many of them both have direct antiviral effects and the ability to activate cells of the immune system. In man, the type I IFNs comprise IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω (Samuel, 2001), and lately a type I IFN related protein named IFN-ζ was found (Kotenko *et al.*, 2003). Interestingly, there are 13 different subtypes of IFN-α in man. In pigs, the type I IFNs include IFN-α, IFN-δ and IFN-ω but also a novel type I IFN called trophoblastic IFN (IFN-τ) (La Bonnardière, Lefèvre & Charley, 1994). Pigs have about 12 IFN-α and 3 IFN-ω subtypes. In both human and pigs, the type II IFN is represented by IFN-γ, which is involved in both the innate and adaptive immune response (Farrar & Schreiber, 1993). The roles of IFN-α and IFN-β in the immune system are by far the most studied among the type I IFNs and the term type I IFN will hereafter refer to IFN-α and IFN-β only. All type I IFNs seem to act through the same receptor, IFNAR, that has two subunits, IFNAR1 and IFNAR2 (Novick, Cohen & Rubinstein, 1994; Uzé, Lutfalla & Mogensen, 1995). The receptor is anchored in the cell membrane from where it transduces signals upon activation, but a soluble form may also exist that regulates the active levels of type I IFN (Hardy *et al.*, 2001).

The multiplicity of the IFN-α genes may be a result of the need to produce large amounts of especially IFN-α from a small number of cells in a short period of time. All type I IFNs are encoded by intronless genes, which may facilitate the process of transcription and translation. However, during the course of evolution, the type I IFN genes have undergone much diversification, which is indicative of a selective pressure favoring different functions. The function of human IFN-α7 is for instance different from many other subtypes in that it cannot activate NK-cells (Ortaldo *et al.*, 1984). Furthermore, although IFN-β and all IFN-ω subtypes act through the same receptor, IFNAR, the latter can mediate different signals dependent on the activating type I IFN (Brierley & Fish, 2002).

Activation and regulation of type I IFN gene expression

The spectrum of type I IFN inducers includes virus, bacteria and components of microorganisms such as glycoproteins, LPS, dsRNA and IS-DNA. The inducers also include immune complexes (IC) containing nucleic acids and synthetic inducers such as imiquimod, the dsRNA analogue poly I:C and oligodeoxyribonucleotides (ODN) containing unmethylated CpG dinucleotides (CpG-ODN) (Fitzgerald-Bocarsly, 2002). Depending on the nature of the IFN-inducer, different regulating mechanisms are in play. Specific receptors responsible for the activation of type I IFN by whole viral particles or bacteria are not known, but recently the Toll-like receptors (TLR) have been shown to mediate expression

of type I IFN induced by poly I:C, LPS, imiquimod and DNA containing unmethylated CpG dinucleotides (CpG-DNA). TLRs are receptors that recognize pathogen associated molecular patterns (PAMP), but also host-derived ligands that are indicative of infection, such as the heat-shock proteins (Takeda, Kaisho & Akira, 2003). Upon triggering of TLR, signalling pathways leading to cytokine production are activated (see below).

The multiplicity of type I IFN genes is also reflected in that different cell types and /or tissues express unique patterns of type I IFN. The different expression levels of individual IFN-α mRNA transcripts in different cell types is regulated at the transcriptional level. The explanation for this can either be found in single mutations within the promoter and/or the presence of negative regulatory domains (Pitha & Au, 1995). The expression is regulated by different repressors and activators of transcription that compete for binding to the type I IFN promoters (Hiscott, Nguyen & Lin, 1995). For example, in a non-induced cell, the IFN regulatory factor (IRF)-2 is tightly bound to the IFN-β promoter, which prevents leakage. Upon activation, IRF-2 and other transcriptional repressors are replaced by activating IRFs and NFκB that bind to the positive regulatory domains (PRD) of the IFN-β promoter. A tight regulation of IFN-β production is achieved by a feedback loop where new synthesis of the repressor IRF-2 is activated by type I IFN. In the early phase of a virus-mediated activation of IFN-α/β, the amplification signal is enhanced by the activation of several IRFs, which allows transcription of many different subtypes of IFN-α (Barnes, Lubyova & Pitha, 2002; Servant, Tenoever & Lin, 2002). For example, virus-induced phosphorylation of the constitutively expressed IRF-3 will allow its translocation to the nucleus where it can activate transcription by binding to PRDIII of the IFN-β promoter and to the corresponding sequence in the virus response element of distinct IFN-α promoters. Secreted IFN-β and IFN-α will in turn activate new synthesis of IRF-5 and IRF-7, which together with IRF-3 activate a much broader spectrum of IFN-α subtypes. Furthermore, more cells will be activated to produce type I IFN, because the IFN produced from the initial activation turn on IRF transcription in other cells, which then become more proficient producers of IFN. These amplifying mechanisms will allow a prompt and massive production of many different subtypes of type I IFN. Recently, the two kinases TBK1 and IKKε which have been linked to the TLR pathway (Barton & Medzhitov, 2003), have been shown to activate IRF-3 and IRF-7 (Fitzgerald *et al.*, 2003; Sharma *et al.*, 2003) (see below).

Because the transcription factors regulating the initial type I IFN expression are constitutively expressed and activated by post-translational modification, the IFN-response is rapidly initiated. This allows efficient inhibition of the replication of microorganisms, especially viruses.

Effects of type I IFN

Antimicrobial effects of type I IFN

In general, a virus depends on the cellular machinery of its host to replicate its genome and to synthesize new viral proteins and finally to assemble viral progeny. The type I IFNs interfere with many steps in this infectious cycle, including

transcription, translation, and by activating apoptosis in infected cells. There are several signalling pathways that are activated upon binding of type I IFN to the IFNAR. Constitutively expressed Jak1/Tyk2 tyrosine kinases are always bound to the cytoplasmic part of the receptor. When the receptor is triggered by its ligand, these kinases are activated, leading to the further activation of the signal transducer and activator of transcription (Stat) 1 and Stat2 proteins (Brierley & Fish, 2002). Activated Stats can form many different transcription factor complexes, e.g., by forming hetero- or homodimers with other Stat molecules or by interactions with other proteins. One of the best studied transcription factor complexes activated by type I IFN is the IFN stimulated gene factor 3 (ISGF3) that is composed of Stat1, Stat2 and IRF-9. ISGF3 recognizes the conserved IFN stimulated response element (ISRE) present in the promoters of many IFN stimulated genes (ISG). Other Stat-containing transcription factors activated by type I IFN recognize the gamma-activated sequences present in many ISGs (Brierley & Fish, 2002; Samuel, 2001)

Among the most important IFN-inducible antiviral molecules are the dsRNA-dependent protein kinase (PKR), 2', 5' oligoadenylate synthetase (OAS) and the Myxovirus resistance (Mx) protein (Brierley & Fish, 2002; Samuel, 2001). The expression of these molecules is regulated by transcription factors that bind to ISRE in their promoters. Both PKR and OAS expression is induced by type I IFN, but the enzymatic activity is activated by the viral product dsRNA. This dual regulation of expression and subsequent activation is indicative of a control mechanism assuring activity only when it is required. Activated OAS will catalyze the formation of 2'-5' linked ATP molecules of varying length. These oligomers will in turn activate RNaseL, which in a dimerized form cleaves single stranded (ss) RNA, thereby preventing translation. Binding of dsRNA to PKR will induce a conformational change that activates the catalytic domain of PKR. Activated PKR will inhibit the eukaryotic initiation of translation factor (eIF2α) and thereby prevent translation of mRNA to protein. In general, the defence against viruses rarely depends on one single mechanism, but rather on many in concert that will interfere with viral replication. An illustrating example of this is the type I IFN-activated Mx proteins that can interfere with many different stages of viral replication, e.g. by disrupting viral RNA polymerase, sequestering of ribonucleoproteins or inhibition of nuclear export of viral RNA, to name a few (Haller & Kochs, 2002). The individual and collective effects of the ISGs PKR, OAS and Mx protein are however not the only antiviral systems because mice devoid of all three genes still have some type I IFN-inducible antiviral activity (Brierley & Fish, 2002).

Type I IFN can also activate functions of cells that are important in both innate and adaptive immunity against bacteria (see below). While the importance of type I IFN in antiviral immunity is well established, the physiological role of type I IFN in the defence against bacteria is less explored.

Antiproliferative, apoptotic and immunological effects of type I IFN

Apart from directly interfering with viral replication, the vertebrate immune system has also evolved mechanisms to kill infected cells, thereby eliminating the

whole machinery on which viral replication relies. For instance, the IFN-inducible, dsRNA-activated enzymes PKR (Balachandran *et al.*, 1998) and OAS (Ghosh *et al.*, 2001) can activate apoptosis in infected cells. Type I IFN will also enhance killing of infected cells by up-regulation of major histocompatibility complex (MHC) class I that is recognized by cytotoxic T (Tc) cells (Belardelli & Ferrantini, 2002) and by enhancing the cytotoxic effect of NK-cells (Biron *et al.*, 1999). Furthermore, type I IFN can affect every step in the cell cycle by indirect inhibition of cyklin-dependent kinases, enzymes necessary for the transition of a cell cycle step to another (Brierley & Fish, 2002). A possible role for the anti-proliferative effect of type I IFN is in the control of hematopoetic neoplasms.

The immunological aspects of type I IFN effects on host cells include the regulation of DC, helper T (Th) cells, B-cells and Tc cells. The activation of naive T-cells requires presentation of antigenic peptides complexed to MHC on the surface of APC. Effective APC are often DC that have matured from precursor cells. Presentation of antigen by immature DC that lack co-stimulatory molecules can lead to T-cell anergy and tolerance to the antigen in question. Furthermore, in the absence of the correct chemokine receptor expression, the immature APC are incapable of homing to the lymphoid organs where the T-cells reside. Type I IFNs have important functions on the maturation of DC (Penna *et al.*, 2002; Santini *et al.*, 2002). For instance, immature DC will upregulate their expression of chemokine receptors, e.g., CCR7 in the presence of IFN-α. This makes DC responsive to chemokines such as MIP3-β and allows homing of activated DC to the T-cell areas of lymphoid organs. The effects of type I IFN on immature DC also include up-regulation of co-stimulatory molecules such as cluster of differentiation (CD) 86, CD80 and CD40 that will ensure activation of T-cells. Also, maturation of DC is associated with upregulation of both MHC class I and MHC class II (Montoya *et al.*, 2002; Santini *et al.*, 2002), which further increases the antigen presenting ability of DC.

Apart from facilitating activation of Th cells by activated APC, type I IFN in concert with interleukin-12 (IL-12) can promote the development of type 1 Th (Th1) cells (Cella *et al.*, 2000; Krug *et al.*, 2001a). The cytokines produced by Th1 cells, e.g. IFN-γ and tumor necrosis factor-α (TNF-α), stimulate strong cell-mediated immunity and production of effective opsonizing and complement-fixing IgG antibodies. In contrast, type 2 Th (Th2) cells produce e.g. IL-4, which stimulates production of IgE antibodies that are associated with atopic reactions and helminth infections. Th2 cells also produce cytokines that can down-regulate cell-mediated immunity, e.g. IL-10. T-cells are also affected by the anti-proliferative effects of type I IFN (see above), dependent on their activation state (Marrack, Kappler & Mitchell, 1999). For instance, the proliferation of naive T-cells is prevented by IFN-α, whereas activated T-cells can divide even in the presence of IFN-α (Dondi *et al.*, 2003). This may represent a control mechanism to assure the expansion of antigen-specific T-cell clones.

An efficient adaptive immune response against microbial infections includes production of antibody classes and subclasses with distinct effector mechanisms that contribute to the eradication of the pathogen (Butler & Howard, 2002; Crawley & Wilkie, 2003). For instance, certain IgG subclasses activate

complement and bind with high affinity to Fc-receptors (FcR), both enhancing for instance phagocytosis of pathogens by macrophages. Type I IFNs promote Ig class-switching in B-cells in several ways. It has thus been demonstrated that type I IFN can induce differentiation of Th1 cells, which promotes class-switching to IgG2a in mice (Sinigaglia, D'Ambrosio & Rogge, 1999). Furthermore, it has also been demonstrated that DC exposed to type I IFN enhance antibody production in general and class-switching in B-cells (Le Bon *et al.*, 2001). Such DC can in fact directly induce class switching in B-cells, independently of T-cells (Litinskiy *et al.*, 2002). Apart from affecting the antibody production in B-cells, type I IFN also promotes survival and differentiation of B-cells (Ruuth *et al.*, 2001) and enhances B-cell receptor dependent responses by lowering the threshold of activation (Braun, Caramalho & Demengeot, 2002).

Type I IFN also stimulates the development of Tc cells. For instance, type I IFN caused the development of Tc cells from naive CD8+ T-cells via induced expression of the chemokine IP-10 and its receptor CXCR3 (Ogasawara *et al.*, 2002). Furthermore, type I IFN can stimulate proliferation of memory Tc cells via induction of the cytokine IL-15 (Sprent *et al.*, 1999).

The capacity of type I IFN to activate both accessory immune cells, such as DC, and effector cells, such as B- and T-cells, is beneficial in combating infections. However, sustained and prolonged activation of type I IFN or treatment of virus infections or tumors with IFN-α can also cause development of autoantibodies and autoimmune disease (Ioannou & Isenberg, 2000; Raanani & Ben-Bassat, 2002; Rönnblom & Alm, 2003). In fact, type I IFN may be an important pathogenic factor in certain autoimmune diseases, as will be discussed below.

The cells producing type I IFN

In humans many different cell types can produce type I IFN in response to microbial stimuli. Fibroblasts produce mainly IFN-β, whereas leukocytes are the major source of IFN-α. Among leukocytes, the NIPC/PDC, monocytes and to some extent myeloid DC are the main producers of type I IFN. Monocytes producing IFN-α constitute approximately 1 cell per 100 peripheral blood mononuclear cells (PBMC) and produce 0.1-0.2 antiviral units (U) IFN per cell in response to certain RNA viruses, such as Sendai virus (SV), but cannot produce IFN-α to most other types of virus or other type I IFN inducers (Fitzgerald-Bocarsly, 1993, 2002; Gobl, Funa & Alm, 1988). The myeloid DC produce intermediate levels of IFN-α in response to the dsRNA analogue poly I:C (Kadowaki, Antonenko & Liu, 2001). The NIPC constitute only 1-2 cells per 1000 PBMC but produce 1-2 U IFN per cell in response to a broad spectrum of inducers including herpes simplex virus (HSV), SV (Feldman *et al.*, 1994; Fitzgerald-Bocarsly, 1993, 2002), bacteria (Funa *et al.*, 1985; Svensson *et al.*, 1996a) and also parasites (Rönnblom *et al.*, 1983). The NIPC/PDC were initially defined as null lymphocytes, because they lacked specific lineage markers for T-cells, B-cells, NK-cells and monocytes (reviewed in Fitzgerald-Bocarsly, 1993, 2002). Further analysis of the HSV-stimulated NIPC revealed that they express e.g. MHC class II, CD4, CD36, CD40, CD83, CD72 and CD45RA but not CD5, CD80, CD86, CD11b or CD11c (Svensson *et al.*, 1996b). This phenotype

suggested that the NIPC corresponded to previously described immature DC (O'Doherty *et al.*, 1994). Further characterization of such immature DC in blood revealed that they were precursors of DC with a unique phenotype and morphology (Grouard *et al.*, 1997; Olweus *et al.*, 1997) and they were termed plasmacytoid monocytes/T-cells. Functional analysis of their antigen-presenting ability showed that they preferentially promote Th2 cell development and they were therefore named precursors of type 2 DC (Rissoan *et al.*, 1999), but are now more commonly termed plasmacytoid DC (PDC). When it was shown that the PDC had the same ability to produce IFN-α as the NIPC, and had a similar phenotype (Cella *et al.*, 1999; Siegal *et al.*, 1999), the cellular identity of these cells merged and they are now sometimes referred to as NIPC/PDC. Another characteristic of these cells is their high expression of the IL-3 receptor (CD123), which is important for IL-3 induced survival and maturation (Grouard *et al.*, 1997; Rissoan *et al.*, 1999).

The blood dendritic cell antigen (BDCA) molecules 2 and 4 are also rather selectively expressed by NIPC/PDC, which have facilitated their purification (Dzionek *et al.*, 2000). The BDCA-2 is a type II C-type lectin that can capture and internalize antigens and the BDCA-4 appears important in the interaction between DC and T-cells (Dzionek *et al.*, 2002), but their importance for special functions in NIPC/PDC remains to be elucidated. Interestingly, however, binding of antibody to BDCA-2 can down-regulate production of IFN-α (Blomberg *et al.*, 2003; Dzionek *et al.*, 2002). In addition, the NIPC/PDC express chemokine receptors such as CXCR3, CXCR4 and CCR5 that are important in recruiting DC to lymphoid organs. For instance, in the presence of the chemokine SDF-1/CXCL12 that interacts with CXCR4, the NIPC/PDC migrate to secondary lymphoid organs (Penna *et al.*, 2002). The NIPC/PDC also express TLR1, 6, 7, 9 and 10 (Hornung *et al.*, 2002), receptors involved in the recognition and response to microbial products such as CpG-DNA (see below). The Fc-receptor (FcR) type II (FcRII/CD32) may also be expressed on these cells (Olweus *et al.*, 1997) but subsequent phenotyping of NIPC/PDC have failed to detect this receptor (Dzionek *et al.*, 2000; Svensson *et al.*, 1996b). Furthermore, the NIPC/PDC also express low levels of the mannose receptor, which is involved in scavenging and possibly also HSV-mediated activation of type I IFN production (Fitzgerald-Bocarsly, 2002).

The human NIPC/PDC have a unique ability to produce large amounts of type I IFN, but they are also capable of producing IL-12 if stimulated in the presence of CD40 ligand (Krug *et al.*, 2001b). Other cytokines produced by these cells are IL-6 and TNF-α (Fitzgerald-Bocarsly, 2002) and the chemokines IL-8, IP-10 (Krug *et al.*, 2001b), CCL3/MIP-1α and CCL4/MIP-1β (Penna *et al.*, 2002). Considering the wide range of agents that activate NIPC/PDC and the spectrum of cytokines they produce, the NIPC/PDC are likely to have an important activatory and regulatory role in the immune system.

Cells that correspond to the human NIPC/PDC and produce high levels of IFN-α in response to virus have also been identified in mice (Asselin-Paturel et al., 2001; Nakano, Yanagita & Gunn, 2001). The murine NIPC/PDC differ from the human counterpart (Hochrein, O'Keeffe & Wagner, 2002) in that they express

CD11c, can express CD8 but do not express CD123 (IL-3R). In contrast, spleen IPC activated by HSV were localized in the marginal zones and had a heterogenous expression of metallophilic and marginal zone macrophage markers, lacked surface markers for T-cells and B-cells, but also MHC class II and CD11c (Eloranta & Alm, 1999). These cells therefore clearly differ from the murine NIPC/PDC.

In pigs, blood leukocytes that produce IFN-α after stimulation with the coronavirus transmissible gastroenteritis virus (TGEV) (Nowacki & Charley, 1993) or Aujeszky's disease virus (ADV) (Artursson *et al.*, 1995) have been characterized. The porcine cells producing IFN-α in response to TGEV are approximately 1-2 in 10,000 PBMC, lack markers for T-cells and B-cells and express MHC class II and CD4 (Charley & Lavenant, 1990; Nowacki & Charley, 1993). Furthermore, purification of porcine blood cells expressing the myeloid marker SWC3 revealed that a subpopulation of these cells that expressed CD4 and MHC class II, but not markers for T-cells, B-cells or monocytes, were the only cells capable of producing IFN-α in response to TGEV (A. Summerfield, personal communication). In vivo, IPC have been found in porcine lymph nodes after intradermal administration of ADV-infected cells (Artursson *et al.*, 1995). Phenotypical analysis of porcine IPC localized in lymphoid tissues after stimulation with TGEV showed that these cells phenotypically resemble the TGEV-activated cells in porcine blood cultures and that they expressed the myeloid marker SWC3 (Riffault *et al.*, 1997; Riffault *et al.*, 2001). Although the frequency of the porcine cells producing IFN-α in response to coronavirus is lower than the frequency of HSV-stimulated human NIPC, the two cell types phenotypically resemble each other. Important cell surface molecules expressed on human/porcine NIPC/PDC and cytokines produced by these cells as well as principal activating agents are illustrated in Figure 1.

Immunostimulatory DNA

When Yamamoto et al investigated the active component in a mycobacterial extract with potent anti-tumor activity, they found that neither the RNA, nor protein content, but instead the DNA content could confer anti-tumor activity (Yamamoto *et al.*, 2002). Short ODNs containing sequences from mycobacteria, especially those containing a hexameric palindrome with a 5' CpG 3' dinucleotide, could mimic the effect of the bacterial extracts. Since this discovery, it has been demonstrated that bacterial DNA or ODNs have many effects on the immune system that is dependent on the presence of unmethylated CpG dinucleotides (Krieg, 2002). Interestingly, CpG dinucleotides normally occur at the expected frequency in bacterial DNA, 1 in 16 nucleotides, but are much less frequent in eukaryotic DNA. Whereas the CpGs of bacterial DNA are mostly unmethylated, the CpGs in eukaryotic DNA are frequently methylated on the cytosine (Bird, 1987; Tweedie *et al.*, 1997). Due to this difference, the ability to react to unmethylated CpG has been interpreted as an innate ability to detect foreign DNA. Such CpG-motifs have been identified in many bacterial plasmids that are employed in DNA vaccination, which may explain their adjuvant potential (Tighe *et al.*, 1998) (see below). Since the initial discovery of IS-DNA it has been shown

that IS-DNA, and many other bacterial and viral constituents, activate innate immunity through pattern recognition receptors (PRR) that are present on cells of the immune system (Akira & Hemmi, 2003). The effects of IS-DNA on the host immune system include activation of B-cell proliferation and production of cytokines that will potentiate the antigen-presenting function of macrophages and DC and favor the development of Th1 cells (Krieg, 2002). Because of this enhancement of immune responses, IS-DNA is considered an effective adjuvant in vaccine formulations (see below).

Sequence specificity and chemical modification of IS-DNA

The initial analysis of IS-DNA established the concept of a palindromic hexameric sequence with at least one CpG dinucleotide as the optimal motif in an ODN for activation of NK-cells and production of IFN (Yamamoto *et al*., 1992a; Yamamoto *et al*., 1992b; Yamamoto, Yamamoto & Tokunaga, 2000). It was further demonstrated that the bases surrounding the CpG dinucleotide were of importance for the activity. The CpG dinucleotide plus one or two bases on the 5' and 3' side are termed a CpG motif. For instance, the optimal CpG-motif for murine B-cell stimulation is 5' purpurCpGpyrpyr 3' (Krieg *et al.*, 1995). Since then, it has been shown that the optimal CpG-motif may differ both for the parameter studied (Krieg, 2002), and between species (Bauer *et al.*, 2001; Takeshita *et al.*, 2001). In pigs, among several motifs tested, the CpG-motif 5' ATCGAT 3' was most efficient for induction of IL-6, IL-12, TNF-□ and blood leukocyte proliferation (Kamstrup, Verthelyi & Klinman, 2001). Furthermore, the CpG motif 5' GACGTT 3' also efficiently induced proliferation of blood leukocytes in several veterinary species including pigs, whereas in mice and rabbits, the CpG-motif 5' GACGTT 3' was the strongest inducer (Rankin *et al.*, 2001). Although the concept of an unmethylated CpG dinucleotide as a basis for immune stimulation by DNA holds true in most situations, introduction of vertebrate dsDNA without a CpG motif into cells can activate Stat1, Stat2 and MHC gene expression (Suzuki *et al.*, 1999). In addition, not all CpG-motifs are activatory and some can actually inhibit the stimulatory effects of CpG-DNA (Krieg, 2002), which has been interpreted as a mechanism whereby pathogens prevent activation of the vertebrate immune system (Hasslung *et al.*, 2003; Krieg *et al.*, 1998). Furthermore, DNA sequences without CpG dinucleotides can also have a suppressive effect on immune activation (Zeuner *et al.*, 2003).

The immunostimulatory activity of CpG-DNA in the form of ODN can be altered by using phosphorothioate instead of phosphodiester nucleotides. This increases the resistance to nucleases and thereby protects the ODN from degradation (Krieg, 2002). In a phosphorothioate-stabilized nucleotide, one of the non-bridging oxygens of the phosphate group is converted to sulphur. Phosphorothioate ODNs are 200 times more efficient than phosphodiester ODNs at B-cell activation (Krieg *et al.*, 1995) and they are more efficiently taken up by cells (Zhao *et al.*, 1993). The potency of a given CpG-motif may differ between the two forms and the phosphorothioate form has intrinsic non-sequence specific biological activity (Krieg, 2002). Another way to improve the efficacy of an ODN is to add poly-G sequences in the 5' and/or 3' ends, which is thought to facilitate

uptake, possibly by binding to scavenger receptors (Kimura *et al*., 1994; Lee *et al*., 2000; Pearson, Rich & Krieger, 1993; Pisetsky, 1996).

Mechanisms involved in the activation by IS-DNA

In contrast to free CpG-DNA molecules, immobilized CpG-DNA cannot activate lymphocytes (Krieg *et al*., 1995; Manzel & Macfarlane, 1999), which indicates that cell uptake is necessary. After uptake, CpG-DNA appears to locate to the endosomal compartment (Krieg, 2002). Treatment with bafilomycin A, monensin or chloroquine completely block CpG-signalling, which indicate that maturation and/or acidification of the endosomal compartment is a prerequisite for CpG-mediated activation of immune cells (Häcker *et al.*, 1998; Yi *et al.*, 1998). Many DNA-binding cell surface proteins have been found, but still no one with specificity for immunostimulatory sequences. A non-specific entry mechanism for CpG-DNA is supported by the findings that CpG-ODN and non-CpG-ODN equally well bind to cell membranes (Häcker *et al.*, 1998; Krieg *et al.*, 1995) and are incorporated into cells (Ahmad-Nejad *et al.*, 2002), and that an excess of non-CpG-ODN can abrogate the activity of CpG-ODN (Häcker *et al.*, 1998). The acidification that is required for signalling has however been associated with increased binding of CpG-ODN, but not of non-CpG-ODN, to cells (Hu, Sun & Zhou, 2003). Also, upregulation of TLR9, the receptor mediating CpG-signalling (see below), also increases the specific uptake of CpG-ODN (Takeshita *et al.*, 2001).

The role of TLR9 in activation by CpG was first shown using TLR9 knock-out mice that could not respond to CpG-DNA, but had unaltered responses to other PAMPs, such as LPS (Hemmi *et al.*, 2000). In human cells, transfection of non-TLR9 expressing cells with TLR9 confers responsiveness to CpG-DNA (Bauer *et al.*, 2001; Takeshita *et al.*, 2001), which demonstrates that TLR9 is involved in cellular activation by CpG-ODN in human cells. The TLR family, initially identified in Drosophila, is PRRs that confer responsiveness to many different conserved pathogen-associated molecules, synthetic compounds and endogenously derived ligands (Akira & Hemmi, 2003). The signalling pathways leading to altered gene transcription after triggering of a TLR are well worked out (Akira & Hemmi, 2003). For instance, activation of TLR will activate both NFκB and MAPK signalling pathways. Moreover, activation of NFκB-dependent genes by CpG-DNA has been shown to occur via activation of the PI3 kinase DNA-dependent protein kinase (DNA-PK) (Chu *et al.*, 2000). If and how signals activated by TLR9 and DNA-PK converge remains to be elucidated (Aderem & Hume, 2000), but recently the involvement of DNA-PK has been questioned (Hemmi *et al.*, 2003), possibly favoring the involvement of another PI3 kinase in CpG-mediated activation of immune cells (Ishii *et al.*, 2002).

Final proof of TLR9-mediated activation of type I IFN by CpG in human cells is still missing. Nevertheless, the ability to produce IFN-α in response to CpG-DNA correlates well with expression of TLR9 (Krug *et al.*, 2001b). The MAPK pathway known to be activated by TLR ligands, is involved in the CpG-mediated activation of Stat1, that independently of type I IFN signalling activates ISGF3, which initiates transcription of IRF7, thereby contributing to the induction of type

I IFN by CpG-DNA (Takauji *et al.*, 2002). The mechanisms that link activation of TLR9 and the activation of type I IFN genes remain to be identified. However, two kinases, IKK and TBK1, that are likely triggered by TLR were recently shown to activate the type I IFN transcription factors IRF3 and IRF7 (Fitzgerald *et al.*, 2003; Sharma *et al.*, 2003). Further studies will tell if this pathway is involved in the activation of type I IFN by CpG-DNA.

In humans, TLR9 is mainly expressed on B-cells and NIPC/PDC (Krieg, 2002). Some of the immunostimulatory effects of CpG-DNA are therefore direct effects due to the activation of these cells, but many are indirectly mediated by the cytokines, including type I IFN, produced by the TLR9-bearing NIPC/PDC (see above). In this way, the human NIPC/PDC are key cells in mediating the effects of CpG-DNA on the immune system. The situation may be somewhat different in mice where TLR9 expression is not limited to NIPC/PDC and B-cells. Little is known of the relevant expression of TLR9 in pigs (Shimosato *et al.*, 2003).

Use of IS-DNA in DNA vaccination

In general, an effective vaccine should activate both humoral and cellular immunity to the antigen in question, preferentially with a Th1 profile that includes efficient antibodies and Tc cells. Tc cells are activated by antigenic peptides in the context of MHC class I, which generally requires that the protein is synthesized in or introduced to the cytoplasm. On the other hand, Th cells are activated by peptides presented in the context of MHC class II on APC. Dependent on the cytokines produced by APC, including NIPC/PDC, Th1 or Th2 cells develop. The principle of immunization with DNA vaccines is that the antigen is delivered as a gene that will be expressed within the cytoplasm of the host cell. The antigen can therefore theoretically be presented on MHC class I, but also on MHC class II by APC that have endocytosed secreted antigen. In this way, DNA vaccination may provide a tool to effectively activate both cellular and humoral responses to delivered antigens as (reviewed in Srivastava & Liu, 2003).

Unlike purified protein antigens, infectious agents typically induce strong immune responses without the addition of adjuvants. An adjuvant, be it a separate molecule or an innate property of the antigen itself, will increase the immunogenicity of an antigen. There are many steps in the immunization process that are subject to improvement by adjuvants, including activation of innate immunity (Lövgren-Bengtsson & Fossum, 2002; Schijns, 2002). In the absence of adjuvants activating innate immunity, presentation of antigen may lead to Th2 development or tolerance to the antigen. Due to CpG-motifs in the plasmid backbone, DNA vaccines often have an intrinsic ability to activate innate immunity, resulting in production of cytokines and upregulation of costimulatory molecules on antigen-presenting DC. Cytokines, including type I IFNs, play an important role in this activation (Cull *et al.*, 2002; Rizza *et al.*, 2002; Tudor *et al.*, 2001).

Adjuvant IS-DNA can be used in several forms. A CpG-containing ODN can be used either in phosphodiester or phosphorothioate form (see above) and administered neat or encapsulated in liposomes, free or covalently linked to the

antigen (Krieg & Davis, 2001). At present, a limited number of studies have been performed in the pig, where CpG-ODNs were used as adjuvants in vaccines (Alcón *et al.*, 2003; Van der Stede *et al.*, 2002). Although many different formulations of antigen and DNA-based adjuvants have been tested, it is difficult to predict the immunological result of a given formulation, but the increasing knowledge of the molecular mechanisms involved will facilitate the design of new vaccines.

IFN-α inducing immune complexes and autoimmune disease

The type I IFN system may have an etiopathogenic role in the development of autoimmune disorders, such as systemic lupus erythematosus (SLE) (reviewed in (Rönnblom & Alm, 2003). Patients with the autoimmune rheumatic disease SLE often have an ongoing production of IFN-α, which in addition correlates to disease activity. Furthermore, treatment of patients with tumors or viral infections with IFN-α can give rise to autoantibodies and occasionally SLE. An etiopathogenic role of IFN-α in SLE is further suggested by the many immunomodulatory effects of this cytokine (see above). The elevated levels of IFN-α in SLE patients is likely due to the presence of endogenous IFN-α inducers in the patients. For instance, interferogenic IC in sera have been shown to activate production of IFN-α in PBMC from normal donors (Vallin *et al.*, 1999a). These IC consist of autoantibodies and DNA and are termed IFN-α inducing factor in SLE (SLE-IIF).

The exact origin of the non-antibody part of SLE-IIF is not known. The interferogenic activity of SLE-IIF could be mimicked by combining either SLE-IgG or anti-dsDNA antibodies with plasmid DNA, which could indicate that microbial DNA is the actual IFN-α inducer (Vallin *et al.*, 1999b). Indeed, methylation of all CpG in the plasmid abrogated the interferogenic activity, which indicates a similar mechanism of IFN-α activation by CpG-DNA and SLE-IIF, the antibody part mediating cell uptake of interferogenic DNA.

The non-antibody part of SLE-IIF could also be derived from dying cells that release material containing DNA. A possible role of apoptosis in the generation of SLE-IIF was investigated by Båve et. al., who showed that apoptotic cells in combination with purified IgG from SLE-patients could induce production of IFN-α in PBMC from normal donors (Båve, Alm & Rönnblom, 2000). Also material released from necrotic cells can activate production of IFN-α if combined with SLE-IgG (T. Lövgren, unpublished observations) In both cases, RNA seems to be of crucial importance because interferogenic activity of SLE-IgG was positively correlated to the presence of antibodies with specificity for RNA-binding proteins, and the interferogenic activity of the cell derived material was destroyed by RNase. However, apoptotic cells also released interferogenic material that was sensitive to Dnase (T. Lövgren, unpublished observations). Therefore, DNA and RNA released from dying cells in complex with autoantibodies may be the actual IFN inducers in SLE. Interestingly, SLE-patients have a wide range of autoantibodies with specificities for DNA, RNA and associated proteins that may form such interferogenic IC. Such interferogenic IC are especially prone to occur in

SLE patients, who have an abnormal a high rate of apoptosis and a defective clearance of apoptotic material (Herrmann *et al.*, 1998).

A common property of all SLE-related inducers named above is that they selectively activate production of IFN-α in NIPC/PDC (Båve *et al.*, 2001; Vallin *et al.*, 1999a; Vallin *et al.*, 1999b). Moreover, NIPC/PDC are also activated to produce IFN-α in response to poliovirus in combination with anti-poliovirus antibodies (Palmer *et al.*, 2000). The presence of antibodies in all these inducers activating NIPC/PDC suggests the involvement of antibody-binding proteins, such as FcR, in the activation process. Interestingly, it has been demonstrated that antibodies to FcγRII (CD32) can inhibit the activation of IFN-α production in PBMC elicited by serum from SLE patients (Batteux *et al.*, 1999).

FcγR on NIPC/PDC

Whereas the NIPC/PDC appear to lack expression of FcγRI (CD64) and FcγRIII (CD16) (Dzionek *et al.*, 2000; Svensson *et al.*, 1996b), the expression of FcγRII is unclear (Båve *et al.*, 2001; Olweus *et al.*, 1997). A low but functional expression can however not be excluded, considering the inhibitory effect of antibodies to FcγRII on the IgG-assisted IFN-α production in PBMC (see above). Also, depletion of cells binding to IgG decreased the IFN-α production induced by bacteria (Rönnblom, Forsgren & Alm, 1983).

Membrane bound FcR are expressed by the majority of immune cells and enable them to react to antibodies bound to antigens. The outcome of triggering an FcR that harbors the immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic part is cell activation, including antibody-dependent cell-mediated cytotoxicity, release of cytokines and proteases, as well as clearance of IC and phagocytosis of opsonized antigens. Conversely, activation of FcR with an immunoreceptor tyrosine-based inhibition motif (ITIM) will down-regulate the immune response, e.g. by antibody feed-back in B-cells. In this way, FcR are responsible for many effector functions initiated by humoral immunity, but also regulation of the immune response (Ravetch & Bolland, 2001). The FcγRII is in humans represented by three different genes, a, b and c which in turn have individual isoforms (Zola *et al.*, 2000). FcγRIIa and FcγRIIc are ITAM-bearing activatory receptors whereas FcγRIIb is an ITIM-bearing inhibitory receptor. Activation of FcR with ITAM results in the phosphorylation of a residue in ITAM which will activate kinases of the SYK and SRC families thereby connecting the transduced signal to common activation pathways, including the MAPK and PI3K pathway (Daëron, 1997; Takai, 2002). Activation of inhibitory FcγRIIb induces phosphorylation of the ITIM that will recruit SH2-domain containing proteins such as SH2 containing inositol phosphate phosphatase (SHIP) that will down regulate cellular activities (Daëron, 1997; Ravetch & Bolland, 2001; Takai, 2002). These different properties of FcγRIIa/c and FcγRIIb make it important to determine their presence on NIPC/PDC and possible function in the activation of IFN-α production in these cells.

Aims of the study

The present thesis focuses on the activation of IFN-α production by IS-DNA or interferogenic immune complexes. One reason for this is to better understand the mechanisms involved in the activation of production of IFN-α, which is an important cytokine in regulation of the immune response against microbial infections, but also against 'self' in autoimmune disorders. Another reason is to identify more efficient interferogenic IS-DNA that can be used as adjuvants in vaccines. The specific aims in this thesis were the following:

To determine if the vaccine vector pcDNA3 can induce cytokine production in porcine PBMC and to what extent certain CpG-motifs and the methylation status of CpG dinucleotides in the plasmid contribute to the IFN-α inducing ability (Paper I).

To determine if CpG-ODNs identified in the serum of SLE-patients could induce production of IFN-α in human PBMC from normal donors and to further examine the structural requirements in terms of strandness, sequence and cytosine methylation for the IFN-α inducing ability (Paper II).

To further examine the interferogenic properties of CpG-containing ODNs in porcine PBMC with respect to the importance of strandness, sequence, cytosine methylation, phosphorothioate/phosphodiester composition and presence of poly-G sequences (paper III).

To determine the identity of the human and porcine PBMC that produce IFN-α in response to IS-DNA, especially if they correspond to the NIPC/PDC (paper II and III).

To study the role of FcγR in the IFN-α production induced by interferogenic immune complexes and to identify the different isoforms of FcγRII on the responding human NIPC/PDC (paper IV).

Table 1. *Inducers used to activate production of IFN-α*

Name	Type of inducer	Comments or Sequence 5' to 3' [1,2]
pcDNA3	Plasmid DNA	Two AACGTT motifs in ampR gene
Mutant A	Plasmid DNA	Two AAGCTT motifs in ampR gene
Mutant B	Plasmid DNA	AACCTT and AAAGAT in ampR gene
PcDNA3kH	Plasmid DNA	AmpR gene replaced by kanR gene
pET11c	Plasmid DNA	Two AACGTT motifs in ampR gene
Poly I:C	dsRNA	
H	ODN	TTT TCA ATT CGA AGA TGA AT
H^{A}	ODN	TTT TCA ATT AGA AGA TGA AT
H^{GC}	ODN	TTT TCA ATT GCA AGA TGA AT
H^{met}	ODN	TTT TCA ATT Me^5CGA AGA TGA AT
$H^{G\text{-}tail\ 1}$	ODN	TTT TCA ATT CGA AGA TGA ATG GGG G
$H^{G\text{-}tail\ 2}$	ODN	GGT TCA ATT CGA AGG GGG GG
$H^{G\text{-}tail\ 3}$	ODN	GGT TTT CAA TTC GAA GAT GAA TGG GGG G
I	ODN	ATT CAT CTT CGA ATT GAA AA
I^{T}	ODN	ATT CAT CTT CTA ATT GAA AA
I^{GC}	ODN	ATT CAT CTT GCA ATT GAA AA
I^{met}	ODN	ATT CAT CTT Me^5CGA ATT GAA AA
I^{metx3}	ODN	ATT Me^5CAT Me^5CTT Me^5CGA ATT GAA AA
RIII	ODN	ATT GGA AAA CGT TCT TCG GG
A2	ODN	GCT AGA CGT TAG CGT
$A2^{GC}$	ODN	GCT AGA GCT TAG GCT
2216	ODN	ggG GGA CGA TCG TCg ggg gG
2216ddiester	ODN	GGG GGA CGA TCG TCG GGG GG
D19	ODN	ggT GCA TCG ATG CAG ggg gg
D19diester (D25)	ODN	GGT GCA TCG ATG CAG GGG GG
MM1	ODN	ggG GTC ATC GAT GAg ggg gG
A.pp	Bacteria	Actinobacillus pleuropneumoniae
HSV	Virus	Herpes simplex virus
ADV	Virus	Aujeszky's disease virus
SV	Virus	Sendai virus
SLE-IgG+apoptotic cells	Interferogenic IC	SLE-IgG in combination with apoptotic U937 cells

[1] Relevant CpG dinucleotides, the modified CpG dinucleotides and methylated cytosines (Me^5) are underlined.

[2] Nucleotides with phosphodiester backbone are indicated with capital letters and phosphorothioate nucleotides with small letters.

Results and discussion

DNA sequences that induce IFN-γ in human and porcine PBMC (papers I, II, III)

At the beginning of these studies, plasmid-based DNA vaccines were evaluated in pigs (Gerdts *et al.*, 1997; Somasundaram *et al.*, 1999; van Rooij *et al.*, 1998), but little was known about the cytokine inducing capacity of the vectors used. In human cells, the concept of IS-DNA was established (Pisetsky, 1996), but there was incomplete information regarding the characteristics of IS-DNA required to induce production of IFN-γ (see below). Therefore, nucleotide sequences that contribute to the interferogenicity of DNA were studied, using plasmid DNA in porcine PBMC (paper I) or using ODNs in human and porcine PBMC (paper II and III. The examined inducers are listed in Table 1.

The IFN-γ inducing capacity of the plasmid pcDNA3 in porcine PBMC

Early studies in the field of IS-DNA highlighted the DNA in the ampicillin resistance (ampR) gene as the carrier of immunostimulatory sequences (ISS) of many DNA vaccine vectors . The presence of two hexameric ISS (5' AACGTT 3') in the ampR were shown to be important for several immunostimulatory effects in man and mice, including up-regulation of type I IFN and IL-12 mRNA synthesis in human macrophages (Roman *et al.*, 1997; Sato *et al.*, 1996). Therefore, the ability of plasmid DNA to activate cytokine production in pigs and the relative importance of the earlier identified ISS in the ampR gene were studied (paper I).

The DNA vaccine vector pcDNA3 was found to readily induce production of IFN-γ in porcine PBMC, but only if pre-incubated with lipofectin. Whether pre-incubated with lipofectin or not, the plasmid only induced low levels of IL-6. This is contrast to the induction of IL-6 found when porcine cells were stimulated by CpG-ODN D19 (Kamstrup, Verthelyi & Klinman, 2001), or when murine spleen cells were exposed to bacterial DNA (Yi *et al.*, 1996). The levels of IFN-γ induced were generally higher than those obtained with ADV, lipofected *Actinobacillus pleuropneumoniae* or lipofected poly I:C. To evaluate the importance of the earlier identified ISS (5' AACGTT 3') on the interferogenic ability of pcDNA3, two mutants were generated by site-directed mutagenesis. In mutant A both ISS were changed to 5' AAGCTT 3' and in mutant B the two ISS were changed to 5' AACCTT 3' and 5' AAAGAT 3', respectively. Both mutated forms of pcDNA3 induced IFN-γ production to the same extent as the original plasmid, or even higher. Thus, the interferogenic activity of pcDNA3 in porcine PBMC is not dependent on the earlier identified ISS (5' AACGTT 3') in the ampR gene. To determine whether other sequences in the ampR gene were of importance, the entire ampR gene was replaced with the kanamycin resistance (kanR) gene, but this did not alter the IFN-γ inducing capacity of the plasmid. Apart from the two ISS in the ampR gene, the DNA sequence of pcDNA3 contains many other potential immunostimulatory CpG-motifs, with other flanking bases. To evaluate their overall impact on the interferogenicity of the plasmid, all CpG dinucleotides in the plasmid were methylated by SssI

methylase. This modification totally abolished the IFN-α inducing ability of the plasmid, demonstrating that unmethylated CpG dinucleotides are crucial for the activation of IFN-α production in porcine PBMC by plasmid DNA. Taken together, other still unknown CpG-motifs must contribute to the IFN-α inducing capacity of the plasmid pcDNA3. These in vitro results have been confirmed in vivo using a tissue chamber model where the plasmid pcDNA3 did not affect IL-6 production and induced production of IFN-α only in the unmethylated form (Johansson *et al.*, 2002). Furthermore, plasmid expression was not necessary for the interferogenic activity of plasmid DNA, because a prokaryotic expression vector induced IFN-α in porcine PBMC to the same extent as pcDNA3. Taken together, these results show that the induction of IFN-α by plasmid DNA depends on unmethylated CpG-motifs and that porcine cells may have the same type of detection system for bacterial DNA as reported for man and mice.

The IFN-α inducing ability of ODN in human and porcine PBMC (paper II and III)

Experiments with CpG-DNA are often carried out using ssODN to determine the optimal sequence. Eukaryotic DNA, as well as both bacterial and plasmid DNA, are generally in the ds form, whereas viral DNA exists in both forms. Furthermore, eukaryotic ssDNA could occur as a result of apoptosis or necrosis. Therefore, we tested both ds and ss forms of ODNs when we evaluated the importance of CpG dinucleotides in the IFN-α inducing ability. The initial ODN sequence studied (designated ODN H) was based on a DNA sequence identified in the circulation of a patient with SLE (Sato *et al.*, 1999), an autoimmune disease where IC containing IS-DNA are thought to act as endogenous type I IFN inducers (Vallin *et al.*, 1999b). The sequence of ODN H is 5'-TTTTCAATTCGAA-GATGAAT 3' and was chosen because it contains a CpG motif and it had earlier been shown to induce proliferation and production of IL-12 and IFN-γ as well as up-regulation of MHC class II and ICAM I expression in human mononuclear cells. These activities were dependent on the CpG dinucleotide, because inversion of the CpG to GpC abolished them (Sato *et al.*, 1999).

Both in human (paper II) and porcine (paper III) PBMC cultures, the ODN H and its complementary strand ODN I, as well as derivatives of them (see Table 1), required preincubation with lipofectin in order to induce production of IFN-α. The importance of lipofectin indicates that cellular uptake of IS-DNA is necessary for activation of IFN-α production, which is further discussed below. The optimal concentrations of lipofectin varied greatly between the two experimental systems. In general, in human PBMC higher concentrations of both components were required to achieve optimal induction of IFN-α (50 μg/ml ODN and 25μg/ml lipofectin). In the porcine system, titration of ODN and lipofectin revealed that 2.5 μg/ml lipofectin was optimal for all ODN concentrations tested and more than 50 μg/ml ODN did not further increase the ability of ODN to induce IFN-α. In general, ODN H, both in ss and ds form, induced 10-20 per cent of the levels of IFN-α activated by the viral inducer HSV in human PBMC. In contrast, in the porcine system, ODN H, and all other CpG-ODNs tested, induced similar or higher levels than the herpes virus ADV.

The importance of the CpG dinucleotide in ODN H for IFN-α induction was evaluated by studying the effect of various alterations of the ODN on its IFN-α inducing capacity. Replacement of the cytosine with an adenine in the CpG motif of ssODN H (H^A), or the guanine with a thymine in the CpG motif of the complementary ssODN I (ODN I^T), severely reduced the induced levels of IFN-α in human PBMC, and essentially abolished the induction of IFN-α in porcine PBMC. Furthermore, the dsODN H^A-I^T, formed by combining ODN H^A and ODN I^T, had a severely impaired IFN-α inducing ability in both human and porcine PBMC cultures. Thus, cytosines and/or guanines are important for the interferogenic activity of both ss and ds forms of these ODNs in both human and porcine PBMC.

In the human experimental system, inversion of the CpG dinucleotide to GpC in ssODN H had no effect, whereas the same modification in ssODN I increased the interferogenic activity in comparison with the unmodified ODNs. This clearly shows that the IFN-α inducing capacity in human PBMC of lipofected ssODN H or ssODN I is not dependent on the CpG dinucleotide. In the porcine experimental system, inversion of the CpG to GpC in ssODN H and ssODN I severely hampered the IFN-α inducing capacity, but some of the activity was clearly retained in comparison with the ODN H^A and ODN I^T. However, inversion of CpG to GpC in the interferogenic CpG-ODN A2 totally abolished the IFN-α inducing ability in porcine cells. The finding that the GpC-modification had an much more pronounced effect on the interferogenic ability of ssODN in porcine cells may indicate species differences in the recognition of IS-DNA.

The interferogenic activity of ssODN H^{GC} and ssODN I^{GC} in human PBMC was essentially abolished when these two ODNs were hybridized to form the dsODN H^{GC}-I^{GC}. In the porcine system, dsODN H^{GC}-I^{GC} had the same impaired ability to induce IFN-α as ssODNs with this modification. This illustrates an importance of CpG dinucleotides for the full interferogenic ability of dsODN H-I in both human and porcine PBMC.

A major difference between microbial DNA and vertebrate DNA is not only the frequency of CpG dinucleotides, but also the methylation status of the cytosine in the CpGs (see the Introduction). The importance of methylation on the IFN-α inducing ability of CpG-ODN was examined, using derivatives of ODN H and the complementary ODN I, where the cytosine in the CpG had been methylated. The results showed that, the ODN H^{met} had a reduced ability to induce IFN-α in human PBMC compared to ODN H, whereas the potency of ODN I was paradoxically strongly increased by methylation. This confirms that the IFN-α inducing capacity of lipofected ssODN I in human PBMC is not dependent on the unmethylated CpG dinucleotide. Furthermore, the requirement for an unmethylated CpG for the interferogenic ability differs between ssODNs. In contrast, methylation of the CpG inhibited the IFN-α inducing capacity of both ssODN H and ssODN I in the porcine system. Again, this indicates species differences in the reactivity to ss IS-DNA. When the ODN H^{met} and ODN I^{met} were combined to form dsODN H^{met}-I^{met}, the interferogenic ability was clearly reduced in comparison with the unmodified dsODN H-I, in both human and porcine

PBMC. This illustrates the importance of unmethylated CpG dinucleotide for the interferogenic ability of dsODN H-I in both human and porcine PBMC.

In the human system, combining the inactive ODN H^{met} with the interferogenic ODN I^{met} to dsODN H^{met}-I^{met} resulted in a loss of interferogenic ability (see above). This prompted us to investigate whether methylation of the CpG in only one of the strands in the dsODN H-I was sufficient to impair the interferogenic activity in human PBMC. It was found that the hemimethylated dsODN H-I^{met}, but not the hemimethylated dsODN H^{met}-I retained the ability to induce IFN-α. The interferogenic activity of dsODN H-I is thus dependent on the unmethylated CpG in ODN H. The results furthermore revealed that the silencing effect of ODN H^{met} was not due to a general inhibitory effect, because the interferogenic ability of an unrelated ODN (ODN RIII, see Table 1) was not affected (Fig. 3, paper II).

The fact that certain ssODNs without unmethylated CpG dinucleotides had potent IFN-α inducing ability in human PBMC suggests that eukaryotic DNA should contain numerous potentially interferogenic sequences. When cells die, either by apoptosis or necrosis, the DNA is fragmented and released, which could expose the immune system to interferogenic sequences. These findings shed light on the possible origin of the nucleic acids in the IFN-α inducer found in the serum of SLE-patients, which is discussed below (Results and discussion of paper IV).

The impact of nucleotide backbone and poly-G sequences on the interferogenic ability of ODNs in porcine PBMC (paper III)

Phosphorothioate ODNs are often used to increase nuclease-resistance, which potentiates and prolongs the adjuvant effects. To evaluate the importance of phosphorothioate nucleotides in interferogenic ODNs, we used earlier identified interferogenic ODNs that consist of a chimera of phosphodiester and phosphorothioate nucleotides. The interferogenic activity of these ODNs was compared to ODNs with the same nucleotide sequence, but with a complete phosphodiester backbone. The chimeric CpG-ODNs 2216 (Krug *et al.*, 2001a) and D19 (Verthelyi *et al.*, 2001) induced high levels of IFN-α in porcine PBMC in comparison with ADV and lipofected dsODN H-I. They were furthermore strikingly independent on preincubation with lipofectin and potent inducers of IFN-α at low concentrations (5μg/ml). To evaluate the importance of phosphorothioate nucleotides in this activation, complete phosphodiester ODNs with the same sequence (2216d and D19d) were synthesized. Also these ODNs were potent IFN-α inducers and independent of lipofectin. These results are in line with the described activation of IFN-α production in human PBMC cultures by ODN 2216, both as chimera (Krug *et al.*, 2001a) and complete phosphodiester (unpublished observation). Therefore, the backbone composition, phosphorothioate/-diester chimera vs. complete phosphodiester of these particular ODNs was not decisive for the IFN-α inducing capacity.

The fact that ODN H and I, but not 2216d and D19d required lipofectin to induce IFN-α prompted us to further investigate the requirements for IFN-α induction. Lipofectin forms cationic liposomes with nucleic acids, which increases

cellular uptake of ODN (Bennett *et al.*, 1992; Hartmann *et al.*, 1998). The need for lipofection therefore suggests that cellular uptake of ODN is required for IFN-α induction, which is in line with the finding that endocytosis of CpG-ODNs is required for their immune stimulatory effect (Ahmad-Nejad *et al.*, 2002; Häcker *et al.*, 1998). The ODNs 2216d and D19d contain a central sequence with a CpG motif and flanking poly G-sequences. These poly-G sequences are not present in the CpG-ODNs ODN H or ODN I. Poly G-sequences may form quarternary structures that facilitate uptake of ODNs, possibly by binding to scavenger receptors (Kimura et al., 1994; Lee et al., 2000; Pearson, Rich & Krieger, 1993; Pisetsky, 1996). This may explain why 2216d and D19d, and not ODN H and ODN I could induce production of IFN-α without preincubation with lipofectin. To test the effect of poly G-sequences, various numbers of flanking guanines were added to ODN H (ODNs $H^{G\text{-}tail\ 1}$, $H^{G\text{-}tail\ 2}$ and $H^{G\text{-}tail\ 3}$; see Table 1) and the modified ODNs were tested for their ability to induce IFN-α production in porcine PBMC. In the absence of lipofectin, no or very low levels of IFN-α were detected in cultures stimulated by ODN H or by ODN H that contained additional guanines. However, the presence of poly-G sequences strongly increased, approximately 1.5 to 5-fold, the IFN-α inducing capacity of lipofected ODN H. Thus, the addition of poly-G sequences does not seem to eliminate the need for lipofectin, but increases the levels of IFN-α induced by the ODN.

In conclusion, the exact role of phosphorothioate nucleotides or poly G-sequences for the interferogenicity of a CpG-ODN is still elusive. The presence of poly G-sequences nevertheless seemed to increase the interferogenic ability of CpG-ODNs. Phosphorothioate ODNs have been used as efficient vaccine adjuvants (Zhang *et al.*, 2003), but are also associated with long-lasting IFN-α and IL-12 production, that can be harmful or even break tolerance (Segal, Chang & Shevach, 2000). The potent interferogenic phosphodiester ODNs that were identified may therefore be of value as vaccine adjuvants, because they are more short-lived in vivo and may therefore have fewer side effects.

The identity of the human and porcine cells producing IFN-α in response to CpG-DNA (paper II and III)

Both human and porcine PBMC produced considerable amounts of type I IFN in response to CpG-DNA, but the responding cell types were unknown at the start of the present investigations. In human blood, there are two major IPC, the monocytes and the NIPC/PDC. In pigs the situation is less clear, but a cell type that resembles the human NIPC has been identified (Charley & Lavenant, 1990; Nowacki & Charley, 1993) (see the Introduction). In papers II and III the IPC were identified by simultaneous flow cytometric (FCM) analysis of the expression of cell surface markers and intracellular IFN-α in cells stimulated with CpG-DNA. The human PBMC responding to CpG-DNA were characterized using antibodies previously employed to phenotype the NIPC/PDC (Olweus *et al.*, 1997; Rissoan *et al.*, 1999; Svensson *et al.*, 1996b). The porcine PBMC producing IFN-α in response to CpG-DNA were phenotyped using antibodies reactive to specific lineage markers and markers used in the earlier phenotyping of porcine IPC activated by TGEV (Nowacki & Charley, 1993; Riffault *et al*., 1997; Riffault *et*

al., 2001). Furthermore, the porcine cells expressing IFN-α mRNA were detected by in situ hybridization (ISH), using a biotinylated poIFN-α cRNA probe and a sensitive catalyzed amplification system.

Human PBMC were stimulated with CpG-DNA in the form of lipofected ssODN H or lipofected dsODN H-I, using HSV as a control (paper II). Porcine PBMC were stimulated with CpG-DNA in the form of ODN 2216 or lipofected pcDNA3, using ADV as a control (paper III). The results demonstrate that the human IPC did not express CD19 and the porcine IPC did not express CD21, and none of them expressed CD3. This indicates that these IPC are not B- or T-cells. Both the porcine and human IPC studied here expressed MHC class II and CD4, although the human IPC had a weak CD4 expression compared to that on other human PBMC (Fig. 4 paper II). Porcine IPC expressed high levels of CD4, comparable to the brightest CD4+ populations of porcine PBMC (Fig. 7 paper III). CD4 is a marker present on many cell types, including Th cells, DC and monocytes. Neither porcine nor human IPC expressed CD14, which shows that they are not monocytes. Taken together, the human and porcine IPC responding to CpG-DNA phenotypically clearly resembles each other.

Further analysis of the human IPC stimulated by CpG-DNA revealed that they also expressed CD40, CD83, IL-3R and CD36, but not CD11c (paper II), which shows that they are identical to the earlier described human NIPC/PDC (Siegal *et al.*, 1999; Svensson *et al.*, 1996b). In paper IV, expression of FcεRII on human NIPC/PDC was also demonstrated. FcγRIIIA has been demonstrated on porcine cells (Sweeney, Halloran & Kim, 1996), including myeloid DC (Carrasco *et al.*, 2001), but the expression of this and other FcγR on porcine IPC has not been reported. Given the pivotal role of FcγRIIa in the activation of human NIPC/PDC (see Introduction and paper IV), it is of great interest to examine the expression and function of this FcR on porcine IPC. Interestingly, early studies on porcine leukocytes indicated that ability to produce IFN correlated with expression of FcRs (Salmon *et al.*, 1989).

Analysis of the porcine IPC responding to CpG-DNA furthermore showed that they expressed intermediate levels of SWC3 (Fig. 7 paper III). The SWC3 molecule is a 90-115 kDa protein whose function is unknown, although it exhibits homology to members of the family of signal regulatory proteins (Alvarez *et al.*, 2000). The expression of SWC3 on porcine IPC is in agreement with earlier studies using TGEV as inducer (Riffault *et al.*, 1997; Riffault *et al.*, 2001). TGEV-activated porcine IPC were recently thoroughly characterized by Summerfield and colleagues (A. Summerfield, personal communication). They observed that porcine PBMC producing IFN-α in response to TGEV had an intermediate expression of SWC3 and MHC class II and a strong expression of CD4. The TGEV-activated IPC lacked the monocytic marker CD14 and markers for T- and B-cells. Therefore, the porcine PBMC producing IFN-α in response to CpG-DNA phenotypically resemble the IPC activated by TGEV.

The porcine IPC responding to CpG-DNA also expressed CD2, which has not been reported for porcine IPC before. Seemingly in contrast with this are earlier studies, where depletion of CD2+ cells did not hamper the ability of porcine PBMC to produce IFN-α in response to TGEV (Nowacki & Charley, 1993). The

reason for this discrepancy is not known. The CD2 expression by porcine IPC responding to CpG-DNA is however in agreement with the expression of CD2 on human NIPC/PDC (Comeau *et al.*, 2002). The CD2 molecule is expressed by many different cell types including porcine T- and NK-cells (Yang & Parkhouse, 1996), but also on porcine thymic DC (Salmon *et al.*, 2000) and human monocyte-derived DC (Di Pucchio *et al.*, 2003).

The hematopoietic origin of human NIPC/PDC has been debated (Brière *et al.*, 2002). Recent work has shown that NIPC/PDC, but not monocyte-derived DC expressed immunoglobulin-like transcripts (Rissoan *et al.*, 2002), which is indicative of a lymphoid origin of NIPC/PDC. This is now a fairly accepted view, but it has also been suggested that NIPC/PDC can convert to a myeloid phenotype (Comeau *et al.*, 2002). The porcine surface molecule SWC3 is regarded as a pan-myeloid marker (Summerfield & McCullough, 1997) that is present on 80-90 per cent of monocytes and granulocytes, but also on 5 per cent of lymphocytes (Thacker *et al.*, 2001). Therefore, the expression of SWC3 on porcine IPC does not permit a clear assignment of the cells as myeloid or lymphoid. However, the porcine IPC activated by CpG-DNA are not likely monocytic DC, because monocytes or monocyte-derived porcine DC failed to produce IFN-α in response to CpG-DNA and ADV (Johansson *et al.*, 2003). The porcine monocyte-derived DC could however produce IFN-α in response to SV. This is reminiscent of the situation in human blood, where monocytes produce IFN-α in response to SV, but not in response to CpG-DNA (Fitzgerald-Bocarsly, 2002).

The human NIPC responding to CpG-ODN constituted approximately 0.1 per cent of total PBMC, as determined by FCM, which is in line with earlier estimations of this population (Svensson *et al.*, 1996b). The frequency of IPC detected by in situ hybridization in porcine PBMC was ≤ 0.1 per cent, when CpG-ODN, lipofected plasmid DNA or ADV were used as inducers. All IPC were strongly labelled suggesting a uniformly high content of IFN-α mRNA. Estimation of the frequency of IPC by FCM revealed that among porcine PBMC activated by CpG-ODN or lipofected plasmid, up to 0.3 per cent were stained by anti-IFN-α antibodies. In contrast, approximately ten-fold lower frequencies were obtained by this method when PBMC were activated with ADV. In general, ADV induced much lower levels of IFN-α than CpG-DNA, which may reflect the lower numbers of cells activated (paper III). This low frequency of virus-activated IPC is in line with the frequency of IPC activated in porcine PBMC stimulated with TGEV (1 per 10^4 PBMC) (Nowacki & Charley, 1993) and also with that of a recent study of the phenotype of TGEV-activated IPC (A. Summerfield, personal communication). This suggests that CpG-DNA is a stronger activator of porcine NIPC than virus.

In summary, the characterization of IPC has revealed that the earlier described NIPC/PDC are the only human cell type in PBMC producing IFN-α in response to CpG-DNA. This is line with other recent studies (Krug *et al.*, 2001a; Krug *et al.*, 2001b) using phosphodiester/phosphorothioate chimeric CpG-ODNs that do not require lipofectin for activity. Our data showed that the same NIPC/PDC are also selectively activated by lipofected CpG-containing phosphodiester ODNs, that by themselves are inactive. The porcine blood cells producing IFN-α in response

to phosphodiester/phosphorothioate chimeric CpG-ODN or lipofected plasmid DNA resembles the human NIPC/PDC and may therefore constitute the porcine counterpart to this infrequent but highly efficient IPC, identified in both man and mice. It appears that the NIPC/PDC in both man and pigs are unique in their ability to produce IFN-α in response to DNA, provided that the DNA is taken up by cells and/or that certain signals are delivered to the NIPC/PDC. This necessary assistance can be provided to the NIPC/PDC by lipofectin or antibodies (see below).

The mechanism of IFN-α induction in human NIPC/PDC by interferogenic immune complexes (paper IV)

The interferogenic IC in SLE serum, commonly referred to as SLE-IIF, models of these IC, or poliovirus in combination with anti-poliovirus antibodies all selectively activate production of IFN-α in NIPC/PDC (see the Introduction). The requirement for IgG-assisted activation prompted us to further investigate the role of SLE-IgG and FcγR on NIPC/PDC in the activation of IFN-α production.

First, we investigated the importance of the Fc-part of SLE-IgG in the activation of IFN-α production in cultures of human PBMC by apoptotic U937 cells in combination with SLE-IgG. The SLE-IgG was pretreated with papain or pepsin to generate Fab fragments or $F(ab')_2$ fragments, respectively. The combination of apoptotic cells and intact SLE-IgG, but not Fab or $F(ab')_2$ fragments of SLE-IgG, induced IFN-α production, which suggests that the Fc-part of SLE-IgG is important in this activation.

Aggregated IgG is known to bind to and block even low affinity FcR. To test if aggregated IgG could inhibit IFN-α production in PBMC, untreated or heat-aggregated normal IgG was added to the PBMC cultures. It was found that such IgG, especially in the heat-aggregated form, severely hampered the interferogenic activity of SLE-IgG in combination with apoptotic cells. This suggests that FcγR binding by other IgG molecules can inhibit the activation of IFN-α production by interferogenic IC. Interestingly, also the IFN-α production induced by CpG-ODN 2216 and HSV was inhibited by aggregated IgG, suggesting that FcR-binding can inhibit the IFN-α production stimulated by principally different IFN-α inducers that are not know to depend on FcR for induction. The aggregated IgG may therefore generally down-regulate production of IFN-α in NIPC/PDC .

To determine if any particular types of FcγR were involved in the activation of IFN-α by the combination of SLE-IgG and apoptotic cells, we added antibodies that recognize and block FcγRI (CD64), FcγRII (CD32) or FcγRIII (CD16) to the PBMC cultures. The interferogenic activity of SLE-IgG in combination with apoptotic cells was efficiently inhibited by the anti-FcγRII mAb, whereas anti-FcγRI or anti-FcγRIII mAb hade no inhibiting effect. F(ab) fragments of anti-FcγRII mAb were sufficient to block the activation, which suggests that cross-linking of the FcR or the Fc-portion of the mAb was not necessary to achieve the inhibition. The monoclonal antibody (IV:3) used to inhibit the IFN-α production recognizes FcγRIIa, but probably not FcγRIIc (Metes *et al.*, 1998) and not FcγRIIb (Van Den Herik-Oudijk *et al.*, 1994). This suggests the involvement of

especially the FcγRIIa in the activation of IFN-α by the combination of SLE-IgG and apoptotic cells. In contrast, the induction of IFN-α induced by HSV or 2216 was not affected by the mAb IV:3, indicating that they are not using FcγR to induce IFN-α. However, the anti-FcγRII mAb AT:10 that recognizes all three isoforms FcγRIIa/b/c, strongly inhibited the IFN-α induction by all three inducers. This suggests that the mAb AT:10 acted in a similar way as aggregated IgG, causing a general inhibition of the production of IFN-α in NIPC/PDC, possibly by activating the inhibitory FcγRIIb.

The involvement of a FcγRII in the activation or inhibition of IFN-α production suggested that the responding cells should express this receptor. Earlier studies on the expression of FcγRII on NIPC/PDC have not been conclusive (see the Introduction). Therefore, our findings prompted us to reinvestigate the expression of FcγRII on NIPC/PDC. This was done by simultaneous staining of fresh PBMC for FcγRII using the mAb FLI8.26 that recognize FcγRIIa, b, c combined with staining for the specific NIPC/PDC marker BDCA-2. We could demonstrate that the NIPC/PDC constituted about 0.5 per cent of PBMC, and that approximately 50 per cent of NIPC/PDC expressed intermediate levels of FcγRII. Because only a subpopulation of NIPC/PDC expressed FcγRII, we examined whether the actual cells producing IFN-α expressed FcγRII. To this end, PBMC were stimulated with HSV, an inducer not known to engage this receptor, and then simultaneously stained for FcγRII (mAb FLI8.26 or IV:3) and intracellular IFN-α. A clear but low expression of FcγRII was seen on essentially all IPC, which demonstrates that NIPC/PDC in fact are capable of expressing FcγRII. Because all actual IPC expressed FcγRII, the NIPC may constitute a subpopulation of the BDCA-2-defined PDC, possibly defined by FcγRII expression. In accordance, it has been shown that only 10-50 per cent of the BDCA-2+ cells are capable of producing IFN-α in response to HSV (Blomberg *et al.*, 2003).

The expression of the three different isoforms FcγRIIa/b/c on NIPC/PDC was further analyzed by RT-PCR. A cell population enriched for BDCA-4 positive NIPC/PDC was first generated from a pool of PBMC by magnetic separation. These cells were further purified by fluorescent activated cell sorting (FACS) for BDCA-2 positive cells, resulting in almost 100 per cent pure BDCA-2+ cells. Messenger RNA prepared from these cells or from unsorted PBMC was then reverse transcribed to cDNA. Measurement of the three different FcγRII transcripts by PCR showed that cDNA from unsorted PBMC contained transcripts for all three isoforms, whereas cDNA from the BDCA-2+ cells only contained the FcγRIIa transcript. Consequently, NIPC/PDC appear to selectively express the FcγRIIa, but not other FcγRs.

The unique expression of FcγRIIa on NIPC/PDC and the inhibitory effect of the mAb IV:3 suggest that FcγRIIa is involved of in the activation of IFN-α production induced by the combination of SLE-IgG and apoptotic cells. The absence of the inhibitory FcγRIIb on NIPC/PDC furthermore suggests that this FcγRII is not involved in the general inhibition of IFN-α production by the mAb AT:10 or heat-aggregated IgG. Instead the activatory FcγRIIa may under certain circumstances be inhibitory. In fact, it has recently been shown that the ITAM of

FcγRIIa can also activate the phosphatase SHIP (Huang *et al*., 2003; Nakamura, Malykhin & Coggeshall, 2002), which can inhibit various types of cellular activation pathways e.g. NFκB-induced gene transcription (Tridandapani *et al.*, 2002). Therefore, strong activation of FcγRIIa by aggregated IgG or the pan anti-FcγRII mAb AT:10 may cause activation of SHIP that mediates down-regulation of IFN-α production in NIPC/PDC.

The active components in the interferogenic IC are possibly DNA or RNA, as demonstrated by DNAse (Vallin *et al.*, 1999a) or RNAse (T. Lövgren, unpublished observations) treatment. The antibodies in interferogenic IC may trigger FcγRIIa-mediated phagocytosis, followed by intracellular recognition of immunostimulatory nucleic acids. Such activation may be conferred by TLR9 that binds CpG-DNA at the inside of intracellular vescicles (Ahmad-Nejad *et al.*, 2002), or possibly by other TLRs present in NIPC/PDC. The signalling pathways from FcγRIIa may also be important in the eventual activation of IFN-α gene expression induced by interferogenic IC (see the Introduction).

The FcγRIIa on NIPC/PDC probably has a more general function than mediating activation by the special IC in autoimmune diseases, such as SLE. It may participate in the activation of NIPC/PDC by IC formed by Ig and virus (Palmer *et al.*, 2000) and perhaps other microorganisms. Viral infection may also activate formation of autoantibodies (Hunziker *et al.*, 2003) that can react with material from dying cells. Tissue damage and concomitant release of apoptotic or necrotic material, as a result of e.g. viral infection, may in the presence of autoantibodies give rise to interferogenic IC that can activate production of IFN-α in NICP/PDC. Therefore, the NIPC/PDC have the ability to react to both exogenous danger signals from microorganisms, and endogenous danger signals from damaged tissues (Gallucci & Matzinger, 2001). The acute phase reactants C-reactive protein and serum amyloid P that can opsonize microbes and apoptotic cells (Mold, Baca & Du Clos, 2002), and bind DNA (Pepys & Butler, 1987) or ribonucleoproteins (Du Clos, 1989), have also been showed to bind to and activate FcγRIIa (Bharadwaj *et al.*, 2001; Chi *et al.*, 2002). This suggests the existence of other complexes that can activate NIPC/PDC via FcγRIIa.

General summary and conclusions

The aim of this thesis was to study the activation of type I IFN production by inducers containing nucleic acids in human and porcine blood cells. The main findings and conclusions were:

Immunostimulatory DNA in the form of the DNA vaccine vector pcDNA3 can activate production of IFN-α in porcine PBMC. The in vitro stimulatory capacity probably required uptake of the pcDNA3 by cells, because pretreatment of the plasmid with the transfecting agent lipofectin was necessary for activation of IFN-α production. The plasmid only induced low levels of IL-6, regardless of lipofection. Unmethylated CpG motifs were a prerequisite for activation of IFN-α production, because specific methylation of CG sequences abolished the IFN-α inducing capacity of the plasmid. However, the earlier defined two ISS (5'AACGTT 3') in the ampR gene were not essential for the IFN-α inducing ability of the plasmid, indicating that other CpG-motifs in the plasmid must contribute. Expression of the plasmid was not necessary for induction, which indicates that it is the DNA that harbors the immunostimulatory activity and not RNA or protein expressed by the plasmid. These results show that the induction of IFN-α by plasmid DNA depends on unmethylated CpG-motifs and that porcine cells may have the same type of detection system for bacterial DNA as reported for man and mice.

The activation of IFN-α by IS-DNA was further investigated in human PBMC using ODNs derived from DNA sequences identified in the serum of SLE patients (ODN H: 5' TTTTCAATTCGAAGATGAAT 3') in ss or ds form. Cellular entry of DNA was probably a requirement, because pretreatment of ODNs with lipofectin was necessary for activation of IFN-α production. The importance of the CpG dinucleotide in ODN H and its complementary strand ODN I for IFN-α induction was evaluated by studying the effect of various alterations of the ODN on its IFN-α inducing capacity. It was found that certain ss forms of ODN H could induce IFN-α in human blood cells in the absence of unmethylated CpG dinucleotides, which contradicts the widely held concept of unmethylated CpGs being necessary for cytokine activation by DNA. However, in the ds form, an unmethylated CpG was necessary for full activity, indicating differences in the recognition of ss and dsDNA. Because unmethylated CpG was not always required for the interferogenic activity, many immunostimulatory DNA sequences should be present in eukaryotic DNA, and not only in prokaryotic DNA. Such eukaryotic DNA sequences might be formed by decomposing genomes of dying cells and constitute potential endogenous IFN-α inducers. They may become active when complexed with autoantibodies, as in SLE.

In porcine PBMC, the full interferogenic activity of ss and ds forms of ODN H required lipofectin and unmethylated CpGs. Certain CpG-ODNs with flanking poly G-sequences (e.g. ODN 2216 and ODN 2216d) were potent inducers of IFN-α production in the absence of lipofectin, both as phosphorothioate/phosphodiester chimeras or as phosphodiesters. Such phosphodiester ODNs may be particularly useful as immunostimulatory adjuvants. The poly-G sequences may enhance the interferogenic ability of ODNs by facilitating their uptake by cells, but may also act in other ways. For instance, it was found that addition of poly-G sequences to ODN H enhanced its IFN-α inducing ability, but did not eliminate the need for lipofectin. The mode of action of poly-G sequences therefore merits further study.

The NIPC/PDC were the only cells among human PBMC producing IFN-α in response to lipofected ssODN H or dsODN H-I. This was shown by flow cytometry, using simultaneous detection of intracellular IFN-α and cell surface markers. The same approach revealed that the porcine PBMC producing IFN-α in response to ODN 2216 or lipofected pcDNA3 expressed MHC class II, CD2, CD4 and SWC3 but not markers for T-cells, B-cells or monocytes. These IPC constituted 1-3 cells per 1000 PBMC and this low frequency was confirmed by in situ hybridization, detecting cells containing poIFN-α mRNA. The principal porcine IPC activated by CpG-DNA therefore appear to be the NIPC/PDC and correspond to earlier identified virus-activated porcine IPC.

The human NIPC/PDC are also activated to produce IFN-α by apoptotic cells in combination with IgG autoantibodies from SLE patients. The involvement of FcγRII in this activation, but not FcγRI or FcγRIII, was demonstrated using mAbs blocking these receptors. The mAb IV:3, which may preferentially block FcγRIIa, inhibited production of IFN-α induced by SLE-IgG in combination with apoptotic cells, but not that induced by HSV or ODN 2216. The responding NIPC/PDC had a significant but weak expression of FcγRII as determined by flow cytometry, and purified BDCA-2 positive NIPC/PDC were by RT-PCR shown to express the isoform FcγRIIa, but not FcγRIIb or FcγRIIc. While FcγRIIa can mediate the activation of IFN-α production in NIPC/PDC by interferogenic IC, FcγRIIa may also convey inhibitory signals. This can explain the general inhibitory effect of heat-aggregated IgG on the IFN-α production in NIPC/PDC induced by interferogenic IC, HSV or ODN 2216.

The human and porcine NIPC/PDC may be pivotal cells in the activation of type I IFN production by exogenous and endogenous inducers containing nucleic acids, e.g. CpG-DNA or interferogenic IC containing DNA and/or RNA. The NIPC/PDC can for instance recognize CpG-DNA by means of TLR9, but other mechanisms may be involved in their activation by RNA and other forms of DNA. Furthermore, certain molecules on NIPC/PDC may be important, such as scavenger receptors that can interact with poly-G sequences and FcγRIIa that can

interact with antibodies in IC. They may like lipofectin facilitate uptake of the IFN-α inducers or provide accessory signals that are necessary for the activation of the NIPC/PDC. It is important to further clarify the mechanisms whereby NIPC/PDC are activated, because this can allow development of more efficient adjuvants for use in vaccines, and also provide targets for new therapies aiming at inhibition of the pathologic activation of NIPC/PDC in autoimmune diseases, such as SLE.

Figure 1. Selective surface molecules expressed by human and/or porcine NIPC/PDC and cytokines produced by the cells in response to exogenous and endogenous inducers. Exogenous IFN-α inducers include virus and bacteria and their products such as CpG-DNA. Endogenous IFN-α inducers include immune complexes (IC) containing IgG and nucleic acids (RNA or DNA). Anti-virus antibodies and virus can also form interferogenic IC. The inducers may also trigger production of other cytokines (IL-12, chemokines, etc.). The figure is based on published findings and results presented in this thesis.

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