Porcine Immunoregulatory Cytokines

With special reference to their induction by CpG-containing DNA

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2003

Acta Universitatis Agriculturae Sueciae
Veterinaria 148

ISSN 1401-6257
ISBN 91-576-6367-X

Tryck: SLU Service/Repro, Uppsala 2003

Abstract

Domeika, K. 2003. *Porcine immunoregulatory cytokines. With special reference to their induction by CpG-containing DNA.* Doctor's dissertation.
ISSN 1401-6257, ISBN 91-576-6367-X.

This thesis describes the possible adjuvant effects of bacterial DNA, in the form of a DNA vaccine plasmid (pcDNA3) or oligodeoxyribonucleotides (ODN), in the pig. The effects were assessed mainly as their ability to induce production of immunoregulatory cytokines. Furthermore, the cells producing type I or type II interferon (IFN) at various stimuli were studied.

The plasmid pcDNA3 was shown to induce a local production of IFN-α *in vivo*, in subcutaneous tissue chambers. The *in vivo* effects of plasmids, expressing the cDNA for the cytokines GM-CSF or IL-6, were also tested. Such cytokine-expressing vectors could have adjuvant effects in DNA vaccines.

The cDNA for porcine IL-12 (poIL-12) fusion protein was cloned into pcDNA3. The plasmid was shown to express poIL-12 with high *in vitro* biological activity in a bioassay. IL-12 and huIL-18 synergized in the induction of IFN-γ production in porcine peripheral blood mononuclear cells (poPBMC), and the IFN-γ producing cells were by flow cytometric analyses shown to be T-cells and NK cells.

The antigen-presenting dendritic cells (DC) are important target cells in vaccination. Monocyte-derived dendritic cells (moDC) were differentiated from adherent monocytes in the presence of GM-CSF and IL-4 *in vitro*, and were able to produce different cytokines depending on stimuli. IL-12 was produced by the moDC mainly at stimulation with pcDNA3 (preincubated in lipofectin), *Actinobacillus pleuropneumoniae* and Sendai virus. IFN-α was only produced in response to Sendai virus, and IL-6 and IL-10 mainly in response to *A. pleuropneumoniae*.

The presence of an unmethylated CpG dinucleotide in ODN was essential for the *in vitro* induction of IFN-α in poPBMC. The presence of a poly-guanine sequence in the ends of the ODN was shown to enhance its IFN-α inducing capacity. The IFN-α producing cells in response to CpG-ODN, lipofected pcDNA3 or Aujeszky's disease virus were by flow cytometry and *in situ* hybridisation shown to be a rare cell population, resembling the human plasmacytoid DC, also referred to as natural IFN producing cells. The results show that certain forms of bacterial DNA have adjuvant effects in the pig, which could be of importance in the development of more efficient vaccines.

Key words: CpG-DNA, cytokines, dendritic cells, DNA vaccines, interferon-γ, interleukin-12, interleukin-18, porcine, type I interferon.

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Contents

Appendix

Papers I-IV

This thesis is based on the following publications and manuscripts, which are referred to by their Roman numerals:

I. Johansson, E., Wallgren, P., Fuxler, L., Domeika K., Lefevre, F. & Fossum, C. 2002. The DNA vaccine vector pcDNA3 induces IFN-α production in pigs. *Veterinary Immunology and Immunopathology 87*, 29-40

II. Domeika, K., Berg, M., Eloranta, M-L. & Alm, G.V. 2002. Porcine interleukin-12 fusion protein and interleukin-18 in combination induce interferon-γ production in porcine natural killer and T cells. *Veterinary Immunology and Immunopathology 86*, 11-21

III. Johansson, E., Domeika, K., Berg, M., Alm, G.V. & Fossum, C. 2003. Characterisation of porcine monocyte-derived dendritic cells according to their cytokine profile. *Veterinary Immunology and Immunopathology.* In press

IV. Domeika, K., Magnusson, M., Fuxler, L., Eloranta, M.-L., Alm, G.V. & Fossum, C. Characteristics of oligodeoxyribonucleotides that induce interferon-α production in infrequent porcine blood leukocytes. Manuscript.

Abbreviations

ADV	Aujeszky's disease virus
APC	antigen presenting cell
CD	Cluster of differentiation
CpG	cytosine-phosphate-guanine dinucleotides
CTL	cytotoxic T lymphocyte
DC	dendritic cells
moDC	monocyte-derived DC
Diester	phosphodiester
DNA	deoxyribonucleic acid
GM-CSF	granulocyte-macrophage colony-stimulating factor
HSV	Herpes Simplex virus
IFN	interferon
IFNAR	interferon-α receptor
Ig	immunoglobulin
IL-	interleukin
IL-12Rβ2	IL-12 receptor β2
IPC	interferon producing cell
IRAK	IL-1 receptor-associated kinase
IRF	Interferon regulatory factor
ISGF3	IFN-stimulated gene factor 3
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MLC	mixed leukocyte culture
MLR	mixed leukocyte reaction
mRNA	messenger RNA
NFκB	nuclear factor κB
NIPC	natural interferon producing cells
NK cell	natural killer cell
ODN	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cells
PDC	plasmacytoid DC
PRR	pathogen racognition receptors
RT-PCR	reverse transcriptase polymerase chain reaction
SPF	specific pathogen free
STAT	signal transducer and activator of transcription
TCF	tissue culture fluid
TCR	T cell receptor
TGF	transforming growth factor
TNF	tumour necrosis factor
Th cell	T helper lymphocyte
Th1	T helper cell type 1
Th2	T helper cell type 2
Thioate	phosphorothioate
TLR	toll like receptor
TNF	tumour necrosis factor
U	units
UV	ultra violet

Introduction

Innate and adaptive immunity

The initial immune response generated at the first contact with antigens such as microorganisms belong to the non-specific innate (natural) immunity. This response is primarily mediated by phagocytic cells, natural killer (NK) cells, and soluble molecules such as complement factors, acute phase reactants and type I interferon (IFN). The phagocytic cells, especially granulocytes and monocytes/macrophages, eliminate the microorganisms by phagocytosis and subsequent degradation. The professional antigen presenting cells (APC), that is the various types of dendritic cells (DC), are also capable of phagocytosis when immature, but their main function is to process and present antigens to antigen-specific T lymphocytes, key cells in the adaptive immunity. In this way, the DC form a bridge between innate and adaptive immunity. The adaptive immunity improves on repeated exposure to a given infection and includes a specific immunological memory. This ability to develop an immunological memory is utilized at vaccination, when the host is immunized by a killed or attenuated living microorganism or its components, in order to evoke specific immunity to natural infection with the same microorganism.

Cells involved in the innate immune response can, depending on the nature of the antigen, stimulate specific properties of the adaptive immunity. For instance, the development of the two major subsets of T-helper (Th) lymphocytes, Th1 and Th2, can be modulated depending on the cytokines initially produced by DC and phagocytes (see below). The concept of Th1 and Th2 immunity and its regulation by DC has primarily been demonstrated in mice and humans, and is not yet firmly established in pigs. One aim of the present thesis was therefore to study porcine DC and their ability to produce immunoregulatory cytokines involved in Th1 and Th2 lymphocyte development.

The role of dendritic cells (DC) in the immune response

The DC are characterized by a typical morphology with 'dendritic' processes, extremely active endocytosis and macropinocytosis and have a high antigen processing and presenting capacity (Mellman & Steinman, 2001; Banchereau *et al.*, 2000). The immature DC are present in high numbers in many tissues, and lower numbers are also present in blood. They migrate to sites of inflammation, take up antigen, and maturate upon inflammatory signals to reach an activated stage with increased ability to present antigen. Maturation of the DC occurs as they migrate to lymphoid organs where they activate naïve T cells to antigen-specific clonal expansion (Sousa *et al.*, 1997; Banchereau *et al.*, 2000; Romani *et al.*, 2001). The DC is the only type of APC capable of stimulating both naïve and activated T-cells. The lymphoid organs also contain many DC, that also can capture and present any antigens that may arrive via lymph or blood (Banchereau *et al.*, 2000).

The mature DC have reduced phagocytic ability, upregulate co-stimulatory molecules (e.g. CD40 and CD80/86) as well as MHC II, and change morphology

(Banchereau *et al.*, 2000; Hart, 1997). The mature DC downregulate the chemokine receptor 6 (CCR6) that responds to chemokines produced at the site of inflammation. Instead, they upregulate the CCR7, and may therefore respond to chemokines produced by cells in the T cell areas of secondary lymphoid organs such as lymph nodes and spleen (Kellermann *et al.*, 1999; Dieu *et al.*, 1998).

Exogenous antigens can be presented by DC on both MHC I and MHC II molecules. By presentation of exogenous antigen on MHC I molecules, a process referred to as cross presentation, DC are able to perform a direct activation of CTL (Cho *et al.*, 2002; Fonteneau *et al.*, 2002; Maurer *et al.*, 2002). In addition, DC can present glycolipid antigens on CD1 molecules that are recognised by T cells expressing the γδ T cell receptor (TCR) (Gumperz & Brenner, 2001). Recently, C-type lectin and lectin-like receptors have been shown to be expressed on the surface of DC, and can serve as antigen receptors and also regulate migration of DC and their interaction with T cells (Figdor *et al.*, 2002)

The T cells are activated by the antigen presenting DC when the TCR and CD4 or CD8 of the T cells are interacting with the MHC class I or II in complex with antigen peptide (Matzinger, 2001; Kalinski *et al.*, 1999). However activation also requires recognition of co-stimulatory molecules such as CD80/86 (B7) on the DC by receptors on the T cell such as CD28 and a signal to T cells by cytokines produced by especially the DC, for instance IL-12 and IFN-α is also required for activation of the T cells (see below). In the absence of the appropriate co-stimulatory molecules and cytokines, the DC may induce immunological tolerance by causing anergy or apoptosis in T cells (Mahnke *et al.*, 2002; Shortman & Liu, 2002)

Th1- and Th2-types of immune responses

The Th1-type of immune response is initiated by the production of IL-12 by DC, and by the ability of DC to stimulate proliferation of naïve T-cells. The latter is due to production of IL-2 by T cells and the simultaneous expression of high affinity IL-2 receptor. The presence of IL-12 influences the stimulated Th cells to Th1 differentiation, including production of IFN-γ (Ma & Trinchieri, 2001). The Th1 cells, by production of cytokines such as IFN-γ and TNF-β and IL-2, stimulate cell-mediated immunity, including activation of phagocytes, development of cytotoxic T-lymphocytes (CTL), and also development of B lymphocytes producing opsonizing and complement fixing antibodies of the immunoglobulin G (IgG) isotype. Furthermore, IL-12 and IFN-γ enhances the cytotoxity of CTL and NK cells. The IL-12 production by APC is in turn enhanced by production of IFN-γ by activated Th1 cells (Ma & Trinchieri, 2001). The expression of the IL-12R on the Th1 cells is maintained in the presence of IFN-γ in mice (Szabo *et al.*, 1997). In man, however, type I IFNs (se below) have this function, which preserves the capacity of Th1 cells to respond to IL-12 (Rogge *et al.*, 1997). Th1 responses are also associated with inflammation and tissue injury, because TNF-β and IFN-γ recruit and activate inflammatory leucocytes.

When DC are not producing Th1-inducing cytokines, they instead promote a Th2 cell development. The Th2 cells are able to produce cytokines such as IL-3, IL-4, IL-5, IL-6, IL-10, IL-13 and therefore stimulate development of B cells producing antibodies of the IgM, IgA, IgG, and IgE isotypes and furthermore cause proliferation and/or recruitment of tissue mast cells and eosinophilic granulocytes. A Th2 dominated immune reaction is present in allergies and helminth infections. Several cytokines produced by Th2 cells such as IL-4 and IL-6 and IL-10 have anti-inflammatory actions, which inhibit acute and chronic inflammation by inhibiting pro-inflammatory cytokine production by macrophages (Abbas *et al.*, 1996).

The two sub-types of Th cells cross-regulate each other. Thus, the IFN-γ produced by Th1 cells inhibits development of Th2 cells, whereas IL-4 and IL-10 produced by Th2 cells block development of Th1 cells (Abbas *et al.*, 1996). It is therefore possible to redirect a Th2 type of immune response towards a Th1 response by induction of e.g IL-12, or IFN-α production (Biron, 2001; Schopf *et al.*, 1999; Sinigaglia *et al.*, 1999).

Although there are dissimilarities between porcine and human T cells, there are a few studies indicating the presence of Th1 and Th2 polarized T cell subsets in the pig. A Th1-like cytokine profile was detected when peripheral blood mononuclear cells (PBMC) from immunized pigs were responding to the herpes virus Aujeszky's disease virus ADV *in vitro* (Fischer *et al.*, 2000). In addition, mRNA for typical Th2 associated cytokines were found in liver and intestinal tissue from pigs infected with the parasite *Schistosoma japonicum* (Oswald *et al.*, 2001). A typical Th1 type of immune response in the pig was observed after oral immunization with cholera toxin, which resulted in cholera toxin-specific delayed type hypersensitivity responses that associated with increased secretion of IFN-γ (Foss & Murtaugh, 1999). Furthermore, a vaccine against ADV induced a Th1 type of immune response, as indicated by the induction of antigen-specfic IFN-γ producing cells (Zuckermann *et al.*, 1998). Recently, Laval et al showed that a DNA vaccine against ADV induced IFN-γ production in antigen specific CD8+ CTL (Laval *et al.*, 2002).

Pathogen Recognition Receptors

The cells of the innate immune system contain a set of molecular pattern recognition receptors (PRR) that recognize certain structures termed pathogen associated molecular patterns (PAMP), present on pathogens but normally not in healthy tissue. Activation of the PRR generate the first signals that indicate the presence of infection (or danger) to DC and other cells of the innate immune responses. The cells contain several different receptors that have affinity to different general structures on pathogens, e.g. the Toll-like receptors (TLR) (Ozato *et al.*, 2002; Medzhitov, 2001), scavenger receptors (Peiser *et al.*, 2002), and lectin receptors such as the mannose-receptor (Stahl & Ezekowitz, 1998). For instance, the TLR family has ten members (TLR1-10), that recognize distinct microbial components and they also recognize endogenous ligands induced during the inflammatory response. Examples of ligands for TLRs are bacterial lipoproteins, which are recognized by TLR1, TLR2, TLR4 and TLR6,

lipopolysaccharides and heat shock protein 60 by TLR4, flagellin by TLR5, dsRNA by TLR3, imidazoquinolin (e.g. imiquimod) by TLR7 and TLR8 and unmethylated CpG-DNA is recognized by TLR9.

The TLRs triggers different signalling pathways in the cell, leading to different biological responses by the various stimuli (Ozato *et al.*, 2002; Medzhitov, 2001). Especially prominent is the production of cytokines associated with inflammatory responses such as IL-1, IL-2, IL-6, IL-10, IL-12, IL-15, TNF-α, GM-CSF and IFN. To our knowledge there are no reports on the presence of TLRs in the pig, but the cDNA for bovine TLR2 and TLR4 and chicken TLR2 have been cloned (Werling & Jungi, 2003). However, because the TLRs are highly evolutionary conserved, they are most likely also present in the pig and have the same important role in the immune response as in humans and mice.

Subsets of dendritic cells

The capacity of DC to stimulate T lymphocytes is linked to their stage of differentiation (immature vs. mature) and activated production of cytokines. Furthermore, there are several different subsets of DC that differ in specific surface markers and functional capacity depending on lineage, origin and stage of maturation The biology of DC has been extensively studied in man and mice (Banchereau *et al.*, 2000; Shortman & Liu, 2002), and the classification of dendritic cell subsets in mice and man differs (Shortman & Liu, 2002).

DC populations originate from $CD34^+$ hemopoetic stem cells in the bone marrow that develop into immature DC. The human immature DC lack typical lineage-specific markers and are divided into CD4+CD11c+ myeloid DC, and CD4+CD11c- immature plasmacytoid DC (PDC) (Kadowaki & Liu, 2002). The immature myeloid DC express myeloid surface markers, they undergo spontaneous maturation in culture and may induce allogeneic T cell proliferation. The immature plasmacytoid DC has plasmacytoid morphology and is dependent on IL-3 for survival and differentiation in culture. Based on the ability to secrete IL-12 and thereby preferentially induce a Th1 type of immune response the human myeloid DC has been referred to as DC1, whereas human plasmacytoid DC was designated DC2 (Kadowaki & Liu, 2002; Liu *et al.*, 2001; Grage-Griebenow *et al.*, 2001). In the mouse, lymphoid DC, also referred to as DC2, directs the immune response towards a Th1 type by their high IL-12 producing capacity (Shortman & Liu, 2002). The different DC subsets are also named according to their localization, in humans for instance epidermal Langerhans cells, dermal interstitial DC, splenic marginal zone DC, T-zone interdigitating cells, germinal center DC, thymic DC, liver DC and blood DC (Banchereau *et al.*, 2000; Shortman & Liu, 2002).

Myeloid DC

Myeloid DC precursors can be transported via the blood into tissues where they become resident immature DC. These cells, in skin, are referred to as Langerhans cells. Alternatively, the precursors remain circulating in the blood until attracted by chemokines to a site of inflammation. Then they may migrate through endothelium and differentiate into immature myeloid DC. Myeloid DC can be generated *in vitro* from bone marrow and from monocytes. The presence of GM-

CSF alone, or in combination with IL-4, differentiated murine bone marrow cells into DC (Hart, 1997). Immature myeloid DC have also been generated *in vitro* by differentiation of blood monocytes in the presence of GM-CSF and IL-4. The presence of LPS, TNF-α, IL-1, IFN-α or necrotic cells induce maturation of such immature myeloid DC (Shortman & Liu, 2002; Luft *et al.*, 2002). These agents were used to mimic the signals to which the DC are exposed *in vivo* during inflammation. The myeloid DC express several TLR, especially TLR2, 3, 4, 5, 7 and 8 (Kadowaki & Liu, 2002; Krug *et al.*, 2001b). These and other PRR, are involved in the recognition of for instance microbial constituents and activate maturation and cytokine production in the DC.

Plasmacytoid DC

The immature plasmacytoid DC are characterised by high IFN-α producing capacity (Fitzgerald-Bocarsly, 2002; Cella *et al.*, 1999; Kadowaki & Liu, 2002) and at least a major part of them constitute the 'natural IFN-α/β producing cells' (NIPC; see below). They can be identified by their selective expression of the markers blood dendritic cell antigen (BDCA) 2 and 4 (Dzionek *et al.*, 2002). These PDC express TLR7 and TLR9 (Hornung *et al.*, 2002; Krug *et al.*, 2001b). Recently, oligodeoxyribonucleotides containing cytidine-phosphate-guanine dinucleotides (CpG-ODN; see below), a TLR9 ligand, and imiquimod, a ligand for TLR7, were shown to induce IFN-α production and to cause maturation of PDC (Bauer *et al.*, 2001a; Bauer *et al.*, 1999; Krug *et al.*, 2001b; Ito *et al.*, 2002). The IFN-α can in turn cause maturation of immature myeloid DC (Gursel *et al.*, 2002). Although characterized by extremely high IFN-α producing ability, the PDC can also produce high amounts of IL-12 when stimulated by CpG-ODN in combination with ligation of CD40 (Krug *et al.*, 2001b). Recently, a murine NIPC/PDC was described (Shortman & Liu, 2002). That the myeloid and plasmacytoid DC express different sets of TLRs, may explain why these DC respond differentially to microbial stimuli, the NIPC/PDC preferentially being activated by CpG-DNA, and the myeloid DC by several microbial components and dsRNA.

Porcine DC

In the pig, DC has been isolated from thymus (Salmon *et al.*, 2000), skin (Bautista *et al.*, 2002; Vana & Meingassner, 2000), spleen (Chun *et al.*, 1999), Peyers patches (Makala *et al.*, 1998) and jejunal lamina propria (Haverson *et al.*, 2000). Porcine DC have also been generated *in vitro* from bone marrow cells (West *et al.*, 1999; Carrasco *et al.*, 2001) and from blood monocytes (Carrasco *et al.*, 2001; Paillot *et al.*, 2001). The latter papers describe the generation of porcine monocyte-derived DC (moDC) using porcine (po)GM-CSF and poIL-4 for differentiation. In that system, presence of TGF-β1, TNF or LPS during differentiation altered the cell morphology and increased their ability to stimulate an allogeneic MLR (Carrasco *et al.*, 2001; Paillot *et al.*, 2001). The bone marrow derived DC (bmDC) and the moDC expressed the myeloid marker SWC3, MHC class II, CD1 and CD80/86. The DC also expressed CD14 and CD16, before and after maturation (Carrasco *et al.*, 2001). Conversely, a marked down regulation of CD14 during differentiation of monocytes to DC was observed by Paillot et al

(Paillot *et al.*, 2001). In their study, the moDC were shown to also express the integrins CD11a, CD18, CD11b/c, as well as CD36, CD68 and p55 facsin (Paillot *et al.*, 2001). The presence of transforming growth factor β1 (TGF-β1) together with IL-4 and GM-CSF induced the porcine monocytes to differentiate into Langerhans cells. Furthermore, an IFN-α producing cell, resembling the NIPC/PDC has been demonstrated (see below).

The immunoregulatory cytokines IL-12 and IL-18

Due to the essential role of IL-12 in the regulation and maintenance of a Th1 type of immune response, IL-12 could be an important adjuvant in vaccines. Human IL-12 (huIL-12) was first known as NK cell stimulatory factor or cytotoxic lymphocyte maturating factor. The bioactive form of IL-12 is a heterodimeric protein of 70 kDa, which consists of a 35 (p35) and a 40 (p40) kDa subunit. Co-expression of the two subunits of IL-12 is required for generation of active cytokine. Furthermore, a homodimeric form of p40 may act as an IL-12 receptor antagonist (Ma & Trinchieri, 2001).

The poIL-12 was cloned, and the amino acid sequences of the p35 and p40 subunits showed approximately 85% homology compared to the human counterparts (Foss & Murtaugh, 1997). A recombinant heterodimeric bioactive porcine IL-12 was produced by expression of the cDNA for the p35 and p40 subunits using a monocistronic baculovirus as vector (Kokuho *et al.*, 1999). In vivo effects of the poIL-12, such as increased IgA titers in mucus, were also shown in an oral immunization model (Foss *et al.*, 1999). Differential expression of mRNA in various porcine tissues indicates that the poIL-12 subunits are regulated independently (Foss & Murtaugh, 1997). The p35 subunit in mice was constitutively expressed in several tissues (Bost & Clements, 1995), but the expression is regulated in certain cell types (Vaidyanathan *et al.*, 2001).

Interleukin-18 (IL-18), first known as interferon-γ inducing factor (Okamura *et al.*, 1995), has structural homology to IL-1β, and both the human and porcine IL-18 is produced in an inactive precursor form which is processed by IL-1β converting enzyme to its active form (Muneta *et al.*, 2000b; Gu *et al.*, 1997; Fonteneau *et al.*, 2002). The poIL-18 has been cloned and showed an 81% homology to the human amino acid sequence (Muneta *et al.*, 1999). Recently, also the IL-18 receptor has been cloned (Muneta *et al.*, 2002). The production of IL-18, can be induced by the interaction between human Th1 cells and APC in the presence of antigen and also occurs in activated human and porcine macrophages (Kohno & Kurimoto, 1998; Foss *et al.*, 2001). In the pig, poIL-18 was detected in plasma and bronchoalveolar lavage fluid at experimental infection with *Actinobacillus pleuropneumoniae* (Muneta *et al.*, 2000a). Constitutive and induced expression of IL-18 mRNA was detected in various tissues, including lymphoid organs of the intestinal mucosa (Muneta *et al.*, 2000a; Foss *et al.*, 2001; Fournout *et al.*, 2000).

IL-18 resembles IL-12 in the ability to induce production of IFN-γ by NK cells and T-cells, and to increase NK cell cytotoxity (Tominaga *et al.*, 2000; Nakanishi *et al.*, 2001). The differentiation of $CD4^+$ T-cell into Th1 cells by IL-12 is

promoted by the presence of IL-18, and IL-18 may also induce proliferation of activated Th1 cells without help from IL-12 (Tominaga *et al.*, 2000; Kohno & Kurimoto, 1998; Kohno *et al.*, 1997; Okamura *et al.*, 1998). IL-12 and IL-18 synergize to stimulate IFN-γ production from human and murine Th1 cells.

IFN-γ (Type II IFN)

IFN-γ is a central immunoregulatory cytokine that promotes and mediates a Th1 type of immune response (Abbas *et al.*, 1996). The IFN-γ binds to the IFN-γ receptor (IFN-γR) and activates the STAT 1 signal transduction pathway, resulting in expression of multiple genes. In this way it influences the immune system in many ways. It enhances production of IL-12 by macrophages and DC, and stimulates the microbicidal effects of macrophages. IFN-γ upregulates MHC class I and II molecules and thereby enhances antigen presentation for both Th and CTL. It suppresses Th2 cell development and promotes Th1-type of Ig class switching in B cells.

Work in mice and man have identified Th1 cells, CD8 positive T cells and NK cells as the major IFN-γ producers, but other cells such as B cells and macrophages have also been described as producers (Okamura *et al.*, 1998; Tominaga *et al.*, 2000; Nakanishi *et al.*, 2001; Otani *et al.*, 1999). As described in the previous section, IL-12 especially in combination with IL-18 is a potent inducer of IFN-γ, and production of IFN-γ, at least by T cells, may require interaction with DC and upregulation of the IL-12R.

Little is known concerning the identity of the IFN-γ producers in the pig, and their activation by e.g. IL-12/IL-18. The proportion of porcine PBMC producing IFN-γ after polyclonal activation by phorbol myristic acid and calcium ionophore A23187 was shown to be significantly higher in adult than in young animals (Rodriguez-Carreno *et al.*, 2002). The subpopulations of T lymphocytes producing IFN-γ were mainly αβ T cells. In young pigs, also a high proportion of γδ T cells produced IFN-γ. In adult animals, T cells double positive for CD4 and CD8α and also CD4-positive/CD45RA-negative cells, including memory T cells (Saalmuller *et al.*, 2002), produced IFN-γ. The IFN-γ production by PBMC derived from pigs immunized by a DNA vaccine against ADV and restimulated *in vitro* by antigen loaded autologous DC mainly resided in T cells with high expression of CD8α, that is CD8 positive CTL (Laval *et al.*, 2002).

Type I interferons

Type I IFN genes

The ability of IFN to protect cells from viral infection was first described in 1957 (Isaacs & Lindenmann, 1957). They discovered a soluble factor, produced by cells exposed to inactivated influenza virus that protected cells against infection with live influenza virus. The term interferon originally comes from this interference with viral replication. Today, it is known that the IFN are products of several different genes and can be divided into type I IFN and type II IFN. Type I IFN are further divided into six subfamilies (IFN-α, -β, -δ, -τ, -ω, and -κ) while only one type II IFN member has been described (IFN-γ). In man, 13 nonallelic genes for

different IFN-α subtypes and four pseudogenes have been described (Diaz & Testa, 1996). In the pig, there are about 12 genes for IFN-α subtypes located on chromosome 1 (La Bonnardiere *et al.*, 1994). Only one IFN-β gene has been described in man (Diaz & Testa, 1996) and in the pig (Artursson *et al.*, 1992). There are at least three functional IFN-ω genes in pig, but only one is present in man (La Bonnardiere *et al.*, 1994; Diaz & Testa, 1996). A new human IFN, the IFN-κ, was recently identified and was found to be produced by keratinocytes (LaFleur *et al.*, 2001). This IFN has not been identified in pigs. The IFN-δ and IFN-τ are constitutively expressed in trophoblast cells of pigs and ruminants, respectively (Lefevre *et al.*, 1998; Alexenko *et al.*, 2000). The IFN-δ has so far only been described in pigs (Lefevre *et al.*, 1998). IFN-τ is present during pregnancy in ruminants and is important for the implantation and early embryonic development (Alexenko *et al.*, 2000) and is not inducible by virus. IFN-γ and perhaps IFN-δ may have the same effect in the pig (La Bonnardiere *et al.*, 2002).

Effects of type I IFN

There is one common receptor for the type I IFN that is termed IFNAR and consists of two subunits, the IFNAR-1 and IFNAR-2. Activation of the IFNAR leads to subsequent signalling into the cell via the Janus protein tyrosine kinase (Jak)-STAT signal transduction pathway (Taniguchi & Takaoka, 2001; Taniguchi & Takaoka, 2002). Activation of the receptor leads to tyrosine phosphorylation of STAT1 and STAT2. The activated STATs together with interferon regulatory factor 9 (IRF9), form a transcriptional activation complex, the IFN-stimulated gene factor 3 (ISGF3). Furthermore a STAT1 homodimer is formed. Both ISGF3 and the STAT1 homodimer translocate to the nucleus and activate expression of a large number of genes, the products of which mediate the many effects of type I IFNs outlined below (Schlaak *et al.*, 2002; Der *et al.*, 1998). The type I IFN are produced in response to e.g. virus infections (see below), and cause inhibition of virus replication at several stages. For example viral penetration, uncoating of virions, transcription, translation or the assembly of progeny virus may be affected. There are several molecular systems described that are activated by type I IFN and in various ways interfere with viral replication (Stark *et al.*, 1998). They include the enzyme 2'-5' oligoadenylate synthetase in the 2'-5'A system, dsRNA dependent protein kinase (PKR) and the Mx protein. Furthermore, type I IFNs induce apoptosis of cells infected by virus.

Apart from the antiviral effects, the type I IFNs may also have other effects on cells. These effects may differ depending on cell type, and can for instance be anti-proliferative, cytotoxic, or anti-tumoral (Belardelli & Ferrantini, 2002; Biron, 1999; Stark *et al.*, 1998).

Type IFNs also exert a large number of immunoregulatory effects and especially IFN-α may be a key cytokine not only in the innate immune system but also in adaptive immune responses (Biron, 1999; Biron, 2001; Belardelli & Ferrantini, 2002; Bogdan, 2000; Akbar *et al.*, 2000). IFN-α has maturing effects on the immature myeloid DC, enhancing their ability to present antigen to and activate T cells (Santini *et al.*, 2000; Luft *et al.*, 2002; Le Bon & Tough, 2002; Gallucci *et al.*, 1999). Type I IFNs also increase the activity of CTL by inducing up-

regulation of MHC class I on virus infected cells, and increase the cytotoxic effects of NK-cells. Furthermore, type I IFNs also increases the numbers of Fc-receptors on macrophages, enhancing their phagocytic activity.

The type I IFN, especially IFN-α, can have an important role in the regulation of the Th1 or Th2 types of immune responses. As described above, IFN-α may promote a development of a Th1 type of immune response by up-regulation and maintenance of a functional IL-12 receptor on Th cells in man (Rogge *et al.*, 1998). Type I IFN in mice was a potent enhancer of the primary antibody response to the antigen chicken gammaglobulin, with both long-lasting antibody responses and development of memory (Le Bon *et al.*, 2001). The type I IFN was in this respect comparable to Freund's complete adjuvant (CFA). This adjuvant effect of IFN could be mediated by DC (Le Bon & Tough, 2002). Furthermore, some of the adjuvant effects of CpG-ODN *in vivo* in mice, especially in priming of antigen specific CTL, have recently been shown to be dependent on the effects of IFN-α on DC (Cho *et al.*, 2002). Type I IFN can also stimulate differentiation of T cells and inhibit apoptosis associated with activation, as well as promote proliferation of memory CTL and maintain immunological memory (Akbar *et al.*, 2000; Sinigaglia *et al.*, 1999; Marrack *et al.*, 1999). In addition, type I IFN can promote survival and differentiation of B cells and enhance B cell receptor-dependent responses (Braun *et al.*, 2002; Ruuth *et al.*, 2001). Obviously, other cytokines such as IL-12 and IL-15 can be further involved, and IL-15 can actually be induced by IFN-α (Mattei *et al.*, 2001; Zhang *et al.*, 1998).

Induction of type I IFN production

The genes for the type I IFN have common regulatory sequences and may be induced in many different cell-types, mainly as a response to virus infection. The expression of IFN-α/β genes is induced by transcription factors for the IFN-α/β genes that include several members of the IRF family. Of the 9 members of the IRF family of proteins, the IRF-3, -5 and -7 are important for induction of high levels of IFN-α/β (Taniguchi & Takaoka, 2002; Barnes *et al.*, 2002). These proteins are present in the cytoplasm of uninfected cells, but translocate into the nucleus at viral infection. Type I IFN can upregulate expression of for instance IRF-1, and IRF-7 (Taniguchi & Takaoka, 2002; Taniguchi & Takaoka, 2001). In this way, type I IFN upregulates its own synthesis, a phenomenon termed priming. Also other cytokines such as GM-CSF and IL-3 have priming effects (Cederblad & Alm, 1991). The different IFN-α genes are expressed at different levels in different cell types and this may be due to differences in expression of the relevant IRFs (Barnes *et al.*, 2002).

Many different microorganisms can trigger production of type I IFN in cells, including viruses, bacteria, parasites, nucleic acids such as dsDNA and dsRNA, short ODNs (see below) and also certain chemicals, such as imiquimod (Fitzgerald-Bocarsly, 2002). The pathways they utilize to induce and activate the essential transcription factors (see above) involved in IFN-α/β gene expression is however incompletely known.

With respect to viruses, viral glycoproteins seem to be important for induction of type I IFN by certain viruses (Ankel *et al.*, 1998; Baudoux *et al.*, 1998; Charley *et al.*, 1991; Zeng *et al.*, 2002). It has been argued that viral nucleic acid is not required for this induction. However, RNA- and DNA-containing products of necrotic or apoptotic cells when combined with antibodies or lipofectin can trigger IFN-α production in NIPC/PDC (Båve *et al.*, 2001 ; Lövgren *et al*, to be published). In fact, double stranded RNA (dsRNA), which is produced by most viruses at some point during their replication cycle is the classical inducer of type I IFN production (Stark *et al.*, 1998). The Toll-like receptors have a special relation to IFN-α/β induction (see below), because the IFN-α/β production induced by ODNs requires TLR9, while induction by dsRNA and imiquimod requires TLR3 and TLR7, respectively (Ozato *et al.*, 2002).

Induction of IFN-α by bacteria, or components of bacteria such as lipopolysaccharide (LPS) and bacterial DNA has also been described (Bogdan, 2000). In the pig, low levels of IFN-α were induced by heat inactivated *A. pleuropneumonae* in cultures of PBMC (Wattrang *et al.*, 1998), approximately 100-fold lower than the levels of IFN-α induced by ADV. The ability of bacteria to induce IFN-α production may in part be due to the presence of CpG-DNA (see below).

IFN-α producing cells

In humans, infrequent cells referred to as NIPC/PDC have been shown to produce high amounts of IFN-α, as discussed above (Fitzgerald-Bocarsly, 2002; Kadowaki & Liu, 2002). The NIPC/PDC produce IFN-α upon stimulation by a wide variety of bacteria and viruses, including CpG-DNA, while IFN-α production by myeloid DC and monocytes can only be triggered by a limited number of stimulators, such as Sendai virus or dsRNA (Fitzgerald-Bocarsly, 2002; Rothenfusser *et al.*, 2002).

When porcine PBMC were stimulated by the transmissible gasteroenteritis virus (TGEV) or ADV *in vitro*, only approximately 0.01% of the cells produced IFN-α, but with a high quantity of IFN-α per cell (Nowacki & Charley, 1993; Nowacki *et al.*, 1993; Artursson *et al.*, 1992). The frequenαcy of IPC increased with the age of the animals tested (Nowacki *et al.*, 1993). These IPCs might constitute a porcine counterpart to the human NIPC/PDC and were shown to be non-adherent cells expressing swine leukocyte antigen (SLA) class II antigens and CD4, but not CD2, CD8 or a B cell marker (Charley & Lavenant, 1990; Nowacki & Charley, 1993). The SLA class II expression was upregulated at stimulation by virus. A population of IPC was also detected in porcine intestinal mucosa and spleen of TGEV infected animals (Riffault *et al.*, 1997; Riffault *et al.*, 2001). The expression of SWC3 was not found in the IPC detected in the gut, but some of the IPC detected in the spleen were SWC3 positive in pigs after intravenous injection with TGEV (Riffault *et al.*, 1997). The porcine IPC were found mainly in the mesenteric lymph nodes close to virus-infected cells, but the IPC appeared not to be infected (Riffault *et al.*, 2001). Also after intradermal administration of Aujeszky's disease virus (ADV) infected cells, the IPC were mainly detected in the regional lymph nodes (Artursson *et al.*, 1995). Large quantities of IFN-α are therefore produced in the lymphoid organs, and the influence of this IFN-α on the

initiation and development of a specific immune response in the pig should be profound and merits further study.

Immunostimulatory CpG-DNA

As mentioned before, DNA that contains unmethylated CpG dinucleotides (CpG) stimulates the innate immune system of vertebrates and has been termed immunostimulatory DNA. Unmethylated CpGs are present at the expected frequency in bacterial DNA, but are decreased in number and also often methylated in vertebrate DNA (Bird, 1980). Immunostimulatory DNA was originally shown to be an important adjuvant component of the Bacillus Calmette Guerin. Purified bacterial DNA was shown to induce IFN-α production by PBMC, increase NK cell activity and to stimulate B-cell proliferation and antibody production (Yamamoto *et al.*, 2002). Oligodeoxyribonucleotides containing unmethylated CpG (CpG-ODN) were shown to mimic these immunostimulatory effects of bacterial DNA (Krieg & Davis, 2001), and promote antigen-specific cellular and antibody responses. The immunostimulatory effect of CpG-ODN are now evaluated as an adjuvant in vaccines and also for the treatment of cancer and allergies (Krieg, 2001; Krieg & Davis, 2001).

The immunostimulatory capacity of CpG-ODNs appears dependent on the presence of TLR9 on the target cells, in humans NIPC/PDC and B cells (Hornung *et al.*, 2002; Kadowaki & Liu, 2002). In humans, CpG-DNA stimulates NIPC/PDC to upregulate co-stimulatory molecules and to produce e.g. IL-12 and IFN-α. The B cells are also directly stimulated to proliferation and to antibody production. The activated NIPC/PDC can promote a Th1-type of immune response. Furthermore, the cytokines produced by NIPC/PDC can cause maturation of other types of DC, for instance moDC, that also can drive the development of Th1 cells and CTL. These cytokines can obviously also stimulate the immune system in many other ways, as described above. In porcine PBMC (poPBMC), CpG-ODN induced production of IL-6, IL-12 and TNF-α (Kamstrup *et al.*, 2001) and the unmethylated CpG-containing vaccine plasmid pcDNA3 caused production of IFN-α and IL-6 (Johansson *et al.*, 2001; Magnusson *et al.*, 2001a). The target cells for the CpG-DNA were however not determined.

CpG-ODNs are considered to activate TLR9 via a process involving initial receptor mediated endocytosis via scavenger receptors, which is CpG-independent. TLR9 is present in endosomes, where it may interact with CpG-DNA, and is dependent on endosomal acidification (Wagner, 2002; Ahmad-Nejad *et al.*, 2002; Bauer & Wagner, 2002; Häcker *et al.*, 1998). The activation of the TLR9 requires an unmethylated CpG (Bauer *et al.*, 1999; Bauer *et al.*, 2001b; Bauer & Wagner, 2002; Ozato *et al.*, 2002; Hemmi *et al.*, 2000), as detailed below. The structure and function of the TLR9 and the signal transduction pathways involved in cytokine gene expression have recently been reviewed (Bauer & Wagner, 2002; Wagner, 2002).

The importance of the CpG motifs for the immunostimulatory effect of DNA has been studied in man, mice and primates (Krieg, 2002a) and recently also in farm animals, dogs and cats (Brown *et al.*, 1998; Rankin *et al.*, 2002; Rankin *et*

al., 2001). The optimal motif varies between species, but a general consensus motif of XCGY where X is any base but C and Y is any base but G (Krieg, 2002a) has been suggested. In general, an increase in the number of CpG in an ODN enhanced the immunostimulatory activity. However, the addition of CpG in the end of an ODN or in an unfavourable sequence context reduced the degree of cell activation. Elimination of CpG dinucleotides from ODN abolished their stimulatory activities, as did methylation of the cytosine in the CpG.

The immunostimulatory capacity of ODN is also affected by the chemical structure of the ODN backbone. ODNs can be made more resistant against nucleases by the use of a complete phosphorothioate backbone (Agrawal *et al.*, 1995; Zhao *et al.*, 1993), or phosphorothioate nucleotides at the 5' and 3'end and central phosphodiester nucleotides (thioate/diester ODN) (Krieg, 2002b). In general, ODNs containing phosphorothioate nucleotides are more potent at activating B-cells compared to the same sequence with a phosphodiester backbone (Krieg, 2002a). In contrast, thioate/diester ODNs were superior to other ODNs in their induction of IFN-α in plasmacytoid DC (Jarrossay *et al.*, 2001; Krug *et al.*, 2001a) and in activation of NK cells (Ballas *et al.*, 1996).

The thioate/diester ODNs mentioned above contained consecutive guanines (poly-G sequences) in the ends. Such poly-G sequences can also enhance the uptake of ODNs by the cells (Kadowaki & Liu, 2002; Kadowaki *et al.*, 2001; Jarrossay *et al.*, 2001; Hemmi *et al.*, 2000; Hemmi *et al.*, 2002). In fact, in most studies ODNs lacking poly-G sequences were extremely poor inducers of IFN-α production. However, they were strong inducers when combined with lipofectin. The lipofectin forms cationic liposomes with nucleic acids and protects them from degradation by nucleases (Thierry & Dritschilo, 1992), and perhaps more importantly increases their uptake by cells (Pisetsky & Reich, 1999; Thierry & Dritschilo, 1992; Bennett *et al.*, 1992; Hartmann *et al.*, 1998). Also the plasmid pcDNA3 induced high levels of IFN-α in porcine PBMC *in vitro*, but only when lipofected (Johansson *et al.*, 2001; Magnusson *et al.*, 2001a). In human NIPC/PDC, the IFN-α production induced by pcDNA3 was dependent on the presence of anti-DNA antibodies, that probably acted by increasing the uptake of the plasmid in the same way as lipofectin (Vallin *et al.*, 1999).

DNA vaccines

Vaccines consisting of DNA plasmids expressing the cDNA for specific antigens can induce protection against infectious agents in humans, mice and farm animals (Babiuk, 2002; Babiuk *et al.*, 2000; Gurunathan *et al.*, 2000a). Compared to DNA vaccines, traditional vaccines based on killed or attenuated organisms as immunogen may have limitations, including poor CTL mediated immunity, reversion to virulence, spread of the organisms and pathogenesis in immuno-compromised animals (Murtaugh & Foss, 2002). One important feature of DNA vaccines is their ability to mediate antigen presentation on both MHC class I and MHC class II molecules. When vaccine plasmids transfect host cells, the protein antigen is expressed, processed and presented in association with MHC class I molecules and in this way mimic natural infection with intracellularly replicating pathogens. Furthermore, if the antigen is released from transfected cells, both B

cells and Th cells may be stimulated via MHC class II restricted antigen presentation by DC, that have taken up the antigen. It is also possible that the DC become directly transfected, and DC have the ability to present antigen on both MHC class I and II (Gurunathan *et al.*, 2000a; Cho *et al.*, 2002). Thus, different mechanisms have been suggested for the generation of immune responses by DNA vaccines, either by direct transfection of DC, or by the transfer of antigen from e.g. transfected myoblasts to DC, as free antigen released by transfected cells or as apoptotic transfected cells (Gurunathan *et al.*, 2000a; Albert *et al.*, 1998). The presentation of antigen by MHC class I and II molecules thus promotes both CTL and Th cell development. It is well known that the way of delivery, the amount of antigen, and if the encoded antigen is secreted or not, have effects on the type of immune response generated (Liu & Ulmer, 2000; Gurunathan *et al.*, 2000a; Ulmer & Liu, 2002). It has also been demonstrated that co-delivery of the vaccine vector pcDNA3 encoding a HIV antigen with a plasmid encoding Fas, a gene involved in the induction of apoptosis, promoted the infiltration of DC to the site of injection (Chattergoon *et al.*, 2000). In this way the delivery of plasmid encoded antigen to the antigen-presenting DC could be facilitated.

It is to be expected that new approaches will improve on the results so far obtained with DNA vaccines, for instance the often-low antibody responses. For this purpose, the combination of a primary immunization by a DNA vaccine with recombinant protein as booster immunization can induce stronger cellular and humoral immunity and more complete protection (Liu & Ulmer, 2000; Gurunathan *et al.*, 2000a). In accordance, multiple injections of plasmid followed by a boost of plasmid plus human immunodeficiency virus (HIV) Env protein induced a strong CTL response and increased neutralizing antibody activity in rhesus monkeys, as well as protection against challenge (Letvin *et al.*, 1997; Baig *et al.*, 2002).

CpG-DNA and cytokines as adjuvant in DNA vaccines

It has been shown that plasmid vectors could have adjuvant activities themselves through the presence of CpG dinucletides (Gurunathan *et al.*, 2000a; Klinman *et al.*, 2000; Sato *et al.*, 1996). Also the number of CpG motifs may affect the adjuvant activity DNA vaccines (Gurunathan *et al.*, 2000a; Krieg *et al.*, 1998; Cohen *et al.*, 1998). In fact, addition of CpG motifs enhanced both cell-mediated and humoral immunity to a DNA vaccine against HIV (Kojima *et al.*, 2002), and the response was dependent on the number of CpG motifs included. Also, the cellular immune response to a DNA vaccine against bovine herpesvirus was correlated to the CpG content in the plasmid vector (Pontarollo *et al.*, 2002a). In the pig, the adjuvant capacity of an unmethylated CpG-containing vaccine plasmid pcDNA3 was shown by induction of high levels of IFN-α *in vitro* (Johansson *et al.*, 2001). Furthermore, methylation of the cytosine in the CpGs of the pcDNA3 plasmid abolished the IFN-α inducing activity (Magnusson *et al.*, 2001a), but exchange of the CpG motif-containing ampicillin resistance gene to a kanamycin resistance gene (lacking CpG motifs) did not affect the amounts of IFN-α induced by pcDNA3, which therefore may be due to the presence of the many other CpG motifs in other parts of the plasmid.

The importance of CpG-induced cytokines such as IFN-α, IFN-γ, IL-12 and IL-18 for the induction of Th1 type of immune responses in DNA vaccination has recently been pointed out (Song *et al.*, 2000; Cohen *et al.*, 1998; Gurunathan *et al.*, 2000a; Gurunathan *et al.*, 2000b; Kim *et al.*, 1998). In mice, the presence of IFN-α was shown to be essential for the induction of antigen-specific Th1-associated IgG isotypes as well as for IFN-γ production by spleen cells after immunization with a DNA vaccine against ADV (Tudor *et al.*, 2001). Also activation of B cells and CD8 positive T cells by the DNA vaccine were shown to be dependent on the presence of a functional IFN-α receptor in mice, which is in line with findings that CpG can induce upregulation of adhesion and activation molecules in an IFN-α dependent manner (Kranzer *et al.*, 2000; Sun & Sprent, 2000; Sun *et al.*, 1998).

The immunogenicity of DNA vaccines in mice and pigs can also be enhanced by addition of cytokines and co-stimulatory molecules, in the form of recombinant protein or expressed from a DNA plasmid (Song *et al.*, 2000; Cohen *et al.*, 1998; Gurunathan *et al.*, 2000a; Gurunathan *et al.*, 2000b; Kim *et al.*, 1998). The ability of IL-12 to promote Th1 immune responses has been utilized in this respect (Gurunathan *et al.*, 2000a; Kim *et al.*, 1998). For a DNA vaccine against porcine ADV, co-administration of a plasmid encoding GM-CSF increased antibody titers and resulted in faster clearance of virus at challenge (Somasundaram *et al.*, 1999; Dufour *et al.*, 2000). In contrast, the immune response to another porcine ADV vaccine was not improved by plasmids expressing IFN-γ or IL-12 (van Rooij *et al.*, 2002). The latter finding may be explained by the fact that, as mentioned above, IL-12 alone may have a relatively poor immunostimulatory effect that is strikingly increased by combination with IL-18. In fact, addition of plasmids encoding IL-12 and IL-18 in DNA vaccines against feline immunodeficiency virus (Hanlon *et al.*, 2001) and against *Mycobacterium tuberculosis* in mice (Triccas *et al.*, 2002) favoured a Th1 type of immune response and increased protection to challenge. Furthermore, combining an ADV vaccine vector with plasmids encoding IL-12 and IL-18 restored the Th1 response in type I IFN receptor deficient mice (Tudor *et al.*, 2001). An IL-6 expression vector was also shown to enhance the efficacy of a DNA vaccine against influenza virus in mice, but was without effect in pigs (Larsen & Olsen, 2002; Larsen *et al.*, 1998).

Aims of the present study

The aims of the present study were to:

- Evaluate the possible adjuvant effects of the vaccine plasmid pDNA3, by studying the ability of the plasmid to induce production of cytokines *in vivo*.
- Study the expression *in vivo* of IL-6 and GM-CSF from pcDNA3 containing cDNA for these cytokines.
- Construct the cDNA for a porcine IL-12 fusion protein in pcDNA3 and to verify the expression of biologically active IL-12 *in vitro*.
- Characterize the IFN-γ producing cells after stimulation of porcine PBMC by IL-12 and IL-18.
- Achieve differentiation of porcine monocytes to DC using IL-4 and GM-CSF *in vitro* and to study effects of IFN-α, TNF-α and pcDNA3 on the moDC.
- Evaluate the ability of moDC, differentiated with or without the presence of IFN-α to produce cytokines at various stimuli.
- Study the properties of ODNs required for IFN-α induction in cultures of PBMC.
- Characterize the IPC after stimulation of PBMC with ODN, pcDNA3 or ADV, using flow cytometry and *in situ* hybridization.

COMMENTS ON METHODS

Experimental animals and implantation of tissue chambers

The studies were performed using specific pathogen free (SPF) pigs (Landrace x Yorkshire; Serogrisen®, Ransta, Sweden) (Wallgren *et al.*, 1999; Wallgren & Vallgårda, 1993) or conventionally reared Yorkshire pigs The SPF pigs were pre-selected for similar IFN-α producing capacity *in vitro* in response to the plasmid pcDNA. In comparison to pigs housed under SPF conditions, the conventionally reared pigs may have different health status that might complicate the interpretation of the immunological response. Other factors that might influence e.g. the IFN-α response are for instance age and the breed of the animal (Nowacki *et al.*, 1993). The ability to produce IFN-α at certain stimuli has been shown to vary between parental offspring groups (Edfors-Lilja *et al.*, 1994). Therefore, PBMC from animals from different litters were used to avoid a bias towards high or low IFN-α response to various stimuli (papers III and IV), and also in the mixed leukocyte reactions (MLR; papers II and III) to avoid the use of SLA class II matched individuals. The tissue chambers, implanted in the SPF pigs, consisted of silicone rubber tubes (70 mm) that were perforated at the ends, sealed and implanted subcutaneously close to regional lymph nodes (Wattrang *et al.*, 1997).

Cytokine-expressing plasmid vectors

The cDNA for poIL-12 p35, p40 and a poIL-12 fusion protein were cloned into pcDNA3. For construction of the cDNA for the fusion protein, the open reading frames for poIL-12 p40 and p35 were PCR amplified from plasmids encoding the respective cDNA, but omitting the p40 signal peptide. A $(Gly_4Ser)_3$ linker sequence (L) (Huston *et al.*, 1988) was ligated between the p40 and p35 cDNAs, and the whole cDNA cassette was then cloned into pcDNA3 (pcDNA/p40-L-p35) and also into a pMT/V5-HisA plasmid. This latter construct encoded a porcine IL-12 fusion protein (designated pMT/IL-12 fusion protein) with a polyhistidine (his) tag at the C-terminus. The pcDNA3 constructs were transfected into COS cells and the pMT/his-p40-L-p35 construct was transfected into *Drosophila melanogaster* Schneider 2 (S2) cells. The IL-12 biological activity in culture supernatants was determined using the IL-12/IL-18 bioassay (see below). The histidine tagged pMT/IL-12 fusion protein was purified on Ni-NTA agarose and the purity and concentration was estimated by SDS-PAGE.

In addition, the cDNA for poIL-6 was cloned into pcDNA3 (pcDNA3/IL-6) and and the expression of bioactive IL-6 was confirmed by bioassay after transfection of COS cells (paper I). This plasmid was subsequently used to immunize mice for generation of monoclonal antibodies (mAbs). The poGM-CSF (pcDNA3/GM-CSF) vector has been described before (Somasundaram *et al.*, 1999). In paper III, the cDNA for IFN-α_1 was cloned in the pcDNA3 plasmid and expressed in COS cells.

Differentiation of monocytes to dendritic cells *in vitro*

Porcine monocytes were obtained by plastic adherence of PBMC and cultivated for 5 days in the presence of recombinant human GM-CSF (*r*huGM-CSF) and *r*huIL-4. Such cells obtained a dendritic morphology and were defined as immature monocyte-derived DC (moDC). The effect of poIFN-α on the differentiation was also studied. The maturing effects of TNF-α and of the plasmid pcDNA3 on DC were studied, and the stimulated DC were analyzed for upregulation of MHC class II and B7 molecules as determined by flow cytometric analysis, and also for their ability to induce allogeneic and syngeneic MLR. In the MLR, the proliferative response of PBMC to irradiated moDC from syngeneic or allogeneic donor pigs was measured using H^3-thymidine uptake.

In vitro induction of cytokines

The cytokine producing ability of stimulated porcine DC, was compared to that of monocytes or fresh PBMC. The cytokine induction was performed using the expression plasmid pcDNA3. This plasmid has previously been shown to induce IFN-α production by porcine PBMC (Magnusson *et al.*, 2001a). Heat inactivated *A. pleuropneumoniae*, which was known to induce production of IL-6 in pigs *in vitro* and *in vivo* (Fossum *et al.*, 1998; Johansson *et al.*, 2001) and of IFN-α *in vitro* (Magnusson *et al.*, 2001a; Johansson *et al.*, 2001; Wattrang *et al.*, 1998) was also used.

The UV-inactivated herpesvirus Aujeszky's disease virus (ADV) was used because of its documented IFN-α inducing ability on porcine cells (Artursson *et al.*, 1995; Artursson *et al.*, 1992), possibly in NIPC/PDC. ADV is an enveloped virus containing linear dsDNA that may cause neurological and respiratory disorders in the pig. In humans, the herpes simplex virus (HSV) induces IFN-α production exclusively by NIPC/PDC, while both NIPC/PDC and monocytes respond to stimulation with Sendai virus (Magnusson *et al.*, 2001b; Fitzgerald-Bocarsly, 2002). Sendai virus, that is an enveloped paramyxovirus with a negative stranded RNA genome, was therefore included in paper III.

Furthermore, the IFN-α inducing ability of various ODN was studied in cultures of PBMC, with or without pre-treatment with lipofectin (paper IV). IFN-α producing cells after stimulation of PBMC with the ODN 2216, ADV or pcDNA3 were characterized using flow cytometry and in situ hybridization. For detection of IFN-γ producing cells by flow cytometry, porcine PBMC were stimulated with rpoIL-12 and rhuIL-18 (paper II).

Immunoassay and bioassay

Cytokine production was measured using bioassays and by immunoassays. The bioassays measure the biological activity of the protein, but the results may be affected by other components in serum or culture supernatants having similar or counteracting effects as the protein measured. The immunoassays are more specific because the protein is detected with antibodies, but nothing is then known about its biological activity. Levels of IFN-α (papers I, III and IV) and IFN-γ (papers II and III) were measured by two immunoassays based on the dissociation-enhanced

lanthanide fluoroimmunoassay (DELFIA) principle as previously described, using pairs of mAbs (Artursson *et al.*, 1995; Wattrang *et al.*, 1997). A laboratory IFN-α standard obtained from Sendai virus induced porcine PBMC was used (Diaz de Arce *et al.*, 1992), and the IFN-γ standard consisted of rpoIFN-γ expressed in *E. coli*.

The levels of IL-6 were determined by a DELFIA (paper I) using a polyclonal goat antibody to porcine IL-6 and a biotinylated anti-poIL-6 mAb (LF159, produced in our laboratory) combined with europium-labeled streptavidin. A commercially available ELISA for IL-6 was also used. Furthermore, IL-6 activity was detected by a bioassay (papers I and III) using B9 cells (Fossum *et al.*, 1998), using *r*poIL-6 as standard.

Two bioassays were used for detection of GM-CSF (paper I), either measuring effects on the human GM-CSF dependent TF1 cell-line, or proliferation of porcine bone marrow cells (Somasundaram *et al.*, 1999).

The IL-12/IL-18 bioassay (papers II and III) was based on the ability of IL-12 in combination with *r*huIL-18 to induce IFN-γ production by PBMC in mixed leukocyte cultures (MLC) established from two pigs. An anti-huIL-12 mAb, cross-reacting with poIL-12 (Kokuho *et al.*, 1999), was used to determine whether the IFN-γ production was caused by IL-12.

Detection of cytokine mRNA by RT-PCR

To verify the results obtained by bioassay for IL-6 and IL-12 (paper III) reverse transcriptase polymerase chain reactions (RT-PCR) were applied to mRNA purified using oligo-dT coated magnetic beads (Dynal, Oslo, Norway). The cytokine cDNA was amplified using the primer pairs described in Table 1 (paper III). The primer recognized sequences that were, in comparison to the human IL-6 and IL-12 genes, most likely located on separate exons. To increase the sensitivity and specificity of the RT-PCR for IL-12, nested PCR was performed using the full length PCR product as template and internal primers.

Analysis of IFN producing cells by flow cytometry

Flow cytometric methods were developed for the study of the IFN-α and IFN-γ producing cells (papers II and IV). Staining for intracellular IFN-γ was carried out using a phycoerythrin (PE)-conjugated anti-poIFN-γ mAb on cells fixed with paraformaldehyde and permeabilized with saponin (paper II). The IFN-γ producing cells were also analyzed for the presence of cell surface antigens by staining, before fixation and permeabilization, using mAbs directed to CD3 (FITC-labelled), CD2 (biotinylated) and CD14 (unlabelled). The binding of the latter two mAbs was visualised using RPE-Cy5-labelled streptavidin (CD2) and FITC-labelled goat-anti-mouse Ig (CD14).

Detection of intracellular IFN-α was performed on paraformaldehyde-fixed cells that were permeabilized with tween-20. A biotinylated anti-IFN-α mAb was used in combination with PE-labelled streptavidin (paper IV). It was important to block unspecific binding, by using 5 % normal mouse serum in the case of staining for

IFN-γ, or by using 5 μg/ml mouse IgG and 10 % of specific pathogen free (SPF) pig sera when staining for IFN-α.

Detection of IFN-α mRNA-containing cells by *in situ* hybridization

To further characterize the IPC, a method to detect cells expressing IFN-α mRNA by *in situ* hybridization was established (paper IV). Briefly, stimulated PBMC were fixed in paraformaldehyde and transferred to glass slides. The cells were hybridized with a biotinylated cRNA probe, prepared by *in vitro* transcription from a 829 bp DNA for poIFN-α_1 in pGEM in the presence of biotin-16-UTP and T7-RNA polymerase or Sp6-RNA polymerase. After hybridization, the mRNA positive cells were detected by means of the sensitive GenPoint™ catalyzed amplification system, using a primary horseradish peroxidase (HRP) labelled streptavidin, biotinyl tyramide and secondary HRP-streptavidin. The frequencies of positive cells, and the intensity of labelling were estimated.

Statistical methods

When indicated, data are presented as mean values ± SEM of all pigs tested. In paper III, pairwise comparisons between the different cell types for each stimulator:responder ratio in the MLR and calculation of p-values were performed using Wilcoxon signed rank test. The Bonferroni-Holm method (Holm, 1979) was used for adjustment for multiple testing.

Results and discussion

The vector pcDNA3 induces production of IFN-α *in vivo* (paper I)

DNA vaccines have recently been shown to induce a protective Th1-type of immune response, including antigen specific CTL responses, in many species (Gurunathan *et al.*, 2000b; Gurunathan *et al.*, 2000a; Liu & Ulmer, 2000). The immune response to vaccines may be biased towards a Th1 type of response by using immunoregulatory cytokines as adjuvant, included as plasmids encoding the cytokine cDNA (Song *et al.*, 2000; Cohen *et al.*, 1998; Gurunathan *et al.*, 2000a; Gurunathan *et al.*, 2000b; Kim *et al.*, 1998). Furthermore, it has been shown that vectors used in DNA vaccination, by the presence of CpG motifs, may have adjuvant effects (Klinman *et al.*, 2000). In porcine PBMC, the ability of the vaccine vector pcDNA3, but not the methylated form of the plasmid to induce production of IFN-α and IL-6 had earlier been described (Johansson *et al.*, 2001; Magnusson *et al.*, 2001a). To study if the same immunostimulatory effect could be achieved in vivo, SPF pigs were injected with pcDNA3, the methylated form of the plasmid and plasmids encoding the cDNA for IL-6 (pcDNA3/IL-6) or GM-CSF (pcDNA3/GM-CSF). The plasmids were injected into subcutaneous tissue chambers, located close to the lymph nodes of pigs as previously described (Wattrang *et al.*, 1997).

The plasmid pcDNA3 (with or without the addition of cytokine cDNA) had an immunostimulatory effect in pigs as demonstrated by a local production of IFN-α in tissue culture fluid (TCF) (Fig. 1 and 2), while no IFN-α could be detected in serum. Methylation of pcDNA3 abolished the IFN-α inducing capacity of the plasmid. The IFN-α response was higher after the second injection with plasmid, which could be explained by recruitment of IPC to the injection site. Furthermore, the locally produced IFN-α at the first injection may also have a priming effect on the IPCs (see Introduction), resulting in enhanced IFN-α production at the second injection in the same tissue chamber. The kinetics of the IFN-α response *in vivo* was similar to that induced by e.g. ADV *in vitro* (Wattrang *et al.*, 1995) and *in vivo* (Wattrang *et al.*, 1997; Artursson *et al.*, 1995). The plasmid pcDNA3 had earlier been shown to require preincubation with lipofectin for induction of IFN-α *in vitro* (Magnusson *et al.*, 2001a), whereas induction with the dsRNA analogue poly(I):poly(C) *in vivo* (Wattrang *et al.*, 1997) or with pcDNA3 in the present study did not. The mechanisms for uptake of the plasmid pcDNA therefore seem to differ *in vivo* and *in vitro*. Lipofectin may facilitate the cellular uptake of the plasmid and protect against degradation by nucleases (Pisetsky & Reich, 1999; Thierry & Dritschilo, 1992; Bennett *et al.*, 1992; Hartmann *et al.*, 1998). Thus, the levels of IFN-α induced could be affected by insufficient uptake of the plasmid into the cells, by degradation of the plasmid and also depend on which cell type is transfected.

No production of IL-6 was detected that clearly was induced by the various plasmids, because injection with saline induced similar levels of IL-6 in the TCF (Fig. 3). The increased levels of IL-6 were most likely due to the sampling

procedure, and for instance elicited by endothelial injury (Krishnaswamy *et al.*, 1999). The presence of IL-6 in serum was detected 48h after injection with pcDNA3/IL-6 and 12h after injection with pcDNA3 using bioassay, but these results were not confirmed by immunoassay. However, a local production of IL-6 was detected in the lungs after instillation of plasmid DNA in mice (Yew *et al.*, 1999). Furthermore, injection of plasmid DNA or CpG ODN induced IL-6 responses in lung lavage fluid or serum (Schwartz *et al.*, 1999; Yi *et al.*, 1996). Expression of plasmid-encoded IL-6 has been detected in murine sera after intradermal injection of a plasmid encoding this cytokine (Sun *et al.*, 1995; Larsen *et al.*, 1998), and the expressed IL-6 enhanced the effect of influenza DNA vaccination (Larsen *et al.*, 1998).

In the present study, the expression of GM-CSF by pcDNA3/GM-CSF could not be detected in TCF or serum by available bioassays. The failure to detect plasmid-encoded cytokines may be due to that the plasmids were not expressed in the tissue chambers or at levels below detection. In mice, plasmid-encoded GM-CSF was shown to attract antigen-presenting cells to the site of injection. Both mature (Kusakabe *et al.*, 2000) and immature (Haddad *et al.*, 2000) DC have been detected in the muscle after intramuscular injection of plasmids encoding GM-CSF and the immature DC migrated further to the local draining lymph node. The pcDNA3/GM-CSF construct used in the present study has in previous vaccine studies improved the protective effect at DNA vaccination of pigs against ADV (Dufour *et al.*, 2000; Somasundaram *et al.*, 1999), which indicates that GM-CSF actually can be expressed by the plasmid cDNA *in vivo*.

Injection with pcDNA3/GM-CSF did not affect the numbers of mononuclear leukocytes and neutrophilic granulocytes in the TCF (Fig. 4). Increased cellular influx of neutrophils was detected after injection of plasmid as well as saline (Fig. 4) and probably reflects a post-operative effect that enhanced the inflammatory response to the injections in general.

The adjuvant effects of pcDNA3 have been studied in mice immunized with a DNA vaccine against ADV, and co-delivery of pcDNA3 enhanced the antigen-specific antibody response in both wild-type (WT) and IFNAR-deficient mice (Tudor *et al.*, 2001). The DNA vaccine induced a Th1 type of immune response in WT mice, with high IgG2a:IgG1 ratio and IFN-γ production by spleen cells after restimulation *in vitro*, but not in the IFNAR deficient mice. This indicates an importance for IFN-α in the generation of Th1 immune responses at DNA vaccination. Furthermore, a role for IFN-α was shown for the activation of CD8+ T cells and B cells. When testing plasmids expressing IL-12 and IL-18 as adjuvant, the Th1 responses was strongly increased also in IFNAR deficient mice.

In conclusion, the use of tissue chambers provides means to study the local immunostimulatory effects of plasmid DNA *in vivo* in the pig. The model could also be used to estimate side effects induced by vaccine adjuvants, such as local inflammatory responses. Efficient *in vivo* induction of IFN-α by pcDNA3 and other vaccine vectors could be an important goal when DNA vaccines are designed, because IFN-α has an impact on the early innate immunity as well as on the generation and maintenance of the specific immune response (see Introduction).

Construction and *in vitro* evaluation of porcine IL-12 vectors (paper II)

Cytokines expressed by vaccine plasmids have been shown to have adjuvant effects in DNA vaccination (Song *et al.*, 2000; Kim *et al.*, 1997; Gurunathan *et al.*, 2000a). Co-administration of plasmids expressing vaccine antigen and e.g. plasmids encoding IL-12 or IFN-γ resulted in protective antigen-specific cellular immune responses in mice (Kim *et al.*, 1998; Song *et al.*, 2000). The ability of IL-12 to direct the immune response to development of antigen specific Th1 cells and CTL indicates that IL-12 can be an important adjuvant in DNA vaccination, also in the pig. We therefore constructed pcDNA3 plasmids encoding the cDNA for IL-12 p35 and p40 subunits, and also for a fusion protein of porcine IL-12.

It is essential to be able to evaluate the biological activity of poIL-12 expressed by plasmids. We therefore developed a bioassay, the IL-12/IL-18 bioassay, based on the ability of IL-12 to induce IFN-γ production in porcine PBMC. We found that huIL-12 induced a dose dependent production of poIFN-γ in PBMC, especially in MLC cultures (Fig. 1a). This production was strongly enhanced in a dose dependent fashion by rhuIL-18, but the rhuIL-18 alone was a poor IFN-γ inducer. In a similar manner, IL-12 enhanced the IFN-γ inducing activity of rhuIL-18. Such synergistic effects of IL-12 and IL-18 have previously been described in human and murine systems (Tominaga *et al.*, 2000), and are probably related to the ability of IL-18 to up-regulate the IL-12Rβ2 (Chang *et al.*, 2000) and of IL-12 to increase the IL-18Rβ expression (Yoshimoto *et al.*, 1998). Furthermore, binding of IL-18 to the IL-18 receptor activates IL-1 receptor-associated kinase (IRAK), leading to activation of nuclear factor κB (NFκB). IL-12, when binding to the IL-12 receptor, activates STAT3 and 4 to induce gene transcription in Th1 cells. This may therefore also explain why IL-12 and IL-18 synergize in the induction of IFN-γ, because both NFκB and STAT4 are known to be transcription factors for the IFN-γ gene (Okamura *et al.*, 1998; Nakanishi *et al.*, 2001).

Porcine IL-12 was expressed by co-transfecting COS cells with two pcDNA3 plasmids containing IL-12 p40 and p35, respectively. Such poIL-12 heterodimers were able to induce IFN-γ in the IL-12/IL-18 bioassay (Fig. 2). No activity was induced after transfection of the plasmids separately. This indicates that a functional heterodimer of p40 and p35 was necessary for biological activity, which is in agreement with earlier studies of poIL-12 (Foss & Murtaugh, 1999; Kokuho *et al.*, 1999)

A fusion protein of porcine IL-12 was also constructed, containing the p40 domain followed by a linker and the p35 domain (pcDNA3/p40-L-p35). This plasmid expressed a protein in transfected COS cells that was highly active in the IL-12/IL-18 bioassay (Fig. 3), indicating that the linker sequence does not interfere with the functionally relevant folding of the poIL-12 subunits.

The cDNA for the IL-12 fusion protein was also transferred to the plasmid pMT-His (pMT/His-p40-L-p35), and transfected *Drosophila melanogaster* Schneider 2 cells (S2 cells) showed both transient and a stable expression of biologically active

poIL-12 as determined by the IL-12/IL-18 bioassay (Fig. 4). A relatively good expression was obtained in the *Drosophila* S2 cells, about 10 μg per ml medium. The His-tagged poIL-12 fusion protein was purifed using Ni-NTA agarose and analysed by SDS-PAGE and Western blotting (Fig. 5). The purification resulted in a pure and biologically active poIL-12, but the yield was low (5-10%).

The poIL-12 fusion protein should be valuable as research reagent and as adjuvant. The *in vivo* bioactivity of a similar poIL-12 p40-L-p35 fusion protein has recently been demonstrated in a cholera toxin vaccination model in swine (Foss *et al.*, 1999). Because of the documented adjuvant effects of IL-12 (Kim *et al.*, 1998; Gurunathan *et al.*, 2000a), the pcDNA3/p40-L-p35 expression vector could be evaluated as adjuvant to enhance the immune response to DNA vaccines in pigs. Recently, a plasmid capable of expressing poIL-12 heterodimers was tested in pigs and found to have poor activity as adjuvant in a DNA vaccine against ADV (van Rooij *et al.*, 2002). Because of the pronounced synergy between IL-12 and IL-18 in the pig observed in the present study, combinations of plasmids expressing poIL-12 and poIL-18 should therefore be evaluated as adjuvant in DNA vaccines. A further motivation for using the combination poIL-12 and poIL-18 is the low biologic activity of poIL-12 on activated porcine lymphocytes, which may be due to a low expression of the IL-12Rβ2 (Solano-Aguilar *et al.*, 2002).

Phenotype of the IFN-γ producing cells induced by IL-12 and IL-18 (paper II)

Many different cell types, such as NK cells, T cells, macrophages and DC, have the ability to produce IFN-γ in mice and humans (Okamura *et al.*, 1998; Tominaga *et al.*, 2000; Nakanishi *et al.*, 2001; Otani *et al.*, 1999), and at the initiation of this study it was not known which cells produce IFN-γ in the pig. In paper II it was demonstrated that the combination of IL-12 and IL-18 was a potent inducer of IFN-γ production in poPBMC. The cell types responsible for this IFN-γ production were identified, and for this purpose we established a three colour flow cytometry method involving staining for intracellular IFN-γ and cell-surface antigens.

When poPBMC in MLC were stimulated by poIL-12 and huIL-18, approximately 3.0% of the cells were stained for intracellular IFN-γ. Staining for the surface antigens CD2, CD3 and for intracellular IFN-γ revealed that the IFN-γ containing cells were mainly CD2+CD3+ T cells (69%) and CD2+CD3- cells (31%) (Fig. 6). The latter cells are considered to be NK cells (Summerfield *et al.*, 2001; Yang & Parkhouse, 1996). The CD2-CD3+ T cells, which constituted about 50% of the CD3+ cells completely failed to produce IFN-γ. These cells should to a large extent be CD4-CD8- TCRγδ+ cells, which have been identified in high frequency in young pigs (Yang & Parkhouse, 1996). Furthermore, the IFN-γ producing cells did not express CD14. This antigen is present on porcine monocytes and myeloid DC (Carrasco *et al.*, 2001). It was recently demonstrated by a similar flow cytometric method and polyclonal activation of PBMC, that approximately half of the T cells that produced IFN-γ were γδ T cells in pigs of the same age as the pigs in the present study (Rodriguez-Carreno *et al.*, 2002).

Although the exact identity of the IFN-γ producing cells in the pig must be further evaluated, our findings that T cells and NK cells are the major producers agree with results obtained in other species (Okamura *et al.*, 1998; Tominaga *et al.*, 2000; Nakanishi *et al.*, 2001; Otani *et al.*, 1999).

Characterization of monocyte-derived DC according to their cytokine profile (paper III)

The DC have an important role in the defence against infection by their ability to take up, process and present antigens, and their ability to stimulate and regulate the adaptive immune response, e.g. by production of cytokines (see Introduction). The DC are therefore also the most important target cell for antigen expression and presentation in vaccination. Human monocytes have been differentiated *in vitro* into moDC in the presence of GM-CSF and IL-4 (Hart, 1997). Further maturation of human moDC *in vitro*, with consequent upregulation of co-stimulatory and MHC class II molecules and enhanced antigen- presenting capacity has been induced by the use of the cytokines TNF-α and IFN-α (Shortman & Liu, 2002; Luft *et al.*, 2002) and by PAMPs such as LPS or CpG-ODN (Kadowaki & Liu, 2002; Shortman & Liu, 2002).

In paper III, a method was established to differentiate porcine adherent monocytes to moDC *in vitro* in cultures with huIL-4 and huGM-CSF. The effects of IFN-α during differentiation were studied. The maturing effects of TNF-α and pcDNA3 were determined as e.g. the enhanced ability of the DC to stimulate proliferation of PBMC in an MLR. Furthermore, moDC cultured with or without IFN-α during the differentiation were characterized according to their ability produce cytokines at exposure to pcDNA3, ADV, Sendai virus or *A. pleuropneumoniae*.

The adherent monocytes obtained a dendritic morphology during differentiation in huGM-CSF and huIL-4 (Fig. 1). No clear effect of IFN-α during differentiation was observed on the cell morphology. Expression of MHC class II and B7 (CD80/CD86) molecules was high, regardless of whether the moDC were differentiated in the presence of IFN-α or not. That porcine monocytes exposed to GM-CSF and IL-4 acquired antigenic and morphologic characteristics of myeloid DC is in accordance with recently published studies (Carrasco *et al.*, 2001; Paillot *et al.*, 2001).

The moDC could stimulate proliferation of allogeneic PBMC and the presence of IFN-α during differentiation increased their ability to stimulate cell proliferation (Fig. 2a). Such effects of IFN-α has also has been observed for human moDC (Santini *et al.*, 2000; Radvanyi *et al.*, 1999; Paquette *et al.*, 1998). Similar results were obtained when the DC were tested in syngeneic MLR. Interestingly, it has recently been shown that the proliferation induced in syngeneic MLR is a response to autologous apoptotic material processed by DC (Chernysheva *et al.*, 2002).

No enhancing effects of TNF-α could be observed on the ability of moDC to stimulate an allogeneic MLR (Fig. 3a), which is in contrast to results obtained by

others with porcine moDC differentiated in poGM-CSF and poIL-4, where addition of TNF-α, TGF-β1 or LPS increased the allogeneic MLR response (Carrasco *et al.*, 2001; Paillot *et al.*, 2001). Also human moDC displayed a strongly increased ability to stimulate allogeneic MLR after stimulation with TNF-α (Yang *et al.*, 1999).

Furthermore, stimulation of the moDC with pcDNA3 did not increase their capacity to evoke an allogeneic MLR (Fig. 3b). Our results are in contrast to those of one study where plasmid DNA increased the ability of human moDC to stimulate an MLR (Schattenberg *et al.*, 2000). Other studies show, however, that NIPC/PDC, but not moDC respond to induction by CpG-ODN (Schattenberg *et al.*, 2000; Kadowaki & Liu, 2002; Fitzgerald-Bocarsly, 2002). The discrepant results may be explained by the fact that TLR9 is expressed by plasmacytoid DC, but not by myeloid DC (Kadowaki & Liu, 2002; Hornung *et al.*, 2002), and that the plasmid DNA stimulate NIPC/PDC to produce cytokines such as IFN-α that cause maturation of the moDC.

The moDC, or moDC differentiated in the presence of IFN-α, were compared to PBMC and adherent monocytes for their ability to produce the Th1 associated cytokines IL-12 and IFN-α and the Th2 cytokines IL-6 and IL-10 (Table 2 and Fig. 5). The plasmid pcDNA3 and Sendai virus induced production of IL-12 in PBMC, adherent monocytes and DC. However, the plasmid pcDNA3 was only active when preincubated with lipofectin. *A. pleuropneumoniae* induced IL-12 only in the DC, while ADV failed to stimulate IL-12 production in these cells. The induction of IL-12 bioactivity was confirmed by detection of p40 mRNA in DC stimulated by *A. pleuropneumoniae* or by pcDNA3 using RT-PCR (Fig. 4). A constitutive expression of p35 was observed, which had previously been described in mice (Bost & Clements, 1995), humans (Cassatella *et al.*, 1995) and pigs (Foss & Murtaugh, 1997). Interestingly, RT-PCR for IL-12p35 after stimulation with *A. pleuropneumoniae* revealed an additional larger PCR-product, which contained an additional 75 bp that were homologous to the entire fourth intron of the human p35 gene (the sequence of the poIL-12p35 gene was not available). This longer transcript may be explained by alternative splicing of this intron at stimulation with *A. pleuropneumoniae*. The relevance of these preliminary data requires further study. However, it was reported that differential splicing of the murine p35 transcripts occurred when spleen cells were stimulated with LPS or a combination of IFN-γ and CD40 (Hayes *et al.*, 1998). Also from the human p35 gene, different transcripts from IFN-γ and LPS stimulated monocytes or unstimulated lymphoblastoid cells have been reported, due to expression from different promoters (Vaidyanathan *et al.*, 2001).

The plasmid pcDNA3, when preincubated with lipofectin, and the herpesvirus ADV induced IFN-α production in PBMC but not in moDC or monocytes. In contrast, Sendai virus induced IFN-α production in PBMC, monocytes and was the only inducer of IFN-α production in moDC. Thus, the viruses tested induced IFN-α production in different cell types. Also human monocytes and moDC produce IFN-α at induction with Sendai virus, while only the NIPC/PDC produces IFN-α at stimulation with herpesvirus, such as HSV (Fitzgerald 2002). In the pig, a cell population with several characteristics of NIPC/PDC has been

described (Nowacki & Charley, 1993; Nowacki *et al.*, 1993). It remains to be determined if the porcine NIPC/PDC are responsible for the IFN-α production at exposure to plasmid DNA or ADV observed in the present study.

The moDC were also able to produce Th2 associated cytokines. The *A. pleuropneumoniae*, and to a lesser degree also Sendai virus, induced production of both IL-6 and IL-10. The ability of moDC to produce IL-6 at stimulation with *A. pleuropneumoniae* was confirmed by determination of IL-6 mRNA by RT-PCR (Fig. 4). Also monocytes and PBMC produced IL-6 and IL-10, especially when exposed to *A. pleuropneumoniae*. Production of IL-6 and IL-10, has also been demonstrated in human moDC (Salio *et al.*, 1999; Reid *et al.*, 2000), and may be one way for microorganisms to inhibit cell-mediated immunity by skewing the response towards the Th2 pathway. For instance, IL-10 has potent inhibitory effects on the production of Th1 cytokines, such as IL-12 and IFN-α (D'Andrea *et al.*, 1993; Fickenscher *et al.*, 2002; Payvandi *et al.*, 1998).

The *in vitro* differentiated moDC were thus capable of producing both Th1 and Th2 associated cytokines at appropriate stimuli. In general, no clear effect of IFN-α during DC differentiation was observed on the cytokine production. The ability of porcine moDC to induce T cells producing IFN-γ and IL-4 has been demonstrated in one study (Carrasco *et al.*, 2001), and by their production of Th1 and Th2 associated cytokines, the moDC should be able to polarize this Th cell development. The porcine moDC generated *in vitro* therefore can be used for basic studies of immune responses to porcine vaccines. The ability of the monocyte-derived porcine DC to produce high amounts of IL-12, and to produce IFN-α at stimulation by Sendai virus, but not ADV, indicates that the porcine moDC resemble the human myeloid DC. Pig models could therefore be relevant also in human vaccine research.

Induction of IFN-α by CpG-ODN in infrequent porcine PBMC (paper IV)

As discussed above, the human NIPC/PDC are the major producers of IFN-α in response to CpG ODN and HSV (Fitzgerald-Bocarsly, 2002). A rare population of IPC, resembling the human NIPC/PDC, has also been described in the pig, producing IFN-α in response to the herpesvirus ADV (Artursson *et al.*, 1995), and TGEV (Nowacki *et al.*, 1993; Nowacki & Charley, 1993; Riffault *et al.*, 1997; Riffault *et al.*, 2001). The results presented in paper III indicated that cells present among PBMC, but not monocytes or moDC, did produce IFN-α in response to the plasmid pcDNA3. It was therefore of interest, in paper IV, to study the ability of PBMC to produce IFN-α at exposure to the plasmid pcDNA3 as well different defined ODNs with unmethylated CpGs (Table I). We also characterized the IPC by flow cytometric analysis and by *in situ* hybridization.

Initially, the IFN-α inducing capacity of phophodiester ODN H-I, that previously had been shown to induce IFN-α in human PBMC (Magnusson *et al.*, 2001b), was estimated (Figs. 1a and b). The levels of IFN-α induced by ODNs were comparable to, or higher than those induced by the plasmid pcDNA3, *A. pleuropneumoniae* or ADV (Fig. 1c). The induction of IFN-α by pcDNA3 and the

phosphodiester ODNs required preincubation of the ODN with lipofectin, which is in accordance with earlier studies (Magnusson *et al.*, 2001b). Also the activity of *A. pleuropneumoniae* was increased by preincubation with lipofectin (Wattrang *et al.*, 1998) (Johansson *et al.*, 2001; Magnusson *et al.*, 2001a).

By modifying the ODN H and the complementary ODN I (Table 1), it was shown that the induction of IFN-α in poPBMC was dependent on presence of a central unmethylated CpG (Fig. 2). Thus, inversion of the CpG, substitution of either the bases C or G, or methylation of the cytosine in the CpG, strongly inhibited the IFN-α production by these ODNs. In contrast, similar alterations of ODNs H and I did not consistently have the same effect on their ability to cause IFN-α production in human PBMC (Magnusson *et al.*, 2001b), indicating species differences in the mechanism for IFN-α induction by ODNs. Furthermore, the concentration of ODN and lipofectin was different in these two studies, which also may contribute to the discrepancies.

Inversion of the CpG in ODN A2 also resulted in a strong reduction of the IFN-α production in the present study, while both ODNs A2 and $A2^{GC}$ enhanced the antigen specific antibody production and cell proliferation in pigs (Van der Stede *et al.*, 2002). However, injection of ODN A2 completely protected mice to challenge with *Listeria monocytogenes* whereas those injected with $A2^{GC}$ were not protected (Klinman *et al.*, 1999). Thus the importance of CpG motifs might be different *in vitro* and *in vivo* and seems to vary between species.

The effects of the chemical composition of the ODN backbone for IFN-α inducing capacity was then evaluated using ODNs having either a backbone consisting of central phosphodiester nucleotides (including the CpG) and phosphorothioate nucleotides in the ends (thioate/diester ODNs) or a complete phosphodiester backbone. It was found that the thioate/diester ODNs (ODN 2216 and D19) induced somewhat higher levels of IFN-α than ODNs with phosphodiester backbone (Fig. 3). These ODNs did not require lipofectin for the induction of IFN-α. Thus, the backbone composition did only moderately influence the IFN-α inducing capacity of these ODN.

One major difference between the ODNs 2216, D19 and MM1 from ODNs H and I and A2 was the presence of poly-G sequences in the ends of the former. ODNs with poly-G sequences may form quartenary structures that enhance their uptake by cells, possibly by binding to scavenger receptors (Pearson *et al.*, 1993; Kimura *et al.*, 1994; Lee *et al.*, 2000). This may explain why the ODNs containing poly-G sequences were able to induce IFN-α without the help of lipofectin to enter the cells. However, addition of poly-G sequences to the ODN H (Table 1) increased its IFN-α inducing capacity when lipofected, but did not obviate the need for lipofectin (Fig. 4).

Studies on the IFN-α induction by ODNs in human PBMC have revealed that e.g. ODN 2216 exclusively induces IFN-α production in NIPC/PDC (Bauer *et al.*, 2001a; Krug *et al.*, 2001a). In the present study, the estimation of the frequency of porcine IFN-α producing cells, by staining for intracellular IFN-α and subsequent flow cytometric analyses, revealed that between 0.03 to 0.07% of the PBMC produced IFN-α at stimulation with ADV, lipofected pcDNA3 and

ODN 2216 (with or without lipofectin). The light scatter characteristics of IPC stimulated by the various inducers were similar, and they were relatively small cells with low granularity. The validity of the flow cytometric determination of IPCs was verified by detection of IFN-α mRNA containing cells among PBMC using *in situ* hybridization. By their presence at a low frequency, and their light scatter characteristics, the IFN-α producing cells detected in the present study resembled the NIPC/PDC previously described in humans (Fitzgerald-Bocarsly, 2002) and also in pigs (Nowacki *et al.*, 1993; Nowacki & Charley, 1993). Attempts to further analyse these IPC by staining with mAbs to cell surface markers are currently undertaken.

The role of CpG-containing ODNs as adjuvant and immuno-modulators in vaccination has been described in mice and primates and recently also in veterinary animals such as cattle (Pontarollo *et al.*, 2002b; Rankin *et al.*, 2002) and pig (Van der Stede *et al.*, 2002). The present study shows that ODNs with poly-G sequences have high IFN-α inducing capacity and such ODNs may be used to increase the immunostimulatory effects of vaccines designed for pigs. The results also indicate that immunostimulatory DNA containing unmethylated CpG, either as plasmid DNA or defined ODNs, can activate IFN-α production in porcine leucokytes that resemble the human NIPC/PDC. The finding that ODNs with poly-G sequences were strongly IFN-α inducing, even in the absence of lipofectin, may be explained by improved uptake of DNA by the IPC. Therefore, an engineered increased uptake of vaccine plasmids may result in both increased vaccine antigen production and adjuvant action.

General summary and conclusions

The most important aim of these studies was to study the adjuvant properties of unmethylated CpG-containing DNA, such as vaccine plasmids and CpG-ODNs, especially the ability to induce immunoregulatory cytokines in the pig. The cells producing such cytokines were also studied. The possibility to further enhance the effects of DNA vaccines, e.g. by addition of plasmids expressing immunostimulatory cytokines also received some attention.

The main results were the following:

- The vaccine plasmid pcDNA3 was shown to induce a local IFN-α production *in vivo*, when injected into subcutaneously implanted tissue chambers in pigs. The IFN-α production was detected within 8 to 24 h, was dependent on the presence of unmethylated CpG-dinucleotides in the plasmid and was achieved in the absence of lipofectin. However, the expression of GM-CSF or IL-6 after injection with pcDNA3 plasmids containing the cDNA for these cytokines could not be verified. Still, the cytokines may have been expressed at levels below detection or produced by DC, or other cells, that had migrated away from the tissue chambers, especially to the local lymph node.

- The adjuvant effect of DNA vaccines can be further enhanced by co-administration of plasmids expressing immunoregulatory cytokines. For such purpose, the cDNA for a porcine IL-12 fusion protein was constructed and inserted into pcDNA3, and the expression of biologically active IL-12 protein was verified in COS cells. The fusion protein was biologically active also after expression in Drosophila (S2) cells and subsequent purification. The ability of IL-12 to induce IFN-γ production in poPBMC was synergistically enhanced by huIL-18, an effect that earlier had been shown in other species. The results suggest that combinations of plasmids expressing IL-12 and IL-18 should have enhanced adjuvant effects on DNA vaccines. Furthermore, flow cytometric analyses after intracellular staining of poPBMC stimulated with IL-12/IL-18 showed that the cells producing IFN-γ were mainly T cells and NK cells.

- It was of importance to study the effect of e.g. immunostimulatory DNA on moDC because these cells are major targets in vaccination. Therefore, porcine moDC were differentiated from adherent blood monocytes *in vitro* with IL-4 and GM-CSF. A possible role of IFN-α in promoting maturation of the immature moDC to highly antigen presenting moDC was also demonstrated, confirming previous results with human moDC, differentiated in a similar way. The porcine moDC responded to Sendai virus, *A. pleuropneumoniae* and lipofected pcDNA3 with production of IL-12 and to Sendai virus with production of IFN-α. It is notable that the moDC did not produce IFN-α in response to CpG-DNA in form of the plasmid pcDNA3 or to the herpesvirus ADV. The moDC were also able to produce the Th2 associated cytokines IL-6 and IL-10 at stimulation with *A. pleuropneumoniae* and Sendai virus. The

cytokine production by the moDC was thus highly dependent on which type of inducer the cells were exposed to.

- The previous results revealed that the plasmid pcDNA3 induced IFN-α production in PBMC, but not in the moDC. The characteristics of the CpG-DNA, in the form of ODNs, contributing to the IFN-α production and the cells involved were studied. The ability of ODN to induce IFN-α production in poPBMC was dependent on a central unmethylated CpG. It was shown that the phosphodiester ODNs H and I required preincubation in lipofectin for IFN-α induction, like the plasmid pcDNA3. In contrast, the thioate/diester ODNs with poly-G sequences in the ends, and the corresponding ODNs with complete diester backbone, were potent inducers of IFN-α, also in the absence of lipofectin. Thus, the backbone composition had no major effect on the IFN-α inducing capacity of ODNs. The ability to induce IFN-α independently of lipofectin may be due to the poly-G sequences, that are known to facilitate the uptake of ODNs by cells. The presence of a poly-G sequence was also shown to enhance the IFN-α inducing ability of the phosphodiester ODN H, but could not in this case circumvent the need for lipofectin.

- Among human PBMC, the thioate/diester ODN 2216 (with poly-G sequences) is known to induce IFN-α production exclusively in NIPC/PDC. The porcine IPC induced by CpG-DNA in the form of lipofected pcDNA3 and 2216 were therefore characterized by flow cytometry and *in situ* hybridization. The IPC were present at a low frequency (less than 0.1 %), had a high labelling intensity and had the light scatter typical for small cells with low granularity. In this way the porcine IPC resemble the human NIPC/PDC.

The findings in the present thesis indicate similarities between subtypes of DC in porcine and human blood, especially in their ability to produce the key immunoregulatory cytokines IL-12 and IFN-α in response to CpG-DNA. The results will furthermore facilitate development of improved adjuvants for use in porcine vaccines, and also suggest that the pig may be a relevant model for human vaccine research.

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Acknowledgements

This work was carried out at the Department of Veterinary Microbiology, Section of Immunology at the Swedish University of Agricultural Sciences. It was supported by grants from the Swedish Council of Forestry and Agricultural Research, and from the European Commission (FAIR3-CT96-1317 and FAIR5-CT97-3732). I would like to thank the Department of Veterinary Microbiology for placing recourses of the department at my disposition.

I would like to express my sincere gratitude to:

Prof. Gunnar Alm, my supervisor, for sharing your time and extensive knowledge in immunology with me. Thank you for your help, patience and support during my time at the department.

My co-supervisor prof. Caroline Fossum, who was the senior author of the majority of the articles, for support and help. I learned a lot from our discussions while writing paper IV.

Maija-Leena Eloranta, for excellent guidance in the development of new methods and for sharing my worries when the work did not seem to proceed.

Mikael Berg, for valuable scientific support and for being interested to discuss the general aim of my project.

Elin Johansson, for invaluable support and for being strong enough to fight for what you think is right. I really enjoyed working with you, when we in the end got the opportunity to collaborate.

Lisbeth Fuxler, for caring and for sharing some of your laboratory skills. Thank you for good colaboration and for always performing the laboratory work in an excellent way. Also for double-checking of tables and figures, “one can always rely on Bettan”.

Anne Riesenfeld, for running an endless ammount of IFN-γ assays, and for creating a positive atmosphere at work.

Anders Perers, for sharing your knowledge in antibody-purification and labelling. You were an understanding “room-mate”, while I was writing and spreading references around the room.

Mattias Magnusson, for participation in the attempts to further characterize the porcine IPC, and for company during some late nights at the lab.

Stina Blomberg, for your ability to handle so many things at the same time. I remember our meetings with our baby-boys in the ’singing group’ with joy.

Ullvi Båve for good company during daily work.

Tanja Lövgren for friendship, and for allways lending a helping hand in the mystery world of computers. I wish you the best of luck with your future studies.

Frida Hasslung, our new recruit in the field of veterinary immunology. I wish you good luck

Sirje Timmusk, for making the beautiful houses out of gingerbread.

Per Wallgren for good collaboration, especially with the experimental animals.

Brita Cederblad, for inspiration and enthusiasm about the field of immunology.

Helena Vallin, for sharing your knowledge in how to culture hybridoma cells.

Eva Wattrang, whose early work in pig immunology has provided useful knowledge for me.

Anders Johannisson, for technical assistance with the flow cytometric analyses.

Ulla Schmidt, for assistance with drawing blood samples from the pigs. Carina Lindström-Backeman, for doing the administrative work and for interesting discussions about Montessori pedagogics.

Students that have spent longer or shorter periods in the lab. Linda Tillmar, the first of us to show IFN-α induction with plasmid DNA in poPBMC. Linnea Nygren, Sara Magnusson, Anna Ledin, Lotta Enerud, Mona Carlsson and Mats Karlberg for pleasant company in the daily work.

My husband Marius, for your love and for a lot of practical support at home during this last intence period. I hope we will have more time for each other soon.

My son Niklas, you are the greatest love of my life.

Family, for support and love.