FACULTEIT DIERGENEESKUNDE

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# Modulation of a systemic induced immune response towards a mucosal one in pigs using $1,25(OH)_2D_3$ and CpG-motifs ?

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| $1\alpha,25(OH)_2D_3$ | 1 alpha, 25-Dihydroxy vitamin $D_3$ |
| ABTS | 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonaat) |
| AEC | Anion exchange chromatography |
| APC | Antigen-presenting cell |
| ASC | Antibody-secreting cell |
| BM | Bone marrow |
| CD | Cluster of differentiation |
| ConA | Concanavalin A |
| CpG-ODN | CpG-Oligodeoxynucleotides |
| Cpm | Counts per minute |
| CTL | Cytotoxic T lymphocyte(s) |
| DHEA | Dehydroepiandrosteron |
| DNA | Deoxyribonucleic acid |
| Dppi | Days post primary immunisation |
| Dpsi | Days post secundary immunisation |
| E. *coli* | *Escherichia coli* |
| EDTA | Ethylenediaminetetraacetaat |
| ETEC | Enterotoxigenic *Escherichia coli* |
| ELISA | Enzyme-linked immunosorbent assay |
| Elispot | Enzyme-linked immuno spot |
| F4R | F4 receptor(s) |
| FCS | Foetal calf serum |
| FITC | Fluoresceïn isothiocyanate |
| GALT | Gut-asssociated lymphoid tissue |
| GM-CSF | Granulocyte/macrophage colony-stimulating factor |
| HSA | Human serum albumin |
| ID | Intradermal(ly) |
| IFA | Incomplete Freund's adjuvant |
| IFN-γ | Interferon-gamma |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IL2-R | Interleukin-2 receptor |
| IM | Intramuscular(ly) |
| IPP | Ileal Peyer's patches |
| JPP | Jejunal Peyer's patches |

| | |
|---|---|
| LNN | Lymph node |
| LP | Lamina propria |
| MAb | Monoclonal antibody |
| MHC II | Major histocompatibility complex class II |
| NK cell | Natural killer cell |
| mRNA | Messenger ribonucleic acid |
| ND | Not done |
| OD | Optical density |
| OVA | Ovalbumin |
| PAGE | Polyacrylamide gel electrophoresis |
| PBMC | Peripheral blood monomorphonuclear cells |
| PBS | Posphate buffered saline |
| PCR | Polymerase chain reaction |
| PI | Propidium idodide |
| PP | Peyer's patches |
| SC | Subcuteneous (ly) |
| SDS | Sodium dodecyl sulfate |
| sIgA | Secretory IgA |
| SEM | Standard error of the mean |
| TGFβ | Transforming growth factor-β |
| Th | T-helper |
| TNFα | Tumour necrosis factor α |

# PART I

## INTRODUCTION

# INTRODUCTION

Mucosal infections with enterotoxigenic *E. coli* (ETEC) affect neonatal and recently weaned animals. ETEC infections are a cause of diarrhoea and significant economical losses. In general, most neonatal infections can be prevented by passive colostral and lactogenic immunity. However, this passive protection decreases with aging and at weaning lactogenic immunity disappears. So, newly weaned animals become highly susceptible for enteropathogens. In order to protect newly weaned piglets, an active mucosal immunity is needed in the form of antigen-specific secretory IgA (sIgA) in secretions. The only way this could be obtained immediately after weaning is by vaccination during the suckling period. Vaccination via the oral route is difficult especially in suckling piglets due to milk antibodies. Parenteral vaccination (intramuscular (IM), subcutaneous (SC), etc) is an other option. However, available parenteral vaccines stimulate the systemic (IgG antibodies) rather than the mucosal immune system. This emphasizes the need for the inclusion in these vaccines of immunomodulating adjuvants.

The term immunomodulation is generally used to describe the pharmacological manipulation of the immune system. Normally this involves non-specific and antigen-specific immunostimulation with following objectives: (i) promoting a greater and more effective immune response and if administered with vaccines it should exert an adjuvant effect (ii) enhancing local protective immune (IgA) responses at mucosal surfaces in neonatal and young susceptible animals and (iii) selectively stimulating the immune system in order to modulate the immune response towards a specific direction, or stimulating non-specific immune mechanisms. The use of immunomodulators in vaccination must enhance a protective immune response. Immunomodulators (immunomodulating adjuvants), which are often used in parenteral vaccination, can be divided into three categories: physiological products (hormones), substances of microbial origin (cell wall, LPS, DNA,…) and synthetic compounds (Levamisole, β-1,3-glucan, indomethacin, synthetic polynucleotides,…) (Mulcahy and Quinn, 1986).

In the present thesis the immunomodulating properties of , $1\alpha,25(OH)_2D_3$ (calcitriol) and Cytidine-phospate-Guanosine oligodeoxynuclotides (CpG-ODN) after an intramuscularly (IM) induced immune response in piglets are studied. Both agents have well been studied in rodents and man but not in domestic food animals such as pigs. The steroid hormone $1\alpha,25(OH)_2D_3$ , the active form of vitamin D, appears to modulate a systemic immune response towards a protective mucosal (IgA) immune response (Daynes et al., 1994, 1996). This was accompanied with the presence of a Th2-cytokine profile. CpG-ODN, which are DNA-sequences characteristic for many bacteria, have potent immune enhancing properties and are classified as Th1-modulating adjuvants. Co-administration of CpG-ODN with a particular antigen showed enhanced antigen-

specific humoral and cellular immune responses with even protection upon challenge (Krieg et al., 2001). Chapter 1 (Part I) reviews the literature on 1α,25$(OH)_2D_3$ and CpG-ODN and their immunomodulating characteristics. Part II describes the specific aims of the study. The experimental work is presented in part III (chapter 2 to 7). Part IV contains a general discussion and the overall conclusions (chapter 8).

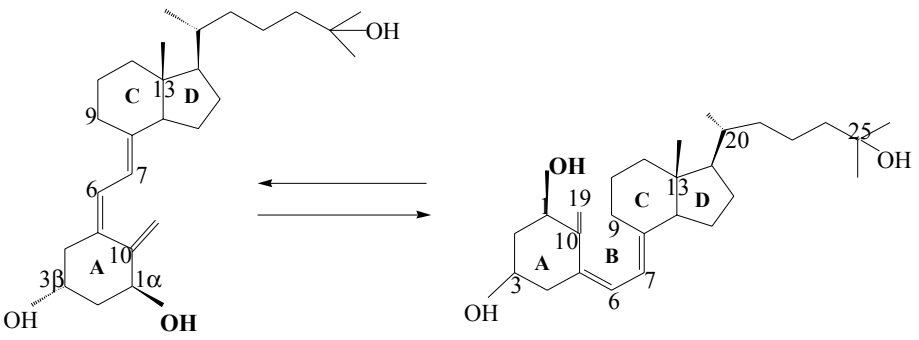
# CHAPTER 1:

# THE IMMUNOMODULATING PROPERTIES OF CALCITRIOL AND CPG-OLIGODEOXYNUCLEOTIDES: A REVIEW

## 1.1. Calcitriol

### 1.1.1. Calcitriol: structure and Vitamin D metabolism

1α,25-Dihydroxyvitamin D$_3$ [1α,25(OH)$_2$D$_3$ or calcitriol] is the biologically active form of vitamin D and is required for the homeostasis of calcium and phosphorus (De Luca, 1976). It is a hydrophobic molecule with a molecular weight of 416,6 Da and is soluble in alcohol and acetone. The molecular structure of 1α,25(OH)$_2$D$_3$ (Fig.1.1) is similar to that of cholesterol and steroid hormones such as progesterone, aldosterone and testosterone except that the B-ring of the structure has been opened between C9 and C10. This allows the A-ring to rotate from the folded (steroidal or cisoid) to the extended (vitamin-like or transoid) conformation.




**1,25(OH)$_2$D$_3$ vitamin conformation** **1,25(OH)$_2$D$_3$ steroid conformation**

**FIGURE 1.1:** Structure of 1,25(OH) $_2$D$_3$ in both conformations.

Only the extended conformation can interact with the nuclear vitamin D receptor (nVDR, Norman et al., 1993, Bouillon et al., 1995).

Vitamin D is a complex of steroids however the name is often used for vitamin $D_3$, found in mammals. Vitamin $D_3$ is synthesized in the skin out of a cholesterol-like precursor (7-dehydrocholesterol) by exposure to ultraviolet light (Lips et al., 1996, Fig. 1.2) or can be administered in feed. Vitamin $D_3$ is biologically inert and requires two successive hydroxylations: one in the liver (at $C_{25}$ making 25-hydroxyvitamin $D_3$ (25(OH)$D_3$)) and one in the kidney (at $C_1$ in the α position), to form the hormonally active 1α,25(OH)$_2D_3$ (Gascon-Barré 1997).

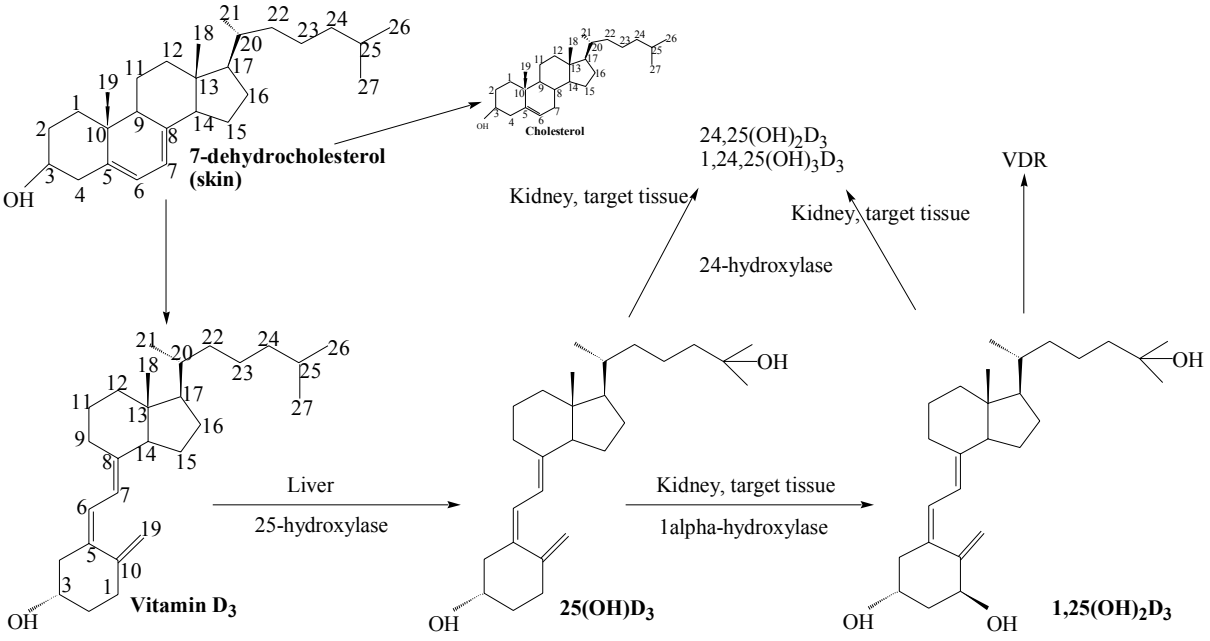



**FIGURE 1.2:** Synthesis and metabolism of vitamin D

The production of 1α,25(OH)$_2D_3$ in the kidney is controlled by the parathyroid hormone (PTH) which directly stimulates the 1α-hydroxylase activity during hypocalcemia and inhibits its activity during hypercalcemia (Garabedian et al., 1972). Other major regulators of the 1α,25(OH)$_2D_3$ concentration are calcium, phosphorus (negative regulation via suppression of PTH secretion), calcitonin, insulin-like growth factor (positive regulation, Henry et al., 1997) and 1α,25(OH)$_2D_3$ itself (negative feedback) (Bell et al., 1998). Acidosis also decreases the level of 1α,25(OH)$_2D_3$ by raising the serum calcium and by decreasing the responsiveness of the kidney to PTH. The negative feedback of 1α,25(OH)$_2D_3$ on its own synthesis and the positive feedback on its catabolism provide an important mechanism to prevent vitamin D intoxication. 1α,25-Dihydroxyvitamin $D_3$ activates its major catabolic enzyme 24-hydroxylase that converts 1α,25(OH)$_2D_3$ to 24,25(OH)$_2D_3$ as well as to 1,24,25(OH)$_3D_3$. Furthermore it initiates the oxidation at $C_{24}$ (Beckman et al., 1996), which is followed by second hydroxylation, and

oxidation at $C_{23}$ and subsequent cleavage of the side chain at $C_{23}$. The final cleavage product of 1α,25(OH)$_2$D$_3$ is calcitroic acid which is biologically inert (Bouillon et al., 1998).

Transport of vitamin D steroids is associated with vitamin D-binding proteins (DBP) which bind 25-hydroxyvitamin $D_3$ with a higher affinity than the bio-active 1α,25(OH)$_2$D$_3$ DBP are synthesized in the liver and circulate in the plasma at concentrations 20 times higher than the total amount of vitamin D compounds. So, under normal physiological conditions nearly all-circulating vitamin D compounds are protein bound. This makes the metabolites less susceptible to metabolism and prolongs their half-life time (Cooke and Haddad et al., 1976). Actually the DBP-unbound vitamin D compounds have greater accessibility to target cells and therefore have a higher biological response (Bikle et al., 1989).

### 1.1.2. Mechanism of actions by 1α,25(OH)$_2$D$_3$

#### *1.1.2.1. Genomic actions of 1α,25(OH)2D3*

Most biological activities of 1α,25(OH)$_2$D$_3$ are mediated largely, if not exclusively, through the high-affinity receptor nVDR (Fig. 1.3).

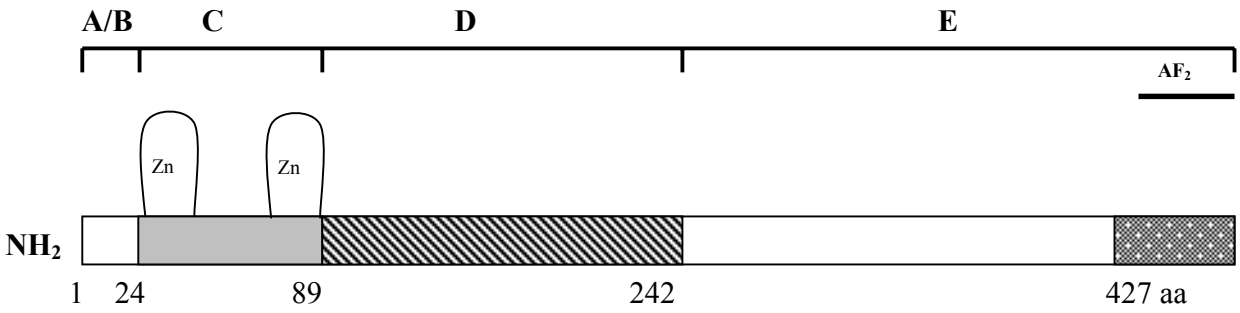



**FIGURE 1.3:** Structural organization of the human nuclear vitamin D receptor (nVDR). The nVDR is composed of 427 amino acids (aa) and consists of several domains (A/B, C, D, E/F and $AF_2$ domain).

This receptor has a molecular weight of 50 to 60 kDa depending on the species. It is a member of the steroid hormone-activated-transcription factor family and acts by binding as homodimer or heterodimer to vitamin D-responsive elements (VDREs) found in the promotor region of several target genes (Haussler et al., 1998). The nVDR consist of several domains: the A/B domain at the N-terminus (transcription-activating domain), the C-domain which contains 2 conserved zinc finger DNA binding motifs which interact with the VDREs. The D-domain, serves as a hinge region and is followed by the E-domain. The latter domain contains the 1α,25(OH)$_2$D$_3$ (ligand) binding domain (consisting of 12 helices) and a ligand-dependent activation function

(AF2), represented by helix 12 (Mangelsdorf and Evans 1995), which is located at the C-terminus. The major steps involved in control of gene transcription by the nVDR are shown in Fig. 1.4.

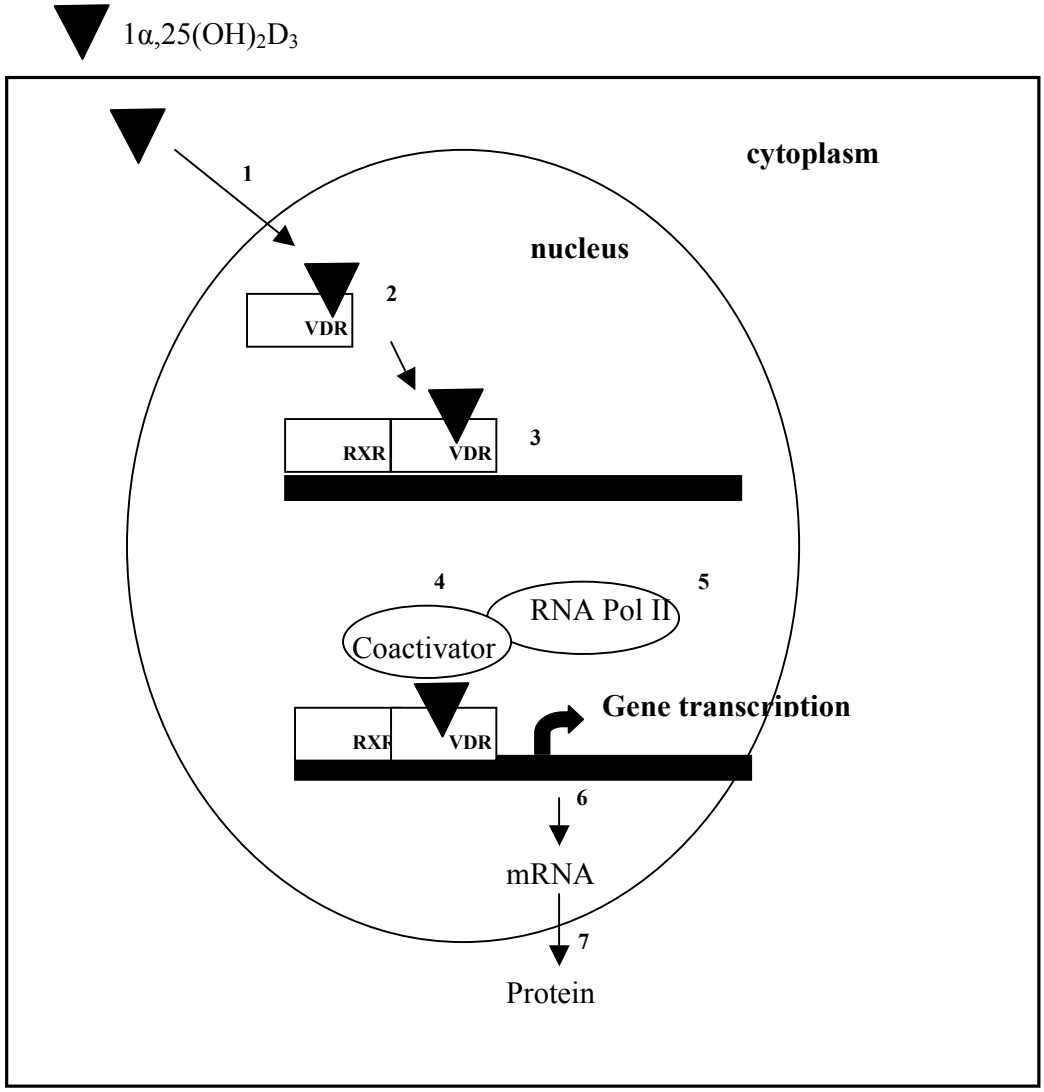



**FIGURE 1.4:** Genomic action of $1\alpha,25(OH)_2D_3$. (1) The steroid hormone $1\alpha,25(OH)_2D_3$ enters the cytoplasm and nucleus of the target cells by diffusion. (2) $1\alpha,25(OH)_2D_3$ binds in the nucleus with the nVDR which results in a conformational change of the nVDR. (3) The nVDR forms a homodimer or heterodimer with RXR which interacts with specific DNA sequences VDRE in the promoter region of the target genes. (4) Subsequent, recruitment of co-activators by the activated heterodimer results in (5) the activation of the RNA-polymerase II complex (RNA-pol II). (6) This leads to transcription of the target genes followed by translation into a protein. (7) The expressed proteins exert the biological effects observed.

The binding of nVDR to its VDRE requires the presence of its ligand 1α,25(OH)$_2$D$_3$ and is favored by RXR. RXR belongs to the group of retinoic acid (Vitamin A) receptors. Although the active derivatives of vitamin A are not related to vitamin D$_3$, RXR belongs to the same family of hormone-activated-transcription factors. This indicates that vitamin D and vitamin A are linked in their signalling. Indeed, the natural ligand for RXR, 9-cis retinoi acid, suppresses VDR-RXR binding to VDREs and subsequently 1α,25(OH)$_2$D$_3$-stimulated transcription. Examples of some 1α,25(OH)$_2$D$_3$ actions in different target tissues are shown in Table 1.1.

**TABLE 1.1.:** 1α,25(OH)$_2$D$_3$ genomic and non-genomic actions in target tissues

| Target tissues | Action |
|---|---|
| Parathyroid gland | Inhibition of cell growth and PTH synthesis. |
| Intestine | Enhancement of calcium and phosphate absorption. |
| Thyroid/C-cells/Follicular cells | Inhibition of calcitonin synthesis. |
| Bone/osteoblast | Enhancement of bonematrix protein synthesis, bone mineralisation, and synthesis of mediators with osteoclastic activity. |
| Kidney (proximal and distal) | Inhibition of 1α,25(OH)$_2$D$_3$ and induction of 24-hydroxylase. |
| Immune system/monocytes macrophages /T cells/B cells | Enhancement of macrophage function to control viral and bacterial infections. Enhancement of Th2-like cytokines (IL-4, IL-10 and IL10-R) and Th3-like cytokines (TGF-β). Downregulation of Th1-like cytokines (IFN-γ, IL-12 and IL-2) and inflammatory cytokines (TNF-α, IL-1, IL-6, IL-8). |
| Skin/Muscle/Heart | Antiproliferative and prodifferentiating. |
| Cancers cells/c-myc (proto-oncogene) | Antiproliferative and prodifferentiating. |
| Pituitary | Control of T3-induced growth hormone and prolactin. |
| Cartilage/Chondrocyte | Antiproliferative and prodifferentiating. |
| Pancreas/β-cells | Enhancement of insulin synthesis and secretion. |

PTH: parathyroid thormone; Th: T-helper cells; IL: Interleukin; TNF: Tumor necrosis factor; IFN: Interferon; TGF: Transforming growth factor; T3: Thyroid hormone

1.1.2.2. Non-genomic actions of $1\alpha,25(OH)_2D_3$

Besides its genomic action, $1\alpha,25(OH)_2D_3$ can also elicit rapid responses via a non-genomic action. There is still discussion on whether the receptor for this non-genomic action is located near (Barsony et al., 1997) or on the plasma membrane (mVDR). Potential membrane receptors are a 66 kDa protein found on chicken duodenal cells (Nemere et al., 1994) and annexin-2 (36 kDa) found on rat osteoblast-like cells (ROS24/1, Baran et al., 2000). The major non-genomic effect of $1\alpha,25(OH)_2D_3$ is calcium mobilization (Fig.1.5).

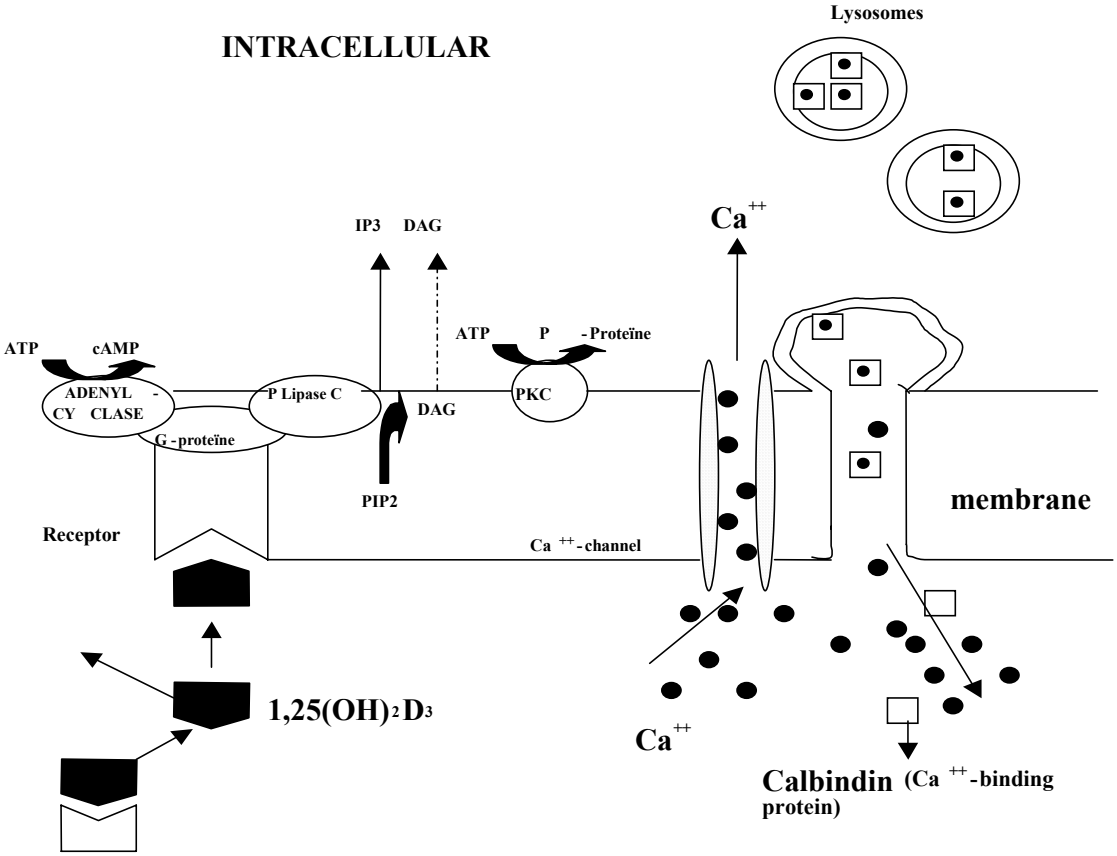



**FIGURE 1.5:** Non-genomic action of $1,25(OH)_2D_3$ (transcaltachia = rapid hormonal stimulation of intestinal $Ca^{++}$ absorption). cAMP: cyclic adenosine monophosphate, P-lipase C: phospho-lipase C, $IP_3$: inositol triphosphate, DAG: diacyl glycerol, $PIP_2$: phospho-inositol-diphosphate, PKC: protein kinase C.

The binding of the hormone to the receptor initiates the activation of a pathway that leads to opening of voltage-gated $Ca^{++}$-channels, increases the absorption of calcium from the small intestinal lumen and together with PTH, the reabsorption of calcium in the distal tubuli of the kidney. Protein kinase A, protein kinase C, phospholipase C/diacyl glycerol and

inositoltriphosphate ($IP_3$) seem to be involved in this mechanism as second messengers. Even so the extra cellular signal-regulated kinase (ERK, Norman et al., 1998) as well as the c-Jun $NH_2$-terminal kinase-pathway (JNK, Caelles et al., 1997) are activated. However, the exact non-genomic pathway remains unclear. It is hypothesized that some of the second messengers may function as a sort of 'cross-talk' between the non-genomic and genomic pathways so modulating the activity of the nVDR. Indeed, phosphorylation of the VDR by PKC has been shown to decrease its transcriptional activity (Matskovits and Christakos., 1995).

### 1.1.3. Calcitriol and the immune system

#### *1.1.3.1. The Th1,Th2 and Th3 cytokine profiles*

T-helper (Th)1 and Th2 $CD4^+$ cells were originally described in mice and later in humans (Mosmann et al., 1991). Th1 and Th2 cells differentiate out of the Th0 cells. Mouse Th1 cells produce interleukin (IL)-2, interferon-γ (IFN-γ) and tumor necrosis factor (TNF)-β which are involved in cellular responses, such as clonal expansion of cytotoxic T-lymphocytes (CTL), macrophage and natural killer (NK) cell activation and class switching to IgG isotypes (IgG2a) that can mediate complement lysis of sensitised cells. Th1 immune responses are important in controlling infections by intracellular organisms (Heinzel et al., 1989; Sher et al., 1992). However, Th1 responses also appear to play a central role in autoimmune diseases such as multiple sclerosis (Voskuhl et al., 1993). In contrast, Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 providing more efficient help for B-cell activation and production of IgG1 and IgE antibodies (von Hertzen et al., 2000). Products of one subset may negatively regulate the development of the other as IFN-γ, produced by Th1 cells, inhibits Th2 proliferation while IL-10 and IL-4, secreted by Th2 cells, inhibits the synthesis of Th1 cytokines probably through inhibition of IL-12 production by the APC (the Th1/Th2 paradigm) (Fig. 1.6) (Muraille and Leo 1998).

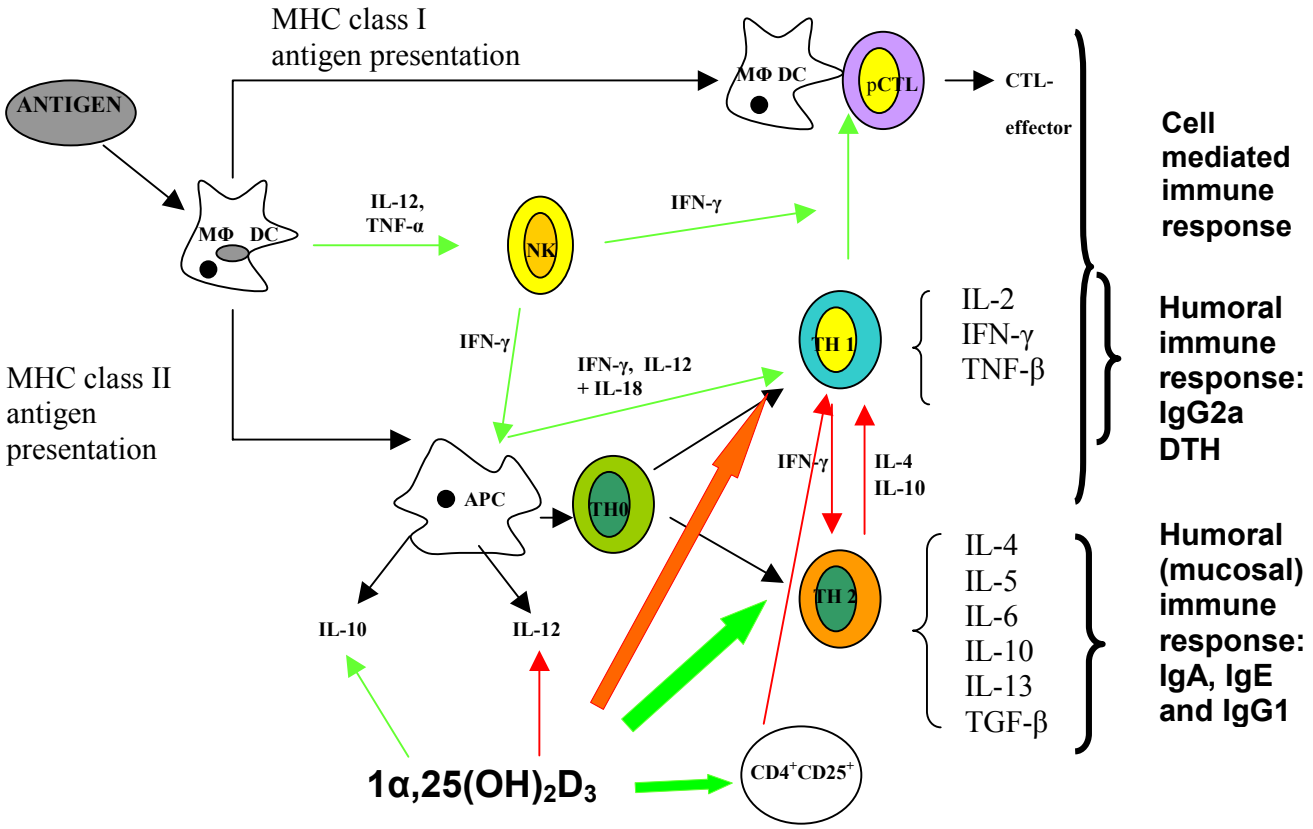



**FIGURE 1.6.:** Orchestration of an immune response with differentiation of naïve $CD4^+$ and $CD8^+$ T cells into armed effector cells (T-helper (Th)1, Th2 and Cytotoxic T cells (CTL)). The released cytokines influence the Th1/Th2 differentiation (Th1/Th2 paradigm) as well as the isotype specific immunoglobulins by activated B cells. Green arrow: activation/differentiation; red arrow: inhibition. The immunomodulating properties of $1\alpha,25(OH)_2D_3$ and its influence on the cytokine-profile is shown. APC: antigen presenting cells, MΦ:Macrophage, DC: Dendritic cellls, DTH: Delayed type of hypersensitivity, pCTL: precursor cytotoxic (CD8+) T-cell, Th: T-helper (CD4+) cell. According to Mathieu and Adorini (2002): $1,25(OH)_2D_3$ inhibits IL-12 and stimulates IL-10 production and thus inhibiting the development of Th1 cells. In addition $1,25(OH)_2D_3$ favors the induction of $CD4^+CD25^+$ regulatory T cells which inhibit the development of Th1 cells.

The $CD4^+$ Th-mediated response is certainly more than just Th1 and Th2, but these represent two extremely polarised forms. Another T-cell subset, the Th3 cells, has been identified more recently and produces high amounts of transforming growth factor β (TGF-β) but no IL-2, IFN-γ, IL-4 or IL-10 (Mosmann and Sad., 1996). TGF-β is an important cytokine in the development of mucosal immune responses (provides help for switch towards IgA) and oral tolerance in the gut (Coffman et al., 1989, Paul and Ceder 1994; MacDonald 1997). Another T-cell subset is the T regulatory (Tr) cell. They are also essential for induction of tolerance (Roncarolo et al., 2001). Tr cells suppress immune responses via cell-to-cell interactions and/or the production of interleukin (IL)-10 and TGF-β. Many types of Tr cells have been described in a number of systems. Type-1 T regulatory (Tr1) cells are defined by their ability to produce high levels of IL-10 and TGF-β but their relationship with other T-cell subsets such as Th3 cells remains unclear.

There are several factors affecting the Th1/Th2/Th3 differentiation, e.g. cytokines produced by 'surrounding' cells such as dendritic cells (DC), macrophages and B cells. Interleukin-12 produced by macrophages and/or DC promotes Th1 differentiation while IL-4 promotes the differentiation towards Th2 cells. The effect of IFN-γ may be variable (Sad et al., 1994; Seder et al., 1993). Furthermore, co-stimulatory molecules on antigen presenting cells (APC, Thompson et al., 1995), and the nature as well as the concentration of an antigen (Pfeiffer et al., 1991) can influence the Th1/Th2 differentiation pathway. During the last decade extensive studies have been performed on the immunomodulating effects of several molecules such as CpG-ODN and 1α,25(OH)$_2$D$_3$, for their capacity to switch the immune response towards Th1 or Th2. The intention is to selectively use these molecules as vaccine adjuvants or in the treatment of non-infectious diseases (Schijns et al., 2000).

It is hypothesized that similar Th1/Th2/Th3-profiles as in mice exist in other mammals such as the pigs. However, the relationship between the cytokine profile and the secretion of different immunoglobulin isotypes as shown in mice has not been proven in pigs. The currently known porcine cytokines with their main functions are summarized in table 1.2.

**TABLE 1.2.:** Porcine cytokines and their function.

| Cytokine (Genebank number) | Source | Function |
|---|---|---|
| INF-α (M28623) | MΦ and monocytes but any cell type can | Inhibition of viral replication, antiproliferative activity and increase of MHC I. |
| IFN-β (M 86762) | Fibroblasts but any cell type can | Inhibition of viral replication, antiproliferative activity and increase of MHC I. |
| IFN-γ (X53085, S63967) | T cells, NK cells and MΦ | MΦ activation; viral protection and increased MHC II and I expression. |
| TNF-α (M29079) | MΦ | Induces inflammation and co-stimulates lymphocyte proliferation. |
| TNF-β (X54859) | T cells | Activates tumor apoptosis, neutrophils, MΦ and B cells. |
| IL-1α(X52731)<br>IL-1β (M 86730) | Many cells | Co-stimulators of Th2 cells and stimulation of acute-phase response. |
| IL-2 (X5842, X56750) | Th1 cells | Activation of T, B and NK cells. |
| IL-4 (X68330) | Th2 cells | Activates B-cell growth and their differentiation ; inhibition of IL-1, IL-6 and TNF-α. |
| IL-6 (M86722,M802258) | MΦ, T- and B cells, bone marrow stroma cells, fibroblasts and keratinocytes. | Promote IL-2 production and T-cell differentiation. |
| IL-8 (M99367) | MΦ, fibroblasts, lymphocytes, granulocytes, endothelial cells, hepatocyes, keratinocytes | Chemoattractant for neutrophils (activation), basophils and T cells. |
| IL-10 (L20001) | MΦ and Th2 cells | Inhibits activation of Th1 and NK cells and suppresses MΦ. |
| IL-12 (U08317) | MΦ | Stimulate Th1 cells with secretion of IFN-γ and IL-2. Activation of NK cells and T cells. |
| IL-15 (U58142) | Activated lymphocytes | Enhances the T-cell proliferation. |
| IL-18 (U68701) | MΦ | Growth and differentiation factor for Th1 cells. Induction of IFN- γ. |
| GM-CSF (D21074) | T cells, MΦ, endothelial cells and fibroblasts | Stimulates proliferation of granulocytes, MΦ, erythrocytes progenitors and activates neutrophils, MΦ and eosinophils. |
| TGFβ-1, TGFβ-2, TGFβ-3 (M23703, M70142,X14150) | Many cell types | Inhibitors of cell proliferation. Increase of cell density, fibrosis and angiogenesis. Switch towards IgA ? |

GM-CSF= granulocyte colony stimulating factor; TNF = tumor necrosis factor; IFN = interferon; IL= interleukin; TGF = transforming growth factor. MΦ = macrophages; NK cells = Natural killer cells. Adapted from Blecha 2001. In Biology of the Domestic Pig. 2001. Ed. Wilson G. Pond and H.J. Mersmann. p 689-711.

### *1.1.3.2. The nVDR in cells of the immune system*

The concept that 1α,25(OH)$_2$D$_3$ has immunomodulatory functions arises from the finding that certain cells of the immune system possess the nVDR. It was shown that activated peripheral lymphocytes, CD4$^+$ Th cells as well as CD8$^+$ CTL and thymocytes contain the nVDR (Manolagas et al., 1985; Provvedini et al., 1987). Recent studies however indicate that the nVDR is present in lymphocytes irrespective of their activation status (Veldman et al. 2000). They used a capture ELISA with two different monoclonal antibodies (MAb's) against the nVDR and demonstrated that basal levels of nVDR in CD8$^+$ cells (86,2 ± 10,4 fmol/mg) were significantly higher than those in CD4$^+$ lymphocytes (40 ± 7,1 fmol/mg) and suggested that CD8$^+$ cells may be the major target for 1α,25(OH)$_2$D$_3$ The amount of measurable nVDR is upregulated after *in vitro* stimulation with concanavalin A (ConA) co-administered with 1α,25(OH)$_2$D$_3$ probably due to an improved longevity and stability of the receptor as suggested by Veldman et al. (2000).

Among the APC, monocytes and macrophages have significant levels of nVDR (Polly et al., 1996) and higher levels were detected after stimulation with LPS and 1α,25(OH)$_2$D$_3$ (Veldman et al., 2000). Also dendritic cells (DC) possess a nVDR. DC are highly specialised APC, playing a central role in activation of naïve T cells and in initiation of cellular immune responses (Banchereau et al., 1998).

Initial reports suggested that nVDR are expressed in B cells upon activation (Provvedini 1983, 1986). Yet, no nVDR were detected by the capture ELISA in normal resting as well as activated and/or 1α,25(OH)$_2$D$_3$ treated B cells (Veldman et al., 2000). Although one research group has shown that nVDR is present in B cells if these B cells are activated in a specific way. This group detected nVDR mRNA expression in human tonsillar B cells after activation with anti-κ/λ MAb and/or anti-CD40 MAb supplemented with IL-4. This was also found in Epstein-Barr virus (EBV)-immortalized B cells (Morgan et al., 1994, 1999, 2000). However, there is discussion about their results and it is currently believed that B cells do not have a nVDR.

#### 1.1.3.2.1. Role of 1α,25(OH)2D3 and the nVDR in the immune function.

In order to define the effect of 1α,25(OH)$_2$D$_3$ and the role of the nVDR in the immune system nVDR-deficient mice (VDR-knockout (VDR-KO) mice) were generated (Yoshizawa et al., 1997). These VDR-KO mice grow up normally until weaning, but after weaning they developed rickets-like symptoms. They had severe hypocalcemia, high serum levels of 1α,25(OH)$_2$D$_3$, alopecia and impaired bone formation. The distribution of their lymphocyte subset

in different immune organs appeared normal in comparison with wild-type mice but their macrophage chemotaxis was reduced. (Mathieu et al., 2001). Proliferation of splenocytes following stimulation with anti-CD3 MAb, a $Ca^{++}$-dependent activation, was significantly reduced in comparison with the wild-type mice. This was not the case after stimulation with phorbol-12-myristate-13-acetate (PMA), which is a $Ca^{++}$-independent mitogen. This suggests a $Ca^{++}$-dependent defect in VDR-KO mice. Indeed, normocalcemic VDR-KO mice show a normal anti-CD3-stimulated splenocyte proliferation and a normal macrophage chemotaxis.

VDR-KO mice are almost completely protected against low-dose streptozotocin-induced diabetes (LDSDM), a model for cell-mediated experimental autoimmune diabetes, in comparison with wild type mice. This indicates a defect in the cellular immunity of the VDR-KO mice. The protection against LDSDM disappeares by restoring serum calcium level in the VDR-KO mice. However, treatment with $1\alpha,25(OH)_2D_3$ of the wild-type and the VDR-KO mice resulted in complete protection against LDSDM of the wild-type mice with no change in the VDR-KO mice. The observed protection in the wild-type mice confirms the role of $1\alpha,25(OH)_2D_3$ as pharmacological immunomodulator, probably by the nVDR. Besides the nVDR, it can be concluded that calcium also may play an essential role in the immune system (Mathieu et al., 2001).

### *1.1.3.3. Effect of $1\alpha,25(OH)_2D_3$ on APC.*

The effects of $1\alpha,25(OH)_2D_3$ on cells of the monocyte-macrophage lineage depends on their stage of activation. Abe and co-workers (1981) were the first to discover that $1\alpha,25(OH)_2D_3$ causes the differentiation of resting HL-60 (a human promyelocytic leukemia cell line) and M1-cells (a murine promyelocyte line) towards phenotypically mature monocytes after several days of culture (prodifferentiation effect) but inhibits, in a dose dependent fashion, the proliferation of these cells (antiproliferative effect). The differentiation of these cell lines involves morphological maturation as well as physiological changes: increased non-specific esterase staining, enhanced cytotoxicity and phagocytosis, production of reactive oxygen intermediates in response to PMA stimulation and increased expression of CD14, CD18, CD11a CD11b and CD11c. CD18 and CD11 are cell surface antigens, which form the dimeric β2-integrin cell-adhesion molecules (Rigby et al., 1984, 1985). The production of cytokines is also affected displaying increased IL-1, TNF-α mRNA expression and IL-6 cytokine production (Miyaura et al., 1989). Later it was found that $1\alpha,25(OH)_2D_3$ also accelerates the maturation of resting human blood monocytes towards macrophages with an increased ability to control the intracellular proliferation of *Mycobacterium*

*tuberculosis* (Rook et al., 1986). Treatment of resting purified human monocytes with 1α,25(OH)$_2$D$_3$ during 48 hours in the absence of other cytokines results in reduced expression of human MHC class II antigens (Rigby et al., 1990; 1992). However, this effect has not been observed in all studies (Poulter et al., 1987, Xu et al., 1993). Using a culture of monomorphonuclear cells (MC, mix of monocytes, macrophages, T- and B cells) the overall effect of 1α,25(OH)$_2$D$_3$ is a reduced antigen-dependent T-cell proliferation (Rigby et al., 1984).
In contrast with resting monocytes, LPS-activated monocytes show *in vitro* a dose dependent inhibition of their IL-1α, TNF-α and IL-6 secretion by a 24 hours incubation with 1α,25(OH)$_2$D$_3$ (Muller et al., 1990, Panchini et al., 1998). Another monocyte-derived cytokine, IL-8 is also reduced by 1α,25(OH)$_2$D$_3$ (Larsen et al., 1991). Comparison of the inhibitory effects
of 1α,25(OH)$_2$D$_3$ obtained in cultures of MC and purified monocytes suggested a direct action of 1α,25(OH)$_2$D$_3$ on the monocytes rather than an indirect one via interference with lymphocyte functions (Muller et al., 1992).

Monocytes and macrophages possess 1α-hydroxylase which intracellularly converts 25(OH)D$_3$ to 1α,25(OH)$_2$D$_3$. As a consequence, 1α,25(OH)$_2$D$_3$ locally produced by macrophages can influence cellular events (lymphocyte reactions) in an autocrine (the secreting cells) and a paracrine way (surrounding cells) (Bouillon et al., 1995). Indeed, activation of monocytes leads to the production of inflammatory cytokines as well as to a high local concentration of 1α,25(OH)$_2$D$_3$, which then may limit further cytokine release by these cells.

In contrast with its effect on monocytes, 1α,25(OH)$_2$D$_3$ inhibits the differentiation, maturation and survival of DC (Penna et al., 2000, Griffin et al. 2001). In fact the steroid hormone directs DC towards an immature state with decreased levels of IL-12 (Adorini et al., 2001). Immature DC have the capacity to take up antigen and migrate towards secondary lymphoid tissues to initiate an immune response. A preference for migration of DC towards mucosal surfaces (Peyer's patches of the gut) with subsequent activation of mucosal immune responses has been observed in mice by Enioutina et al. (1999, 2000).

### *1.1.3.4. Effect of 1α,25(OH)$_2$D$_3$ on T cells*

1α,25-Dihydroxyvitamin D$_3$ inhibits *in vitro* the mitogen- or antigen-induced T-cell proliferation in cultures of MC (Tsoukas et al., 1984; Rigby et al., 1984). Cell cycle analysis revealed that 1α,25(OH)$_2$D$_3$ blocks the transition from the G1a phase to the late G1b phase but 1α,25(OH)$_2$D$_3$ has no effect on the expression of the IL-2 receptor (IL-2R, Rigby et al., 1990). As

described above the reduced T-cell proliferation is indirectly mediated by APC. However, it was also shown that 1α,25$(OH)_2D_3$ directly inhibits the proliferation of $CD4^+$ and CD45 $RO^+$ T-cell lines, activated by anti-CD3 MAb (Muller et al., 1993). Flow-cytometric analysis of mitogen-activated MC showed no effect of 1α,25$(OH)_2D_3$ on the percentage of $CD4^+$ and $CD8^+$ lymphocytes indicating that the proliferation of both subsets is equally affected. Within subsets, 1α,25$(OH)_2D_3$ inhibits the proliferation of a pure culture of activated (memory) T cells ($CD45RO^+$) while this of naïve ($CD45RA^+$) was unaffected during 6 days of culture (Muller and Bendtzen., 1992). It was not shown if this was also the case when the subsets were cultured together. 1α,25$(OH)_2D_3$ failed to affect the $CD45RA^+$/$CD45RO^+$ ratio of cultured MC.

1α,25-Dihydroxyvitamin $D_3$ also affects the Th cytokine profiles. The steroid hormone reduces directly the transcription, and subsequently the secretion of several cytokines including IFN-γ (Cipitelli et al., 1998), IL-2 (Alroy et al., 1995; Takeuchi et al., 1998), IL-8 (Harant et al., 1997), IL-12 (D' Ambrosio et al., 1998, Adorini et al., 2001) and granulocyte-macrophage colony stimulating factor (GM-CSF, Towers and Freedman., 1998). IL-12 is the most important cytokine for promoting differentiation of Th0 cells towards Th1 cells. Furthermore, 1α,25$(OH)_2D_3$ enhances the production of Th2-cytokines such as IL-4 and IL-10 (Daynes et al., 1994, 1996; Cantorna et al., 1998) which in turn indirectly inhibit Th1-responses by acting on monocytes and APC. This indirect inhibition of Th1 probably occurs via decreased levels of IL-12 (D' Andrea et al., 1993). 1α,25-Dihydroxyvitamin $D_3$ is also known to stimulate TGF- β (Weinreich et al., 1999). In mice and man TGF-β, a Th3-cytokine (Fukaura et al., 1996), is involved in mucosal immunity, isotype-switching towards IgA as well as IgG2b and in tolerance regulation (Letterio et al., 1998). So the steroid hormone can be classified as a Th2 (Th3?)-modulating-adjuvant (see also Fig. 1.6). A recent review (Mathieu and Adorini 2002) describes in secondary lymphoid tissues the inhibition of IL-12 and stimulation of IL-10 production by 1α,25$(OH)_2D_3$ with downregulation of costimulatory molecule expression (CD40, CD80 and CD86) by DCs, thus inhibiting the development of Th1 cells. The steroid hormone favored the induction of $CD4^+CD25^+$ regulatory T cells and of Th2 cells, which are able to further inhibit Th1 cells. The immune deviation towards a Th2-pattern was also described in nonobese diabetic (NOD) mice but this was limited to diabetic inducing antigens (pancreatic auto antigens, Overbergh et al., 2000).

### *1.1.3.5. Effect of 1α,25$(OH)_2D_3$ on B cells and immunoglobulin secretion*

The role of 1α,25$(OH)_2D_3$ on B-cell function has yet to be fully elucidated. First of all reports concerning expression of nVDR yielded conflicting evidence as described above.

Furthermore conflicting data came on the effect of 1α,25(OH)$_2$D$_3$ on B cells. Some groups showed a direct inhibition of proliferation and Ig production of EBV-transformed and purified B cells (Provvedini et al., 1986, Iho et al., 1986). Others were unable to detect a direct 1α,25(OH)$_2$D$_3$-mediated inhibition of B-cell function (Chen et al., 1987). Later there was evidence to believe that inhibition of Ig production is not caused by a direct effect of 1α,25(OH)$_2$D$_3$ on B cells, but rather mediated through impairment of T-cell and monocyte functions (Muller et al., 1992). Indeed, the inhibitory effect of 1α,25(OH)$_2$D$_3$ on the Ig production was seen after activation with poke weed mitogen (PWM, T cell dependent mitogen), but not after activation with EBV (T cell independent mitogen). In addition, 1α,25(OH)$_2$D$_3$ was not effective in T-lymphocyte- or monocyte-depleted cultures and thirdly the effect of 1α,25(OH)$_2$D$_3$ on PWM-activated MC was reversed by recombinant monokines (IL-1, IL-6) as well as lymphokines (IL-2). So 1α,25(OH)$_2$D$_3$ seems to inhibit Ig production in response to T-cell-dependent antigens but not to T-cell-independent antigens. This is accordance with a previous observation (Komoriya et al., 1985).

As mentioned earlier, the action of 1α,25(OH)$_2$D$_3$ on T cells in mice results in a Th2 response with increased IgG1 and decreased IgG2a concentrations (Lemire et al., 1995). However, in man no effect was observed on the humoral immune response after IM injection of influenza with 1 µg of the steroid hormone. This suggest that factors such as dose, antigen, species, activation stage in vivo, etc. can influence the outcome of 1α,25(OH)$_2$D$_3$ on B-cell responses.

### 1.1.4. Toxicity of 1α,25(OH)$_2$D$_3$

Vitamin D intoxication is often accompanied with hypercalcemia. Multiple factors may influence susceptibility to vitamin D toxicity: concentration of vitamin D metabolites itself, the nVDR expression by target tissues, the activity of 1α-hydroxylase, the metabolism and the capacity to bind with DBP. Furthermore vitamin D toxicity can be due to an increase of 1α,25(OH)$_2$D$_3$ and/or vitamin D metabolites but also by several diseases such as sarcoidosis (Sharma et al., 1996), tuberculosis ( Need et al., 1980), leprosy (Hoffmann et al., 1986), histoplasmosis (Walker et al., 1977) but also lymphoproliferative neoplasm such as Hodgkin's lymphoma (Davies et al., 1985).

Physiological routes and sources of vitamin D$_3$ normally do not cause vitamin D intoxication as pharmacological and high doses (40,000-200,000 International Units (IU with 40 IU = 1 µg 1α,25(OH)$_2$D$_3$) daily for weeks to months) are required as shown in humans (Jubiz et al., 1977). In contrast with the vitamin D 'precursors', the biological active 1α,25(OH)$_2$D$_3$ can

induce intoxication very quickly. Despite this, the use of 1α,25$(OH)_2D_3$ in treatment of osteoporosis is preferred over high pharmacological dosages of vitamin D 'precursors' because the latter and its main metabolite 25-hydroxyvitamin $D_3$ with a half-life of 15 days (Haddad et al., 1976) are stored in muscle and fat and can be retained in large amount. This can lead on the long term to severe hypercalcemia and hypercalcuria. This is not the case for 1α,25$(OH)_2D_3$ with a half-life time of 15 hours (Kawakami et al., 1979; Klein et al., 1977). The most favorable results from 1α,25$(OH)_2D_3$ treatment have been achieved using dosages in the order of 0.5 to 0.75 μg per day (oral intake). Dosages above 1 μg per day result in increased serum calcium and increased bone resorption due to activation of osteoclasts by the 1α,25$(OH)_2D_3$. At the recommended dosages of 0.5 to 0.75 μg 1α,25$(OH)_2D_3$ per day and with calcium intake restricted to 1000 mg per day, 1α,25$(OH)_2D_3$ appears to be safe (Tilyard et al., 1992).

### 1.1.5. Applications of 1 α,25$(OH)_2D_3$

Besides the 'classical' effects of 1α,25$(OH)_2D_3$ on mineral homeostasis and its use for osteoporosis, the steroid hormone also has other (therapeutic) applications.

An important aspect of the immunosupressive actions of 1α,25$(OH)_2D_3$ is the therapeutic application of the steroid hormone in the control of autoimmune diseases such as experimental autoimmune encephalitis (EAE, model for multiple sclerosis), systemic lupus erythematosus (SLE), and type I (juvenile) diabetes. There is strong evidence that these autoimmune diseases are Th1-mediated (Baron et al., 1993; Bach et al., 1994). Studies using the non-obese diabetic mice showed that 1α,25$(OH)_2D_3$ can prevent the clinical development of type I diabetes (Mathieu et al., 1994). Multiple injections of 1α,25$(OH)_2D_3$ resulted in a reduction of the incidence and severity of EAE (Renno et al., 1995) and was due to the production of Th2/Th3-cytokines (TGF-β1 and IL-4) that suppress TNF-α and IFN-γ secretion (Cantorna et al. 1998). Similar results were obtained with rheumatoid arthritis induced by *Borrelia burgdorferi* (Lyme disease).

The immunosuppressive actions of 1α,25$(OH)_2D_3$ may also be useful in the regulation of transplant rejection. Acute rejection is prevented by immunosuppressants like cyclosporin A or FK 506, but these are toxic (nephrotoxic, hypertension, etc). The use of 1α,25$(OH)_2D_3$ as an alternative for these immunosuppressants was demonstrated in a heart allograft mouse model. Continuous administration of low doses of 1α,25$(OH)_2D_3$ significantly prolonged graft survival but caused hypercalcemia (Lemire et al., 1992). Currently, 1α,25$(OH)_2D_3$ and new 1α,25$(OH)_2D_3$-analogs in combination with cyclosporin A are tested for their capacity to enhance survival of

xenografts. These analogs have the advantage that they do not cause hypercalcemia. In addition, combination of 1α,25(OH)$_2$D$_3$ and cyclosporin or of 1α,25(OH)$_2$D$_3$ and FK 506 allows a 10-to 5-fold reduction in dosage of cylcosporin and FK506, respectively (Mathieu et al., 1994).
Due to its capacity to control growth and differentiation, 1α,25(OH)$_2$D$_3$ has been therapeutically exploited to treat leukaemia, different cancers (Miller et al., 1998; Kawaura et al., 1990) and psoriasis (Morimoto et al., 1986). Moreover, it has been described that 1α,25(OH)$_2$D$_3$ enhanced the nerve growth factor in the central nervous system and may be a therapeutic agent in neuro-degenerative disease like Alzheimer's disease (Wion et al., 1991).

## 1.2. CpG-oligodeoxynucleotides (CpG-ODN)

### 1.2.1 General aspects about CpG-ODN

#### *1.2.1.1. Definition*

Cytidine-phosphate-Guanosine (CpG) are unmethylated dinucleotides present at a frequency of 1 on 16 nucleotides in bacterial DNA, whereas they are underrepresented (1/50 to 1/60) and methylated in the vertebrate (mammalian) genomes (Cardon et al., 1994, Pisetsky 1996). Because of these differences, a nonself pattern recognition mechanism has evolved in the vertebrate immune system using 'pattern recognition receptors' (PRRs) enabling them to encounter invading pathogens and unmethylated CpG-dinucleotides (Krieg et al., 1995,2000, 2001). The biological activity of these CpG-dinucleotides can be mimicked by using *in vitro* and *in vivo* chemically synthesized CpG-oligodeoxynucleotides (CpG-ODN).

CpG-ODN are chemically synthesized single stranded DNA sequences and are able to stimulate macrophages, NK cells, DC and B cells. They were originally synthesized in mice in a specific motif in which the CpG-dinucleotide is flanked preferentially by two purines, adenine (A) or guanine (G) at the 5'-end, and two pyrimidines, cytosine (C) or thymine (T) at the 3'-end, making for example AGCpGTT. The experimental use and success of some DNA-vaccination trials is partly due to the presence of CpG-motifs in many vectors (Babiuk et al., 2000). Based on its backbone and the context in which the CpG-ODN are arranged, they can be divided into 2 classes of which the characteristics are shown in Table 1.3.

**TABLE 1.3.:** Distinct immune effects of different CpG-ODN

| Class[¶] | Backbone[a] | Poly G stretch[b] | Palindrome[c] | B-cell-activation[d] | NK-activation[e] | DC-activation[f] |
|---|---|---|---|---|---|---|
| CpG-A | O, SOS | + | + | +/- | ++++ | ++++ |
| CpG-B | S | - | - | ++++ | + | ++++ |

[a]backbone of the DNA includes phosphodiester (O), nuclease-resistent phosphorothioate (S) or both (SOS, at which 5' and 3' ends are S nucleotides) ; [b]four or more consecutive Gs might enhance its cell uptake ; [c]flanking complementary sequences such as AACGTT; [d]proliferation, expression of CD80 and CD86, Ig secretion and IL-6;[e] increased lysis and IFN-γ secretion; [f]expression of MHC II and of CD80 and CD86. Adapted from Krieg et al (2001).
[¶]According to Verthelyi et al. (2001,2002). Class A is also called "D-ODN", triggers the maturation of APC and induces the secretion of IFN-α and IFN-γ by NK cells. Class B is also called "K-ODN" and triggers the maturation of DC, and stimulates B cells to produce IgM and IL-6.

Normally, the bases are linked by phosphodiester (O-backbone) bridges although several modifications in the backbone of the CpG-ODN are possible (Fig. 1.7). An example is the formation of phosphorothioate oligonucleotides (S-backbone), in which one of the non-bridging oxygens in the phophodiester backbone is replaced by a sulfur atom (Fig. 1.7). This makes the CpG-ODN more resistant to nucleases, resulting *in vivo* in a decreased degradation and an increased cell-uptake (Liang et al., 2000; Sester et al., 2000). Base modifications have been shown to affect the level of immune stimulation of the CpG-ODN (Boggs et al.,1997) such as modification at the 5' position on the cytosine ring.

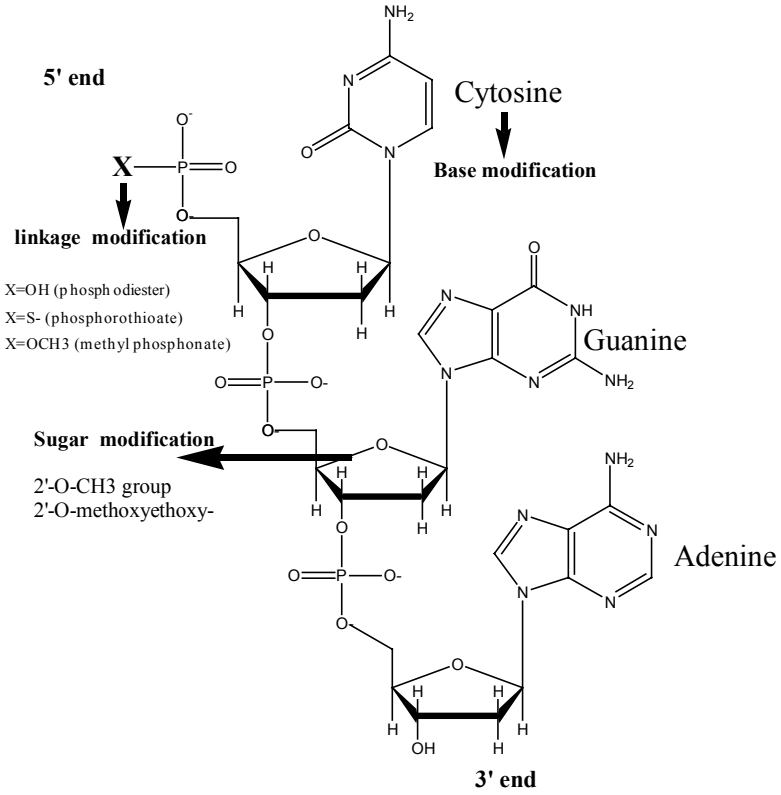



**FIGURE 1.7:** Chemical structure of CpGA-sequence. Chemical modifications of CpG-ODN at the phosphodiester bond, sugars as well as at bases are indicated.

The immunostimulating effects of CpG-ODN also depend on the sequence of the nucleotides flanking the CpG-dinucleotide as well as the target species. Optimal CpG-ODN motifs have been reported for several animal species (Table 1.4.). Comparing these motifs, it was obvious that recognition of a GTCGTT motif is highly conserved. A GACGTT motif however, was optimal for inbred strains of mice and rabbits (Rankin et al., 2001).

Not all CpG-ODN have immunostimulatory properties and may have even neutralizing effects when co-administered with stimulating CpG-ODN. This is the case with CpG-dinucleotide sequences in the genome of adenoviruses in which the CpG-dinucleotide is preceded by a C and/or followed by a G (Krieg et al., 1998a).

**TABLE 1.4.:** Optimal CpG-ODN motifs for humans and several animal species.

| Species | Backbone | ODN-sequence 5'-3' | Reference |
|---|---|---|---|
| Sheep | S | TCGTCGTTTGTCGTTTTGTCGTT | Rankin et al., 2001 |
| Goat | S | TCGTCGTTTGTCGTTTTGTCGTT | Rankin et al., 2001 |
| Horse | S | TCGTCGTTTGTCGTTTTGTCGTT | Rankin et al., 2001 |
| Pig | S, SOS | TCGTCGTTTGTCGTTTTGTCGTT | Rankin et al., 2001 |
| | | ggTGCATCGATGCAGggggg | Kamstrup et al., 2001 |
| Dog | S | TCGCGTGCGTTTTGTCGTTTTGACGTT | Wernette et al., 2002 |
| Cat | S | TCGTCGTTTGTCGTTTTGTCGTT | Wernette et al., 2002 |
| Cow | S | TCGTCGTTTGTCGTTTTGTCGTT | Brown et al., 1998, 2000; Pontarollo et al., 2002., |
| Chicken | S | TCGTCGTTTGTCGTTTTGTCGTT | Rankin et al., 2001 |
| Rat | S | TCGTCGTTGTCGTTTTGTCGTT | Rankin et al., 2001 |
| Rabbit | S | TCCATGACGTTCCTGCAGTTCCTGACGTT | Rankin et al., 2001 |
| Mouse | S | TCCATGACGTTCCTGACGTT | Klinman et al., 1999 Sparwasser et al., 1998 |
| Fish | S | pcDNA3* with ampR gene with 2 repeats of AACGTT | Kanellos et al., 1999. |
| Human/primates | S | TCGTCGTTTTGTCGTTTT | Hartmann et al., 2000. |

Backbone details are abbreviated: SOS (mixture of phosphorothioate and phosphodiester bonds within the same ODN); S (phosphorothioate ODN), O (phosphodiester ODN). Potential stimulatory motifs are underlined. *:pcDNA3 is an eukaryotic plasmid which contains an ampicillin-resistance-gene and is often used in DNA-vaccination trials. g = phosphorothioate Guanosine-stretch.

*1.2.1.2. Molecular mechanisms of CpG-ODN*

Hemmi et al. (2000) demonstrated that the Toll Like Receptor (TLR)-9 is required for the immune activation by CpG-ODN. The TLR-family, first discovered in *Drosophila*, is a phylogenitically-conserved family of receptors that plays a central role in the initiation of cellular innate immune responses and is essential for microbial recognition (Medzhitov et al., 1997). So far 10 members (TLR 1-10) have been identified in mammalia and the current idea is that these members have distinct ligands (Metzhitov et al., 1997, 2001; Vasselon et al., 2002). Toll of *Drosophila* and its mammalian homologues are type I transmembrane proteins, with an extra-cellular domain consisting of leucine-rich repeats (LRRs) and one or two cysteine-rich regions followed by a transmembrane region. The intracellular domain contains a Toll/IL-1 receptor (TIR) domain which is also found in the IL-1 and IL-18 receptor. However, in the latter two, the extra cellular LRR domains are replaced by three immunoglobulin domains in their extra-cellular domain (Fig. 1.8).

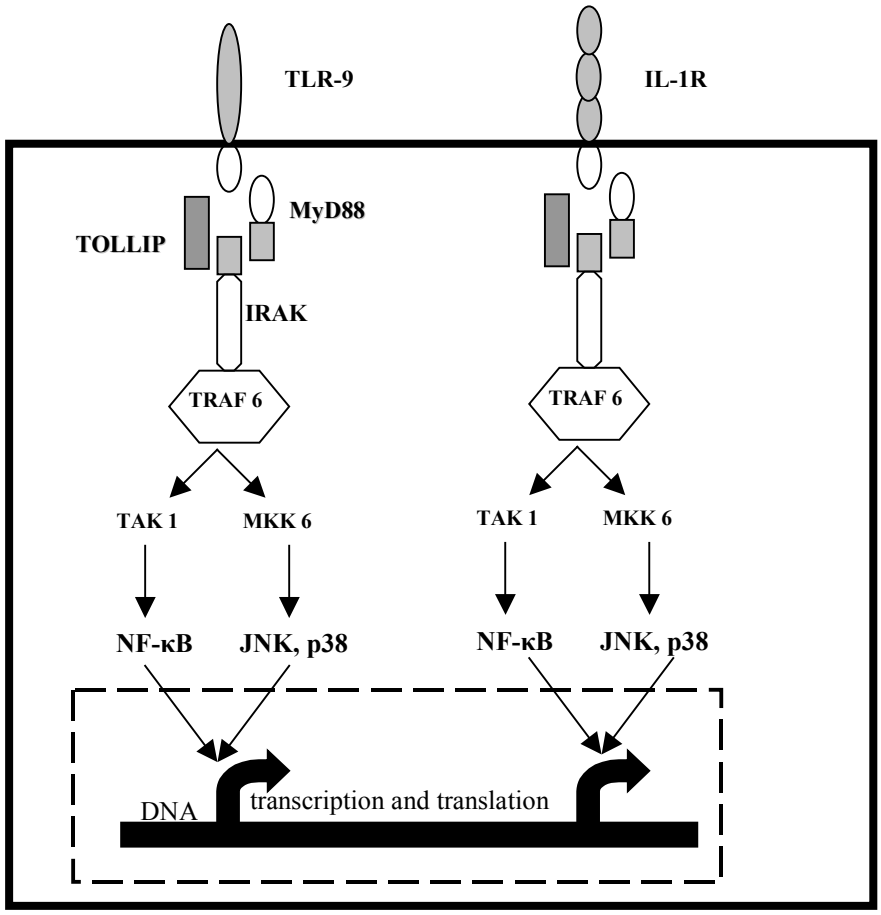



**FIGURE 1.8:** The Toll-like receptor 9 (with leucine rich regions in their extra-cellular domain) and the interleukin-1 receptor (IL-1R)-family members (three immunoglobulin domains extra-cellular) share several signalling components, including MyD88, Toll-interacting protein (TOLLIP), the protein kinase IRAK (ILR-associated kinase) and TRAF 6 (TNF-receptor associated factor 6). MyD88 can bind to activated Toll and IL-1R through interactions with the Toll/IL-1 receptor (TIR) domains of the receptor. Subsequently, MyD88 associates with IRAK and this leads to activation and association with TRAF 6. TRAF 6 can activate nuclear factor-κB (NF- κB) through TAK (TGF-β activated kinase), and JNK (c-jun N-terminal kinase) and p38 MAP kinases through MKK6 (mitogen-activated protein kinase kinase 6) which induce transcription (via different transcription factors). TOLLIP lacks a TIR domain, but contains a C2 domain, which is known to interact with membrane lipids. TOLLIP can also associate with IRAK and the TIR domains of the TLR-9 and binds IRAK to the receptor complex. LRR: Leucine rich regio.

The TIR domain of Toll proteins is a conserved protein-protein-interaction module, which is also found in a number of transmembrane and cytoplasmic proteins in animals and plants. TIR domain-containing proteins have a role in host defence. Signalling pathways activated by TLRs can be divided into 'shared' and 'specific' pathways. A shared pathway is induced by all TLRs as well as by the IL1-R. The specific pathways are activated in some TLRs and may account for differences in signalling between some TLRs and IL-1R. The signalling pathway of TLR-9 includes four essential components: the two adaptor proteins, MyD88 (Aravind et al., 2001) and TOLLIP (Toll-interacting protein, Burns et al., 2000); a protein kinase, IRAK (IL-1 receptor-associate kinase) and an other adaptor molecule TRAF 6 (TNF-receptor-associated factor 6). TRAF 6 induces activation of TAK 1 (=TGF-β activated kinase) and MKK6 (MAP kinase kinase 6), which, in turn activate NF-κB and JNK (c-jun-N-terminal kinase) + p38 MAP kinase, respectively (Hacker et al., 1998, Yi and Krieg 1998). Activation of the signal transduction pathways by TLR-9 leads to the induction of various genes that function in the host defence and results among others in the production of ROS (reactive oxygen species), inflammatory cytokines, chemokines, MHC II and co-stimalatory molecules. JNK phosphorylates the activator protein 1 (AP-1)-family transcription factor Jun while p38 activates the activation transcription factor (ATF)-2 and leads to expression of IL-12 and TNF-α in macrophages. Also the expression of oncogenes such as c-myc is induced by CpG-ODN (Yi et al., 1998). The induction of c-myc may be related to anti-apoptotic effects of CpG-ODN as observed in macrophages and B cells (Yi et al., 1998).

The nature and location of TLR-9 remains controversial. In general, TLRs are found on the cell membrane surface and this was recently confirmed for TLR-9 by flow cytometry (Chuang et al., 2002). However, previous experimental studies by Yamamoto and co-workers (1994) showed that lipofection of murine splenocytes with liposomes containing CpG-ODN enhances IFN-γ and NK-activity. Since lipofection allows CpG-ODN to enter the cell without binding on a cell membrane receptor, it appears that interaction with a cell surface receptor is not essential for CpG-ODN to exert their biological effects. Results of Manzel and Macfarlane (1999) seem to confirm this. They demonstrated that cellular uptake of CpG-ODN was needed for B-cell activation and suggested that CpG-ODN interact intracellularly with a receptor or a signal transduction pathway. An other argument for this intracellular interaction is that the uptake into the cells seems to be sequence (CpG)-independent (Krieg et al., 1995, Yamamoto et al., 1994) whereas the biological effect is not. This indicates that distinction between activating and non-activating CpG-ODN takes place after the uptake. Interestingly, signalling by CpG-ODN requires internalisation into late endosomal or lysosomal compartments. This was shown by addition of

drugs that prevent acidification of endosomes such as chloroquine or monensin (Hacker et al., 1998 ; Yi et al., 1999). The lysosomes or late endosomes are possible candidates for harboring this intracellular receptor. Indeed, MyD88 co-localises with CpG-ODN in endosomal structures and not at the cell membrane as shown by flow-cytometry and confocal microscopy (Schnare et al., 2000; Horng et al., 2001). Also the presence of a transmembrane domain in the TLR-9 suggests that the TLR-9 can interact into the membranes of the endosomes. So at this moment, the mechanism proposed is binding of CpG-ODN to the TLR-9 followed by endocytosis and the release into the cytoplasm. Subsequently, CpG-ODN bind to a 'intracellular' receptor and activate the signalling pathways.

### 1.2.2. CpG-ODN and the Immune system

#### *1.2.2.1. Effect of CpG-ODN on macrophages and DC.*

One of the properties of macrophages and to a greater extend of DC is processing and presenting an antigen to T cells via MHC class II. This is accompanied with the co-expression of co-stimulatory molecules and subsequent secretion of cytokines (Bancherau and Steinman, 1998). Upon stimulation with CpG-ODN, both APC are directly stimulated and subsequently produce IL-12. This in turn activates NK cells into the production of IFN-γ which suppresses Th2 cells and stimulates macrophages to produce more IL-12. All this results in a Th1 microenvironment. Besides IL-12 and IFN-γ, there is also increased secretion of IL-18, which also modulates towards Th1 (Klinman et al., 1995, 1997; Yi et al., 1996, Lipford et al., 1997).

CpG-ODN may also affect T-cell responses by altering antigen processing and presentation functions of macrophages and DC. Theoretically, an increase in antigen presentation can explain the adjuvant effects of CpG-ODN. However, it was shown *in vitro* that CpG-ODN cause a decrease in the synthesis of MHC II molecules by peritoneal macrophages resulting in down regulation of antigen presentation (Chu et al., 1999). How this relates to the *in vivo* effects of CpG-ODN is not clear since the *in vivo* situation is a summary of direct and indirect effects on macrophages. The latter are due to stimulation of other cell types such as NK cells which can produce IFN-γ enhancing antigen processing and presentation.

Macrophage responses to CpG-ODN differ significantly from that of DC. Treatment of DC with CpG-ODN induces maturation, increased expression of both MHC II and co-stimulatory molecules and a transient increase in antigen processing followed by a decline in antigen processing (Jakob et al., 1998, Sparwasser et al., 1998). The presentation of a previously processed antigen is not affected. (Hartman et al.,1999).

*1.2.2.2. Effect of CpG-ODN on T cells.*

CpG-ODN have been shown to act as Th1-modulating adjuvants as shown in mice and man (Forsthuber et al., 1996; Chu et al., 1997).

It is still controversial whether CpG-ODN have a direct effect on T cells. Lipford and co-workers (1997) showed that CpG-ODN do not directly activate a culture of pure T cells. Sun et al. (1998) showed that CpG-ODN could increase proliferation as well as expression of surface-markers (CD69, B7-2, Ly6C and MHC I) of purified $CD4^+$ (Th) and $CD8^+$ (CTL) T cells and that this was dependent on the presence of APC but independent of a TCR/MHC (class I and II) interaction. Later on, they demonstrated that the surface-marker upregulation was mediated by CpG-ODN induced type-I interferon (IFN-I, Sun and Sprent 1999) secretion by APC. The type-I IFN-secretion could not explain the increased proliferation since type I IFN normally inhibit T-cell proliferation. Bendigs et al. (1999) showed that CpG-ODN act as a co-stimulatory signal (signal 2) for T cells activated by cross-linking their T-cell receptors (signal 1 = addition of anti-CD3 MAb). This mechanism could promote antigen-specific T-cell proliferation.

*1.2.2.3. Effect of CpG-ODN on B cells and immunoglobulin (Ig) secretion.*

CpG-ODN are very potent B-cell mitogens (equally stimulatory for both resting as activated B-cell subsets (Krieg et al., 1998)) that drive more than 95% of the B cells into the cell cycle (Krieg et al., 1995). The mechanism of this polyclonal B-cell activation remains unclear.

B-cell stimulation with CpG-ODN also results in the secretion of IL-6 and IL-10 and IgM. Addition of IFN-γ (Th1) results in a higher secretion of IL-6 (Yi et al., 1996). CpG-ODN also seem to rescue B cells (Mower et al., 1994, Yi and Krieg, 1998) from apoptosis. This requires the activation of the NFκB pathway and the expression of c-myc and bcl-$x_l$ (Fischer et al., 1994).

For human B cells it was shown that these CpG-ODN that induced maximal proliferation, also enhanced the production of IgM, IgA and IgG, in the absence of exogenous cytokines or T cells (Liang et al., 1996). Co-administration of IL-2 (Th1), but not IL-4 and IL-10 (Th2), enhanced B-cell proliferation and Ig secretion. *In vivo,* addition of CpG-ODN leads to isotype switch to IgG2a. In mice, this isotype is correlated with a Th1 response and with the presence of NK cells and APC (Mosmann et al., 1991; Davis et al., 1998). More than 80% of CpG-activated B cells show enhanced expression of activation markers including CD69, CD86, IL-2R (CD25) and IFN-γR (Martin-Orozco et al., 1999). Besides the polyclonal activation of B cells, CpG-ODN also

stimulate the antigen-specific B-cell responses. This stimulation occurs indirectly via APC after cross linking the BCR (Krieg et al., 1995).

*1.2.2.4. Effect of CpG-ODN on NK cells*

NK cells protect against viruses and other pathogens by releasing the contents of their granules and killing the infected cells. NK cells seem to be very important in the innate immune response providing a first defence in the immune system. Tokunaga and colleagues (1984) showed that bacterial DNA (CpG-ODN) strongly activates NK cells with enhanced IFN-γ secretion and lytic activity. These effects are not observed using highly purified NK cells indicating that other cells are needed and that the effect on NK cells is indirect. The adding of MAb against IL-12, TNF-α and type I IFN blocked NK cell-activity and indicated that these cells were APC (Cowdery et al., 1996). Furthermore, addition of CpG-ODN together with IL-12 had a synergistic effect on the activity of NK cells in comparison with NK cells incubated with only an equal amount of IL-12. The same seemed to be true for IL-18 (Weiner et al., 2000). The effect of both cytokines (IL-12 + IL-18) enhances the production of IFN-γ by NK cells. In conclusion, CpG-ODN activate NK cells in a Th1-environment (IL-12 and IL18) provided by activated APC. These activated NK cells in turn produce IFN-γ and have an increased lytic activity. The secretion of IFN-γ further maintains and/or enhances this Th1-environment.

1.2.3. Toxicity of CpG-ODN

Weeratna et al. (2000) showed that CpG-ODN , when used as a vaccine adjuvant, are safe and even less toxic than other conventional adjuvants. There has been fear that CpG-ODN could trigger systemic autoimmune diseases, as most autoimmune diseases are mediated by Th1-responses. Indeed, Bachmaier et al. (1999) reported that CpG-ODN can induce autoimmune myocarditis. Moreover, CpG-ODN activate auto-reactive Th1-effector cells specific for myelin basic protein and trigger the development of experimental autoimmune encephalitis (EAE) (Tsunoda et al., 1999). In contrast Boccacio et al. (1999) could reduce the symptoms of EAE with CpG-ODN due the high secretion of IFN-γ. However, this could be due to a different sequence of ODN, dose, etc.

A single dose of 500 µg CpG-ODN does not cause toxic shock in mice. However, repeating this dose within a week can cause death by either enhanced TNF-α, IFN-γ and IL-12 secretion or

by massive proliferation of B cells (Krieg et al., 2000). Toxicity has also been seen in combination with LPS: CpG-ODN seems to prime for a schwartzmann reaction when a sublethal dose of LPS is given a few hours after the CpG-ODN. This schwartzmann reaction is a LPS-mediated-shock due to dramatic increase of TNF-α and IFN-γ (Cowdery et al., 1996; Sparwasser et al., 1997).

### 1.2.4. In vivo applications of CpG-ODN

CpG-ODN can be administered either by injection (intravenous, intraperitoneal, intramuscular or intradermal injection) or via non-invasive methods (gene gun, topical administration on the skin, administration via mucosae). The use of CpG-ODN *in vivo* results in an overall increase of antigen-specific Ig secretion but with a prominent increase of the IgG2a Ig isotype and a Th1-cytokine pattern (Fig. 1.9).

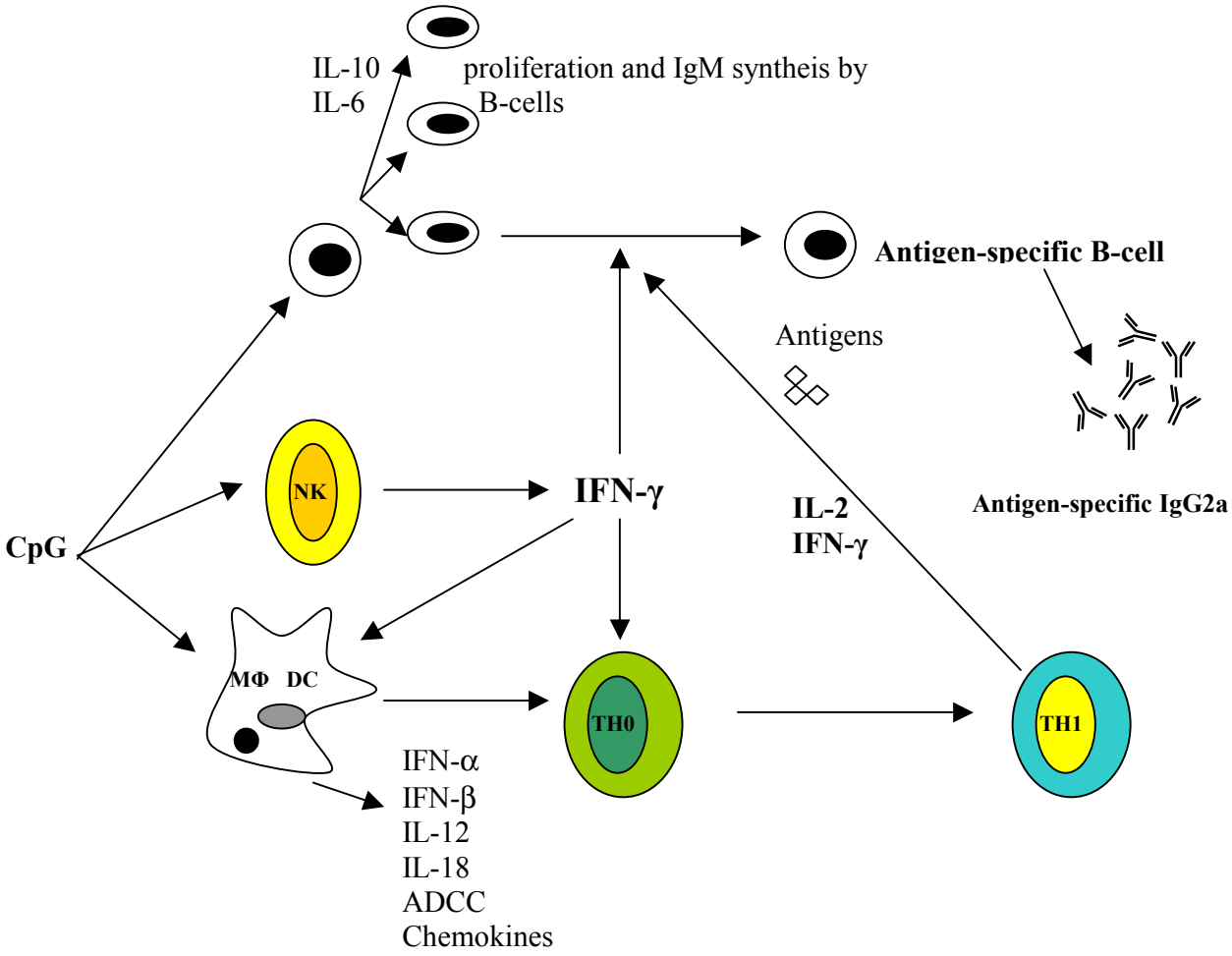



**FIGURE 1.9:** Schematic presentation of the mechanism postulated for the induction of an antigen-specific Th1 and antibody response by CpG-ODN vaccination. Initially CpG-ODN trigger the release of IFN-γ by NK cells as well as inducers of IFN-γ such as IL-12, IL-18 and TNF-α, which are secreted by APC (DC and macrophages (MΦ)). Furthermore CpG-ODN act as direct mitogens for naïve B cells which proliferate and secrete IL-6, IL-10 and IgM antibodies. At the same time CpG-ODN activate and induce maturation of DC resulting in the expression of co-stimulatory molecules (CD80, CD86, MHC II, CD40). The mature DC migrate to the secondary lymphoid organs where APC-T-cell interaction as well as T-cell -B-cell interactions occur. The initial secreted Th1-cytokines (IFN-γ) prime naïve T-helper cells to differentiate towards Th1 cells. Th1 cells secrete Th1-cytokines. These cytokines induce activated B cells to produce antigen-specific IgG2a antibodies. DC =dendritic cells; APC=antigen presenting cells; IFN=interferon.

The modulation towards a Th1-response together with the increased Ig secretion make CpG-ODN very potent adjuvants for vaccines and were shown to be safe after intramuscular injection (Weeratna et al, 2000). Modulation towards a Th1-response can be desirable because (i) in mice Th1-responses are associated with the production of IgG2a which have better neutralizing capabilities than IgG1 antibodies and (ii) Th1 cytokines (IFN-γ, IL-2, IL-12) enhance the cell mediated immunity (CMI) in general and more specific the maturation of CTL-responses. The adjuvant effects of CpG-ODN have been demonstrated in immunisation with several pathogens and antigens such as live attenuated measles virus (Kovarik et al., 1999), *Brucella melitensis* (Al-Mairiri et al., 2001), hen-egg lysozyme (Chu et al., 1997), ovalbumin (Shirota et al, 2000), β-galactosidase (Roman et al., 1997). Adjuvants effects were also seen for responses against T-cell independent polysaccharide antigens (Kovarik et al., 2001).

CpG-ODN can also be used as a mucosal adjuvant. Nasal administration of β-galactosidase (β-gal) together with CpG-ODN resulted in an enhanced β-gal-specific IgA response in serum as well as mucosal secretions (feces, vagina, broncho-alveolar fluid, Horner et al., 1998, 2000). The antibody response was comparable with the response seen after intranasal administration of β-gal together with cholera toxin (CT), a very potent mucosal adjuvant (Lycke et al., 1992).

The neonatal immune system is still immature. Immune responses are Th2 based, the cell-mediated responses are poor but also the B-cell responses are weak and preferentially generate IgM and IgG1 antibodies with low affinity. CpG-ODN might be helpful for the maturation of this neonatal immune system and in controlling infections in young animals. Indeed, CpG-ODN have been shown to be protective in young mice against challenge with *Listeria monocytogenes*, *Anthrax, Malaria, Leishmania* or *Schistosoma* after injection with antigens of these pathogens supplemented with CpG-ODN. (Krieg et al., 1998, Elkins et al., 1999, Zimmerman et al., 1999, Walker et al., 1999, Stacey et al., 1999).

One of the most important therapeutic applications of CpG-ODN could lay in treatment of allergies such as asthma. Allergies are becoming very important problems in Western countries. They are characterized by a Th2-type of immune response involving IL-4, IL-5 and IL-13 (Robinson et al., 1996; Wills-Karp et al., 1998). IL-4 induces the formation of IgE (Del Prete et al., 1994), IL-5 stimulates the proliferation and activation of eosinophils (Clutterbuck et al., 1988) and basophils and IL-13 causes airway hyperresponsiveness and inflammation (Grunig et al., 1998). Studies in animal models (murine models of asthma) revealed that CpG-ODN prevent eosinophylic airway inflammation and bronchial hyperreactivity to non-specific stimuli (Kline et al., 1998). Moreover, less IL-4 but increased IFN-γ and IL-12 were found in lung lavage fluid. The antigen-specific IgE antibody response was also suppressed by CpG-ODN (Roman et al.,

1997). So, CpG-ODN seems to reverse an established Th2-mediated allergic disease towards a Th1 response (Kovarik et al.,1999). However, the Th2-response could only be redirected in older but not in neonatal animals. The new Th1-response remained for a long period even in the presence of antigens, which normally evoke a Th2-response (Goodman et al., 1998).

The ability of CpG-ODN to co-stimulate T cells (signal 2) might be important in situations where no APC are present or where the antigen presentation is not sufficient to evoke a response, as it is the case in some tumor cells. CpG-ODN could bypass this insufficient presentation by acting as co-stimulators for T cells. Moreover, CpG-ODN enhance the production of cytokines such as TNF-α, IL-12 and IFN-γ which all have anti-tumor activity (Ballas et al., 1996; Cowdery et al., 1999; Lipford et al., 1997). Limited data suggest that CpG-ODN themselves can have anti-tumor effects. Smith and Wickstrom (1998) showed that CpG-ODN inhibit the growth of lymphomas. In addition CpG-ODN are able to enhance the efficacy of antibody therapy using MAb against tumor antigens by increasing the cell killing of the antibody-coated tumor cells (ADCC) by NK cells, macrophages and/or monocytes (Maloney et al., 1997, Pegram et al., 1998). Most challenging in the development of cancer vaccines is the induction of a tumor-specific immune response. This requires a cellular rather than a humoral response. Mice immunised with the tumor antigen, an antigen of lymphoma, to which CpG-ODN were added as an adjuvant, were protected against tumor challenge (Weiner et al., 1997). These mice showed enhanced IgG2a antibodies and CTL responses. Normally, exogenously delivered antigens are loaded on MHC II molecules and evoke a humoral response, while intracellular proteins are processed and presented by class I which lead to CTL-responses. Some extra-cellular antigens, however, are taken up by APC and processed in a manner that leads to presentation in class-I molecules, which is termed "cross-priming" (Rock et al., 1996). The results above support the concept that an effective cellular response (CTL responses) can be induced by immunisation with an intact tumor antigen plus CpG-ODN. CpG-ODN activate immune cells and induce cross-priming (Davis et al., 2000). Among the cells activated by CpG-ODN important in cancer therapy are the DC. The combination of CpG-ODN and DC could be useful in cancer-immunotherapy.

## 1.3. Use of Calcitriol and CpG-ODN in veterinary medicine and vaccines

The majority of publications regarding immunoregulatory properties of 1α,25(OH)$_2$D$_3$ and CpG-ODN are based on models in rodents (mice) and man. Little is known about its role in the immune system of domestic animals such as pigs, cattle, dogs, cats, horses, chickens, etc. However, it was already found in the early eighties that T cells in the calf thymus and lymph node

exhibit nVDR (Reinhardt et al., 1982). In cattle, an inhibitory effect of 1α,25$(OH)_2D_3$ on ConA-induced proliferation of lymphocytes was observed in bovine MC. In higher doses however the steroid hormone enhanced the ConA-induced proliferation (Reinhardt and Hustmyer, 1987). Furthermore, 1α,25$(OH)_2D_3$ inhibits Th1-like responses as the secretion of IFN-γ by bovine MC was abolished (Ametaj et al., 1996). This is accompanied with enhanced immunoglobulin secretion as observed by Nonnecke et al. (1992). Indeed, IM vaccination with the J5-vaccin (containing cell wall core antigens of *Escherichia coli* causing coliform mastitis) supplemented with high doses of 1α,25$(OH)_2D_3$ (2 x 200 μg) enhanced the J5-specific IgA, IgG1 and IgG-titer in milk but decreased the IgG2-milk antibody titer (Reinhardt et al., 1999). In sheep, 1α,25$(OH)_2D_3$ altered the ratio between IgG1 and IgG2 but no typical mucosal immune response in a peripheral lymph node, as characterised by IgA antibodies, was induced (Scheerlinck et al., 2001). Little is known about the immunomodulating effects of 1α,25$(OH)_2D_3$ in swine. However, the presence of the nVDR in porcine leukocytes and macrophages is expected (Bondarenko et al., 1994; Reichel et al., 1991). Moreover, it was shown that porcine alveolar macrophages are able to produce 1α,25$(OH)_2D_3$ (Reichel et al., 1991).

As described earlier CpG-ODN may have adjuvants effect in many veterinary species (Rankin et al., 2001). Recently it was demonstrated that even mice-specific CpG-ODN enhance the humoral responses in chickens after IM immunisation (Vleugels et al., 2002). In cattle it was shown that CpG-ODN (GTCGTT-motif) or CpG DNA from *Babesia bovis*, *Trypanosoma cruzi* and *Trypanosoma brucei* sequences stimulates bovine leukocytes with enhanced IL-12, IL-6, NO and TNF-α production. This was accompanied with increased proliferation of B cells (Brown et al., 1998, 2001; Shoda et al., 2001; Zhang et al., 2001; Pontarollo et al., 2001). Recently, Kamstrup et al., (2001) showed *in vitro* that porcine blood MC are stimulated by CpG-ODN. In addition, the plasmid pcDNA 3, containing an AACGTT-motif, induced production of IFN-α and IL-6 in cultures of porcine leukocytes (Magnusson et al., 2001). Methylation of all cytosines in these CpG-motifs abolished the IFN-α inducing capacity. The pcDNA3 is a commonly used expression vector for eukaryotic cells.

# PART II

## AIMS OF THE STUDY

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Intestinal infections caused by enterotoxigenic *Escherichia coli* (ETEC) can lead to neonatal and post-weaning diarrhea in swine and to severe economic losses. Many parenteral vaccines are available for inducing a lactogenic protection against neonatal diarrhea. These vaccines can not be used against post-weaning diarrhea since parenteral vaccines in general do not induce an active intestinal mucosal immunity which is needed to protect the weaned piglets.

The goal of the present work is to study in pigs if a systemic (intramuscular) immunisation can induce or prime a mucosal (IgA) response using immunomodulators and if so to gain insights into the mechanism of the immunomodulation. In this work two different immunomodulators (adjuvants) are used: $1\alpha,25(OH)_2D_3$ and Cytidine-phosphate-Guanosine oligodeoxynucleotides (CpG-ODN). Immunisation is performed using different antigens: (ovalbumin (OVA), human serum albumin (HSA) and purified F4-fimbriae from $F4^+$-ETEC.

In particular for $1\alpha,25(OH)_2D_3$ the following issues are examined:

- Can $1\alpha,25(OH)_2D_3$ enhance the antigen-specific IgA immune response ?
- If so, is the enhanced IgA response correlated with a Th2-like cytokine profile?
- Is it possible by adding $1\alpha,25(OH)_2D_3$ to prime for an intestinal mucosal immune response?

In particular for CpG-ODN the following issues are investigated:

- Can CpG-ODN be used as immunomodulating/immunoenhancing adjuvants for IM immunisation?
- If so, which CpG-ODN sequences are most immunostimulatory for pigs and which immune cells become activated by CpG-ODN ?

In particular for the $F4^+$-ETEC infection in piglets we wondered if:

- Different doses of F4-fimbriae can evoke different isotype-specific antibody responses after intramuscular immunisation ?
- Intramuscular immunisation of piglets with an optimal dose of F4-fimbriae supplemented with $1\alpha,25(OH)_2D_3$ or CpG-ODN could protect against an $F4^+$-ETEC challenge ?

# PART III

## EXPERIMENTAL STUDIES (CHAPTERS 2-7)

# CHAPTER 2

# ENHANCED INDUCTION OF THE IgA RESPONSE IN PIGS BY CALCITRIOL AFTER INTRAMUSCULAR IMMUNISATION

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## 2.1. Abstract

In this study, the immunomodulating effect of two steroid hormones namely 1α,25-dihydroxyvitamin $D_3$ [1α,25$(OH)_2D_3$] and dehydroepiandrosterone [DHEA] was examined on the antigen-specific antibody responses by intramuscular immunisation of pigs with human serum albumin alone (HSA) or supplemented with 2μg of 1α,25$(OH)_2D_3$, 40 μg of DHEA or the combination of both steroids. 1α,25$(OH)_2D_3$ significantly enhanced the antigen-specific IgA and IgM serum response. Higher HSA-specific IgA titers were also found in the mucosal secretions (saliva, feces and nasal) of the steroid treated animals, especially in the 1α,25$(OH)_2D_3$ group. Furthermore, 1α,25$(OH)_2D_3$ and DHEA increased the number of antigen-specific IgA and IgG antibody-secreting cells in the local draining lymph nodes, but only few numbers were detected in lymph nodes draining the mucosa. DHEA decreased the IgM serum response and had the tendency to enhance the IgG2 and IgG serum responses. Strong and comparable IgG, IgG1 and IgG2 serum responses were seen in all groups. Combining both steroids did not result in a higher IgA serum response. On the contrary DHEA seems to neutralize the effect of 1α,25$(OH)_2D_3$ on the IgA response. In conclusion, 1α,25$(OH)_2D_3$ significantly enhanced the antigen-specific IgA and IgM response in serum and the number of antigen-specific IgA and IgG ASC in the local draining lymph nodes following intramuscular immunisation.

## 2.2. Introduction

Mucosal surfaces are continuously exposed to environmental organisms and antigens and have therefore developed a variety of protective mechanisms (McGhee et al., 1992). Mucosal protection is mainly due to the active secretion of IgA antibodies. These antibodies are induced following contact of the mucosae with potential harmful microorganisms resulting in neutralisation and subsequent elimination of these pathogens. Although mucosal immune responses are generally induced at mucosal sites, Daynes and co-workers (Daynes et al., 1996 ; Enioutina et al., 1999) demonstrated in mice that a mucosal immune response can also be induced systemically using immunomodulators, such as the steroid hormone 1α,25$(OH)_2D_3$, the active metabolite of vitamin D. 1α,25$(OH)_2D_3$ influences the production of different lymphokines in the local draining lymph node. Indeed, *in vitro* stimulation with anti-CD3ε of cells from the local draining lymph node of a mouse treated with 1α,25$(OH)_2D_3$ resulted in an enhanced production of IL-4, IL-5 and IL-10 and a reduced production of IL-2 and interferon-gamma (IFN-γ). So the steroid compound appears to modulate a switch of the cytokine-profile from a Th1 towards a Th2-

profile as occurs in the mucosal-associated lymphoid tissue (MALT). This was accompanied with an increase in antigen-specific IgA in serum and mucosal secretions and with the homing of IgA and IgG antibody secreting cells to the lamina propria of the intestine and the lungs.

1α,25$(OH)_2D_3$ exerts its effect via binding to a nuclear receptor which presence is well documented in activated T-cell subpopulations (T-helper (Th) and T cytotoxic cells (CTL)) as well as in activated B-lymphocytes (Baran et al., 1994; Minghetti et al., 1988; Povvedine et al., 1986, 1989). 1α,25$(OH)_2D_3$ modulates via this receptor not only the production of cytokines but also induces a dose-dependent suppression of Th cell proliferation. Furthermore, inhibition of B cell proliferation and a dose-dependent suppression of the IgG1 immunoglobulin production have been described (Lemire et al., 1984).

Dehydroepiandrosterone (DHEA) also seems to have immunomodulatory effects. DHEA is produced by the adrenal glands and is one of the major androgen steroid hormones in men and women between the second and fifth decade of life (Regelson et al., 1994) A specific receptor for this hormone is found in T cells (Meikle et al., 1992). It directly enhances Th1 activity as demonstrated *in vitro* on isolated lymphocytes from peripheral lymph nodes, spleen and Peyer's Patches (PP) with an increased interleukin-2 (IL-2) production (Suzuki et al., 1991).

In the present study, the immunomodulatory activity of both steroids was evaluated on the antibody responses in pigs following intramuscular immunisation with human serum albumin (HSA).

## 2.3. Materials and Methods

### 2.3.1. Experimental animals

Thirteen conventional pigs (Belgian Landrace x Piétrain) were weaned at the age of 4 weeks and were subsequently housed in isolation units where they obtained food and water *ad libitum*. All animals were negative for serum antibodies against human serum albumin (HSA) as determined by ELISA.

### 2.3.2. Experimental procedure

At the age of 7 weeks, the pigs were divided into four groups which were all intramuscularly immunized in the musculus gluteobiceps with 1 mg of HSA (SIGMA, Sigma-Aldrich, Bornem,

Belgium) in 0,5 ml PBS suspended 1/1 [vol/vol] in incomplete Freund's adjuvans (IFA). The first group was immunized with the antigen supplemented with 2 µg $1.25(OH)_2D_3$ (*n=3*, $1.25(OH)_2D_3$ group), the second group with 40 µg of DHEA (*n=3*, DHEA group) and the third group with a combination of both steroids (2 µg of $1\alpha,25(OH)_2D_3$ and 40 µg of DHEA) (*n=3*, combination group). The fourth group served as a control and only received 1 mg HSA (*n=4*, control group). The steroids were dissolved in 95% (vol/vol) ethanol at a concentration of $1.10^{-4}$ M and stored at -20°C until used. Forty-three days after the first immunisation all animals were identically reimmunized.

The immune response after the intramuscular immunisation was evaluated by determining the HSA-specific antibody titers in serum (IgM, IgA, IgG1, IgG2 and IgG), in rectal content, nasal and oral secretions (IgA) weekly or twice a week until 4 weeks post secondary immunisation. Furthermore in some animals ELIspot assays were performed for localizing and quantifying the numbers of HSA-specific IgM, IgA and IgG antibody secreting cells in different lymphoid tissues 16 days post primary immunisation (PPI) and 10 and 43 days post secondary immunisation (PSI).

### 2.3.3. Samples

#### *2.3.3.1. Serum*

Blood was sampled from the jugular vein at different time points PPI and PSI. Time points are presented in Fig. 2.1 and Table 2.1. Serum was collected and inactivated at 56°C during 30 min and subsequently treated with kaolin (SIGMA) as described to decrease the background reading in ELISA (Van den Broeck et al., 1999). Final dilutions of 1/10 (vol/vol) were prepared in ELISA dilution buffer (PBS + 0.05% (vol/vol) Tween 20 + 5% (wt/vol) glycine) whereafter the diluted samples were stored at –20°C until used in ELISA.

#### *2.3.3.1. Mucosal secretions*

Rectal contents, nasal and oral secretions were sampled weekly until 4 weeks PSI using cotton swabs. The mass of the collected secretions was determined by weighing the swabs before and after the sampling. Subsequently the secretions were rapidly diluted 1/10 in PBS (pH 7.4, 150 mM) supplemented with 0.05% [vol/vol] Tween 20 and 20% [vol/vol] fetal bovine serum (FBS, Gibco, BRL, Life Technologies, Merelbeke, Belgium) and stored at -80°C until used in ELISA.

*2.3.3.2. Lymph node monomorphonuclear cells*

Sixteen days PPI, 10 and 43 days PSI, 2 (Fig. 2.2.A), 4 (Fig.2.2.B) and 2 pigs (Fig. 2.2.C) respectively were euthanatized by intravenous injection of an overdose pentobarbital (24mg/kg; Nembutal, Sanofi Sant Animale, Brussels, Belgium) followed by exsanguination. Subsequently the bronchial (BLN), mesenteric (MLN), gluteal (GLN) and iliacal lymph nodes (ILN) were aseptically collected. After removing surrounding fat, the MC were isolated by gently teasing the tissues apart. The MC were collected in RPMI 1640 (Gibco, BRL) on ice, and the erythrocytes were lysed with ammoniumchloride (0.8% [wt/vol]). After centrifugation (380 x g at 4°C for 10 min), the pelleted cells were washed and resuspended in leukocyte medium (RPMI-1640 supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml), kanamycin (100µg/ml), glutamine (200 mM), sodiumpyruvate (100mM), non-essential aminoacids (100mM) and 10% FBS ([vol/vol] Gibco BRL, Life Technologies, Merelbeke, Belgium)) at the concentration of $1.10^7$ cells/ml.

2.3.4. Titration of HSA-serum antibodies

HSA-specific serum IgG1, IgG2 and IgG titers were determined in an indirect ELISA. Briefly, the wells of a 96-well microtitre plate (NUNC®, Polysorb Immuno Plates, Gibco BRL) were coated with HSA at a concentration of 50µg/ml of coating buffer (carbonate-bicarbonate buffer, 50mM, pH=9.4). After 2 hours of incubation at 37°C, the remaining binding sites were blocked with PBS supplemented with 0.2 % Tween®80 (PBS-Tw) during 2 hours at room temperature. Subsequent the plates were incubated for 1 hour at 37°C with twofold dilutions of sera (starting from 1/10) in ELISA dilution buffer (PBS, pH 7.2 + 0.05% [vol/vol] Tween ®20 + 5% glycine), swine-specific IgG1, IgG2 or IgG monoclonal antibody (MAb, (Van Zaane et al., 1987), biotinylated rabbit anti-mouse IgG1 (Zymed laboratories, Sanbio B.V., Uden, Nederland) in ELISA dilution buffer supplemented with 2% [vol/vol] pig serum and horseradish peroxidase-linked (HRP) streptavidin. Between each step, the plates were washed with PBS + 0.2% [vol/vol] Tween® 20. Finally, ABTS, containing $H_2O_2$, was added and after 30 min incubation the optical density was measured at 405 nm ($OD_{405}$).

The HSA-specific IgA (serum and mucosal secretions) and IgM (serum) antibodies were determined by an antibody capture ELISA. Microtitre plates were coated with 5µg/ml of swine-specific IgA or IgM MAb's [13] in PBS (pH 7.4) for 2 hours at 37°C. Subsequent steps were: blocking overnight at 4°C with PBS supplemented with 0.2% Tween ®80, adding twofold

dilutions of the treated sera or the mucosal secretions in ELISA diluting buffer during 1 hour at 37°C, adding 10 µg/ml HSA in PBS (pH 7.4) supplemented with 0.3 M NaCl and 0.2%Tween®80 (1 hour at 37°C). Finally an optimal dilution of sheep anti-HSA polyclonal antibodies (Serotec, Oxford, England) was added. Bound conjugate was visualised as described for the HSA-specific IgG ELISA. Between each step the plates were washed with PBS supplemented with 0.3M NaCl and 0.2% Tween®80. The antibody titer was determined as the inverse of the highest dilution that still had an $OD_{405}$ higher than the cut-off value. The cut off value was determined by calculating the average plus 3 times the standard deviation of the optical densities of the 1/10 diluted samples measured at day 0.

### 2.3.5. ELIspot assay for HSA-specific IgA, IgG and IgM antibody secreting cells (ASC)

The ELIspot test was performed as described previously by Van den Broeck and co-workers (Van den Broeck et al., 1999). Briefly coating and blocking of the plates were similar to the HSA-specific IgG ELISA. Subsequently each MC cell suspensions ($10^7$ cells/ ml leukocyte medium) was added to 10 different wells (100 µl/well), after which the plates were incubated for 3 hours at 37°C in a humidified 5% $CO_2$ atmosphere. The cells were removed by three subsequent washes with washing buffer. Thereafter, the plates were incubated with the swine-specific IgA, IgM and IgG MAb's for 1 hour at 37°C. After three washes the biotinylated rabbit anti-mouse IgG1 was added. Unbound conjugates were washed away and horseradish peroxidase-linked streptavidine was added. Following 3 washes the spots were developed in a substrate solution consisting of 3-amino-9-ethylcarbozole (AEC) [(4 volumes of AEC working solution {0.67 ml AEC stock solution (0.4%,[wt/vol] in dimethylformamide) in 10 ml Na-acetate (0.1 M, pH 5.2) + 10µl 30% $H_2O_2$ and 1 volume of 3% [wt/vol] low-melting point agarose gel (BIOzym, Landgraaf, The Netherland). Spots were counted after overnight incubation in the dark at room temperature. For each MC suspension, spots in 10 wells ($10^6$ MC/well) were counted, so that finally the amount of isotype-specific ASC per $10^7$ MC was obtained.

### 2.3.6. Statistical analysis

Differences in $\log_2$ antibody serum titers (IgM, IgA, IgG1, IgG2 and IgG) and mucosal secretions (IgA) between the groups were tested for statistical significance using General Linear Model (Proc. Mixed, SAS V8, Repeated measures Analysis of variance). A first order autoregressive variance covariance matrix was considered to take into account the correlations of the measurements on the same pig in time. The significance level was set to 5%.

## 2.4. Results

### 2.4.1. The HSA-specific IgM, IgA, IgG1, IgG2 and IgG serum responses

The HSA-specific antibody titers are presented in Fig. 2.1. The first immunisation induced a temporary increase in HSA-specific IgM 14 days PPI in all four groups. This increase was similar in all groups with geometric mean titers (GMT) of 640 ($2^{9.3}$) except for the DHEA group where the IgM response was clearly but not significantly lower ($2^{6.3}$). After the first immunisation a HSA-specific IgA response was only seen in the group supplemented with $1.25(OH)_2D_3$ IgA appeared 4 weeks PPI with antibody titers going from 160 ($2^{7.3}$) at day 28 PPI to 640 ($2^{9.3}$) at day 43 PPI. Following the re-immunisation, a secondary immune response was observed in all groups with 10- to 20-fold increases of the IgM (GMT $2^{8.7}$ to $2^{11.3}$) and IgA (GMT from $2^{10.3}$ to $2^{12}$) antibody titers 5 days PPI. These IgM and IgA serum responses were significantly higher in the $1\alpha,25(OH)_2D_3$ in comparison with the control group ($p = 0.03$). The HSA-specific IgG antibody titers increased in all groups on day 14 PPI and reached a plateau 21 days PPI with GMT going from 12,900 for the control group to 25,800 in the DHEA group. Both IgG subtypes (IgG1 and IgG2) showed slightly different responses but the response for each subtype was similar for the different groups: IgG1 immediately reached a plateau 3 weeks after the immunisation whereas IgG2 gradually increased between 2 and 4 weeks PPI. The booster immunisation induced a 3 to 5-fold increase of HSA-specific IgG, IgG1 and IgG2 in all groups without significant differences between the groups.

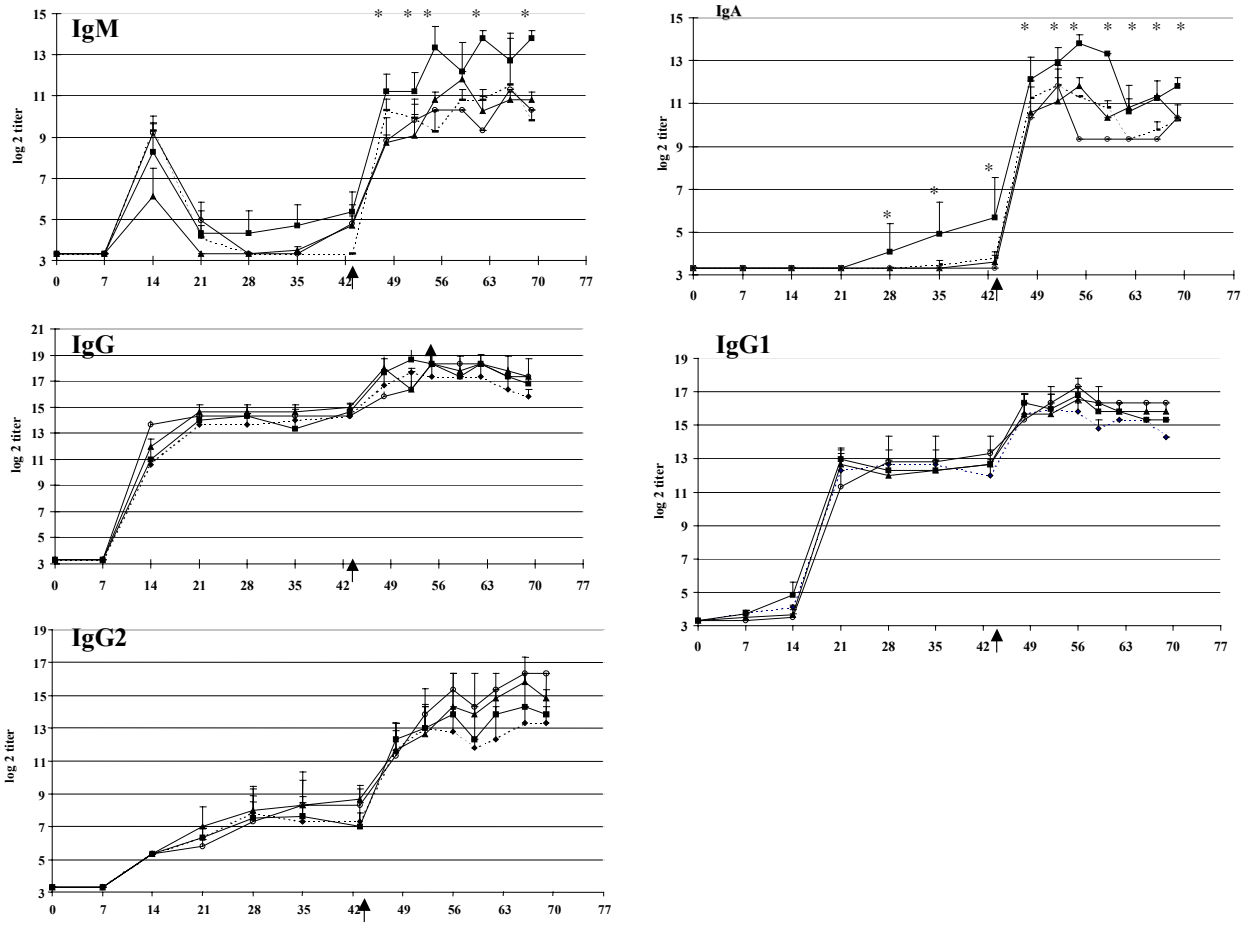


**FIGURE 2.1:** Kinetics of the HSA-specific IgM,IgA ,IgG, IgG1 and IgG2 serum response following intramuscular immunisation of pigs with HSA (◊), HSA + 1α,25(OH)2D3 (■), HSA plus DHEA (▲), HSA + combination of both steroids (O). The booster immunisation was given 43 (arrow) days post primary immunisation. The antibody titers are plotted as mean log 2 titers +/- the SEM. Significant differences ($p<0.05$) between the $1\alpha,25(OH)_2D_3$ and the control group were found for IgM and IgA responses in as indicated with ∗.

2.4.2. HSA-specific IgA response in mucosal secretions (Table 2.1.)

Following the primary immunisation HSA-specific IgA titers were detected 7 and 14 days PPI in the saliva of some animals of all groups except in the DHEA group. One animal of the $1\alpha,25\ (OH)_2D_3$ group and one animal of the combination group showed titers of 10 on day 7 PPI while in the combination group two animals had titers of 10 and 20. One week later all animals of this latter group showed titers of 40. In the DHEA group IgA was found in the saliva of 2 on 3 animals 21 days PPI and in all 3 animals at 35 days PPI (titer of 20). In the nasal secretions and rectal contents however, a low HSA-specific IgA titer of 10 was detected for almost all pigs and no obvious differences could be observed between the groups.

Following the second immunisation, IgA titers appeared already 6 days PSI in the rectal

content of all animals of the 1α,25$(OH)_2D_3$ group and of the combination group. IgA could still be detected one week later in the 1α,25$(OH)_2D_3$ group. At that moment, IgA appeared in the rectal content of one animal of the control group and the DHEA group. The highest IgA titers were found in the saliva of the 1α,25$(OH)_2D_3$ group 14 and 21 days PSI ranging from 40 to 80 which was significantly higher ($p = 0.023$) than for the control animals. At that moment only slightly lower HSA-specific IgA titers were found in the rectal contents of the DHEA group.

**TABLE 2.1.:**HSA-specific IgA antibody titers in saliva, nasal secretions and rectal content following two immunisations (day 0 and 43*) of pigs with HSA, HSA + 1α,25$(OH)_2D_3$, HSA+ DHEA or HSA + combination of both steroid hormones.

| Sample | Immunization | DPPI* / Pigs | IgA titers 7 | 14 | 21 | 28 | 35 | 42** | 49 | 56 | 63 | 70 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SALIVA | HSA | 1 | -*** | 10 | 🖬 | | | | | | | |
| | | 2 | - | 20 | - | 10 | - | - | - | - | 20 | - |
| | | 3 | - | 20 | - | - | - | 10 | - | - | - | - |
| | | 4 | 10 | - | - | - | - | - | 10 | 🖬 | | |
| | HSA+ 1,25$(OH)_2D_3$ | 1 | - | 10 | - | - | - | - | - | 40 | 40 | - |
| | | 2 | 10 | 20 | - | - | - | - | - | 80 | - | - |
| | | 3 | - | - | - | - | - | - | - | 🖬 | | |
| | HSA+ DHEA | 1 | - | - | 10 | - | 20 | 40 | 10 | 30 | 10 | - |
| | | 2 | - | - | 20 | - | 20 | - | - | 🖬 | | |
| | | 3 | - | - | - | - | 20 | - | - | 20 | - | - |
| | HSA+ Combination | 1 | - | 40 | 🖬 | | | | | | | |
| | | 2 | 20 | 40 | 20 | - | - | - | - | - | - | 10 |
| | | 3 | 10 | 40 | - | - | - | - | - | 🖬 | | |
| NASAL SECRETIONS | HSA | 1 | - | - | 🖬 | | | | | | | |
| | | 2 | - | - | - | - | - | 10 | 10 | 10 | 20 | 40 |
| | | 3 | - | - | - | - | 10 | - | - | - | - | - |
| | | 4 | - | - | - | - | - | 10 | - | 🖬 | | |
| | HSA+ 1,25$(OH)_2D_3$ | 1 | - | - | - | - | 10 | - | - | - | 10 | 10 |
| | | 2 | - | - | - | - | - | 10 | - | 20 | - | - |
| | | 3 | - | - | 10 | - | - | - | - | 🖬 | | |
| | HSA+ DHEA | 1 | - | - | - | - | - | - | - | 10 | 20 | 20 |
| | | 2 | - | - | - | - | - | - | - | 🖬 | | |
| | | 3 | 10 | - | - | - | - | - | - | 10 | - | - |
| | HSA+ Combination | 1 | - | - | 🖬 | | | | | | | |
| | | 2 | - | 10 | - | - | - | - | - | - | - | - |
| | | 3 | - | - | - | 10 | - | - | - | 🖬 | | |
| RECTAL CONTENT | HSA | 1 | - | - | 🖬 | | | | | | | |
| | | 2 | - | - | - | 10 | - | - | - | - | - | - |
| | | 3 | - | - | - | - | - | - | - | 10 | - | - |
| | | 4 | - | - | 10 | - | - | - | - | 🖬 | | |
| | HSA+ 1,25$(OH)_2D_3$ | 1 | - | - | - | - | - | - | 10 | 10 | - | - |
| | | 2 | - | - | - | - | - | - | 20 | 20 | - | - |
| | | 3 | - | - | - | - | - | - | 10 | 🖬 | | |
| | HSA+ DHEA | 1 | - | - | - | - | - | - | - | 10 | - | - |
| | | 2 | - | - | - | 10 | - | - | - | 🖬 | | |
| | | 3 | - | - | 10 | 10 | - | - | - | - | - | - |
| | HSA+ Combination | 1 | - | - | 🖬 | | | | | | | |
| | | 2 | 10 | - | - | - | - | - | 10 | - | - | 10 |
| | | 3 | - | 10 | - | - | - | - | 10 | 🖬 | | |

*DPPI: days post primary immunisation; ** boosterimmunisation 43 DPPI
-*** : HSA-specific IgA titre < 10 ; 🖬 euthanatized

### 2.4.3. HSA specific IgA, IgG and IgM ASC in lymphoid tissues

Differences in the number of HSA-specific isotype-specific ASC between groups were determined at different time points for the lymph nodes directly draining the injected muscle (GLN), as well as the secondary draining iliacal lymph node (ILN), the bronchial lymph node (BLN) and the intestinal mucosa draining nodes (MLN). The effect of the primary immunisation was determined at 16 days PPI for one animal of the combination group and one of the control group. The effect of the second immunisation was evaluated for one animal of each group at 10 days PSI and for one control animal and a $1\alpha,25(OH)_2D_3$ treated animal at 43 days PSI. Because results present only one animal of each group at each time point no statistical analysis could be performed.

#### *2.4.3.1. Primary immunisation*

HSA-specific ASC were not observed in the lymphoid tissues of the control animal euthanatised 16 days PPI whereas at that time more than 2000 IgG ASC and 182 IgM ASC were counted in the local draining lymph node and 13 IgG and 10 IgM ASC in the iliacal lymph node of the pig from the combination group (Figure 2.2.A). HSA-specific ASC were not enumerated in the BLN and MLN.

#### *2.4.3.2. Booster immunisation*

Ten days PSI the GLN of the animal of the DHEA group could not be found (Figure 2.2.B). Furthermore HSA-specific ASC were still not detected in tissues of the control animal whereas high number of IgG ASC were detected in the found primary (GLN) and/or secondary draining lymph nodes (ILN) of all three steroid treated animals: 360 IgG ASC in the GLN for the $1\alpha,25(OH)_2D_3$ treated animal and 1000 for the animal of the combination group. In the ILN of the DHEA injected animal even more than 1400 IgG ASC were enumerated. In the MLN of steroid treated animals however only a few IgG ASC were seen: 2, 5 and 13 for the $1\alpha,25(OH)_2D_3$, DHEA and combination animal respectively. Only three IgG ASC could be found in the BLN of the combination animal 10 days PSI. At that time neither IgM nor IgA ASC were found in any of the sampled lymphoid tissues.

Forty-three days PSI the local draining lymph node of the control animal could not be found (Figure 2.2.C). However low numbers of IgG and IgM ASC were observed in the other lymphoid tissues of the control animal with the highest numbers in the secondary draining lymph node (150

IgG ASC in ILN) and few positive cells in the BLN ( 27 IgG and 4 IgM ASC) and the MLN (8 IgG, 3 IgM and 2 IgA ASC). The $1\alpha,25(OH)_2D_3$ treated animal showed much higher numbers of HSA-specific IgG ASC in the different lymphoid tissues. More than 2000 IgG ASC were found in the GLN and ILN while 106 and 34 IgG ASC were counted in the BLN and MLN, respectively. Furthermore, significant numbers of IgA ASC were counted in the local draining lymph nodes (GLN (430) and ILN (17)), but only few in the BLN (2) and MLN (2). The number of IgM ASC cells in the different lymphoid tissues were low: no IgM ASC in the local draining lymph nodes of both animals, while in the $1\alpha,25(OH)_2D_3$ treated animal 5 and 6 HSA-specific IgM ASC were enumerated the MLN and BLN respectively. These numbers were similar to those seen for the control animal (4 in the BLN and 3 in the MLN). At 43 days PSI there was an important enlargement of the GLN of the $1\alpha,25(OH)_2D_3$ injected animal. The size was about 2 to 3 cm in length while the normal size of the GLN is 0.5 to 1.5 cm.

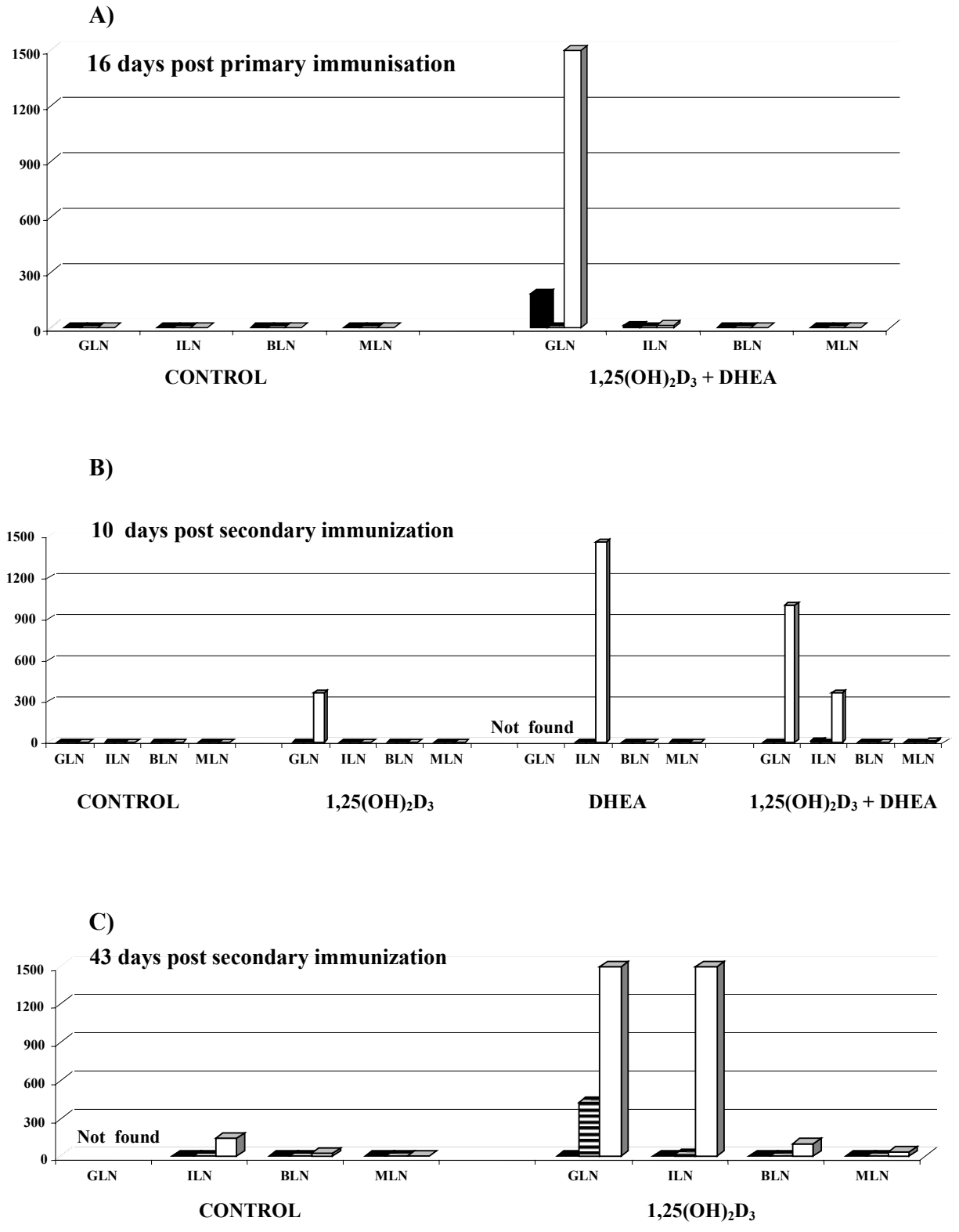


**FIGURE 2.2:** Number of HSA-specific IgM (black bars), IgG (white bars) and IgA (striped bars) antibody-secreting cells (ASC) per $10^7$ monomorphonuclear cells (MC) in the local draining lymph node (GLN), iliacal lymph node (ILN), bronchial lymph node (BLN) and mesenteric lymph node (MLN) of a pig immunized with HSA and a pig immunized with HSA + a combination of $1\alpha,25(OH)_2D_3$ and DHEA at 16 days post primary immunisation (PSI) (Figure 2.2.A), a pig of each group 10 days PSI (Figure 2.2.B) and a pig of the control and 1α,25(OH)2D3 group at 43 days PSI (Figure 2.2.C).

## 2.5. Discussion

This study demonstrated a significantly enhanced antigen-specific IgA and IgM response in pigs after IM immunisation using the steroid hormone 1α,25$(OH)_2D_3$ as immunomodulator. Indeed supplementing HSA with 2µg of 1α,25$(OH)_2D_3$ resulted in an earlier and higher HSA-specific IgA serum response after a first immunisation which consistently remained higher after the booster immunisation. The IgM serum response was most pronounced after the booster immunisation. This could be of importance for mucosal immunity since not only dimeric IgA but also IgM can be secreted at mucosae (Porter et al., 1974, Bianchi et al., 1992). Furthermore there was a tendency that 1α,25$(OH)_2D_3$-treated animals showed more HSA-specific IgA antibodies in the saliva and nasal secretions following the first immunisation and in saliva, rectal content and nasal secretions following the second immunisation. This tendency resulted in significantly higher IgA titres in saliva at 14 and 21 DPSI. These results seem to support the findings of Daynes and co-workers (Daynes et al., 1996) in mice that a subcutaneous or topical administration of 1α,25$(OH)_2D_3$ resulted in a significant increase of the antigen-specific IgA response in serum as well as in the mucosal secretions. A recent study in humans however (Kriesel et al., 1999) did not show an IgA enhancing effect after IM injection of 1µg 1α,25$(OH)_2D_3$ with antigen. This difference in effect is most likely not due to a dose effect, since the 2 µg in the present study induced a clear effect. Results in human and mice could suggest that the effect of 1α,25$(OH)_2D_3$ on IgA is species related with most effect in mice, less in pigs followed by man. Indeed, in mice the use of 1α,25$(OH)_2D_3$ resulted in high numbers of antigen-specific IgA ASC in the local draining lymph nodes but also in the mucosa-associated lymphoid tissue (MALT) whereas in pigs the effect of 1α,25$(OH)_2D_3$ was most pronounced in the local draining lymph node and was only weak in lymph nodes draining the mucosa (MLN). This is consistent with (i) the enlargement of the local draining lymph node of the 1α,25$(OH)_2D_3$ treated animal 43 days PSI, (ii) the absence of this enlargement in the control animal and (iii) with the higher numbers of HSA-specific IgA and IgG ASC in the local draining lymph nodes of steroid-treated animals. This illustrates that steroid hormones, like 1α,25$(OH)_2D_3$ and DHEA, exert their adjuvant effect locally. Whether or not this is due to an increase of MC and/or changes in membrane expression of receptors on the MC or endothelial cells of the local draining lymph node, has still to be elucidated. Conversely, studies in mice showed a decrease of 50% of MC in the local draining lymph node after application of 1α,25$(OH)_2D_3$ (Cantorna et al., 1998).

Even though the control animals lacked IgM and IgG ASC in their local draining lymph nodes they had high serum antibody levels for both isotypes. The spleen could account for these serum responses as 43 days PSI high numbers of HSA-specific IgM (420/$10^7$ MC) and IgG

($50/10^7$MC) ASC were found in this tissue. However, this can not exclude the bone marrow, which has not been looked at in this study, as another site of antibody production (Bianchi et al., 1999; Jaraskova et al., 1978).

In two animals, one control animal and one DHEA animal, the local draining lymph node (GLN) was not found. This indicates that the lymph node was either too small or absent. Absence of lymph nodes from the ischiatic lymphocenter is not an uncommon finding in pigs (Saar et al., 1975). The absence of the GLN in the pig of the DHEA group 10 days PSI could explain why the number of IgG ASC was even higher in the ILN in this animal than in the GLN of the other animals. Indeed the ILN receive lymph from the GLN and the muscles of the lumbosacral region and most likely have overtaken the role of the missing GLN.

The use of 40 µg of DHEA resulted in a decreased serum IgM response and an increased number of IgG ASC. DHEA did not seem to influence the IgA serum response as was shown in mice (Daynes et al., 1996). This seems to indicate that DHEA enhances the switch of IgM to IgG in pigs. The mechanism of this effect has not been examined in the present study. In mice, DHEA enhances the IL-2 production, suggesting that it exerts its effect in mice by inducing a Th1 response (Araneo et al., 1993). Indeed, in mice and humans, Th1 and Th2 cytokines regulate the production of IgG1 and IgG2a antibodies. The use of steroid compounds also seems to modulate the subclass of antibodies. Lemire and co-workers (Lemire et al., 1995) described in mice an enhanced IgG1 and a decreased IgG2a response using $1\alpha,25(OH)_2D_3$. This was also seen in a recent study with midlactating cows using 200 µg $1\alpha,25(OH)_2D_3$ (Reinhardt et al., 1999). In the present study on pigs, $1\alpha,25(OH)_2D_3$ seems to induce a switch towards the IgA subclass with only little effect on the IgG subclasses. There was a tendency towards a higher IgG2 serum response in the DHEA treated animals although the differences again were very small. If cytokines are responsible for the effects in pigs is not investigated in the present study. However, it should be emphasised that the presence of Th1 and Th2 cells and their relationship with the secretion of different isotype-specific immunoglobulins has not been proven in pigs.

Combining DHEA with $1\alpha,25(OH)_2D_3$ abolished the effect of $1\alpha,25(OH)_2D_3$ on the IgA response. This is not in accordance with the observations in mice where DHEA had an additive effect. The reason for this difference is not clear. A possible explanation is an age-related effect of DHEA. Indeed the pigs in the present study were still very young (7 weeks at the moment of immunisation). Endogeneous DHEA synthesis in humans and mamalian species decline in an age-related way so that most immunomodulatory effects of DHEA can be expected in older animals (Parker et al., 1999). Other explanations for the difference in effect in pigs can be the induction of a different cytokine profile, the different dossage and species differences.

In conclusion this study examined the effect in pigs of $1\alpha,25(OH)_2D_3$ and DHEA on the humoral immune response following IM immunisation. $1\alpha,25(OH)_2D_3$ is capable to significantly enhance the IgA and IgM antibody serum response . Also higher numbers of IgA and IgG ASC were found in the local draining lymph nodes of the $1\alpha,25(OH)_2D_3$ treated animals. DHEA had the tendency to decrease the antigen-specific IgM and enhanced the antigen-specific IgG2 and total IgG responses in serum and local draining lymph node, respectively.

# CHAPTER 3

# 1α,25-DIHYDROXYVITAMIN D$_3$ INCREASES IgA SERUM ANTIBODY RESPONSES AND IgA ANTIBODY SECRETING CELL NUMBERS IN THE PEYER'S PATCHES OF PIGS AFTER INTRAMUSCULAR IMMUNISATION

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## 3.1. Summary

Pigs were intramuscularly (IM) injected twice with human serum albumin (HSA) with or without 1α,25-dihydroxyvitamin $D_3$ (1α,25$(OH)_2D_3$) with a 5 weeks interval. The supplementation of 1α,25$(OH)_2D_3$ enhanced the HSA-specific IgA serum antibody response but decreased the IgM, IgG, IgG1 and IgG2 response. Furthermore, higher numbers of HSA-specific IgA antibody secreting cells were obtained in systemic lymphoid tissues (local draining lymph node, spleen and bone marrow) as well as in Peyer's patches and lamina propria of the gut (GALT). In addition, the *in vivo* mRNA expression for Th1 (IFN-γ, IL-2), Th2 (IL-4, IL-6 and IL-10) and Th3 (TGF-β) cytokines as well as the percentage of different cell subsets ($CD2^+$, $CD4^+$, $CD8^+$, $IgM^+$, MHC $II^+$, $CD25^+$) of monomorphonuclear cells from the local draining lymph node were determined at different time points after the IM immunisations. Cytokine profiles didn't resemble a typical Th-cytokine profile using 1α,25$(OH)_2D_3$ : higher levels of IL-10 and significantly lower levels of IL-2 were observed the first day after the primary immunisation but significantly higher levels of IL-2 and significantly lower levels of IFN-γ were observed the first day after the second immunisation. Furthermore, after the second immunisation TGF- β mRNA expression decreased more rapid in the 1α,25$(OH)_2D_3$ group. This difference became significant 7 days after the second immunisation. One week later a significantly higher percentage of $CD25^+$-cells was observed in this group, indicating more activated T- and B cells using the steroid hormone. These results suggest that in pigs the addition of 1α,25$(OH)_2D_3$ to an intramuscularly injected antigen can enhance the antigen-specific IgA-response and prime GALT tissues, but relation with cytokines and cell phenotype in the local draining lymph node needs further clarification.

## 3.2.Introduction

1α,25-dihydroxyvitamin D3 [(1α,25(OH)$_2$D$_3$], the active metabolite of vitamin D$_3$, is a liphophilic steroid hormone which exerts its actions through binding to a nuclear receptor, the vitamin D receptor (Bouillon et al., 1995). Traditionally 1α,25(OH)$_2$D$_3$ has been associated with calcium homeostasis but the discovery of the nVDR in most cells of the immune system such as monocytes, macrophages, dendritic cells (Adorini et al., 2001), T- and B-lymphocytes (Lemire et al., 1984; Veldman et al., 2000; Morgan et al., 1994, 1996, 1999) suggested a role of the hormone in the immune system. Indeed, 1α,25(OH)$_2$D$_3$ stimulates the differentiation and maturation of monocytes (Abe et al., 1981) while it inhibits the differentiation and maturation of dendritic cells (Penna et al., 2000; Griffin et al., 2001). The steroid hormone also diminishes T-cell proliferation (Rigby et al., 1984; Bhalla et al., 1984) as well as production of cytokines required for Th1 differentiation such as interleukin (IL)-12 (D'Ambrasio et al., 1998; Adorini et al., 2001), IFN-γ (Cipitelli et al., 1998), IL-2 (Alroy et al., 1995; Takeuchi et al., 1998) as well as GM-CSF (Towers et al., 1998). On the other hand, 1α,25(OH)$_2$D$_3$ is able to enhance the production of Th2-cytokines such as IL-4 and IL-10 (Daynes et al., 1994, 1996; Cantorna et al., 1998; Adorini et al., 2001) as well as of the Th3-cytokine TGF-β (Fukaura et al., 1996; Weinreich et al., 1999) . The latter cytokine is involved in the induction of a mucosal immune response and/or oral tolerance. Moreover, in man and mice TGF-β is responsible for the switch in B cells towards the IgA isotype (Letterio et al., 1998). Therefore, the steroid hormone is classified as a Th2 and/or Th3-modulating-adjuvant. In addition, 1α,25(OH)$_2$D$_3$ is able to mimic in the local draining lymph node the cytokine pattern which is normally produced by the intestinal Peyer's patches after oral immunisation with replicating antigens. This cytokine pattern is essential for the development of a mucosal protective immune response after oral immunisation (McGhee et al., 1992). In mice, subcutaneous injection of antigen together with 1α,25(OH)$_2$D$_3$ evoked a Th2 pattern in the local draining lymph node, an increased homing of IgA and IgG ASC to the LP of the intestine and the lungs as well as an enhanced IgA response in serum and mucosal secretions (Daynes et al., 1994, 1996). Later it was shown by the same group, that the induction of the mucosal immune response is also due to the migration of antigen-pulsed dendritic cells from the local draining lymph nodes towards the Peyer's patches, where the activation and differentiation of antigen-specific B cells is initiated (Enioutina et al., 1999, 2000).

In pigs it was shown that supplementation of antigen with 1α,25(OH)$_2$D$_3$ increased the antigen-specific IgA serum antibody response as well as the number of IgA and IgG ASC in the local draining lymph node of an IM induced immune response (Van der Stede et al., 2001). This suggested that a similar immunomodulating mechanism for 1α,25(OH)$_2$D$_3$ as described for mice

could also occur in the pig. However, in pigs the effect of 1α,25$(OH)_2D_3$ on the cytokine-profile as well as on homing of antigen-specific antibody secreting cells (ASC) towards mucosal lymphoid tissues was not yet demonstrated. The aim of the present study was to obtain insights into the immunomodulating mechanism of 1α,25$(OH)_2D_3$ in pigs. Therefore, phenotype of and Th1-/Th2-/Th3-like cytokine mRNA expression by MC from the local draining lymph node were examined after IM injection with human serum albumin (HSA) supplemented with 1α,25$(OH)_2D_3$. In addition, the antigen-specific serum antibodies as well as the number of antibody secreting cells (ASC) in systemic and mucosal lymphoid tissues (Peyer's patches and lamina propria) were analysed to determine the effect of 1α,25$(OH)_2D_3$ on homing towards the mucosae.

## 3.3. Material and Methods

### 3.3.1. Pigs

In total 37 conventional pigs (Belgian Landrace x Piétrain) from 6 different litters were used in the experiments. These pigs were weaned at the age of 4 weeks, transported to the faculty and housed in isolation units where they obtained water and food *ad libitum*. All pigs were seronegative for antibodies against human serum albumin (HSA) as determined by ELISA at the start of the experiment.

### 3.3.2. Experimental design

a) Effect of a single injection on immune parameters in the local draining lymph node (the popliteal lymph node): at the age of 7 weeks, pigs were intramuscularly (IM) immunized with 1 mg HSA (Sigma-Aldrich, Bornem, Belgium). Fifteen animals received one IM injection in the right musculus gastrocnemius with 1mg of HSA and 2 µg of 1α,25$(OH)_2D_3$ (Sigma-Aldrich, Bornem, Belgium) ($D_3$-group) and one in the left m. gastrocnemius with HSA only (control group). So each of these animals received one injection at each site to compare different immune parameters at the left (without 1α,25$(OH)_2D_3$) and at the right site (with 1α,25$(OH)_2D_3$) on the same pig.

b) After the first IM immunization cells of the local draining lymph node will also home to other lymphoid tissues. Therefore the effect of a booster immunization on immune parameters was analysed in another 24 animals. These animals received two identical IM injections with a 5 weeks interval: 12 animals received the antigen together with 1α,25$(OH)_2D_3$ (left and right musculus gastrocnemius, $D_3$-group) while the other 12 animals

received HSA only (control group). In these animals not only effects in the local draining lymph node but also on the serum antibody response and the HSA-specific ASC numbers in systemic and mucosal lymphoid tissues were analysed.

The HSA-antigen was dissolved in 0.5 ml PBS and suspended in an equal volume of incomplete Freund's adjuvant (IFA, DIFCO Laboratories). IFA was used as an additional adjuvant allowing a gradual and slow release of the antigen and 1α,25(OH)$_2$D$_3$. The 1α,25(OH)$_2$D$_3$ was dissolved in absolute ethanol at a concentration of 8 µg/ml and stored at –20°C. Only at the moment of immunisation, the HSA antigen in IFA and the dissolved 1α,25(OH)$_2$D$_3$ were mixed. The control group received the same amount of HSA-antigen and an equal volume of ethanol (250 µl) as the D$_3$-group.

The immune response after the IM immunisations was evaluated by (i) determining weekly the HSA-specific antibody titers (IgM, IgA, IgG1, IgG2 and IgG) in serum from the moment of the first immunisation until 14 days post secondary immunisation (dpsi), (ii) quantifying and localising the number of HSA-specific IgM, IgA, IgG1, IgG2 and IgG antibody secreting cells (ASC) in different lymphoid tissues via ELIspot assays: 10 and 30 days post primary immunisation (dppi) and at 1, 2, 7, and 15 dpsi, (iii) defining the phenotype of MC of the local draining lymph node using flow cytometric analyses 1,2, 10 and 30 dppi and at 1, 2, 7 and 15 dpsi and (iv) determining the cytokine mRNA expression for interleukin (IL)-2, IL-4, IL-6, IL-10, interferon(IFN)-γ and transforming growth factor (TGF)-β in the local draining lymph node via real time RT-PCR at 1,2 and 10 dppi and at 1,2 and 7 dpsi. Euthanasia was performed by intravenous injection of an overdose pentobarbital (24 mg/kg; Nembutal, Sanofi Sant Animale, Brussels, Belgium) followed by exsanguination. The number of animals used for each parameter in both groups is shown in table 3.2 and in the legends of figures 3.1. to 3.4.

### 3.3.3. Samples

#### 3.3.3.1. Serum

Blood samples were taken from the jugular vein at the time points shown in Fig. 3.1. Serum was collected and subsequently inactivated at 56°C during 30 minutes. Thereafter, the serum was treated with kaolin (Sigma-Aldrich) to decrease the background reading in ELISA as also described by Van den Broeck et al. (1999a). Finally the serum was diluted 1/10 (vol/vol) in ELISA buffer (Phosphate buffered saline (PBS, 150mM, pH 7.4) + 0.05% (vol/vol) Tween® 20). The diluted samples were stored at –20°C until tested in ELISA.

*3.3.3.2. Monomorphonuclear cells (MC)*

3.3.3.2.1. Spleen and lymph nodes

The spleen and popliteal lymph node (LN) cells were aseptically removed by gently teasing the tissues apart followed by lysis of erythrocytes with ammonium chloride (0.8% (wt/vol)). After centrifugation (380 x g at 4°C for 10 min.), the cells were washed and resuspended at 1 x $10^7$ MC/ml in leukocyte medium (RPMI-1640 supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml), kanamycin (100 µg/ml), glutamine (200 mM), sodiumpyruvate (100 mM), non-essential amino acids (100 mM) and 10% (vol/vol) fetal bovine serum (FBS, Gibco BRL, Life Technologies, Merelbeke, Belgium)).

3.3.3.2.2.Bone marrow MC

The sternum was compressed with a forceps and cells from the bone marrow (BM) were collected in centrifugation tubes. Following centrifugation, the cells were washed 3 times in PBS (150 mM, pH 7.4). The erythrocytes were lysed as described above and the cells were resuspended in leukocyte medium at 1 x $10^7$ MC/ml.

3.3.3.2.3. Lamina propria and Peyer's patches MC

Lamina propria (LP) and Peyer's patches (PP) were only sampled 7 and 15 dpsi. The MC of the LP of the jejunum were isolated as described by Van der Heijden and others (1987) with slight modifications. Fifteen to 20 cm long segments of the mid jejunum were flushed with PBS (150mM, pH 7.4) to remove the intestinal content. Subsequently, these segments were opened longitudinally and cut into pieces of 4 $cm^2$. These pieces were rinsed twice with PBS and twice with $Ca^{2+}$ and $Mg^{2+}$-free balanced salt solution (CMF-buffer, pH 7.2). This was followed by incubation for 15 min at 37°C in the CMF-buffer containing 0.37 mg/ml EDTA (Sigma) and 0.37 mg/ml dithiothreitol (DTT, Sigma) to remove the epithelial cells and intraepithelial lymphocytes. The remaining tissue fragments were rinsed with RPMI 1640 containing 5% FBS and 20 mM HEPES (Gibco) and thereafter incubated with collagenase and DNAse (RPMI -1640 + 0.1 mg/ml DNAse (Roche Diagnostics) + 300 U/ml collagenase (Sigma) + 100 IU/ml penicillin + 100 µg/ml streptomycin) during 30 min at 37 °C and rotating at 250 rounds per minute. A first fraction of cells was collected following filtration through stainless steel sieves (80, 150 and 200 mesh screens, Sigma). A second fraction of MC was collected by mechanical scraping and squeezing the remaining tissue pieces on the sieves. Subsequently, the obtained cell suspension was filtered through a gauze filter. Both cell fractions were combined and washed in RPMI-1640 containing 5% (vol/vol) FBS, 20 mM HEPES and 0.1 mg/ml DNAse. The MC were isolated by Percoll

(Amersham Pharmacia, Uppsala, Sweden) gradient centrifugation. Subsequently, the MC were washed and resuspended in leukocyte medium.

For the isolation of MC from jejunal PP (JPP) and ileal PP (IPP), small intestinal pieces were washed and incubated in CMF-EDTA medium as described for the lamina propria MC isolation. Subsequently, MC were collected by scraping the PP's with glass slides followed by washing, filtration through a gauze filter and resuspending of the MC in leukocyte medium.

### 3.3.4. ELISA for HSA-specific serum antibody responses

HSA-specific serum IgM, IgA, IgG1, IgG2 and IgG titers were determined in an indirect ELISA as previously described (Van der Stede et al., 2001). The antibody titer was determined as the inverse of the highest dilution that still had an $OD_{405}$ higher than the cut-off value. The cut off value was determined by calculating the average plus 3 times the standard deviation of the optical densities of the 1/10 diluted samples measured at day 0. The cut off values were 0.10, 0.14, 0.11, 0.15 and 0.35 for IgM, IgA, IgG1, IgG2 and IgG respectively.

### 3.3.5. ELIspot assay for HSA-specific antibody secreting cells

The ELIspot test was performed as described previously (Van der Stede et al., 2001). For each MC suspension, spots in 10 wells (1 x $10^6$ MC/well) were counted, so that finally the amount of isotype-specific ASC per 1 x $10^7$ MC was obtained.

### 3.3.6. Flow cytometric analysis

MC cell suspensions of the LN were analysed for expression of cell surface antigens by flow cytometry. MC ($1.10^6$/ml) were suspended in ice-cold 100 µl RPMI-1640 supplemented with 2% immunoglobulin free horse serum and 0.02 % sodium azide (staining medium). Subsequently, 100 µl of staining medium containing a swine leukocyte surface-specific MAb was added for 45 min on ice: MSA-4 = anti-CD2 (IgG2a), 74-12-4 = anti-CD-4 (IgG2b, Pescovitz et al., 1984), 76-2-11 = anti-CD8 (IgG2b, Zuckerman et al., 1998B), anti-IgM = anti IgM$^+$B cells (IgG1, Van Zaane et al., 1987), MSA3 = anti MHC II (SLA-DR, IgG2a, Hammersberg et al., 1986), 231.3B2 = anti-CD25 (IL-2R, IgG1, Bailey et al., 1992). Subsequently, the cells were washed twice with staining medium and resuspended in staining medium containing FITC-conjugated (Sheep anti-mouse IgG (whole molecule) Fab-fragment (Sigma-Aldrich). Cells were incubated for 45 min on ice, washed twice with staining medium and once with PBS. Flow cytometric analysis was

conducted using a FACScalibur flow cytometer (Becton-Dickinson, San Jose, California) equipped with a 15mW air-cooled argon ion laser. At least 10,000 cells were analysed per sample. Data are presented as mean percent positive cells ± SEM and as mean fluorescence intensity (MFI) per cell.

3.3.7. RNA extraction and RT-PCR.

RNA was extracted from MC of freshly isolated LN of each pig using the acid guanidinium-isothiocyanate phenol-chloroform-based method (RNAgents®, Promega, Leiden, The Netherlands) as described by Verfaillie et al. (2001). Reverse transcription was performed in a 20 µl reaction mixture containing (i) 3 µg of total RNA (ii) 1 µl of random hexanucleotide primer (50 µM, Perkin Elmer, Oosterhout, The Netherlands) (iii) 4 µl of $MgCl_2$ (25 mM) (iv) 2 µl 10 x PCR II buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3) (v) 1 mM deoxynucleotide triphosphate mix (dNTP-mix) of the four dNTPs (Roche, Mannheim, Germany) (vi) 200 units of Superscript II Rnase $H^-$ reverse transcriptase (GIBCO, BRL, Life Technologies, Merelbeke, Belgium) and (vii) 20 U of RNAsin® Ribonuclease inhibitor (Promega) in diethylpyrocarbonate-treated (DEPC) water. Briefly, the template RNA and the random hexanucleotide primer were preincubated during 10 min at 70°C. After chilling on ice, all other components were added. Subsequently, the reaction mix was incubated at room temperature for 10 min and then at 42 °C for 45 min followed by heating for 5 min at 95 °C to inactivate the reverse transcriptase.

The oligonucleotide primers used for the detection of the porcine interleukins (IL)-2, IL-4, IL-6, IL-10, IFN-γ, TGF-β and of cyclophilin cDNA were designed from the published nucleic acid sequences available from the GenBank/EMBL databases (Table 3.1.).

**TABLE 3.1.:** Sequences of primers with the fragment length of PCR-products for different porcine cytokines.

| **Target mRNA** | **Oligonucleotide sequences 5'-3'** | **Length PCR-fragment** | **Reference** |
|---|---|---|---|
| Cyclophylin<br>S[b]<br>AS[c] | F14571[a]<br>TAA CCC CAC CGT CTT CTT<br>TGC CAT CCA ACC ACT CAG | 368 | (Dozois et al., 1997) |
| Interleukin 2<br>S<br>AS | X56750<br>GAT TTA CAG TTG CTT TTG AAG<br>GTT GAG TAG ATG CTT TGA CA | 338 | (Goodall et al., 1991) |
| Interleukin 4<br>S<br>AS | F68330<br>TAC CAG CAA CTT CGT CCA<br>ATC GTC TTT AGC CTT TCC AA | 311 | (Bailey et al., 1993) |
| Interleukin 6<br>S<br>AS | M86722<br>ATG AGA ATC ACC ACC GGT CTT G<br>TGC CCC AGC TAC ATT ATC CGA | 310 | (Richards et al., 1991) |
| Interleukin 10<br>S<br>AS | L20001<br>CCA TGC CCA GCT CAG CAC TG<br>CCC ATC ACT CTC TGC CTT CGG | 295 | (Blancho et al., 1995) |

| Target mRNA | Oligonucleotide sequences 5'-3' | Length PCR-fragment | Reference |
|---|---|---|---|
| Interferon-γ<br>S<br>AS | X53085<br>A TGT ACC TAA TGG TGG ACC TC<br>C TCT CTG GCC TTG GAA CAT AG | <br><br>360 | (Dijkmans et al., 1990) |
| TGF-β<br>S<br>AS | Y00111<br>GAC CCG CAG AGA GGC TAT AG<br>GAG CCA GGA CCT TGC TGT AC | <br><br>399 | (Derynck et al., 1987) |
| [a]: genebank accesion number, [b]: sense primer, [c]:antisense primer | | | |

Cyclophilin was used as constitutively expressed 'housekeeping control gene' to determine the uniformity of the reverse transcription reactions and as a reference for quantification of cytokine mRNA (Dozois et al., 1997). Cytokine-cDNA was amplified and quantified by real time PCR using the Light Cycler® (Roche, Mannheim, Germany) and the lightcycler-faststart DNA Master SYBR Green I kit (Roche, Mannheim, Germany, nr 2239264). The reaction mixture consists of a master mix containing Taq DNA polymerase, dNTP mixture and SYBR Green I, 3-5 mM $MgCl_2$, 20 pmol of sense and anti-sense primer and 2 µl of template cDNA in a total volume of 20 µl. Subsequent steps were initial denaturation for 10 min at 95 °C, followed by 40 cycles of denaturation for 15 sec at 94 °C, annealing for 5 to 14 sec at 50-60°C depending on the particular cytokine and an extension for 7 to 15 sec at 72 °C depending on the length of the product (~1sec/25 bp). These products were subjected to a melting curve analysis and subsequently agarose gel electrophoresis for confirmation of the specificity of the PCR-products. Quantification occurred using external standards of cytokine cDNA. Calculation was performed with the LightCycler® analysis software. The relative amount of cytokine-expression was plotted as a ratio ((=copy number of target cytokine/copy number of house keeping gene) x $10^5$).

### 3.3.8. Statistical Analysis

Statistical analysis was done using SPSS 7.5 for windows. Differences in $\log_2$ serum antibodies (IgM, IgA, IgG1, IgG2 and IgG) between the 1α,25(OH)$_2$D$_3$ (D$_3$-group) and the control group were tested for statistical significance using the General Linear Model (Repeated Measures Analysis of Variance). For ELIspot assays differences in the number of HSA-specific ASC between the D$_3$ group and the control group as well as differences in the expression of surface antigens (flow cytometer) were tested for statistical significance using a two-sample t test. For RT-PCR experiments, the concentrations of the cytokines were corrected for variations between different samples by using cyclophylin as a housekeeping gene and are presented as a ratio (= copy numbers of target cytokine gene/1 x $10^5$ copy numbers of house keeping gene). Within each

group the ratios of the individual pigs were grouped and the differences in the mean ratios between the $D_3$-group and the control group were tested for statistical significance with a two-sample t test. . Statistical significance was assessed at a P value of $< 0.05$.

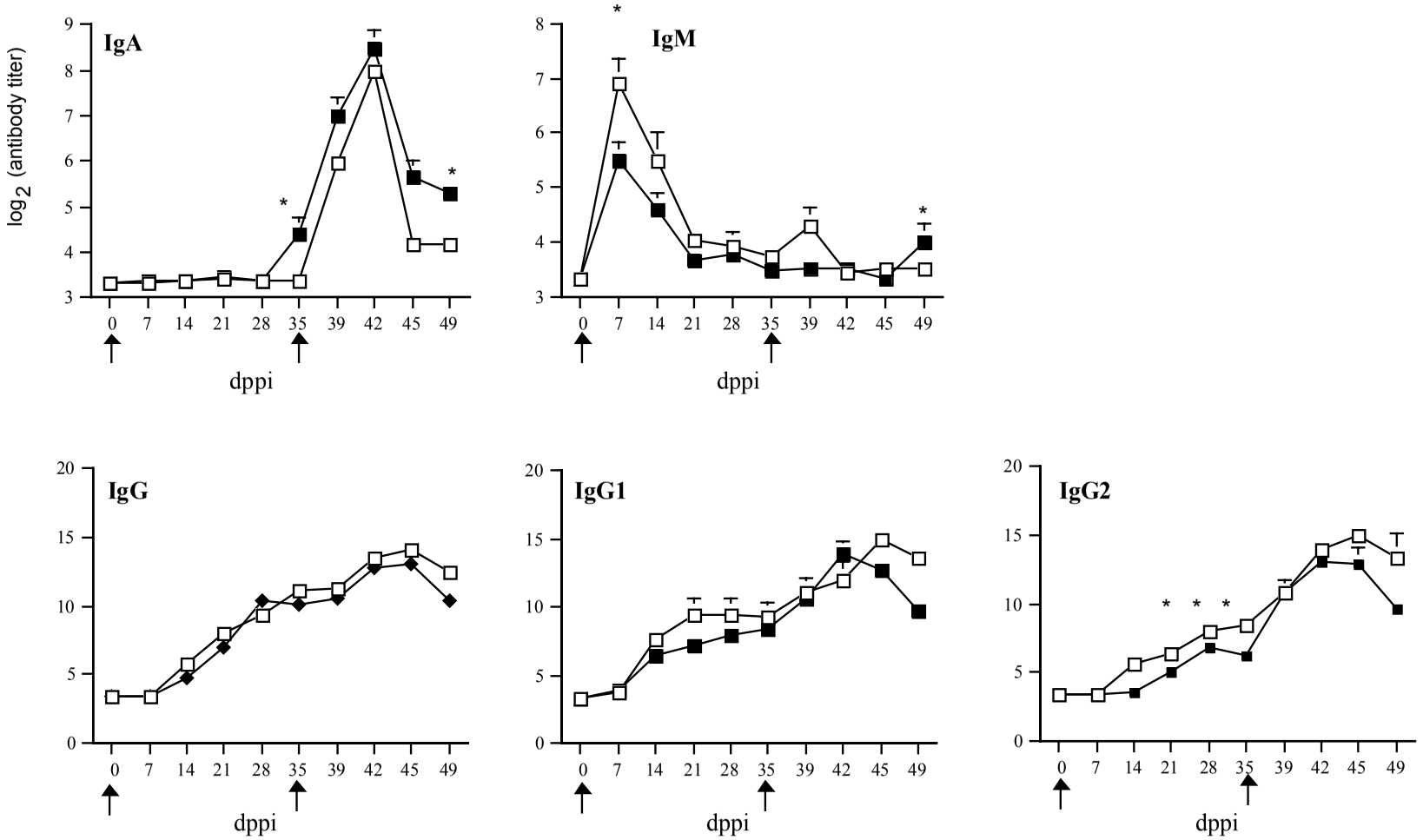
## 3.4. Results

### 3.4.1. The HSA-specific IgM, IgA, IgG1, IgG2 and IgG serum responses

The HSA-specific IgM titer peaked 7 days post primary immunisation (dppi) in both groups. At that time the titer was significantly higher ($P<0.05$) in the control group (Fig.3.1.). The IgM titers declined to base line values at 35 dppi when the second IM immunisation was given. This second immunisation induced temporarily a slight increase of the IgM titer in the control group 4 dpsi but not in the $D_3$-group. In the $D_3$-group, IgM became significantly higher 14 days post second immunisation (dpsi).

Serum HSA-specific IgA titers were continuously higher in pigs of the $D_3$-group than in pigs of the control group between 35 and 49 dppi. This difference was significant ($P<0.05$) at moment of second immunisation (35 dppi) and 14 days later. In contrast, the HSA-specific IgG1, IgG2 and IgG titers in the $D_3$-group were reduced in comparison with the control group and this reduction was significant for IgG2 ($P<0.05$) between 21 and 35 dppi.

**FIGURE 3.1 (next page): :** Kinetics of the HSA-specific IgM, IgA, IgG1, IgG2 and IgG serum response following 2 intramuscular (IM) immunisations. Pigs (the number of pigs sampled/group is 12 between 0 and 35 days primary immunisation (dppi), 6 on 37 and 42 dppi and 3 on 45 and 49 dppi) had been injected in the m. gastrocnemii with 1mg HSA in IFA supplemented with either 2 µg of $1\alpha,25(OH)_2D_3$ ( ) or without steroid hormone supplementation ( ). The antibody titers are plotted as mean log 2 titers ± the SEM. Significant differences ($p<0.05$) between both groups are indicated with an asterisk.


IgA
IgM
IgG
IgG1
IgG2
log$_2$ (antibody titer)
dppi
0 7 14 21 28 35 39 42 45 49

### 3.4.2. HSA specific IgM, IgA, IgG1, IgG2, IgG and IgM ASC in lymphoid tissues

At different time points post primary (10 and 30 dppi) and secondary immunisation (1, 2, 7 and 15 dpsi) pigs were euthanized for quantifying and localising of the HSA-specific IgM, IgA, IgG1, IgG2 and IgG ASC in the LN, the spleen and the BM (systemic lymphoid tissues). The IPP, JPP and LP (gut-associated lymphoid tissues (GALT)) were only sampled at 7 and 15 dpsi.

Ten dppi, only HSA-specific IgM ASC could be detected in the LN. The number of IgM ASC was slightly higher in the $D_3$-group (mean of 23 IgM ASC per 1 x $10^7$ MC) than in the control animals (10 IgM ASC per 1 x $10^7$ MC) (data not shown). The highest numbers of HSA-specific IgM were found in the spleen and this one day after the second immunisation. However there were no significant differences in IgM ASC between the groups (Fig. 3.2.a).

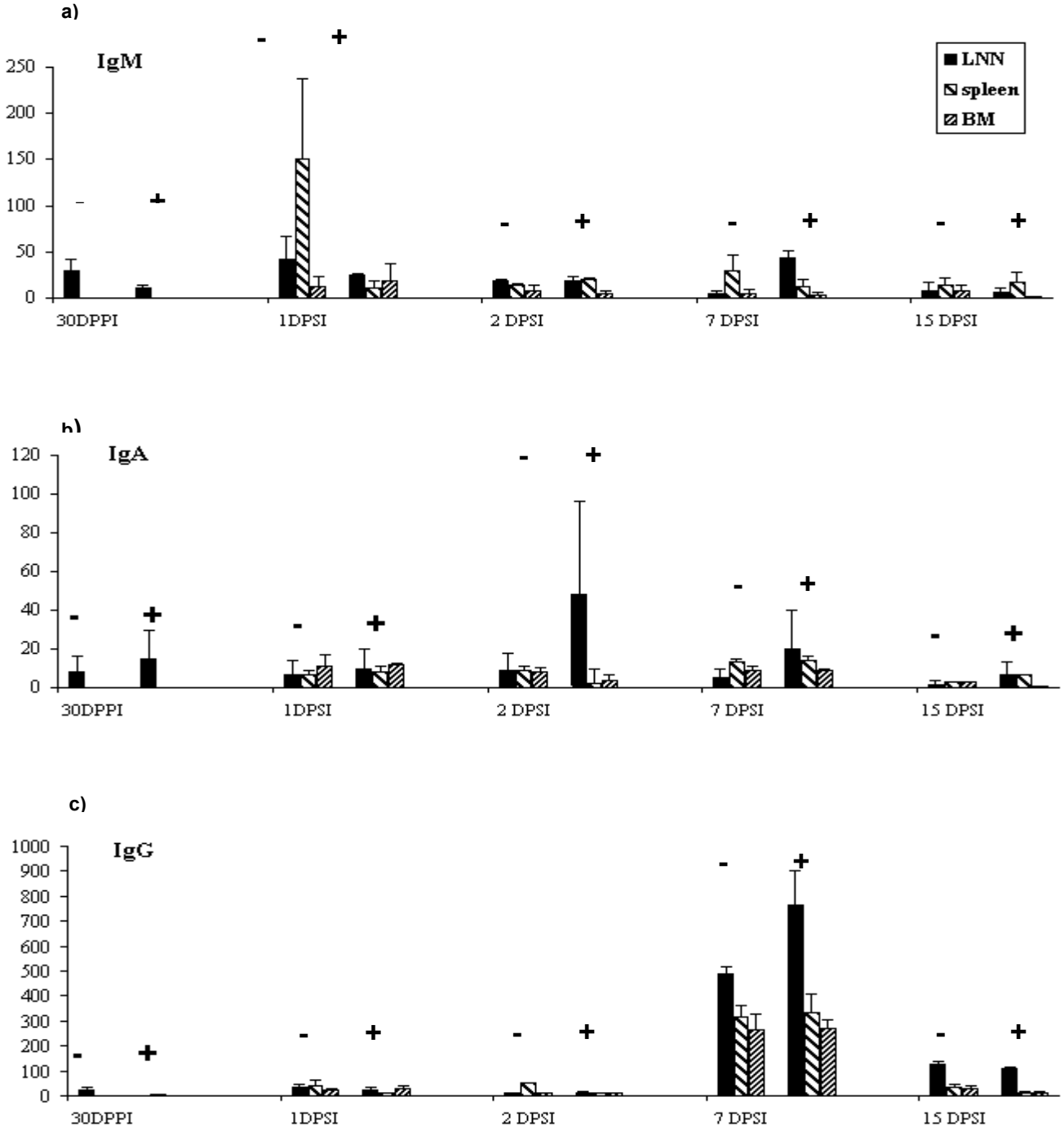



**FIGURE 3.2:** Mean number of HSA-specific IgM (Fig. 3.2a), IgA (Fig. 3.2b) and IgG (Fig. 3.2c) ASC per 1 x $10^7$ MC in different systemic lymphoid tissues [local draining lymph node (LNN), spleen and bone marrow (BM)] at different time points post primary (PPI) and second immunisation (PSI). Animals were IM injected with either 1 mg HSA in IFA supplemented with either 2 µg of 1α,25(OH)$_2$D$_3$ (+) or without steroid hormone (-). The numbers of HSA-specific ASC are the mean of 3 pigs/group ± SEM.

The numbers of HSA-specific IgA ASC were always higher for the $D_3$-group in the LN while this was not the case in the spleen and BM indicating the local effect of the steroid hormone on IgA (Fig. 3.2.b). The numbers of IgA ASC peaked after the second immunisation with more than 50 IgA ASC in the D3-group 2 dpsi. However, this was not significantly higher than in the control group (less than 20 IgA ASC 2 dpsi).

HSA-specific IgG ASC were higher in the 3 systemic lymphoid tissues than in the GALT (Fig. 3.3) and peaked 7 dpsi with no significant differences between the groups (Fig. 3.2.c). Similar results were obtained for the IgG1 and IgG2 ASC (data not shown).

GALT was only collected 7 and 15 dpsi. Seven dpsi higher numbers of HSA-specific IgM, IgA and IgG ASC were found in the IPP of the $D_3$-group (P = 0.268 for IgM, P = 0.242 for IgA and P = 0.238 for IgG) (Fig. 3.3A). Interestingly, in both groups the number of IgG ASC was higher than the number of IgA ASC. Similar tendencies were seen in the JPP (data not shown). At that moment, low numbers of IgM, IgA and IgG ASC were found in the LP of both groups (Fig. 3.3B). One week later, HSA-specific ASC were low in both PP and in the LP in both groups.

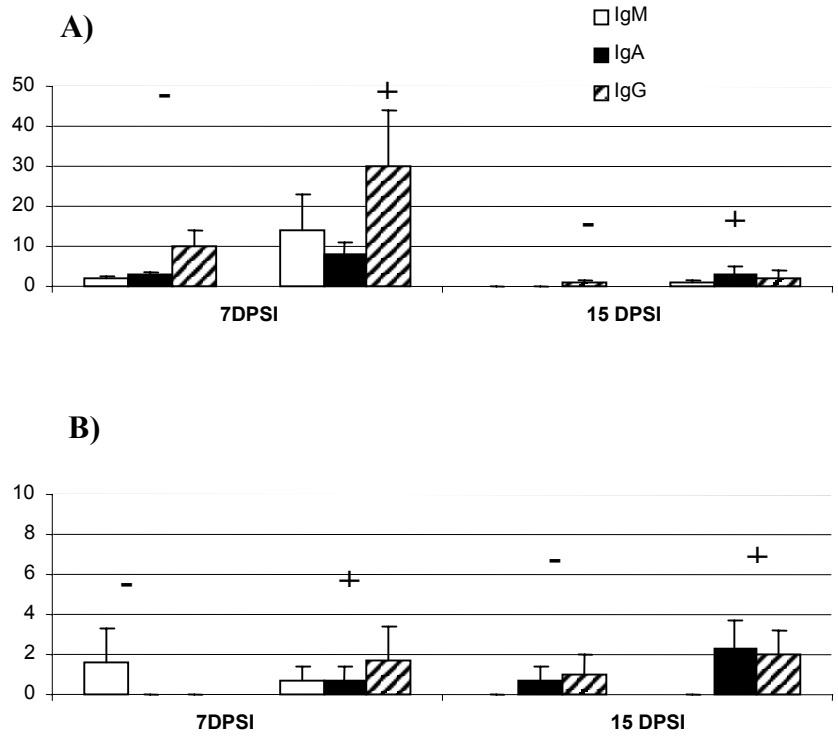



**FIGURE 3.3:** Mean number of HSA-specific IgM (open bars), IgA (filled bars) and IgG (shaded bars) ASC per 1 x $10^7$ MC in ileal Peyer's patches (Fig. 3.3A) and lamina propria (Fig. 3.3B) 7 days psi and 15 days psi. Animals were IM injected with 1 mg HSA in IFA supplemented with either 2 µg of $1\alpha,25(OH)_2D_3$ (+) or without steroid hormone (-).The numbers of HSA-specific ASC are the mean of 3 pigs/group ± SEM.

### 3.4.3. Phenotype of MC from local draining lymph node

No significant differences were detected between both groups either in the percentages of CD2$^+$, CD4$^+$, CD8$^+$, IgM$^+$and MHC II$^+$ -cells at any moment tested (Table 3.2.). However, 7 and 14 dpsi the mean percentage CD8$^+$ cells was clearly lower in the $D_3$-group. Furthermore, 15 dpsi the percentage CD25$^+$ cells was significantly higher in the $D_3$-group and the degree of MHC II-expression was significantly lower (P= 0.019) in this $D_3$-group (MFI 651 ± 38) in comparison with the control group (MFI 917 ± 59). The MHC II expresion after the second immunisation in both groups (MFI of 653 ± 60) was also significantly higher than after the first immunisation (MFI 175 ± 30).

**TABLE 3.2.:** Percentages of positive cells (± SEM) in the local draining lymph node at different times post primary and secondary immunisation

| | | Mean percentage of leukocyte subpopulation | | | | | |
|---|---|---|---|---|---|---|---|
| | 1,25(OH)$_2$D$_3$ | CD2 | CD4 | CD8 | IgM | MHC II | CD25 |
| 1 DPPI[b] | - | 49 ± 12,7 | 34 ± 5,8 | 32 ± 4,5 | 33 ± 8,0 | ND[d] | ND |
| (n=2) | + | 49 ± 8,14 | 33 ± 2,7 | 35 ± 3,4 | 42 ± 4,5 | ND | ND |
| 2 DPPI | - | 69 ± 2,4 | 45 ± 4,1 | 30 ± 5,1 | 26 ± 5,1 | 35 ±14,6 | 43 ± 3,0 |
| (n=5) | + | 68 ± 3,7 | 42 ± 2,2 | 28 ± 5,6 | 30 ± 5,0 | 31 ± 11,4 | 41 ± 1,9 |
| 10 DPPI | - | 61 ± 7,8 | 33 ± 4,4 | 27 ± 1,6 | 38 ± 7,1 | 52 ± 9,9 | 49± 4,8 |
| (n=5) | + | 65 ±4,5 | 37 ± 2,8 | 27 ± 1,7 | 34 ± 5,8 | 48 ± 9,6 | 42 ± 3,6 |
| 30 DPPI | - | 77 ± 1,6 | 35 ± 1,42 | 30 ± 2,5 | 25 ± 3 | 62 ±3,2 | 47 ± 5,2 |
| (n=3) | + | 79 ± 1,9 | 40 ± 3,1 | 29 ± 2,5 | 24 ± 2,2 | 56 ± 6,8 | 50 ± 4,5 |
| 1 DPSI[C] | - | 68[a] ± 2 | 29 ± 3,24 | 29 ± 3,2 | 36 ± 7,6 | 39 ±4,5 | 52 ± 1,8 |
| (n=3) | + | 69 ±2,1 | 36 ± 6,5 | 30 ± 1,8 | 32 ± 1,3 | 39 ± 3,6 | 43 ± 4,1 |
| 2 DPSI | - | 68 ± 3,7 | 31 ± 2,0 | 34 ± 1,2 | 33 ± 4,7 | 47 ± 7,1 | 57 ± 2,3 |
| (n=3) | + | 62 ± 5,5 | 31 ± 3,1 | 33 ± 1,6 | 34 ± 2,7 | 51 ± 5,0 | 56 ± 6,6 |
| 7 DPSI | - | 71± 2,0 | 40 ± 5,6 | 44 ± 3,6 | 30 ± 2,5 | 46 ±3,4 | 54 ± 2,3 |
| (n=3) | + | 65 ± 3,2 | 38 ± 2,4 | 33 ± 2,9 | 27 ± 0,2 | 39 ± 6,4 | 44 ± 6,5 |
| 15 DPSI | - | 67 ± 2,5 | 28 ± 1,5 | 37 ± 3,2 | 23 ± 3,7 | 47 ± 1,6 | 56 ± 2,0 |
| (n=3) | + | 65 ± 4,2 | 32 ± 1,7 | 31 ± 0,6 | 20 ± 3,1 | 56 ± 2,8 | 66† ± 1,7 |

[a]After the first immunisation the percentages were determined on the same animals by comparing left (without 1α,25(OH)$_2$D$_3$) and right (with 1α,25(OH)$_2$D$_3$) popliteal lymph node while different animals were used after the second immunisation. MC suspensions form popliteal lymph nodes were stained with MAbs directed against porcine CD2, CD4, CD8, IgM, CD25 and MHC II. [b] DPPI: days post primary immunisation [c] DPSI: days post secondary immunisation [d]ND: not determined †: P< 0.05

### 3.4.5. In vivo cytokine mRNA-expression by MC from local draining lymph node

The *in vivo* cytokine mRNA-expression was measured 1, 2 and 10 dppi and 1, 2 and 7 dpsi. The cytokine mRNA responses peaked 1 to 2 days after the first (innate response) as well as after

the second (memory response) IM injections. The cytokine mRNA-expression per cytokine revealed the following descending rank irrespective of the use of $1\alpha,25(OH)_2D_3$: TGF-β (1,67 x 1 x $10^5$ ± 1,8 x 1 x $10^5$) > IL-10 (1,38 x $10^4$ ± 2,65 x$10^4$) > IFN-γ (7,9 x $10^3$ ± 1,3 x $10^4$) > IL-2 (2,91 x$10^2$ ± 5,24 x$10^2$) > IL-4 (1,3 x $10^2$ ± 3,17 x $10^2$) > IL-6 (1 x $10^2$ ± 1,2 x $10^2$).

The relative amount of cytokine mRNA-expression in both groups is shown in Fig. 3.4.

One day after the first immunisation IL-10 mRNA was higher and IL-2 was significantly lower ($P<0.05$) in the $D_3$-group than in the control group. The IFN-γ, TGF-β and IL-6 mRNA expression did not differ statistically between the groups whereas IL-4 mRNA was not detected. One day later IL-2 and IL-10 mRNA were almost absent in both groups whereas levels of IFN-γ and TGF-β mRNA expression had not changed. On the other hand IL-6 mRNA expression was higher in the control group and traces of IL-4 mRNA were detected in the $D_3$-group. However, ten dppi IL-4 and TGF-β mRNA expression could only be detected in the control group ($P = 0.26$ and 0.15 respectively) in comparison with the $D_3$-group while the expression of the other cytokines was similar in both groups.

One day after the booster immunisation, the expression of IL-2 and IL-10 mRNA were significantly higher ($P = 0.043$) and lower ($P = 0.14$) respectively, in the $D_3$-group than in the control group, which was opposite to the first immunisation. The other cytokines showed a higher mRNA expression than after the first immunisation but expression was lower in the $D_3$-group than in the control group. Moreover $1\alpha,25(OH)_2D_3$ seems to inhibit significantly the IFN-γ mRNA expression with at least 50 % 1 and 2 dpsi ($P = 0.023$ and 0.013 respectively) whereas the TGF-β mRNA expression became significantly lower ($P = 0.04$) in the $D_3$ group 7 dpsi. Interleukin-2, IL-6 and IL-10 mRNA were low to absent 2 and 7 dpsi. Interleukin-4 could only be detected in the $D_3$-group ($P = 0.37$) 7 dpsi.

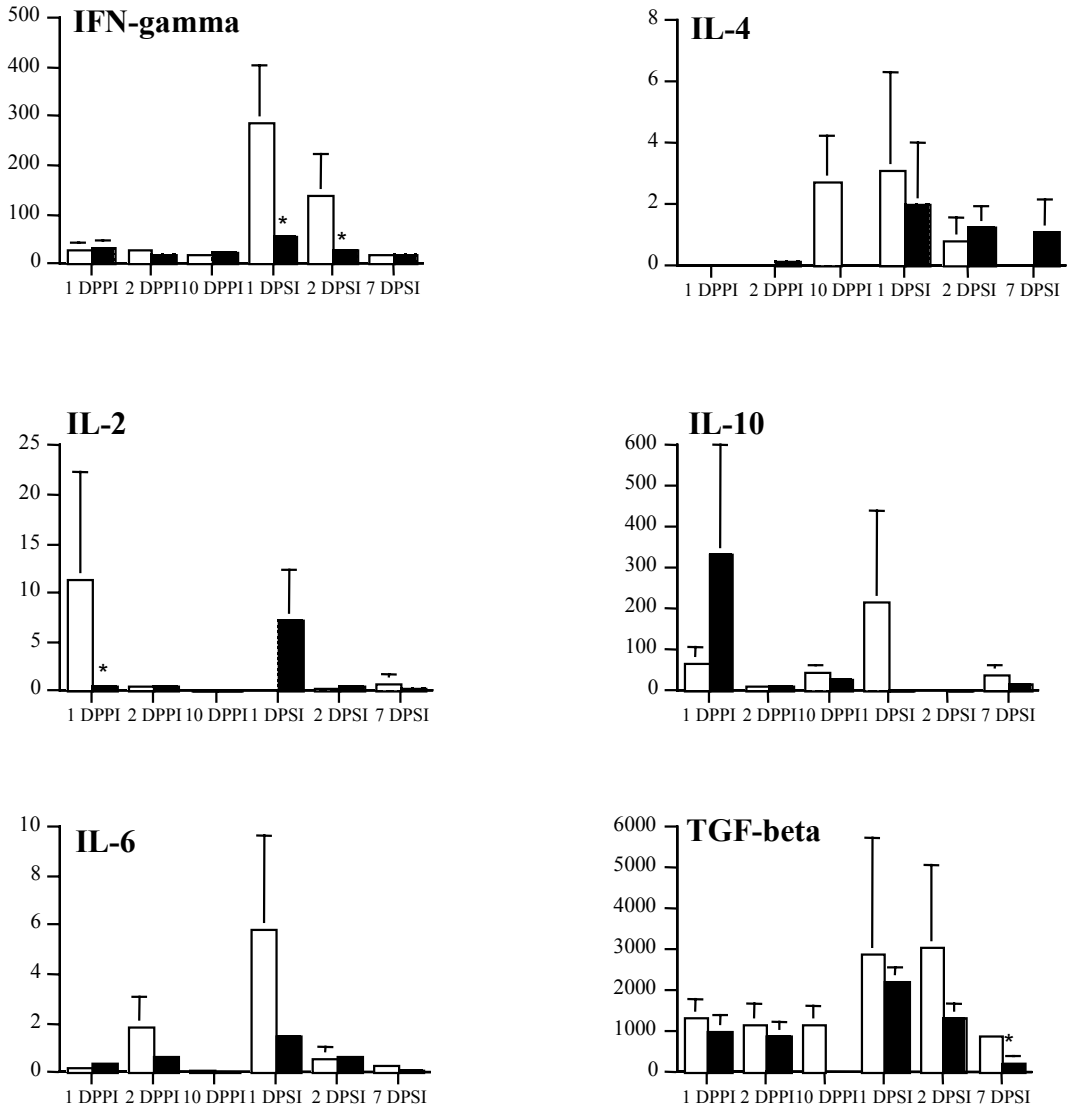



**FIGURE 3.4:** Porcine cytokine mRNA expression 1 (n=5), 2 (n=5) and 10 (n=3) days post primary immunisation (dppi), 1 (n=2) , 2 (n=3) and 7 (n=3) days post second immunisation (dpsi) of MC from the local draining lymph node (lnn popliteus) of pigs IM injected with either 1 mg HSA supplemented with (black bars) or without 2 µg of 1α,25(OH)$_2$D$_3$ (white bars). *In vivo* cytokine mRNA-expression was measured and quantified using real time PCR and corrected for individual variation by making a ratio to a housekeeping gene. The mean ratios/cytokine for the pigs with the same immunisation at the same time point (copy number of particular cytokin/$10^5$ copy numbers of housekeeping gene) were calculated. The cytokine mRNA expression was determined on the same animals after the first IM immunisation by comparing left (without 1α,25(OH)$_2$D$_3$, control) and right (1α,25(OH)$_2$D$_3$-supplemented) popliteal lymph node while different animals were used after the second immunisation. Significant differences ($p<0.05$) between both groups are indicated with an asterisk.

## 3.5. Discussion

In the present study, it was examined whether addition of 1α,25$(OH)_2D_3$ could result in the appearance of antigen-specific ASC in the GALT after IM administration of an antigen and whether the steroid hormone polarises the cytokine response towards a Th2-cytokine profile. As in a previous study (Van der Stede et al., 2001), co-administration of 1α,25$(OH)_2D_3$ significantly enhanced the serum HSA-specific IgA response and increased the numbers of HSA-specific IgA ASC in the local draining lymph node of pigs after IM injection. However, in the present study it decreased the antigen-specific serum IgM, IgG1, IgG and IgG2 ($P<0.05$) serum response. This was most pronounced after the first IM injection. This decreased IgM antibody response was not observed in a previous study (Van der Stede et al., 2001). The reason is not clear but the IM injection occurred at a different place (musculus gastrocnemius as compared to musculus gluteobiceps) and it has been observed in previous experiments with pigs comparing immunisations in the back versus neck (Vanderpooten et al., 1997) that the injection site can influence the isotype profile of the antibody response. So, in contrast to the serum IgA response, the steroid hormone does not consistently enhance the serum IgM response in pigs. A decreased as well as an increased serum IgM response using 1α,25$(OH)_2D_3$ was also observed in man (Lemire et al., 1984). In cattle, milk IgM and IgA titers increased and IgG2 decreased (Reinhardt et al., 1999) and in mice serum IgG2a decreased, whereas IgG1 increased using high doses of 1α,25$(OH)_2D_3$ (Lemire et al., 1995). The situation in the mice led to the interpretation that 1α,25$(OH)_2D_3$ stimulates the Th2 or humoral branch of the immune system as a Th2-cytokine profile preferentially stimulates the formation of IgG1, IgM and IgA (Mosmann et al., 1996). However, it should be emphasized that the correlation between Th1 and Th2 cytokine profiles and the secretion of isotypes is well established in mice and man but not in domestic animals such as pigs and cattle (Blecha, 2001).

In order to know whether IM immunisation with 1α,25$(OH)_2D_3$ directed the response towards the GALT, the number of HSA-specific ASC in the PP as well as in the LP were determined. Intramuscular immunisation induced IgA and IgG ASC first in the PP and later, although in very low numbers, in the LP. This seems to indicate that there is no direct homing of the LN cells to the effector sites in the GALT but a preferential homing to the inductive sites. Others found that memory B cells can reside in the GALT inductive sites after a systemic immunisation (Pierce et al., 1975; Fuhrman et al., 1981; Yuan et al., 2001). Upon mucosal antigen challenge these memory B cells differentiate and migrate towards the lamina propria where they produce antigen-specific IgA (Coffin et al., 1998). In the present study however no mucosal antigen challenge was performed. So, it is not surprising that only low numbers of ASC were

found in the LP. Another reason for these low numbers might be a too short interval of 7 days between both samplings of GALT. Indeed, the time required for activation, differentiation and homing of antigen-specific ASC from PP towards LP is at least 6 days (Coffin et al., 1997). Nevertheless, the low number of ASC may already result in a protective effect. Indeed, IM injection with F4-fimbriae co-administered with 1α,25(OH)$_2$D$_3$ induced a partial protection upon oral challenge with F4$^+$ enterotoxigenic *E. coli* (Chapter 7) which was accompanied with a secondary serum IgA response, indicating priming of the intestinal mucosal immune system. Further studies are necessary in pigs to elucidate if this mucosal priming occurs via migration of dendritic cells towards the GALT as observed in mice (Enioutina et al., 1999,2000).

As 1α,25(OH)$_2$D$_3$ primarily enhances the antigen-specific ASC in the local draining lymph node and clearly enlarges the local draining lymph node (Van der Stede et al., 2001), the distribution of various lymphocyte subsets in the local draining lymph node was analysed at different time points after the IM injection. In the present study however, enlargement of the local draining lymph node due to 1α,25(OH)$_2$D$_3$ was not consistently observed. Moreover no statistically significant changes were observed in neither the percentage of CD2$^+$ (T cells), CD4$^+$ (Th cells), CD8$^+$ (CTL-cells) and IgM$^+$ (B cells) cells nor in the degree of expression of these molecules on the cells. In human studies however a decreased CD4$^+$/CD8$^+$ ratio, due to a significant increase in CD8$^+$-cells, was observed after treatment with 0.5 µg or 2 µg of 1α,25(OH)$_2$D$_3$ for 60 and 14 days respectively (Fujita et al., 1984; Matsui et al., 1985; Zofkova et al., 1997). Although not statistically significant, the CD4$^+$/CD8$^+$ T-cell ratio appeared to be increased 7 and 15 dpsi in the present study. Most obvious differences were observed after the second IM injection. This booster immunisation with 1α,25(OH)$_2$D$_3$ resulted in a significant higher % of CD25$^+$-cells with however a lower expression of MHC II molecules. The latter is consistent with human studies in which a decreased expression of MHC class II molecules on human monocytes was observed due to 1α,25(OH)$_2$D$_3$. This decrease was observed was dose and time dependent (6 days) (Rigby et al., 1992; Xu et al., 1993; Splitter et al., 1997). The higher percentage of CD25$^+$-cells suggest at least an activation of more cells or a better persistance of activated cells by the steroid hormone as the porcine IL-2R (CD25) is expressed on activated T- (Lunney et al., 1994) and B cells in lymph nodes (Denham et al., 1994). For human B cells it was shown that the degree of cellular activation, rather than the differentiation, is a criterion for the biological receptivity to 1α,25(OH)$_2$D$_3$ (Morgan et al., 2000).

The *in vivo* mRNA expression of Th1-like (IFN-γ, IL-2), Th2-like (IL-4 and IL-10) and Th3-like (TGF-β) cytokines showed a high variability among the pigs. Following, the first immunisation with 1α,25(OH)$_2$D$_3$, increased IL-10 and IL-4 mRNA and decreased levels of IL-2 mRNA (Th1-like) were observed indicating a Th2 modulation by 1α,25(OH)$_2$D$_3$. A Th2-like

response could also explain the increased serum IgA and the higher number of IgA ASC in the local draining lymph node. Th2-polarisation by 1α,25$(OH)_2D_3$ was also observed after *in vitro* stimulation of mice lymph node cell suspensions (Daynes et al., 1996; Boonstra et al., 2001) while others observed inhibition of IL-4 by naïve murine T cells (Staeva-Viera et al., 2002). However, in the present study TGF-β, which is involved in the isotype switching towards IgA (Lebman et al., 1990) was not increased.

More striking differences were observed after the secondary immunisation with 1α,25$(OH)_2D_3$. Significant lower levels of IFN-γ mRNA were observed as compared with the control group indicating a Th2-cytokine modulating response using 1α,25$(OH)_2D_3$. However, also lower levels of Th2- (IL-4 and IL-10) and Th3-like (TGF-β) cytokines mRNA together with statistically significant higher levels of IL-2 were observed in the 1α,25$(OH)_2D_3$-treated animals. This indicates a rather Th1-cytokine modulating response. In conclusion, the results of the present study do not allow us to conclude that 1α,25$(OH)_2D_3$ induces a Th2-like response in pigs except possibly after the primary inoculation. The inverse correlation between IL-10 and IL-2 mRNA expression appears consistent in our experiments and suggest that IL-10 is able to down-regulate Th1-mediated immune responses as described for man (Wanidworanun et al., 1993; Randow et al., 1995) as well as for pigs (Blancho et al., 1995). However, in man and cattle, IL-10 is expressed by all Th and not selectively by Th2 cells as in mice (Brown et al., 1994).

In conclusion, supplementation of 1α,25$(OH)_2D_3$ to parenteral immunisation enhanced the IgA serum antibody response as well as the number of antigen-specific ASC in different systemic lymphoid tissues. Moreover, 1α,25$(OH)_2D_3$ administatrion resulted in an enhanced priming of the GALT, especially in the Peyer's patches. This was not correlated with major changes in T/B-cell subsets and a clear Th2-like (IL-4, IL-6, IL-10) cytokine profile in the local draining lymph node.

# CHAPTER 4

# CPG-OLIGODINUCLEOTIDES AS AN EFFECTIVE ADJUVANT IN PIGS FOR INTRAMUSCULAR IMMUNISATIONS

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## 4.1. Abstract

In this study, the effect of two oligodeoxynucleotide (ODN) sequences 5'GCT-AGA-CGT-TAG-CGT-3' (CpG-ODN) and 5'-GCT-AGA-GCT-TAG-GCT-3' (GpC-ODN) on the antigen-specific antibody and cellular immune response after intramuscular immunisations with ovalbumin (OVA) was analysed in pigs. Pigs immunized with OVA supplemented with these ODN's showed a significantly enhanced primary antibody response in comparison with the control group which received OVA without ODN. This enhanced primary antibody response appeared ODN-sequence-independent as similar effects were seen in both ODN-groups. The OVA-specific antibody titers obtained after a single injection of antigen combined with either of both ODN's were as high as the titers in the control group after two injections. Furthermore, the ODN-supplemented animals showed significantly higher OVA-specific IgA antibodies in their saliva and nasal secretions at some time points after the first immunisation. Proliferation assays showed that CpG- as well as GpC-ODN significantly enhanced the antigen-specific as well as the mitogen-induced proliferation in different lymphoid tissues. Furthermore, 48 hours after the third immunisation both the CpG-group showed a significantly decreased IL-6 mRNA expression in cells of the local draining lymph node but no significant difference in TGF-β (Th3-like) and IL-10 (Th2-like). The ODN injected animals showed the tendency to have higher IFN-γ (Th1-like) mRNA-expression in comparison with the control group. To our knowledge, these are the first *in vivo* studies in pigs, which demonstrate the appropriateness of CpG-ODN as immunostimulating adjuvants in vaccines for farm animals.

## 4.2. Introduction

Microbial (plasmid) DNA has potent immunostimulatory properties that result from short sequence motifs called CpG-motifs or immunostimulatory sequences. These motifs consist of an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines and can be synthesized chemically (Krieg et al., 1998). As a consequence these CpG-motifs can be used as adjuvant and/or immunomodulator. The immunomodulatory properties in mice include the induction of different cytokines by APC and T-helper (Th) cells such as interferon-α, IFN-β, IFN-γ, interleukin (IL)-6, IL-12, IL-18, so directing the Th cells towards Th1 and the activation of NK cells (Ballas et al, 1996; Stacey et al., 1996). CpG-oligodeoxynucleotide sequences (ODN) are also capable to directly activate B-lymphocytes triggering the secretion of immunoglobulins (Ig) with more IgG2a and less IgG1 (Krieg et al., 1995; Liang et al., 1996). It has been shown that vaccination with CpG-ODN can lead to protective immune responses against several viral,

bacterial and parasitic infections including hepatitis B, influenza (Davis et al., 1998), tuberculosis (Huygen et al., 1996), malaria (Hoffmann et al., 1995) and leishmania (Zimmermann et al., 1998). Moreover, CpG-ODN's induce anti-tumor responses (Weiner et al., 1997) and decrease the IgE responses in allergic individuals (Slater et al., 1997; Bohle et al., 1999). Recently, Hemmi *et al*. (2000) showed in mice that the Toll-like receptor 9 (TLR –9), present on a variety of immune cells, including macrophages and dendritic cells, is responsible for the uptake of CpG-ODN and subsequenlty the immunostimulatory effects of these motifs.

The majority of publications regarding CpG-vaccination and the use of CpG-motifs is based on rodent models. In literature, almost no information is available for the effects of synthetic CpG-ODN in livestock or domestic animals such as cattle, pigs, horses and poultry. One study by Brown and co-workers (1998) showed that CpG-ODN derived from *Babesia bovis* were mitogenic for bovine B cells and enhanced the antigen-specific IgG1 and IgG2 secretion. Recently, Kamstrup *et al*. (2001) and Magnusson et al. (2001) showed that porcine PBMC can be stimulated *in vitro* by CpG-ODN.

The observation that CpG-ODN can enhance innate as well as adaptive immune responses makes CpG-ODN a promising candidate adjuvant for veterinary vaccines. In the present study, the immunostimulating effects of CpG-ODN on cellular and humoral immune responses are evaluated in pigs, after intramuscular immunisation with the test protein OVA.

## 4.3. Material and methods

### 4.3.1. Pigs and immunizations

Pigs (Belgian Landrace x Piétrain) were selected that were negative for antibodies against OVA as tested by ELISA. The pigs were conventionally housed and received food and water *ad libitum*. Subsequently, pigs were i.m. immunized in the musculus splenius at the age of 5 weeks with 1 mg OVA (Sigma Aldrich, Nr. A-5503 Bornem, Belgium) in IFA (Difco Laboratories) supplemented with either 500 µg CpG-ODN (n=7, CpG-group) or 500 µg GpC-ODN (n=7, GpC-group) or without supplement (n=6, control group). CpG-ODN (ODN containing a CG-motif marked with bold letters 5'GCT-AGA-**CG**T-TAG-**CG**T-3') and GpC-ODN (inverted CG = 5'-GCT-AGA-**GC**T-TAG-**GC**T-3') were chosen from experiments in mice according to Klinman et al. (1999) and were manufactured by Biosource Europe. As it is known that phosphodiester-ODN are rapidly degraded *in vivo* (Krieg et al., 1995) a high dose of 500 µg was used. Thirty-five days and seventy-five days later the pigs received identical booster immunisations. All pigs were euthanatized by intravenous injection of an overdose pentobarbital (24mg/kg; Nembutal, Sanofi

Sante Animale, Brussels, Belgium) followed by exsanguination 48 hours after the third immunisation (77 days post primary immunisation, PPI).

For determining the serum-antibody response, blood was taken from the jugular vein once a week PPI or twice a week post secondary immunisation (PSI). Ten days PSI blood was sampled on Alsever's solution for isolation of PBMC. These PBMC were used for proliferation assays. Three or four pigs of each group were sampled for nasal and oral secretions weekly until 5 weeks PSI using cotton swabs (Novolab, Belgium). The volume of the collected secretions was determined by weighing the swabs before and after the sampling. Subsequently, the saliva and nasal secretions were rapidly diluted 1/2 [vol/vol] and 1/8 [vol/vol], respectively, in PBS (pH 7.4, 150 mM) supplemented with 0.05% [vol/vol] Tween® 20 and 20% [vol/vol] fetal bovine serum (FBS, Gibco, BRL, Life Technologies, Merelbeke, Belgium) and stored at –80 °C until analysis in ELISA. At euthanasia, the local draining lymph node and spleen were aseptically collected and the MC were isolated for use in proliferation assays and total RNA was extracted for quantifying the mRNA expression of IL-6, IL-10, TGF-β and IFN-γ.

### 4.3.2. ELISA

OVA-specific (IgM, IgA, IgG1, IgG2 and IgG) antibodies in serum and OVA-specific IgA antibodies in secretions were measured by an indirect ELISA, as previously described for human serum albumin (Van der Stede et al., 2001). Briefly, microtiter plates (Polysorb, Nunc, Life Technologies, Merelbeke) were coated with OVA (50 µg/ml in 50 mM carbonate-bicarbonate buffer, pH 9.6) during 2 hrs at 37 °C. Subsequently, the plates were blocked overnight at 4 °C with 0.2% (vol/vol) Tween® 80 in PBS and thereafter incubated with series of two-fold dilutions of serum starting at 1/10 (vol/vol) in PBS + 0,05% Tween® 20. Then, the plates were incubated with anti-swine IgM, IgA (27.8.1), IgG1 (23.49.2), IgG2 (34.1.1a) and IgG (23.3.1.b)-specific MAb's (Van Zaane et al., 1987) diluted in ELISA buffer (PBS + 0.2% Tween®20 + 3% BSA) , with biotinylated rabbit anti-mouse Ig (DAKO, Prosan, Merelbeke, Belgium) supplemented with 2% [vol/vol] pig serum and with horseradish peroxidase-conjugated (HRP) streptavidin (Roche, Germany). Between each step, the plates were washed with PBS + 0.2% [vol/vol] Tween® 20. Finally, ABTS, containing $H_2O_2$, was added and after 30 min incubation the optical density was measured at 405 nm ($OD_{405}$).

The antibody titer was determined as the inverse of the highest dilution that still had an $OD_{405}$ higher than the cut-off value. The cut-off values was determined by calculating the average $OD_{405}$ plus 3 times the standard deviation of the serum, oral and nasal secretions before i.m. immunisation, diluted 1/10, ½ and 1/8 respectively.

4.3.3. Preparation of cell suspensions

PBMC were isolated from blood on Alsever's solution by density gradient centrifugation using Lymphoprep (Nycomed, Pharma AS, Life Technologies, Merelbeke).
MC from spleen and lymph nodes were prepared as described by Van den Broeck et al (1999). Briefly, after removing surrounding fat, the MC were isolated by gently teasing the tissues apart. The MC were collected in RPMI 1640 (Gibco, BRL) on ice, and the erythrocytes were lysed with ammoniumchloride (0.8% [wt/vol]). After centrifugation (380 x g at 4°C for 10 min), the pelleted cells were washed and resuspended in leukocyte medium (RPMI-1640 supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml), kanamycin (100 µg/ml), glutamin (200 mM), sodiumpyruvate (100mM), non-essential aminoacids (100mM), 10% FBS ([vol/vol] Gibco BRL, Life Technologies, Merelbeke, Belgium) and β-mercaptoethanol ($5.10^{-5}$ M, Life Technologies) at the concentration of 5 x $10^6$ cells/ml.

4.3.4. Lymphocyte proliferation test

MC from each immunized pig were brought into 4 to 5 wells of a 96-well flat-bottom microtitre plate (Nunc) at 5 x $10^5$ cells/well in 100 µl leukocyte medium whereafter OVA (final concentration 100 µg/ml medium), concanavalin A (Con A, final concentration 10 µg/ml) or medium were added giving a final volume of 200 µl. The plates were incubated at 37.5 °C in a humid atmosphere with 5% $CO_2$. After 72 hours the cells were pulse-labelled with [$^3$H]-thymidine (1 µCi/well, Amersham ICN, Bucks, UK) and 18 hours later the cells were harvested onto glass fibre filters. The radioactivity incorporated into the DNA was measured using a β-scintillation counter (Perkin Elmer, Brussels, Belgium).

4.3.5. RNA-extraction, cDNA synthesis and PCR-amplification of porcine cDNA.

Total RNA was extracted from the cell suspensions of the local draining lymph node 48 hours after the third immunisation using the acid guanidinium-isothiocyanate phenol-chloroform-based method (RNAgents® Total RNA Isolation System, Promega, Leiden, the Netherlands). Three microgram total RNA was pre-incubated with 1 µl (50 µM) random hexanucleotide primer (Perkin Elmer, Brussels, Belgium) during 10 min at 70 °C. After chilling on ice 5mM of $MgCl_2$, 1 x PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl), 1 mM dNTP-mix (Roche, Mannheim, Germany) 20 units of RNAsin® Ribonuclease inhibitor (Promega) and 200 units of Superscript II Rnase H$^-$ reverse transcriptase (GIBCO, BRL) were added. The reaction mix was incubated at

room temperature for 10 min and then at 42 °C for 45 min followed by heating for 5 min at 95 °C to inactivate the reverse transcriptase. The oligonucleotide primers used for detection of the porcine interleukins (IL)-6, IL-10, IFN-γ, TGF-β and cyclophilin cDNA were designed from the published nucleic acid sequences available from GenBank/EMBL databases and described by Verfaillie et al (2001). Cyclophilin was used as constitutively expressed 'housekeeping' gene control to determine the uniformity of the reverse transcription reactions.

Cytokine-cDNA was amplified and quantified via real time PCR using the Light Cycler® and the lighcycler-faststart DNA Master SYBR Green I kit (Roche, Mannheim, Germany, catalogue nr. 2239264). The reaction mixture consists of a master mix containing Taq DNA polymerase, dNTP mixture and SYBR Green I, 3-5 mM $MgCl_2$, 0.3 µM of each primer and 2µl of template cDNA in total volume of 20 µl. Subsequent steps were initial denaturation for 10 min at 95 °C, followed by 40 cycles of denaturation for 15 sec at 94 °C, annealing for 5-7 sec at 60°C sec and an extension for 7-15 sec at 72 °C depending on the length of the product (~1sec/25 bp). Confirmation of the specificity of the PCR-products was performed by subjecting these products to a melting curve analysis and subsequent agarose gel electrophoresis. Quantification occurred using external standards of cytokine cDNA. Calculation was performed with the LightCycler® analysis software. The relative amount of cytokine-expression was plotted as a ratio ((= copy numbers of target cytokine/ copy numbers of house keeping gene) x $10^5$ ). Target cytokines were IFN-γ (Th1-like), IL-10 (Th2-like), TGF-β (Th3-like), and IL-6 (pro-inflammatory cytokine).

### 4.3.6. Statistical anaylysis

Differences in $log_2$ antibody serum titers ( IgM, IgA, IgG1, IgG2 and IgG) between the groups were tested for statistical significance using General Linear Model (Repeated Measures Analysis of Variance). Differences between groups in cytokine-mRNA expression and in T-cell proliferation were analysed for statistical significance with an unpaired t test and a Mann-Whitney U test, respectively. $P < 0.05$ was considered as statistically significant.

## 4.4. Results

### 4.4.1. Effect of ODN on the OVA-specific serum antibody response

Animals injected with OVA and one of both oligonucleotide sequences, CpG-ODN or GpC-ODN, showed significantly higher serum antibody titres than the animals injected with OVA alone (Fig. 4.1).

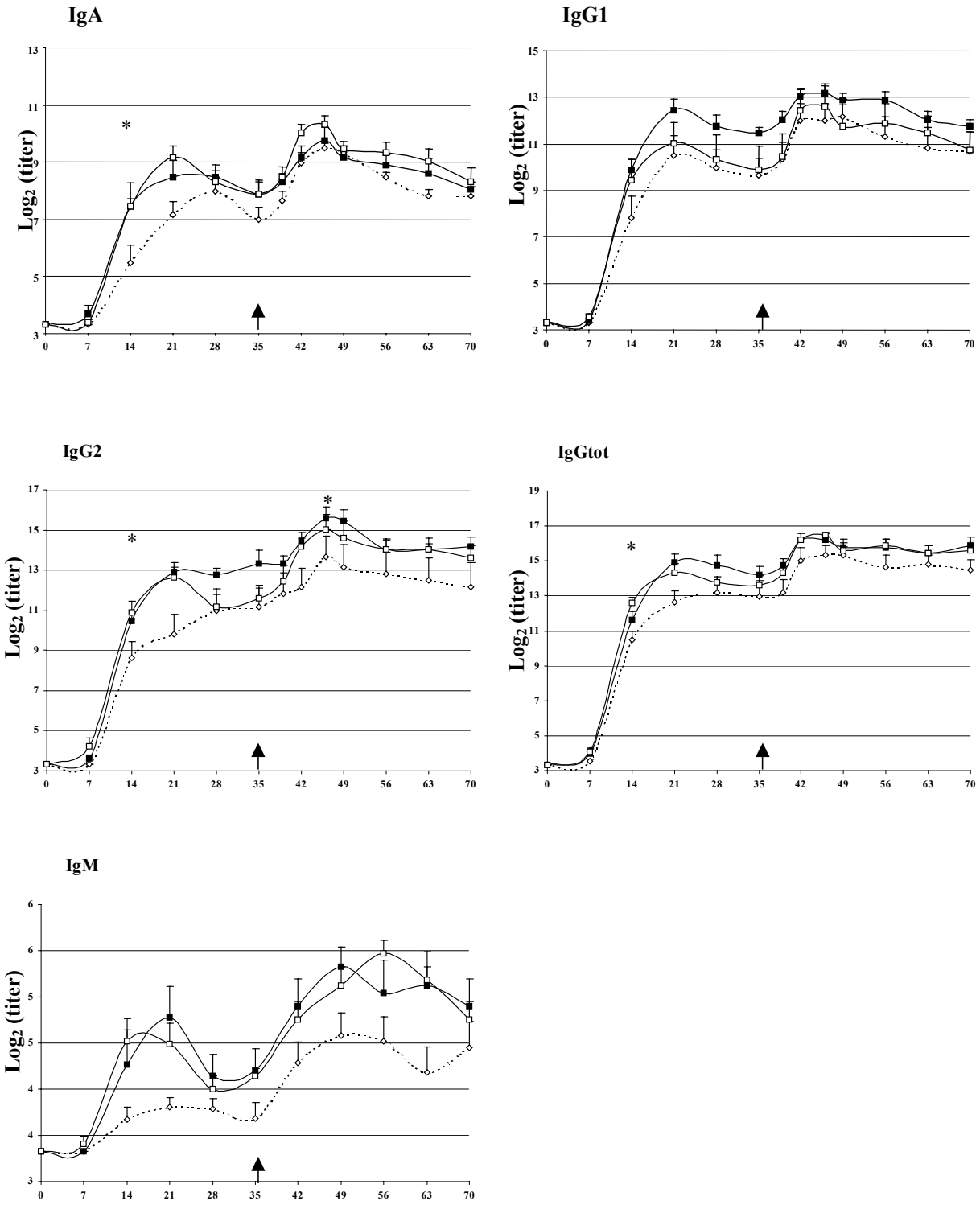


**FIGURE 4.1**: Kinetics of the OVA-specific IgM, IgA , IgG, IgG1 and IgG2 serum response following intramuscular immunisation of pigs with OVA ( ), OVA+ CpG (■), OVA + GpC ( ) The booster immunisation was given 35 (arrow) days post primary immunisation. The antibody titers are plotted as mean log 2 titers ± SEM. Significant differences ($p<0.05$) are indicated with ∗.

There were however no significant differences between the two ODN-groups. The effects of the oligonucleotide-supplement on the antibody response was most obvious after the primary immunisation. This resulted in significantly higher OVA-specific IgA ($2^{7.46}$–$2^{9.18}$) , IgG2 ($2^{10.46}$–$2^{12.89}$) and IgG ($2^{12.2}$–$2^{15.5}$) serum antibody titers 14 and 21 days PPI in comparison with the control group (IgA:$2^{5.49}$–$2^{7.2}$, IgG2:$2^{8.7}$–$2^{9.8}$, IgG:$2^{11}$–$2^{13.2}$ ). No significant differences could be found for the OVA-specific IgG1 and IgM responses, although the mean IgM response was higher for both ODN-groups. The second immunisation induced a secondary antibody response with serum titers increasing 4-to 8-fold in all groups. This resulted in significantly higher OVA-specific IgG2 serum antibody titers for the CpG and GpC groups, 7 days PSI. The titers reached after a single immunisation with CpG or GpC-ODN were as high as titers reached after two injections with OVA alone. This was most obvious for the OVA-specific IgM, IgA and IgG response.

### 4.4.2. Effect of ODN on the OVA-specific antibody response in mucosal secretions

To determine if there was an effect of CpG and GpC-ODN on the IgA response in the mucosal secretions, the OVA-specific IgA in saliva and nasal secretions was measured weekly until 5 weeks PSI in the sampled pigs (Table 4.1.).

**TABLE 4.1.:** OVA-specific IgA antibody titer of each pig in saliva and nasal secretions following immunisation at day 0 and day 35 days PPI with OVA , OVA + CpG and OVA + GpC.
DPPI[a]: days post primary immunization

| Sample | Immunization | Pigs | DPPI[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 7 | 14 | 21 | 28 | 35[b] | 42 | 49 | 56 | 63 | 70 |
| **SALIVA** | **OVA** | 1 | -[d] | - | - | - | - | 64 | 8 | 4 | 4 | 2 |
| | | 2 | - | - | - | - | - | 2 | 4 | - | - | - |
| | | 3 | - | - | - | - | - | 32 | 32 | 64 | 32 | 16 |
| | **OVA + CpG** | 1 | - | - | 2 | 2 | - | 16 | 8 | 4 | 2 | 8 |
| | | 2 | - | - | 4 | 4 | - | 8 | 32 | 16 | 8 | 2 |
| | | 3 | - | 2 | 2 | 2 | - | 16 | 16 | 16 | 8 | 2 |
| | | 4 | | - | 2 | 2 | - | 4 | 2 | 8 | 2 | - |
| | **OVA + GpC** | 1 | - | 2 | 16 | 4 | - | 32 | 16 | 16 | 8 | - |
| | | 2 | - | - | 2 | - | - | 8 | - | - | - | - |
| | | 3 | - | - | 2 | - | - | 8 | 8 | 2 | 4 | - |
| | | 4 | - | - | 2 | - | - | 8 | 8 | 4 | 4 | 2 |
| **NASAL SECRETIONS** | **OVA** | 1 | - | - | - | - | - | - | 16 | 32 | 16 | - |
| | | 2 | - | - | - | - | - | - | 8 | 16 | 16 | 32 |
| | | 3 | - | - | - | - | - | 8 | NT[c] | 64 | 32 | 16 |
| | **OVA + CpG** | 1 | - | - | 8 | - | - | - | - | 8 | 8 | 16 |
| | | 2 | - | - | 8 | - | - | 8 | - | 16 | - | 8 |
| | | 3 | - | 16 | 16 | 8 | 16 | 16 | 16 | 32 | 16 | 16 |
| | | 4 | - | - | - | - | 8 | - | 8 | NT | 8 | - |
| | **OVA + GpC** | 1 | - | 16 | 16 | - | - | 16 | 128 | 64 | 16 | 8 |
| | | 2 | - | 8 | - | - | - | 8 | 16 | 16 | - | NT |
| | | 3 | - | - | 16 | 8 | 8 | 8 | 32 | 8 | 32 | 8 |
| | | 4 | - | - | - | - | - | 8 | 16 | 16 | 8 | 8 |

35[b:] Booster immunisation 35 DPPI; NT[c:] not tested -[d] : lower than detection limit (<2 for saliva and <8 for nasal secretions)

The results demonstrate that CpG and GpC-ODN enhanced the primary OVA-specific IgA response in both secretions: in the control group only one animal had a titer of 2 in its saliva 21 days PPI whereas the pigs of the ODN-groups had significantly higher IgA titers at that moment ($p = 0.021$). Moreover, in the CpG-group all pigs had low OVA-specific IgA titers in their saliva one week later whereas in the GpC-group, 3 out of 4 animals were negative. In nasal secretions no IgA (titer < 8) could be detected in the control group after the first immunisation whereas in both ODN-groups, 3 out of 4 animals had at least on one occasion titers of 8 or 16.

After the second immunisation all animals showed 2- to 8-fold increases of the OVA-specific IgA response in the saliva and nasal secretions. No major differences could be observed between the groups, although slightly higher titers were observed in the GpC-group.

4.4.3. Effect of oligonucleotides on lymphocyte proliferation

OVA-specific (Fig. 4.2A) as well as ConA-induced (Figure 4.2B) proliferation of PBMC 10 days PSI of local draining lymph node cells and spleen cells collected 48 hours after the third immunisation were always significantly higher for the CpG-group ($p<0.01$ or $p<0.05$) ) than in the control group. The OVA-specific and ConA-induced proliferation in the GpC-group was always higher than in the control group but not always at statistically significant levels. Spontaneous proliferation (cpm of control medium cultures) of PBMC, spleen cells and local draining node cells were below 530, 2730 and 1720 cpm, respectively. No significant differences ($P>0.05$) were observed between the groups for this spontaneous proliferation.

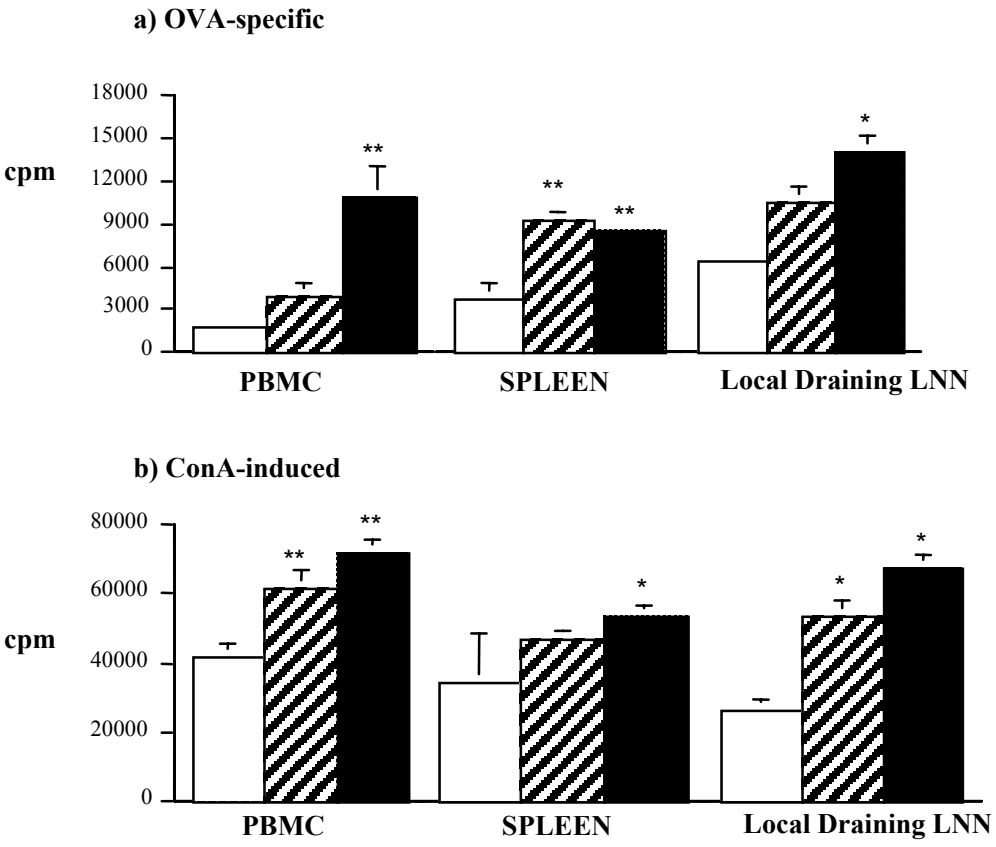


**FIGURE 4.2**: **A)** OVA-specific (100 µg/ml final concentration) **B)** ConA-induced proliferation (10 µg/ml final concentration) of PBMC (n=6 for OVA group (open bars), n=7 for ODN-groups) 10 days PSI, spleen cells (n=3 for OVA-group, n=4 for CpG (black bars) -and GpC-group (hatched bars)) and cells from local draining lymph node (n=1 for OVA-group and n=4 for CpG and GpC-group) 48 hours post tertiary immunisation (=77 days PPI). Results are presented as mean cpm ± SEM Significant differences with the control group are indicated with * ($P<0,05$) or ** ($P<0,01$).

4.4.4. Effect of ODN on cytokine mRNA expression

The relative amount of cytokine mRNA-expression 48 hours after the third immunisation for IL-6, IFN-γ, TGF-β and IL-10 showed a high variation among the animals. No significant differences were observed between the groups for IL-10, TGF-β and IFN-γ mRNA expression. However, the IL-6 mRNA-expression was significantly higher for the control group as compared to the CpG-group (p=0.035) but not to the GpC-group (Fig. 4.3).

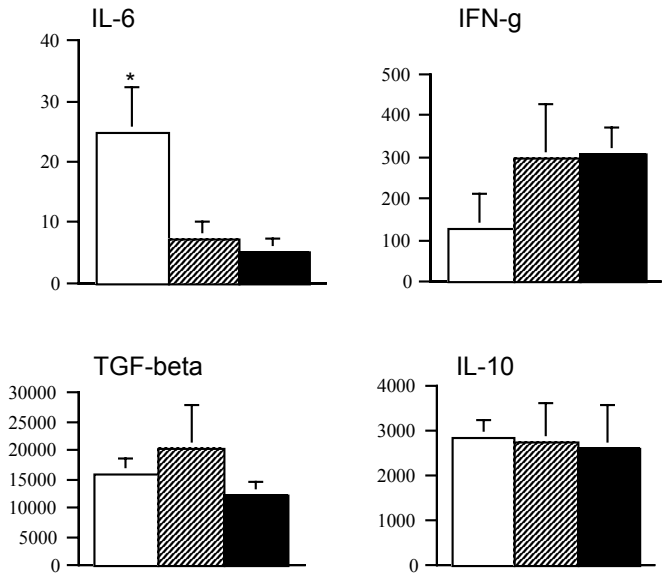


**FIGURE 4.3**: In vivo mRNA-expression of IL-6, IFN-γ, TGF-β and IL-10, 48 hours after the third immunisation with OVA (open bars), GpC-group (hatched bars), CpG-group (black bars). The relative amount of cytokine-expression was plotted as a ratio (= copy numbers of target cytokine/ $10^5$ copy numbers of house keeping gene) ± SEM. Significant differences between the test and control group ($P<0.05$) are indicated with *

## 4.5. Discussion

The ability of CpG-ODN to activate the immune system and its use as potent immune adjuvants has clearly been shown in mice and man *in vivo* as well as *in vitro* (Krieg et al., 1995,1996,1998,2000,2001; McCluskie et al., 1998). Only few studies examined *in vitro* the effect of CpG-ODN on cells of farm animals. Brown et al. (1998) and Shoda et al. (2001) showed *in vitro* that ODN-sequences derived from the parasite *Babesia bovis*, *Trypanosoma Cruzi* and T. *brucei* were mitogenic for bovine B cells and enhanced the antigen-specific IgG1 as well as the IgG2 secretion. Recently, Kamstrup et al. (2001) showed that porcine PBMC can respond to palindromic hexamer motifs containing an unmethylated CpG-ODN. Moreover, they found that ATCGAT was the optimal sequence to stimulate the proliferation and/or the expression of cytokine (IL-6, IL-12, TNF-α) mRNA. Since both studies were performed *in vitro* they do not necessarily reflect the *in vivo* situation. The present study was undertaken to examine in pigs the effects of CpG-ODN on antibody responses and lymphocyte proliferations against intramuscularly injected OVA. The CpG-sequence used in the present study was selected as it appeared the most active in mice whereas a non-active GpC-dinucleotide was used as a control (Klinman et al., 1999). The effect of both sequences on the immune responses was compared with the immune response of pigs injected with OVA without ODN.

Surprisingly, we observed that the in mice active sequence CpG-sequence as well as the in mice non-active GpC-sequence significantly enhanced the primary serum IgA and IgG (IgG2) antibody responses in pigs. Moreover, the serum antibody titers obtained in both ODN- groups after a single injection were as high as those in the control group after two immunisations. So, using oligonucleotides as adjuvant, perhaps only one immunisation, instead of the commonly performed two immunisations schedule, can be enough to induce a protective level of antibodies. These results on the CpG-ODN in pigs confirm those previously seen in mice, clearly showing that CpG-ODN enhance the antigen-specific antibody responses in both species (Sato et al., 1996; Halpern et al., 1996). However, the effect on the antibody response in the present study is not sequence-specific and/or CpG-dependent since the same adjuvant effect was also observed in the GpC-supplemented group. This similar effect was observed in two successive experiments using different batches of oligodeoxynucleotides. This effect might be due to species differences as described by Hartman *et al*. (2000) or to an influence of the nucleotides flanking the CpG dinucleotide. Indeed Kamstrup and co-workers showed in an *in vitro* study that substitution of the flanking purines with pyrimidines or vice versa changed the immunostimulatory properties of the optimal sequence ATCGAT. So in the present study with pigs, the flanking sequences AG and TT might both be stimulating. The *in vitro* study of Kamstrup et al. also supported that the presence

of the CpG-dinucleotide is crucial for activating porcine PBMC. However, the present study in pigs clearly shows that this is not the case *in vivo*, indicating that *in vivo* other factors than the presence of CpG-motifs influence the antigen-specific antibody responses. These factors might include differences in cell-populations activated *in vivo* as compared to the *in vitro* situation such as the dentritic cells (DC). DC cells are primary target cells for the CpG-ODN (Jakob et al., 1998; Sparwasser et al., 1998). CpG-ODN induce on DC-cells an enhanced expression of MHC-II molecules, CD80 and CD86 with better antigen processing and presentation towards Th cells. Moreover, DCs seem to be crucial cells for the induction of primary antibody responses. Differences in stimulated cell populations could have been due to breed or age-related changes. Indeed, the cellular distribution in different lymphoid organs, including blood, is in pigs age-dependent (Bianchi et al., 1992; Yang et al., 1996; Rothkotter et al., 1999). Kamstrup and co-workers used 6 to 10 months old mini-pigs while in the present study much younger conventional pigs (five weeks at moment of first immunisation) were used.

Not only in the serum but also in the saliva and nasal secretions an earlier and higher OVA-specific IgA response was observed in the ODN-supplemented groups. Again, this enhanced response was most obvious after the primary immunisation. Nearly all samples from both oligonucleotide groups were positive. After the second immunisation, this difference was less obvious even though the highest titers were found in the nasal secretions of the GpC-group. This indicates that even systemic administration of CpG or GpC-ODN, and not only oral administration can enhance the antigen-specific IgA response in mucosal secretions (Horner et al., 1998). This effect was not due to an increased switch towards IgA because the IgA/IgG ratio did not differ between the CpG/GpC-groups and the control group.

Besides its effect on the humoral response, the *in vivo* effect of both ODN-sequences was also analysed on the cellular (memory) response by measuring the antigen-specific proliferation of PBMC, spleen cells and cells from the local draining lymph node and by determining the cytokine mRNA expression in this lymph node. The CpG-sequence significantly enhanced the OVA-specific as well as the ConA-induced proliferation in all tissues tested (PBMC, spleen cells or cells from draining lymph node) while this was not always the case for the GpC-ODN. This observation suggest that both sequences, used in this study, have the intrinsic capacity to trigger directly or indirectly via APC the MC to proliferate better. This capacity was more obvious for the CpG-sequence than for the GpC-ODN indicating that the presence of the CpG-motif is important when stimulating the cells *in vitro* with antigen and/or ConA. The observation that CpG-ODN enhance the antigen-specific proliferation is consistent with the observations of Lipford et al.(1997) who demonstrated that CpG-ODN may influence the signal threshold of antigen-reactive T cells *in vivo* and that this effect was due to the CpG-mediated activation of APC like DC and

macrophages. Moreover, it has been shown that CpG-ODN activate B cells and T cells (in the presence of APC and cytokines) in a polyclonal way (Liang et al., 1996). The observation that the CpG-ODN (and to a lesser degree the GpC-sequence) induced a higher OVA-specific proliferation might be due to more activated APC, and subsequently better antigen-presentation. Another explanation for this observation might be a different subset of T(h)-cells and/or cytokine-profile in the cultures of *in vivo* CpG/GpC stimulated cells.

The level of mRNA expression for the different cytokines (IL-6, IFN-γ, TGF-β and IL-10) displayed a lot of variation between animals within a group. Such heterogeneity has also been observed after *in vitro* stimulation of porcine PBMC (Kamstrup et al., 2001). There was no significant difference between the groups in IFN-γ, TGF-β and IL-10 mRNA expression. However, there was a significant decreased IL-6 mRNA expression in the CpG-ODN group as compared to the control group. Consequently, the increased levels of IgA antibodies in the CpG-group were not accompanied by higher levels of IL-6 and TGF-β mRNA expression as we would expect from studies in mice and men (Beagley et al., 1989 ; Lebman et al., 1990). As IL-6 is a pro-inflammatory cytokine, mRNA-expression could have been low as measurements were done 48 hours after the third immunisation.

In conclusion this study shows that single immunisations in pigs with antigen, containing CpG and/or GpC-ODN can be used *in vivo* to enhance the antigen-specific antibody responses and cellular responses. Indeed, a single immunisation containing ODN equals double immunisations without ODN and could render a booster immunisation redundant. Moreover, CpG-ODN have been shown to enhance the IgA in mucosal secretions which plays an important role in mucosal protection. Taken together these immunostimulating properties, ODN might be applicable in vaccines against infectious diseases in pigs.

# CHAPTER 5

# PORCINE-SPECIFIC CpG-OLIGODEOXYNUCLEOTIDE ACTIVATES B-CELLS AND INCREASES THEIR EXPRESSION OF MHC-II MOLECULES.

## 5.1.Summary

Two motifs namely A = 5'-GCTAGACGTTAGCGT-3'and B= 5'-TGCATCGATGCAG-3' were synthesized by two different companies and tested *in vitro* for their capacity to stimulate porcine peripheral blood monomorphonuclear cells (PBMC). Motif B consisting of a nuclease-resistant phosphorothioate guanosines at the 5' and at the 3'-end [$B_s$] consistently enhanced significantly the proliferation of porcine PBMC in comparison with motif A. The latter motif did not induce any proliferation. Methylation of motif B diminished these responses although this was dependent of the company that synthesized this CpG-ODN. Four days of culture with motif [$B_s$] increased the percentage of B cells as well as B cell blasting. Moreover, this CpG-ODN also enhanced the expression of class II MHC in most cultures while there were no changes in percentage of macrophages or in the degree of expression of the macrophage marker. In conclusion, in this study it was shown that 5'-ggTGCATCGATGCAGggggg-3' is a swine-specific CpG-ODN. This motif activates porcine B-cells and deserves further evaluation *in vivo* as a potential immunostimulating adjuvant.

## 5.2. Introduction

The immune system of vertebrates recognizes unmethylated cytidine-phosphate-guanosine (CpG) dinucleotides as a danger signal (Krieg et al., 2001). These CpG's are predominantly present in bacterial DNA. Recently it has been demonstrated that CpG-motifs are recognized by Toll-like receptor (TLR)-9 (Hemmi et al., 2000). Synthetic oligodeoxynucleotides (ODN) containing these CpG-motifs (CpG-ODN) have been extensively studied *in vitro* in mice and man for their immunostimulatory activity on different immune cells. For B cells, CpG-ODN have been shown to directly stimulate their proliferation and their immunoglobulin secretion, to increase the expression of MHC class II antigens and co-stimulatory molecules (Krieg et al., 1995) and to decrease apoptotic cell death (Yi et al., 1998). For macrophages and dendritic cells, activation with induction of IL-12, TNF-α, IFN-α and IFN-γ has been demonstrated (Jakob et al., 1998;

Stacey et al., 1996, Ballas et al., 1996, Cowdery et al., 1996). Activation of human T cells by CpG-ODN requires the presence of APC (Kranzer et al., 2000) while this is not the case in mice (Iho et al., 1999). It is known that a Th1 cytokine-based immune response is induced when CpG-ODN are used as an adjuvant in vaccine formulation (Roman et al., 1997). Although it is believed that CpG-ODN recognition is conserved across mammalian species, there is evidence that the recognition of different CpG-motifs varies between species (Kanellos et al., 1999). Even within species two distinct CpG-motifs may activate different cell populations (Verthelyi et al., 2001). Today, there is limited information concerning the biological effects of CpG-ODN in pigs. Kampstrup et al., (2001) demonstrated *in vitro* that the TGCATCGATGCAG motif was optimal for proliferation of PBMC. This was accompanied with an enhanced secretion of IL-6 and IL-12. We demonstrated that the CpG-ODN GCTAGACGTTAGCGT, that has immunostimulatory properties in mice, is immunostimulating in pigs as well when used as an adjuvant for intramuscular immunisation (IM) (Van der Stede et al., 2002, Chapter 4). The present study was undertaken to screen different CpG-ODN sequences for their *in vitro* capacity to stimulate proliferation of PBMC from outbred conventional pigs and to identify the proliferating cell populations.

## 5.3. Material and Methods

### 5.3.1. Oligodeoxynucleotides

Two motifs (motif A and motif B) of CpG-ODN were synthesized by two different companies (Biosource Europe (Nivelles, Belgium) and Eurogentec SA (Seraing, Belgium)). They are presented in Table 5.1.

**Table 5.1.:** CpG-ODN (motif A and B) and its modifications used for *in vitro* proliferation assays.

| Motif | Name[2] | ODN Euro[1] | ODN Bio[1] | Sequence | Backbone | -$CH_3$[¶] |
|---|---|---|---|---|---|---|
| Motif-A | A | 565 | 572 | GCTAGACGTTAGCGT | O | - |
| | [$A_S$] | 459 | 7972 | gg GCTAGACGTTAGCGTggggg | SOS | - |
| | rev [$A_S$] | 259 | 7974 | ggGCTAGAGCTTAGGCTggggg | SOS | - |
| | $A_m$ | 566 | 573 | GCTAGAZGTTAGZGT | O | + |
| | [$A_S$]$_m$ | 258 | 7973 | gg GCTAGAZGTTAGZGTggggg | SOS | + |
| | rev [$A_S$]$_m$ | 260 | 7975 | ggGCTAGAGZTTAGGZTggggg | SOS | + |
| Motif-B | B | 567 | 574 | TGCATCGATGCAG | O | - |
| | [$B_S$] | 261 | 7976 | gg TGCATCGATGCAGggggg | SOS | - |
| | [$B_S$]$_m$ | 262 | 7977 | ggTGCATZGATCCAGggggg | SOS | + |

[1]the numbers were assigned based on the synthesis run in which the ODN were designed and produced by the company (Eurogentec and Biosource).[2] ODNs are grouped according to sequence and backbone: A = motif A in its phosphodiester backbone, [$A_s$] = motif A in its phosphorothioate (S) backbone followed by its reversed (rev [$A_s$]) and the methylated form ([$A_s$]$_m$). Phosphodiester nucleotides (O) are shown in upper case and phosphorothioate (S) nucleotides are shown in lower cases (g-stretch). Z = 5-methylcytosine.[¶]:methylation is indicated with +

ODN's that contain an unmodified phosphodiester bacbone are indicated as A and B while [$A_S$] and [$B_S$] have a SOS-backbone consisting of a nuclease-resistant phosphorothioate guanosines at the 5' and the 3'-end and with a phosphodiester in the center. Reversed GpC-ODNs (rev [$A_S$]) or methylated ($A_m$, [$A_S$]$_m$, [$B_S$]$_m$ and rev [$A_S$]$_m$) CpG-ODN were used as controls since they should be less immunostimulatory. The motif ATCGAT (motif B) was tested since Kamstrup et al., (2001) demonstrated *in vitro* that it was highly immunostimulatory for pig PBMC. The GACGTT motif (motif A) was *in vitro* shown to be immunostimulatory for mice and *in vivo* for pigs (Van der Stede et al., 2002, chapter 4).

All ODN were diluted in TE buffer (10mM Tris, pH 7, and 1mM EDTA) at a concentration of 1 mg/ml and stored at –20°C. For *in vitro* proliferation assays, the CpG-ODN were diluted to optimal concentrations in leukocyte medium (RPMI-1640 supplemented with penicillin (100

IU/ml), streptomycin (100 µg/ml), kanamycin (100 µg/ml), L-glutamin (200 mM), sodiumpyruvate (100mM), non-essential aminoacids (100mM), β-mercaptoethanol ($5.10^{-5}$ M) and 1% (vol/vol) serum of a colostrum-deprived piglet.

5.3.2. Preparation of cells and cell culture

Blood, from four weaned 5-8 weeks old outbred pigs of both sexes (Piétrain x Belgian Landrace) was sampled from the jugular vein on Alsever's solution (50% vol/vol). PBMC were isolated by density gradient centrifugation using Lymphoprep (Invitrogen, Merelbeke, Belgium) as described previously (Van den Broeck et al., 1999a). After lysis of the erythrocytes in ammoniumchloride (0.8% [wt/vol]), the PBMC were washed 3 times and subsequently resuspended at the concentration of $4.10^6$ cells/ml in leukocyte medium.

5.3.3. Lymphocyte proliferation assays

PBMC were brought into wells of flat bottem microtiter plates (Geiner Bio One) at 4 x $10^5$ cells/well in 200 µl leukocyte medium to which 20 or 10 µg/ml of an ODN with or without a suboptimal final concentration of concanavaline A (ConA) of 0.5 µg/ml. Each condition was tested in triplicate on cells of four different pigs. The cells were incubated for 72 h at 38°C in a humidified atmosphere with 5% $CO_2$ after which one µCi of $^3$H-thymidine (Amersham ICN, Bucks, UK) was added for 18 h. Then, the cells were harvested onto glass fiber filtermats (Perkin Elmer, Life Science, Oosterhout, Netherlands). The radioactivity incorporated into the DNA was measured using a β-scintillation counter (Perkin Elmer). Stimulation indices (SI) were calculated as the quotient of the mean cpm of the stimulated PBMC (triplicates of cultures of cells with addition of ODN with or without ConA) over the mean cpm of the media control (neither ODN nor ConA). PBMC were stimulated with 0.5 µg/ml ConA as a positive control.

5.3.4. Phenotypic analysis of ODN-stimulated PBMC

The PBMC of two pigs were cultured for 4 days in 24-well plates (Nunc) at 1 x $10^7$ cells/well in 2 ml leukocyte medium alone, in leukocyte medium supplemented with 10 µg/ml of an ODN or with 10 µg/ml ConA. On days 0 and 4, the cells were stained for 15 min on ice in 100 µl of staining medium (RPMI-1640 supplemented with 2% immunoglobulin free horse serum and

0,02 % sodium azide) with one of the following monoclonal antibodies (MAb): MAb specific for swine B cells (clone 28.4.1, α-IgM Van Zaane et al., 1987), swine monocytes and macrophages (clone 74-22-15, Pescovitz et al., 1984; Lunney et al., 1993), swine T cells (clone PPT3, α-CD3 , Yang et al., 1996) and swine SLA-DR (MHC II (clone MSA-3, Hammerberg et al., 1986) and a control MAb specific for penicillin (clone 19C9, Cliquet et al., 2001). Subsequently, the cells were washed twice with staining medium and resuspended in staining medium containing FITC-conjugated antibody (sheep anti-mouse $(Fab)_2$, Sigma-Aldrich). Cells were incubated for 45 min on ice, washed twice with staining medium and once with PBS (150 mM, pH 7.4). Flow cytometric analysis was conducted using a FACScalibur flow cytometer (Becton-Dickinson, San Jose, California) equipped with a 15-mW air-cooled argon ion laser with an excitation wavelength of 488 nm. Emission fluorescence was detected with a 530/30 nm bandpass filter for FITC. At least 10,000 cells were analysed per sample. Propidium iodide exclusion (FL3) was used to select for viable cells. Cells expressing a specific antigen are presented as the percent of the total number of viable analysed cells.

### 5.3.5. Statistical analysis

The data from the lymphocyte proliferation assays were analysed using the statistical software program SPSS. A one-way ANOVA was used to estimate the effect of ODN on proliferation. SI was used as the dependent variable and was $\log_{10}$ transformed and treatment was the factor. The Bonferroni adjustment for multiple comparisons was applied to evaluate the significant differences among the ODN-sequences (treatment). $P < 0.05$ was considered as statistically significant.

## 5.4. Results

5.4.1. Immunostimulatory effects of ODN on porcine PBMC

Both CpG-ODN motifs (A and B) and their modifications were synthesized in two different companies and screened for the ability to induce lymphocyte proliferation (Table 5.2.). Spontaneous proliferation (cpm of control medium cultures) varied between 197 (animal 2) and 1224 cpm (animal 4). The cpm after addition of ConA (without ODN) varied between 6,341 and 40,977. ODN [$B_S$] (nrs 7976 and 261) consistently induced a proliferative response in PBMC with mean SIs significantly greater ($P<0.05$) than that induced by other ODNs (Fig. 5.1). For the ODN of Eurogentec (ODN 261), the SIs were even higher using a dose of 10 µg/ml than with 20 µg/ml while this was not the case for the same ODN of Biosource (ODN 7976). Surprisingly, methylation of CpG-dinucleotide in ODN 7976 by Biosource ([$B_S$]$_m$) still induced a significant proliferation using 20 µg/ml in comparison with other ODN (SI ranged from 4.7-21.3) (Fig. 5.1A). However, this was not the case using a dose of 10 µg/ml ODN (SI ranged from 1,4-3.9) (Fig. 5.1B). The mean SI of ODN 7977 was not significantly lower than the mean SI of ODN 7976 while ODN 262 of Eurogentec (equivalent of ODN 7977), showed significantly lower SIs than ODN 261. Here again proliferation was higher using 10 µg/ml ODN 262. All other ODN did not display any proliferation, except for motif B (ODN 574) in animal 1 and motif rev[$A_s$] (ODN 7972) in animal 4.

**TABLE 5.2.**: Lymphocyte proliferation responses by porcine PBMC of four pigs stimulated with ODNs

| Stimulation | | | 20 µg/ml ODN | | | | 10 µg/ml ODN | | | | 10µg/ml ODN + 0.5 µg/ml ConA | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ODN** | | **Pig nr** | **1** | **2** | **3** | **4** | **1** | **2** | **3** | **4** | **1** | **2** | **3** | **4** |
| **MOTIF-A** | | | | | | | | | | | | | | |
| Eurogentec | 565 | A | - | - | - | - | - | 1.1 | 1.2 | 1.5 | 102 | 99 | 34 | 30 |
| | 459 | [$A_s$] | - | - | NT | NT | - | - | NT | NT | NT | NT | NT | NT |
| | 259 | rev[$A_s$] | - | 1.3* | - | - | - | 1.3 | 1.23 | - | 100 | 98 | 31 | 26 |
| | 566 | $A_m$ | - | - | - | - | - | 1.3 | - | - | 108 | 100 | 28 | 25 |
| | 258 | [$A_s$]$_m$ | - | 1.1 | - | - | 1.1 | 1.1 | 1.1 | - | 87 | 91 | 25 | 25 |
| | 260 | rev[$A_s$]$_m$ | - | 1.1 | - | - | - | - | 1.1 | - | 111 | 109 | 34 | 28 |
| Biosource | 572 | A | - | - | - | - | - | - | 1.1 | - | 103 | 83 | 33 | 22 |
| | 7972 | [$A_s$] | - | - | - | 2.6 | 1.1 | - | - | - | 143 | 102 | 44 | 28 |
| | 7974 | rev[$A_s$] | - | - | - | 1.1 | - | - | - | - | 109 | 89 | 35 | 22 |
| | 573 | $A_m$ | 1.1 | 1.1 | - | - | - | - | 1.1 | - | 113 | 80 | 36 | 24 |
| | 7973 | [$A_s$]$_m$ | 1.1 | 1.1 | 1.2 | - | 1.1 | - | - | - | 146 | 137 | 62 | 40 |
| | 7975 | rev[$A_s$]$_m$ | - | - | - | - | 1.1 | - | 1.1 | - | 116 | 77 | 28 | 24 |
| **MOTIF-B** | | | | | | | | | | | | | | |
| Eurogentec | 567 | B | - | - | - | - | - | - | - | - | 118 | 126 | 29 | 30 |
| | 261 | [$B_s$] | 18.5 | 24.9 | 21.1 | 7 | 34 | 50.5 | 28.3 | 8 | 213 | 302 | 215 | 46 |
| | 262 | [$B_s$]$_m$ | - | - | - | - | 2 | 1.6 | 1.4 | 1.1 | 127 | 170 | 138 | 29 |
| Biosource | 574 | B | 2 | - | - | - | - | - | - | - | 145 | 96 | 40 | 26 |
| | 7976 | [$B_s$] | 28.5 | 44.3 | 7.6 | 9.3 | 4.5 | 8 | 2.1 | 2.5 | 234 | 330 | 131 | 45 |
| | 7977 | [$B_s$]$_m$ | 11.5 | 21.3 | 4.7 | 7.6 | 2.4 | 3.9 | 1.4 | 2.3 | 202 | 289 | 91 | 38 |
| 0.5 µg ConA without ODN | | | 127* | 105 | 34 | 28 | | | | | | | | |
| cpm Medium[#] | | | 323 | 197 | 185 | 1224 | | | | | | | | |

-:SI < 1 ; NT: not tested; *: the stimulation index for each animal was calculated by dividing the mean cpm of the stimulated cells by the mean cpm of the medium cultures. #: counts per minute of medium cultures of each pig (1,2,3 and 4)

The same tendency was observed when the PBMC were stimulated with suboptimal concentrations of ConA and supplemented with 10 µg/ml ODN. Significantly higher SIs ($P<0.05$) were observed after addition of motif [Bs] (ODN 261) in comparison with all other ODNs except for $[Bs]_m$ (ODN 7977). Besides ODN 261, also ODN 7976 and their methylated forms (ODN 262 and ODN 7977) showed consistently higher SIs in comparison with PBMC cultures stimulated with ConA only (Table 5.1.), but this was not statistically significant (Fig. 5.1C).

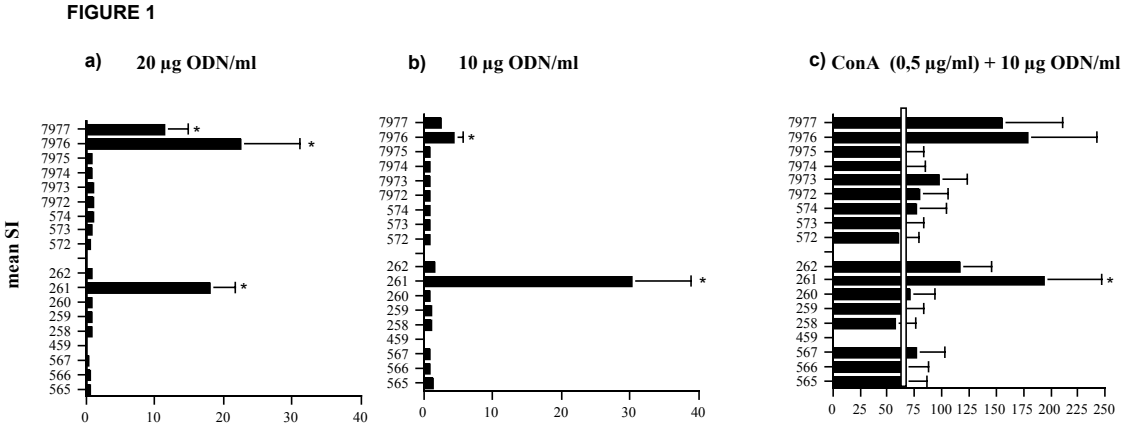


**FIGURE 5.1:** Mean SI ± SEM of porcine PBMC stimulated with 10 µg/ml ODN (Fig 5.1A) , 20 µg/ml ODN (Fig 5.1B) or ConA (0,5 µg/ml) + 10 µg/ml CpG-ODN (Fig 1C). The cells were incubated in triplicate for 72 hours, labelled with $^3$H-thymidine, and incubated for an additional 18 hours. The stimulation index (SI) was calculated by dividing the mean cpm of the stimulated wells by the mean cpm of the medium wells for each animal. For each treatment, the mean SI ± SEM was the mean of the SIs of 4 animals. Fig. 1A:*The mean SI of ODN 7977, ODN 7976 and ODN 261 are significantly higher ($P<0.05$) than all other ODNs. Fig. 5.1.B: *The mean SI of ODN 7976 and ODN 261 are significantly higher ($P<0.05$) than all other ODNs. Fig. 5.1.C :*The mean SI of ODN 261 is significantly higher than all other ODNs except ODN 7977, 7976 and 262. The mean SI for ConA only (73,5 ± 24,5) is shown as a white bar.

## 5.4.2. Phenotypic analysis of CpG-ODN-stimulated PBMC

To define which cell types were responding to the immunostimulatory CpG-motifs, PBMC of 2 pigs were cultured for 4 days with 10 µg/ml ODN of Biosource or with ConA (T-cell activator). On day 0 the PBMC population included 64,2 to 70 % T cells ($CD3^+$) and 13.9 to 19% B cells ($IgM^+$). Fig. 5.2 presents the data for PBMC of one pig. After 4 days of culture with [Bs] (ODN 7976) an increase between 6.3 to 12.1 % (Fig 5.2) of $IgM^+$B cells was observed while the increase was lower after 4 days of culture with $[Bs]_m$ (ODN 7977), with a range between 2 and 4.6

% (Fig 5.2), in comparison with medium alone. An increase of 3.5 % of activated B-lymphocytes was observed in one animal after culture with [Bs] (Fig. 5.2.). There was almost no increase in activated $IgM^+$ B cells in cultures with other ODN (data not shown). The percentage of activated $CD3^+$ T cells was 12.5 % (between 11.8%-13.2%) lower after 4 days of culture with [Bs] in comparison with medium alone while this decrease was lower (mean of 5%) with its methylated form $[Bs]_m$. PBMC stimulated with ConA had a 81 % increase in activated T cells relative to medium alone, whereas almost no B cells could be detected in the ConA stimulated cultures.

The increase in the percentage of MHC $II^+$ cells ranged between 2 and 4.6 % after 4 days of culture with [Bs] in comparison with non-stimulated cultures (no addition of ODN, Fig. 5.3). This was not due to an increase of the percentage of activated MHC $II^+$ cells since less activated MHC $II^+$ cells were observed in ODN-activated cultures. There was however an increased mean relative fluorescence intensity for MHC II on the [Bs] stimulated cultures indicating an increased expression of MHC class II molecules (Fig. 5.3).

There were no changes in percentage or in the degree of expression of the macrophage marker.

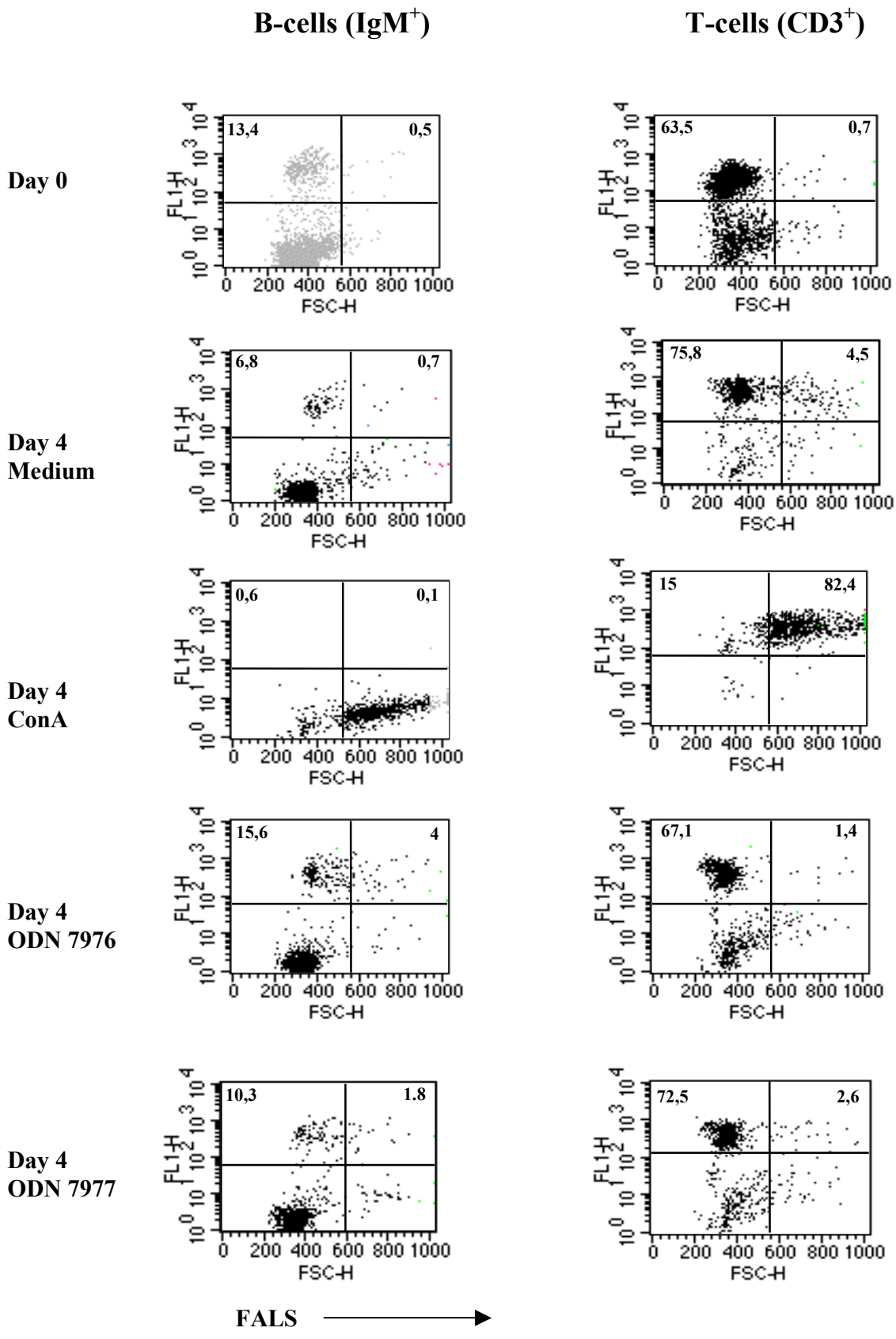


**FIGURE 5.2:** 'Fluorescence-forward scatter profiles' for $IgM^+$ B-cells and $CD3^+$ T-cells among ODN activated PBMC. PBMC stimulated with 10 µg/ml ODN 7976, with 10 µg/ml ConA or not stimulated. The percentage of $IgM^+$ and $CD3^+$viable cells in the upper right quadrant are defined as activated on the basis of increased cell size measured by forward angle light scatter (FALS). Green fluorescence (FL1) was used to identify surface $IgM^+$ and $CD3^+$ cells.

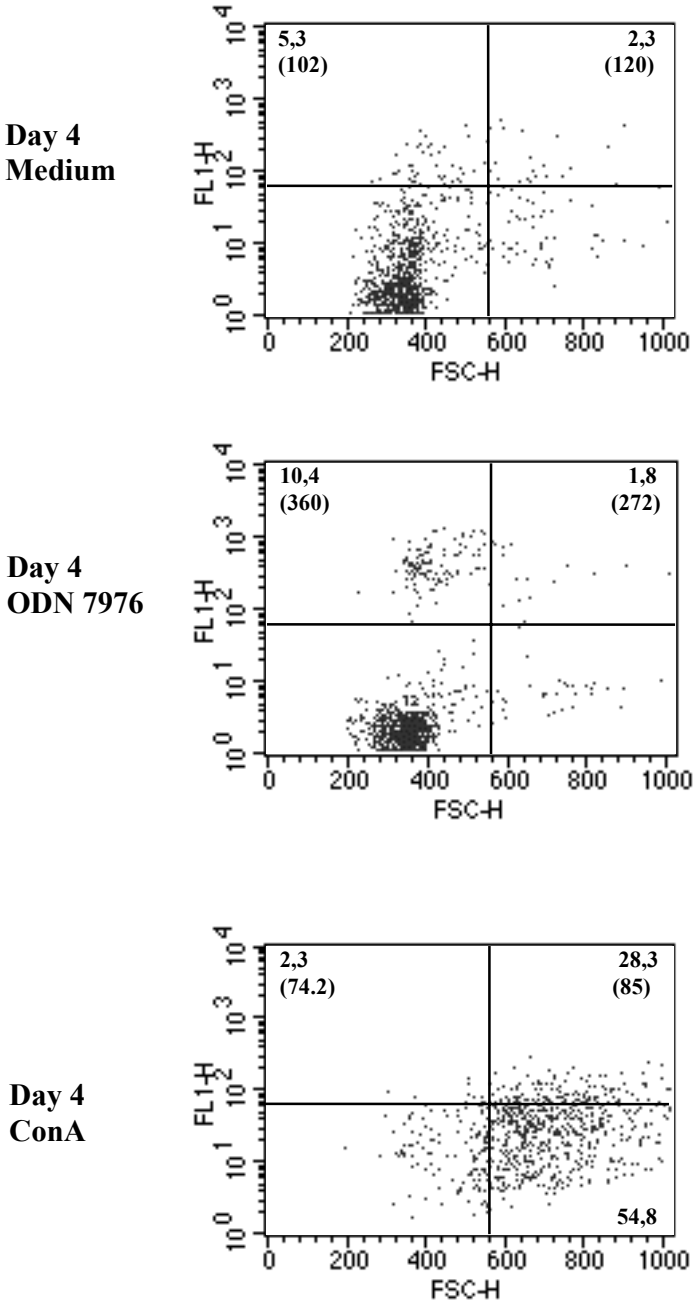


**FIGURE 5.3:** 'Fluorescence-forward scatter profiles' for MHC class II on PBMC 4 days after stimulation with 10 µg/ml ODN 7976, 10 µg/ml ConA or without stimulation (medium). The numbers in the upper quadrants present the percentage of MHC-II$^+$ viable cells. The mean fluorescence intensity (MFI, FL1, log scale) is written between brackets.

## 5.5. Discussion

Originally, it was believed that CpG-ODN recognition is conserved across several mammalian species. However, nowadays typical immunostimulatory CpG-ODN-sequences have been characterized for mice (Krieg et al., 1995), humans (Verthelyi et al., 2001) and primates (Hartmann et al., 2000). Recently, a phosphorothioate CpG-ODN containing the palindromic hexamer 5'-ATCGAT-3' has been shown to induce optimal proliferation of porcine PBMC and induce secretion of IL-6, IL-12 and TNF-α (Kamstrup et al., 2001). Moreover, an *in vivo* study injecting pigs intramuscularly with 500 µg of the phosphodiester mice-specific CpG-ODN containing a 5'-GACGTT-3' motif and ovalbumin (OVA) was able to enhance the OVA-specific antibody serum response as well as the OVA-specific IgA response in mucosal secretions. In addition, the motif induced a significant increase in the proliferation of spleen cells, lymph node cells and PBMC (Van der Stede et al., 2002). The present study evaluated this mice-specific motif A and swine-specific motif B *in vitro* in a lymphocyte proliferation assay by comparing them with control ODN in which the CpG-dinucleotide is reversed into GpC-ODN (rev[$A_s$] and rev[$B_s$] or in which the CpG-dinucleotide was methylated ([$A_s$]$_m$ and [$B_s$]$_m$). Furthermore, the effect of the backbone modification, addition of a nuclease resistant (phosphorothioate nucleotides) leader and tail ([$A_s$] and [$B_s$]) was analysed.

The ODN that induced the highest proliferation of PBMC from the 4 pigs was the one reported by Kamstrup et al. (2001) namely [$B_s$] (= 5'-TGCATCGATGCAG-3'). Our data indicate that the addition of G's at the 5'- and 3'-end, even if these G's are phosphodiester nucleotides, is required for activating PBMC since motif B (=5'-TGCATCGATGCAG-3'), in contrast with ODN D25 (=5'-GGTGCATCGATGCAGGGGGGG-3') as described by Kamstrup et al. (2001) didn't induce any proliferative response. The addition of this G-stretch at both ends may be immuno-enhancing by helping the non-specific uptake of the ODN by APC (Pistesky and Reich 1998; Wloch et al., 1998). Recently, it was shown *in vivo* that even a poly-G stretch at the 3'-end of the ODN strongly enhanced the uptake of CpG-ODN (Dalpke et al., 2002). However, Lipford and co-workers (2000) showed that extension of CpG-ODN with a poly-G strech at either 3' or 5' end impaired the TNF-α, IL-6 and IL-12 secretion by a macrophage cell line (J744). Yet, this G-stretch, and not the CpG-motif itself, is responsible for direct costimulation of purified T cells subjected to TCR cross-linking (Lipford et al., 2000), for moderate B-cell proliferation (Liang et al., 1996) and for NK-cell activation (Ballas et al., 1996) and may account for better proliferation of the PBMC.

The mice-specific phosphodiester motif A (=5'-GCTAGACGTTAGCGT-3') did not induce any proliferation of porcine PBMC at any dose tested. This is a surprising finding as this

sequence and even its control GpC-ODN sequence enhanced the antigen-specific *in vitro* proliferation of PBMC after *in vivo* administration (IM injection of 500 μg, Van der Stede et al., 2002). Moreover, the use of this ODN as adjuvant in an intramuscular vaccination of pigs with F4-fimbriae has been shown to reduce the faecal excretion of $F4^+$ enterotoxigenic E. coli following (ETEC) challenge (Van der Stede et al., 2003 chapter 7). This indicates that CpG-ODN sequences which do not enhance *in vitro* proliferation can effectively been used *in vivo* as an adjuvant. As a consequence, *in vitro* proliferation assays may not be as conclusive to evaluate the immunostimulatory effect of CpG-ODN *in vivo*. Different cell populations may be activated *in vivo* compared to the *in vitro* situation. Dentritic cells (DC) for example, which are primary target cells for the CpG-ODN (Jakob et al., 1998; Sparwasser et al., 1998), are not in high frequency in the circulation (Carrasco et al., 2001). Nevertheless, our and Kampstrup et al. (2001) *in vitro* studies show that 5'-ggTGCATCGATGCAGggggg-3'is an interesting candidate to be analysed *in vivo* for its immunostimulatory properties and its capacity to protect against different infectious diseases.

Considerable heterogeneity was observed in the response of the individual pigs to various CpG-ODN. Using conventional outbred pigs we observed lower proliferation using the same ODN as compared with the *in vitro* proliferation assays of Kamstrup et al. (2001) using NIH inbred minipigs. However, using these NIH inbred minipigs they observed also individual differences indicating no link between MHC II haplotype and CpG-ODN responsiveness. These individual differences are also observed in man, an other outbred population (Verthelyi et al., 2001).

Methylation of the CpG-dinucleotide normally reduces or eliminates the immunostimulatory capacity of the CpG-ODN. In the present study, methylation of [$B_s$] had different results depending on the company that synthesized the ODN: ODN 7977 still induced significant proliferation in a dose dependent way while this was not the case for ODN 262. This indicates that one must be careful interpreting effects of CpG-ODN with the same sequences but produced by different companies. Probably, the methylation in ODN 7977 was not as complete as it was in ODN 262.

ODN [$B_s$] (7976) enhanced the percentage of $IgM^+$ porcine B cells of MHC $II^+$-cells and enhanced B-cell blasting (Fig.5.2). As a consequence there was a decrease in the percentage of porcine T cells This indicates that this CpG-motif induces porcine B cells to proliferate, as has been observed for mice-specific ODN in mice (Yi et al.,1998, Krieg et al., 1995). In mice it has been demonstrated that ODN-stimulated B cells resist apoptosis whereas the decrease in the number of $IgM^+$ B cells in medium cultures is due to spontaneous B-cell death (Illera et al., 1993). Whether the effect of CpG-ODN on B cells occurs directly or indirectly via monocytes is not

clear. Depleting the monocytes in a bovine PBMC culture dramatically reduced the proliferative response induced by an optimal CpG-ODN sequence (Pontarello et al., 2002). This is in contrast with studies in mice (Krieg et al., 1995) and man (Hartmann and Krieg 2000). Results showed that [$B_s$] increased the percentage of MHC $II^+$-cells as well as the expression of MHC II molecules on porcine PBMC. In pigs class II antigens are expressed on B cells, activated macrophages but also on a subset of $CD4^-CD8^+$ and $CD4^+CD8^+$ T cells (Lunney et al., 1987; Saalmüller et al., 1991). As, no difference in the percentage of macrophages (MAb 74-22-15) and a decreased percentage of $CD3^+$-cells (all T cells) was observed, it is expected that clonal expanded B cells are responsible for the increased percentage of MHC $II^+$-cells and the increased expression of MHC class II antigens.

In conclusion, it was shown that motif B (5'-TGCATCGATGCAG-3') in its phosphorothioate backbone is mitogenic for porcine B cells and increases their percentage as well as the expression of MHC class II molecules. Methylation of this motif reduces this effect although this may differ depending on the manufacturer.

# CHAPTER 6

# ANTIGEN DOSE MODULATES THE IMMUNOGLOBULIN ISOTYPE RESPONSES OF PIGS AGAINST INTRAMUSCULARLY ADMINISTERED F4 FIMBRIAE

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## 6.1. Abstract

Parenteral immunisation normally induces a systemic antibody response characterized by high IgG and low IgA responses. In the present study, the effect of different doses of F4-fimbriae on the isotype-specific antibody response after intramuscular immunisation was studied in pigs. Pigs were injected twice with a 9 weeks interval with either 1, 0.1 or 0.01 mg of F4-ETEC fimbriae. The dose of 1mg F4 induced significantly lower primary F4-specific IgG and IgM responses than the doses of 0.1 and 0.01 mg F4 but primed for an enhanced F4-specific IgM serum antibody response after the booster immunisation. Furthermore, the dose of 0.1 mg induced the highest F4-specific IgA serum response which was significantly higher than after injection with 0.01 and 1mg F4. Moreover, both lower doses (0.1 and 0.01 mg) showed a higher number of F4-specific IgA and IgG antibody secreting cells (ASC) in the local draining lymph nodes of the pigs. This study demonstrated that low doses of purified F4-ETEC fimbriae, especially the 0.1 mg dose, are optimal for inducing F4-specific IgA responses after IM immunisation.

## 6.2. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) that express F4 (K88) fimbriae are an important cause of neonatal diarrhea in newborn and post-weaning diarrhea (PWD) in newly-weaned piglets (Klemm et al., 1985; Cox et al., 1987). These F4-fimbriae mediate adhesion to the brush border receptors on porcine intestinal epithelial cells, the initial step in the establishment of the enteric infection (Nagy et al., 1985). The F4-fimbriae are proteinaceous filamentous adhesins that are expressed on the surface of the bacteria. Three serological variants of F4 exist namely: F4ab, F4ac and F4ad (Guinée et al., 1979; Mooi et al., 1984; Gaastra et al., 1986). However, F4ac is most commonly associated with diarrhea in pigs (Gonzalez et al., 1995; Westerman et al., 1988). Rutter and co-workers (1972) were the first to show that neonatal diarrhea can be prevented by passively protecting the piglets via parenteral (intramuscular) immunisation of pregnant dams with fimbriae. This was confirmed by others using commercial vaccines, containing a combination of heat- or formalin-killed *E. coli* bacteria, LT enterotoxin and purified fimbriae (Nagy et al., 1985; Moon et al., 1993; Osek et al., 1994,). At weaning however, this maternal protection stops and pigs become susceptible again for $F4^+$-ETEC and can develop PWD. In order to protect the piglets against PWD, the induction of an acquired mucosal immunity is necessary. This protection is given by IgM, IgA and IgG antibodies against fimbriae and/or enterotoxins (Porter et al., 1974; Bianchi et al., 1992).

It was shown that oral administration of F4ac fimbriae induced a protective mucosal immune response in 6-week-old weaned piglets bearing the F4-receptor on their enterocytes (Van den Broeck et al., 1999a). However, mucosal immunity will be difficult to induce in young pigs in the presence of passively transferred lactogenic antibodies from the sow. Therefore, parenteral vaccination of piglets during the suckling period could be more ideal. It was shown that intramuscular immunisation of piglets with 1 mg of crude F4 fimbriae in incomplete Freund's adjuvant evoked an IgA serum antibody response only slightly lower than that following an ETEC infection (Van den Broeck et al. 1999b).

In the present study, a great effect of the dose of intramuscularly injected F4-antigen was seen on the isotype-specific antibody response in serum and in the local draining lymph node.

## 6.3. Materials and Methods

### 6.3.1. Pigs

Nine conventionally reared pigs (Belgian Landrace x Piétrain) from 1 litter on 1 closed farm were seronegative for antibodies against F4 as determined by ELISA. They were weaned at the age of 5 weeks and immediately housed in isolation units where they obtained water and food *ad libitum*.

### 6.3.2. Purification of F4-fimbriae

The fimbriae were purified as described by Van den Broeck and co-workers (1999c). Briefly, fimbriae were isolated by homogenising a bacterial suspension of strain *E.coli* GIS 26 (O149:K91: F4ac, $LT^+Sta^+STb^+$) using an Ultra Turrax at 24,000 rpm for 20 min. The solubilized fimbriae were precipitated with 40 % (wt/vol) ammonium sulphate. The pellet was dialysed against PBS (150 mM, pH 7,4) and thereafter the fimbriae were further purified by by anion exchange chromatography using a Bio-Scale Q5 column (BIO-RAD Laboratories, Eke, Belgium). The total protein concentration was determined using the Bicinchoninic Acid Protein Assay Kit (SIGMA, Sigma-Aldrich, Bornem, Belgium) and purity was assessed by electrophoresis on SDS-12% polyacrylamide slab gels according to Laemmli et al.(1970) using the BIO-RAD Mini-Protean II Electrophoresis cell. These purified F4-fimbriae were used to immunise the pigs.

### 6.3.3. Experimental design

Nine pigs were intramuscularly (IM, m. gastrocnemius) immunized at the age of 8 weeks with different doses of F4-fimbriae and divided into 3 groups. The pigs received either 1mg (n=3, 1 mg group), 0.1 mg (n=3, 0.1 mg group) or 0.01 mg (n=3, 0.01 mg group) purified F4-fimbriae supplemented with 2 μg of 1α,25$(OH)_2D_3$. The 1α,25$(OH)_2D_3$ has been able to enhance the IgA serum response against human serum albumin (HSA) and to increase the number of HSA-specific IgA and IgG ASC in the local draining lymph node (Van der Stede et al., 2001). The F4-fimbriae were dissolved in 0.5 ml PBS which was suspended 1/1 (vol/vol) in incomplete Freund's adjuvant (IFA, DIFCO Laboratories). The steroid compound 1α,25$(OH)_2D_3$ (ALEXIS Biochemical's, Zandhoven, Belgium) was dissolved in absolute ethanol as a stock solution (8 μg/ml) and stored at -20°C until used. It was mixed immediately with the F4 antigen and IFA at moment of the IM immunisation.

All these animals received a booster immunisation 66 days post primary immunisation (ppi) with the same dose of F4-fimbriae suspended in IFA.

The immune response after the intramuscular immunisations was evaluated by determining the F4-specific antibody titers in serum (IgM, IgA, IgG), weekly or twice a week until 7 weeks post secondary immunisation (psi). Furthermore ELIspot assays were performed on MC of the local draining lymph nodes to localize and quantify the numbers of F4-specific IgM, IgA and IgG antibody-secreting cells (ASC) between 32 (one animal of each group) and 38 days psi (all other animals n= 6).

### 6.3.4. Samples

#### *6.3.4.1. Serum*

Blood was sampled from the jugular vein at different time points ppi and psi as shown in Fig 6.1. Serum was collected and inactivated at 56°C during 30 min and subsequently treated with kaolin (Sigma Aldrich, Bornem, Belgium) as described by Van den Broeck et al (1999b). Final serum dilutions of 1/10 (vol/vol) were prepared in ELISA dilution buffer (PBS, 150 mM, pH 7.4) + 0.05% (vol/vol) Tween® 20 + 3% (wt/vol) bovine serum albumin (BSA)) whereafter the diluted samples were stored at –20°C until use in ELISA.

*6.3.4.2.Lymph node monomorphonuclear cells*

All pigs were euthanatized by intravenous injection of an overdose pentobarbital (24mg/kg; Nembutal, Sanofi Sant Animale, Brussels, Belgium) followed by exsanguination. Subsequently the popliteal lymph nodes were aseptically collected. After removing surrounding fat, the MC were isolated by gently teasing the tissues apart. The MC were collected in RPMI 1640 (Gibco, BRL) on ice and the erythrocytes were lysed with ammonium chloride (0.8% [wt/vol]). After centrifugation (380g, 4°C, 10 min), the pelleted cells were washed and resuspended in leukocyte medium (RPMI-1640 supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), kanamycin (100 µg/ml), glutamine (200 mM), sodiumpyruvate (100 mM), non-essential aminoacids (100 mM) and 10% [vol/vol] fetal calf serum (FCS, Gibco BRL, Life Technologies, Merelbeke, Belgium) at the concentration of $1.10^7$ cells/ml.

6.3.5. Titration of F4-specific serum antibodies

F4-specific IgM, IgA and IgG titers were determined by an indirect ELISA as described by Van den Broeck and co-workers (1999b). Briefly, the wells of a 96-well microtitre plate (NUNC®, Polysorb Immuno Plates, Gibco BRL) were coated with a F4-specific monoclonal antibody (MAb, IMM-01) at a concentration of 1 µg/ml PBS. After 2 hours of incubation at 37°C, the remaining binding sites were blocked overnight at 4°C with PBS supplemented with 0.2 % Tween® 80 (Merck Eurolab, Leuven, Belgium). Subsequently, F4 fimbriae were added to the wells at a concentration of 30 µg/ml in ELISA dilution buffer (PBS, pH 7.4 + 0.05% [vol/vol] Tween® 20 + 3 % BSA), and incubated for 1 hour at 37°C. Then, the treated sera were added in series of twofold dilutions in ELISA dilution buffer, starting at a dilution of 1/10, and the plates were incubated for 1 hour at 37°C. Thereafter, biotinylated-swine-specific IgM, IgA and IgG MAb (Van Zaane et al., 1987) were added and the plates were incubated for 1 hour at 37°C followed by 1 hour incubation at 37°C with peroxidase-conjugated streptavidin. Between each step, the plates were washed with washing buffer (PBS + 0.2% [vol/vol] Tween® 20). Finally, ABTS-solution, containing $H_2O_2$, was added and after 30 min incubation the optical density was measured at 405 nm ($OD_{405}$).

The antibody titer was determined as the inverse of the highest dilution that still had an $OD_{405}$ higher than the cut-off value. The cut off value was determined by calculating the mean plus 3 times the standard deviation of the optical densities of the 1/10 diluted samples measured at day 0. The cut off values were 0.337, 0.280 and 0.250 for F4-specific IgM, IgA and IgG respectively.

### 6.3.6. ELIspot assay for F4-specific IgA, IgG and IgM antibody secreting cells (ASC)

The ELIspot test was performed as previously described by Van den Broeck and co-workers (1999b). Briefly, coating and blocking of the plates were similar to the F4-specific serum ELISA. Subsequently the MC cell suspensions ($10^7$ cells/ ml leukocyte medium) were added in five wells (100 µl/well), after which the plates were incubated for 3 hours at 37°C in a humidified 5% $CO_2$ atmosphere. The cells were removed by three subsequent washes with washing buffer. Thereafter, the plates were incubated with the swine-specific IgA, IgM and IgG MAb (Van Zaane et al., 1987) coupled to peroxidase for 1 hour at 37°C. Unbound conjugates were washed away by 3 washes with washing buffer. The spots were developed in a substrate solution consisting of 4 volumes of 3-amino-9-ethylcarbozole (AEC) working solution (0.67 ml AEC stock solution (0.4%,[wt/vol] in dimethylformamide) in 10 ml Na-acetate (0.1 M, pH 5.2) + 10µl 30% $H_2O_2$) and 1 volume of 3% [wt/vol] low-melting point agarose gel (BIOzym, Landgraaf, The Netherlands)). Spots were counted after overnight incubation in the dark at room temperature. For each MC suspension, spots in 5 wells ($10^6$ MC/well) were counted, to obtain the number of isotype-specific ASC per 5 x $10^6$ MC.

### 6.3.7. Statistical analysis

Differences in $\log_2$ antibody serum titers (IgM, IgA and IgG) between the groups were tested for statistical significance using General Linear Model (SPSS 7.5 for windows, General Linear Model: Repeated Measures Analysis of Variance). Differences in the number of F4-specific ASC 32 to 38 days psi were tested for statistical significance by a one-way ANOVA test. Statistical significance was assessed at a P value of $< 0.05$.

## 6.4. Results

### 6.4.1. Effect of doses of F4 fimbriae on serum antibody responses

The doses of 0.1 and 0.01 mg purified F4 induced a significantly ($P<0,05$) higher F4-specific IgG and higher IgM and IgA responses after the first immunisation in comparison with the 1mg dose group (Fig. 6.1). The IgM titer peaked 10 days ppi in all groups and was highest in the 0.01 mg group (with $P<0.05$ at 15 days ppi) followed by the 0.1 mg group and then declined to baseline values from 25 days ppi onwards. The IgG titer in both lower dose groups reached its peak 5 days later, with geometric mean titers (GMT) ranging from 640 ($2^{9.3}$) to 2560 ($2^{11.3}$) and thereafter they declined until the second immunisation. Both lower dose groups showed also a primary IgA response which was highest for the 0.1 mg group. This group showed a gradually increase of the F4-specific IgA titer until the moment of the booster immunisation while in the 0.01 mg group the IgA titer peaked at 25 days ppi and declined slowly until the moment of the second immunisation. No IgA serum antibody response could be detected in the 1 mg group.

In contrast with the first immunisation, the second immunisation with 1 mg F4 induced a strong IgM, IgA and IgG booster response while lower doses induced an IgA and IgG but no IgM booster response. Animals that received 1mg purified F4 antigen showed a great increase (mean GMT from $2^{4}$-$2^{10.32}$) of the F4-specific IgM with a peak 8 days psi, which was significantly higher ($P<0.05$) than for both lower-dose groups. This IgM titer almost disappeared 30 days psi. All three groups showed an IgG and IgA booster response with their peak 12 days psi. As for the IgM response, the 1mg group showed a significant increase (>500-fold) increase of the F4-specific IgG titer and a 30-fold increase of the F4-specific IgA titer. The 0.01 mg group displayed a 150-fold increase of the IgA titer and a 6-fold increase of the IgG titer. The 0.1 mg group showed the highest increase ( ± 320-fold) of the F4-specific IgA titer and a 25-fold increase of the IgG titer. The F4-specific IgA titers in the 0.1 mg group were significantly higher ($P<0.05$) at some time points (8, 12, 15 and 22 days psi) than in the other groups. Both, the F4-specific IgA and IgG titers, remained high until euthanasia 32 to 38 days psi.

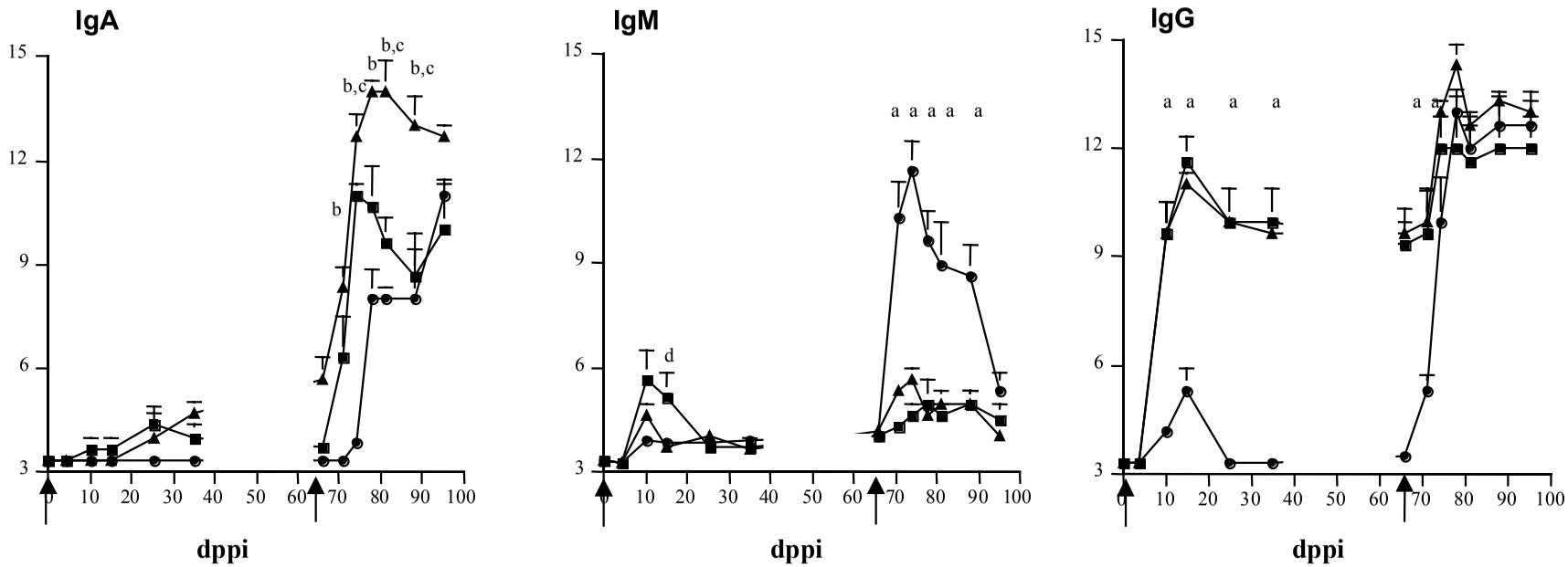


**FIGURE 6.1:** Kinetic of the F4-specific IgM, IgA and IgG serum response following intramuscular immunisation of pigs with either 1mg purified F4-fimbriae (●, n=3), 0.1 mg F4 (□, n=3), and 0.01 mg F4 (■, n=3) all supplemented with 2 µg of 1α,25$(OH)_2D_3$. The booster immunisation was given 66 days post-primary immunisation (dppi) with the same dose of F4-fimbriae (arrow). The antibody titers are plotted as geometric mean $\log_2$ titers ± the SEM. Significant differences ($P<0.05$) are indicated with letters a-d:

a: statistical difference between the 1mg group (●) and the lower dose (□,■) groups.

b: statistical difference between the 0.1 mg group (□) and the 1 mg F4-group (●).

c: statistical difference between the 0.1 mg group (□) and the 0.01 mg group (●,■).

d: statistical difference between the 0.01 mg group (■) and the other groups (●,□)

6.4.2. Effect of dose on the number of F4-specific ASC in the local draining lymph node

Between 32 and 38 days psi all pigs were euthanatised for enumerating the numbers of F4-specific IgM, IgG and IgA ASC in their local draining lymph nodes (Table 6.1.). Slightly higher numbers of F4-specific IgM ASC were detected in the 0.1 mg group (mean 15 IgM ASC) than in the 1 mg group (mean 12 IgM ASC) and the 0.01 mg group (mean 7 IgM ASC). In contrast, the number of F4-specific IgG ASC was highest in the 0.01 mg group (mean of 709 IgG ASC) followed by the 0.1 mg group (mean of 425 IgG ASC) and the 1mg group (mean of 260 IgG ASC and significantly lower than the 0.01 mg group). Although statistically not significantly different from the other groups, the highest F4-specific IgA ASC were detected in the 0.1 mg group.

**TABLE 6.1.:** F4-specific IgM, IgG and IgA ASC in local draining lymph node (Lnn. popliteus) 32 to 38 days after a second intramuscular immunisation (IM) with different doses of F4 fimbriae.

| | **Number of F4-specific ASC in local draining lymph node per $5.10^6$ MC** : | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | IgM | | | | IgG | | | | IgA | | | |
| Immunisation dose | | | | Total[a] | | | | Total | | | | Total |
| 1 mg (n=3) | 17 | 14 | 7 | 38 | 192 | 178 | 408 | 778 | 7 | 19 | 57 | 83 |
| 0.1 mg (n=3) | 1 | 13 | 31 | 45 | 387 | 252 | 637 | 1276 | 63 | 46 | 23 | 132 |
| 0.01 mg (n=3) | 19 | 0 | 3 | 22 | 862 | 804 | 462 | 2128 | 20 | 0 | 65 | 85 |

[a] : sum of the number of F4-specific isotype-specific ASC of three animals.

## 6.5. Discussion

The present study describes the effect of different doses of intramuscularly injected F4 antigen on the isotype-specific antibody response in pigs. The first injection with F4-fimbriae induced a primary antibody response with low IgM and IgA titers peaking between 10 and 25 days post immunisation and IgG titers reaching the highest level 11 to 23 days post immunisation. This is in accordance with a previous study in which 5-week-old piglets were injected with 1 mg crude F4 antigen (Van den Broeck et al., 1999a) containing approximately 0.74 mg pure F4 (Van den Broeck et al., 1999b). In the present study however, a remarkable dose effect was seen using purified F4. One mg of purified F4 induced lower antibody responses than 0.1 and 0.01 mg F4. That 0.01 mg F4 induced a high and quickly appearing primary IgG serum antibody response with even the highest numbers of IgG ASC in the local draining lymph node indicates that F4 is a very potent antigen. However, increasing the dose above the range of 1mg purified F4-antigen suppressed the primary antibody response. The reason for this suppression is not clear. Apparently, the antibody response against IM injected F4-fimbriae seems to differ between species. Intramuscular injection of 1 mg purified F4 induced significant-F4-specific IgG titers in chickens (Yokoyama et al., 1992) while 5 µg seems to be the 'optimal' dose for mice (Bianchi et al., 1996). That 1 mg crude F4 used by Van den Broeck et al (1999a) did not induce such low IgG and IgA responses could have been due to the lower amount of pure F4 or contamination with LPS that activate macrophages, dendritic cells and induce the release of inflammatory cytokines (Raetz 1993). Moreover, Van den Broeck and co-workers injected IM in the neck. This localization was shown to induce better IgA responses than injection in the back (Vanderpooten et al., 1997). In the present study, pigs were injected in the m. gastrocnemius to have a strictly systemic response.

The second intramuscular immunisation resulted again in remarkable differences between the 1mg group and the lower dose groups in IgG and IgM responses. One mg induced a significantly higher IgM response and a significant increase in IgG so that a serum IgG titer comparable to the titers in both lower dose groups was obtained 12 days later. In the local draining lymph node, however, the number of F4-specific IgG ASC was still the lowest in the 1mg group. A reason for these high IgM responses can be the nature of the F4-antigen. As the F4-antigen is composed of identical repeating FaeG protein subunits (Oudega et al., 1989), the F4-antigen might act as a TI-2 antigen (T-cell independent antigen, Vos et al., 2000). In low concentrations, TI-2 can also induce a T-helper dependent antibody response. This could explain the strong IgG responses seen in the lower dose groups after the first immunisation and is consistent with the high antigen-specific proliferation induced in MC of 4-week old IM primed

(100 µg F4-fimbriae) piglets (Van der Stede et al., 2003 chapter 7). In high concentrations however, these TI-2 antigens can even suppress or block the B cells, which could explain the low IgM and IgG response following the first injection. However, this blocking of the B cells was not complete as strong IgM responses were seen after the second immunisation. Another explanation for this IgM response might be the formation of antigen-antibody complexes upon use of high doses of F4-fimbriae. Such complexes can be entrapped by complement receptors on B cells or macrophages which can lead to accumulation of antigen in the local draining lymph node and in turn may attract more $IgM^+$ antigen-specific B cells (Tite et al., 1979; Ochs et al., 1996). The high IgM responses against F4 are not exceptional as has been shown in several studies. Bianchi et al (1996) showed that intramuscular immunisation of 4 week-old piglets with $2.10^9$ killed $F4^{ac+}$ bacteria induced a low primary IgA, IgM and IgG serum antibody response with a peak 5-10 days post immunisation. Oral challenge of these piglets with live $F4^{ac+}$-ETEC resulted in a strong systemic IgM as well as IgG booster response. In addition, Van den Broeck et al (1999a) showed that oral challenge with crude F4 (3 successive days (2.3mg F4/day/pig) of 18 weeks old IM primed pigs resulted in high numbers of F4-specific IgM ASC in the peripheral blood. Also combinations of oral and parenteral administration with F4 resulted in high IgM antibodies in the colostrum (Chidlow et al., 1979) preventing piglets from neonatal diarrhea. Although, whole bacteria cannot be compared with F4-proteins, we suppose that high doses of F4-fimbriae result in high IgM responses and is consistent with the results in the present study. It indicates that at least more $IgM^+$-B cells are stimulated by high doses of F4-fimbriae.

Fimbriae-specific serum IgA may be a useful indicator for mucosal immune responses against fimbriae (Sarrazin et al., 1997; Van den Broeck et al., 1999a). Moreover, it has been shown that a polymeric IgA response in serum can be produced by parenteral immunisation (Mascart-Lemone et al., 1987). The F4-specific serum IgA was significantly higher after two IM injections with 0.1 mg. In contrast, the IgA response was again lower in the 1mg group following the second immunisation as could be seen in serum and the local draining lymph node indicating that this dose is not optimal for induction of lactogenic immunity in sows by intramuscular immunisations. This indicates that, besides the route of immunisation (Vanderpooten et al., 1997; Todryk et al., 1998), also the antigen dose appears to be essential for a good IgA response. It is conceivable that IM injection of different doses F4 induces distinct patterns of cytokines that promote respectively different T-lymphocyte functions suppressing or stimulating IgA, IgM and/or IgG responses. In mice, immunisation with high doses of soluble protein (high ligand density) results in Th1 differentiation, whereas low doses tend to modulate towards Th2-type responses (Constant et al., 1997) which have been shown to promote the expansion of IgA-producing B-cell (Romagnani, 1997). In conclusion, these results stress the importance of the F4-

antigen dose for modulation of immune responses and consequently construction of vaccines against E. *coli*.

# CHAPTER 7

# REDUCED FAECAL EXCRETION OF $F4^+$-E. COLI BY INTRAMUSCULAR IMMUNISATION OF SUCKLING PIGLETS BY ADDITION OF 1α,25-DIHYDROXYVITAMIN $D_3$ OR CpG-OLIGODEOXYNUCLEOTIDES.

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## 7.1. Abstract

In this study it was analysed whether IM immunisation of piglets with F4 during the suckling period could protect against oral challenge with $F4^+$-*E. coli* and whether addition of $1\alpha,25(OH)_2D_3$ or CpG-ODN could improve this protection.

F4-seronegative F4-receptor positive pigs were divided into four groups of 5 pigs each. The pigs were intramuscularly injected with F4 fimbriae only or supplemented with $1\alpha,25(OH)_2D_3$ ($D_3$-group) or CpG-ODN (CpG-group). The control group received PBS in IFA. Seven days after the second immunisation, all pigs were intragastrically inoculated with 1. $10^{10}$ CFU of $F4^+$-*E. coli*. All F4-injected groups, showed a reduced faecal excretion of $F4^+$-*E. coli*. However, this reduction was only statistically significant in the $D_3$-group 2 days post challenge. Pigs in the latter group showed a secondary antibody response upon challenge, indicating that F4-primed memory B cells were present in the gut-associated lymphoid tissues at that moment.

CpG-ODN, on the other hand, did not enhance the F4-specific antibody response. However, CpG-ODN significantly increased the F4-specific as well as mitogen-induced proliferation of pheripheral blood monomorphonuclear cells indicating a direct or indirect overall effect on T-lymphocytes. In conclusion, supplementation with $1\alpha,25(OH)_2D_3$ or CpG-ODN improved protection against an $F4^+$-*E. coli* infection. This protection was most obvious for $1\alpha,25(OH)_2D_3$ and indicates its potential use in veterinary vaccines against enteropathogens.

## 7.2. Introduction

Enterotoxigenic Escherichia *coli* (ETEC) causes neonatal and post-weaning diarrhoea (PWD) in pigs resulting in high economic losses in many piggeries. ETEC involved in neonatal and PWD can produce F4 and/or F18 fimbriae and LT, STa and/or STb (Nagy et al., 1999). The $F4^+$- *E. coli* strains seem to be the predominant serotype in most countries (Hampson 1994). Neonatal infections can be prevented effectively by passive colostral and lactogenic immunity that can be obtained and increased by vaccination of the sows (Rutter et al., 1974; Deprez et al., 1986; Moon et al., 1988). In contrast, no vaccines are available for PWD. Besides the weaning itself, other factors are involved in the PWD-complex such as mixing of the pigs, changes in environmental temperature, humidity and diet (Miller et al., 1984; Kelly et al., 1990; Scheepens et al., 1994; Jones et al., 2001). Furthermore, weaning is accompanied with the disappearance of the lacteal protection provided by the milk antibodies of vaccinated or naturally infected dams. These protective antibodies inhibit the adhesion of ETEC to brush border receptors of small intestinal epithelium of pigs and can neutralise the enterotoxins produced by ETEC. So, to protect piglets

against PWD the induction of an active mucosal immune response is required. Secretory IgA (sIgA) antibodies play an important role in this protective immunity (McGhee et al., 1992). It has been shown that the steroid hormone 1α,25$(OH)_2D_3$ (Van der Stede et al., 2001) and CpG-oligodeoxynucleotides (Van der Stede et al., 2002) can enhance the antigen-specific IgA response in serum as well as in mucosal secretions after IM immunisation. Consequently, CpG-ODN and 1α,25$(OH)_2D_3$ look promising candidate adjuvants for veterinary vaccines.

1α,25$(OH)_2D_3$, the active metabolite of vitamin D, is a lipophilic steroid hormone and acts through binding and activation of the nuclear vitamin $D_3$ receptor which influences the transcription of numerous genes (Carlberg et al., 1998). In mice, it was also shown that 1α,25$(OH)_2D_3$ modulated a systemically induced immune response towards a mucosal immune response with enhanced homing of antigen-specific IgA and IgG antibody secreting cells (ASC) to the lamina propria of the intestine and lungs (Daynes et al., 1994, Daynes et al., 1996). This modulation was accompanied with an enhanced production of IL-4 and IL-10 (Th2-cytokines). In cattle, 1α,25$(OH)_2D_3$ increased milk IgA, IgM and IgG and IgG1 serum antibodies but decreased serum IgG2 serum antibodies (Reinhardt et al., 1999) while in sheep 1α,25$(OH)_2D_3$ enhanced both IgG1 and IgG2 serum antibodies with moderate effects on the IgA response (Scheerlinck et al., 2001). In general, the steroid hormone 1α,25$(OH)_2D_3$ is classified as a Th2-immunomodulating adjuvant.

In contrast CpG-ODN are classified as Th1-modulating adjuvants in mice and man (Roman et al., 1997). CpG-ODN directly activate monocytes, macrophages and dendritic cells to secrete IL-12, TNF-α and IFN-α (Ballas et al., 1996; Stacey et al., 1996). These cytokines enhance NK-activity with the production of IFN-γ (Cowdery et al., 1996). *In vitro* studies in mice have been shown that CpG-ODN directly stimulate B cell proliferation inducing the secretion of immunoglobulins, IL-6 and IL-10 (Krieg et al., 1995; Yi et al., 1996; Redford et al., 1998; Brown et al., 1998) and modulate these B-lymphocytes towards production of IgG2a. Also immune cells (monomorphonuclear cells) of other species like cattle, sheep (Pontarollo et al., 2002) and pigs (Kampstrup et al., 2001) are activated *in vitro* by CpG-ODN.

The aim of the present study was to evaluate whether IM immunisation of piglets with F4 during the suckling period could protect against oral challenge with $F4^+$-*E. coli* and whether addition of 1α,25$(OH)_2D_3$ or CpG-ODN could improve this protection.

## 7.3. Material and methods

### 7.3.1. Animals

Twenty conventional pigs (Belgian Landrace x Piétrain) derived from three different litters on 1 closed farm were used in the experiment. These pigs were F4 seronegative as determined by ELISA. During the suckling period the pigs remained with their dams on the farm. At 29 days of age they were weaned, transported to the faculty where they were housed in isolation units and obtained water and food *ad libitum*.

### 7.3.2. Bacterial inoculum

The *E. coli* strain GIS 26 (O149:K91:F4ac, $LT^+STa^+STb^+$) was cultured during 24 hours on Brain Heart Infusion Agar (Oxoid, Unipath, Drongen, Belgium) and bacteria were collected by washing the agar with phosphate-buffered saline (PBS, 150mM, pH 7,4) Subsequently, the bacteria were washed once with PBS and the concentration was determined by measuring the optical density at 660 nm ($OD_{660}$). An $OD_{660}$ of 1 equals $10^9$ bact/ml, as determined by counting the colony forming units (CFU). The concentration of the suspension was adjusted to $10^9$ bacteria/ml. The production of F4-fimbriae was tested by agglutination using a F4a-specific MAb (IMM-01). The production of *Escherichia coli* heat-lable enterotoxin was tested using the VET-RPLA kit (TD920, Oxoid, Unipath, Drongen, Belgium).

### 7.3.3. Purification of F4 (K88ac)-fimbriae

The fimbriae were purified as described by Van den Broeck and co-workers (1999). Briefly, the bacteria were cultured in Tryptone Soya Broth (Difco Laboratories, Biotrading, Bierbeek, Belgium) at 37°C for 18 hours. Subsequently the bacteria were collected by centrifugation (3,000 x g, 30 min) and washed in PBS, after which the fimbriae were isolated by homogenising the bacterial suspension followed by a precipitation with 40 % (wt/vol) ammonium sulphate. The pellet was dialysed against PBS and hereafter the fimbriae were further purified by anion exchange chromatography using a Bio-Scale Q5 column (BIO-RAD Laboratories, Eke, Belgium). The F4-fimbriae solution was filter sterilised and the total protein concentration was determined using the Bicinchoninic Acid Protein Assay Kit (SIGMA, Sigma-Aldrich, Bornem, Belgium). Purity was assessed by electrophoresis on 12% SDS-polyacrylamide slab gels as described and contained mainly the major subunits of 27.5 kDa (Laemmli 1970). The purified F4-antigen was used to immunize the pigs and for *in vitro* proliferation assays.

## 7.3.4. Experimental design

### *7.3.4.1. Immunisations*

The pigs of the 3 different litters were allocated at random into 4 groups of 5 piglets each. The pigs were intramuscularly (IM, in the neck) injected at 8 and 25 days of age with 100 µg of F4 in incomplete Freund's adjuvant (IFA, F4-group), 100 µg F4 in IFA supplemented with either 2 µg of 1α,25$(OH)_2D_3$ ($D_3$-group) or 500 µg of CpG-ODN (CpG-group). The control group received PBS in IFA.

The $D_3$-, CpG and F4-group received F4 dissolved in 0.5 ml PBS and 1/1 (vol/vol) suspended in incomplete Freund's adjuvant (IFA, DIFCO Laboratories) with or without their specific supplement. The steroid compound 1α,25$(OH)_2D_3$ (Sigma-Aldrich, Bornem, Belgium) was dissolved in absolute ethanol as a stock solution 40 µg/ml and stored at -20°C until used. CpG-ODN (5'GCT-AGA-CGT-TAG-CGT-3') had a phosphodiester backbone and were stored at –20°C (Biosource Europe) at a concentration of 10 mg/ml sterile DEPC treated water.

Eight days post the second immunisation (dpsi, age of 33 days), all pigs were intragastrically infected with the GIS26 strain as further described.

The effects of the IM immunisations upon the $F4^+$-*E. coli*-challenge were evaluated by analysing (i) weight gain (ii) faecal $F4^+$-*E. coli* excretion, (iii) F4-specific antibody response and (iv) F4-specific proliferation of PBMC.

All pigs were weighed at moment of the first IM immunisation (8 days of age), a week before challenge, at moment of challenge infection (33 days of age) and 7 and 14 days later (47 days of age). The average daily weight gain (ADWG) was calculated at moment of challenge (= (weight (kg) on day 33)-(weight on day 8)) and 14 days post challenge (dpc) (= (weight on day 47)-(weight on day 33)) to compare the growth of the different groups during the challenge infection.

In order to know the F4-receptor status (F4R) of the pigs, they were euthanatized 15 dpc by an intravenous injection of pentobarbital (24 mg/kg; Nembutal, Sanofi Sante animale, Brussels, Belgium). Thereafter small intestinal villi were collected for determining the presence of the F4R.

### *7.3.4.2. Challenge with virulent $F4^+$-*E. coli *GIS 26*

The pigs were treated three days with florfenicol (Nuflor, Schering-Plough, Brussels, Belgium) in PBS (300 mg in 5 ml PBS/animal/day) in order to decrease colonisation resistance by the normal gut flora. Before the challenge, the pigs were deprived of food and water for 12 hours whereafter the acidic gastric pH was neutralized with 62 ml $NaHCO_3$ (1,4 % (wt/vol) in distilled

water) to increase the survival of the bacteria during the passage through the stomach (Cox et al., 1991). Fifteen minutes later, the pigs were intragastrically infected with $10^{10}$ $F4^+$-*E. coli*.

### 7.3.5.. Samples

#### *7.3.5.1. Serum*

On days 0 (first immunisation 8 days of age), 10, 17, 25, 29, 33 and 40 of the experiment blood was sampled from the jugular vein, serum was collected and inactivated at 56°C during 30 min and subsequently treated with kaolin (Sigma-Aldrich, Bornem, Belgium) to decrease background reading in ELISA (Arnouts 1994). Therefore, 4 volumes of a kaolin suspension (25% (wt/vol) in PBS) were added to 1 volume of serum and incubated for 30 min at room temperature. The suspension was centrifuged at 5,500 x g for 10 min and the supernatant was diluted in ELISA dilution buffer (PBS + 0,2% (vol/vol) Tween 20 + 3% (wt/vol) bovine serum albumin (BSA)), yielding a final serum dilution of 1/10.

#### *7.3.5.2. Faeces*

Faecal samples were collected daily from day 0 till 7 dpc. The prevalence and severity of the diarrhoea were noted by visually scoring the consistency of the faeces on a scale of 0 to 3 as described by Cox et al (1987) : 0 = no diarrhoea (normal faeces); 1 = slight (pasty faeces); 2 = moderate (semi liquid faeces); 3 = severe diarrhoea (watery faeces).

#### *7.3.5.3. Peripheral blood monomorphonuclear cells (PBMC)*

Blood was collected from the jugular vein 4 dpsi and 14 dpc on an equal volume of Alsever's solution. PBMC were isolated by density gradient centrifugation on Lymphoprep (Invitrogen, Merelbeke, Belgium). After lysis of the erythrocytes in ammonium chloride (0.8% [wt/vol]) and subsequent centrifugation (380 x g for 10 min at 4°C), the PBMC were washed and resuspended at the concentration of $4.10^6$ cells/ml in leukocyte medium (RPMI-1640 supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml), kanamycin (100 µg/ml), L-glutamin (200 mM), sodiumpyruvate (100mM), non-essential aminoacids (100mM), β-mercaptoethanol ($5.10^{-5}$ M) and 1% (vol/vol) serum of a colostrum-deprived piglet). The cells were used for analysis of F4-specific proliferation.

### 7.3.6. ELISA for F4-specific IgG, IgA and IgM serum antibodies

F4-specific IgM, IgA and IgG titers were determined in an indirect ELISA as described by Van den Broeck et al (1999). Briefly, the wells of a 96-well microtitre plate (NUNC®, Maxisorb Immuno Plates, Gibco BRL) were coated with a F4-specific MAb (IMM-01) at a concentration of 1 µg/ml coating buffer (PBS, 150mM, pH=7,4). After 2 hours of incubation, the remaining binding sites were blocked overnight at 4°C with PBS supplemented with 0.2 % (vol/vol) Tween®80 ( Merck Eurolab, Leuven, Belgium). Subsequently, purified F4 was added to the wells at a concentration of 30 µg/ml in ELISA dilution buffer (PBS, pH 7.4 + 0.05% [vol/vol] Tween® 20 + 3 % BSA) for 1 hour at 37°C. Then, the pretreated sera were added in series of twofold dilutions in ELISA dilution buffer, starting at a dilution of 1/10. The plates were incubated for 1 hour at 37°C. Thereafter, biotinylated-swine-specific IgM, IgA and IgG MAb (Van Zaane et al., 1987). were added and plates were incubated for 1 hour at 37°C followed by 1 hour incubation at 37°C with peroxidase-conjugated streptavidin. Between each step, the plates were washed with PBS + 0.2% [vol/vol] Tween® 20. Finally, the ABTS solution (Roche Diagnostic, Brussels, Belgium), containing $H_2O_2$, was added and after 30 min incubation the optical density was measured at 405 nm ($OD_{405}$).

The antibody titer was determined as the inverse of the highest dilution that still had an $OD_{405}$ higher than the cut-off value. The cut off value was determined by calculating the mean $OD_{405}$ plus 3 times the standard deviation of the optical densities of the 1/10 diluted samples measured at day 0. The obtained cut-off values were 0.26, 0.32 and 0.4 for F4-specific IgM, IgG and IgA respectively.

### 7.3.7. Faecal excretion of $F4^+$ E. coli.

The faeces was examined for the presence of $F4^+$-*E. coli* by preparing a 1% (wt/vol) suspension in PBS at 4°C. Subsequently 50 µl of four serial 10-fold dilutions in PBS were plated out on sheep blood agar plates (Difco Laboratories). After 24 hours incubation at 37°C the colonies were blotted onto polyvinylidene fluoride membranes (Gelman Science, Leuven, Belgium) during 2 hours at room temperature. Subsequently, the remaining binding sites were blocked overnight with blocking solution (5% (wt/vol) nonfat dry milk in PBS). After rinsing the membranes in PBS, a peroxidase-conjugated F4-specific MAb (IMM-01) was added for 1 hour at room temperature. The membranes were rinsed again whereafter the substrate solution (0.67 ml of AEC stock solution (0.4% (wt/vol) in dimethylformamide) in 10 ml sodium acetate (0.1 M, pH 5.2) + 10 µl 30 % $H_2O_2$) was added. The enzymatic reaction was stopped after 15 min by rinsing

the membranes in PBS and the brown-red dots were counted. The results are presented as the mean number ($log_{10}$) of $F4^+$-*E. coli* per gram faeces ± SEM (Figure 7.1).

### 7.3.8. F4-specific proliferation assay

The lymphocyte proliferation test was performed by adding F4-fimbriae (final concentration 10 µg/ml) or medium to the wells of a 96-well flat-bottom microtitre plate (Nunc) containing 4 x $10^5$ PBMC/well (final volume of 200 µl). To evaluate the proliferative capacity of the cells, concanavalin A (ConA, final concentration 10µg/ml) induced proliferation was used as a control. Each stimulation was performed in triplicate. The cells were incubated at 38°C in a humidified atmosphere with 5% $CO_2$. After 72 hours the cells were pulse-labeled with 1 µCi/well, of [$^3$H]-thymidine (Amersham ICN, Bucks, UK) and 18 hours later the cells were harvested onto glass fiber filters (Perkin Elmer, Life Science, Brussels, Belgium) The radioactivity incorporated into the DNA was measured using a β-scintillation counter (Perkin Elmer, Life Science, Brussels, Belgium). The results are presented as the mean counts per minute (cpm) calculated by substracting the background cpm (medium) from the cpm of F4-stimulated cultures.

### 7.3.9. In vitro villous adhesion assay for the F4R using $F4^+$ E. coli

In order to determine the presence of the F4R on the small intestinal villous enterocytes, an *in vitro* villous adhesion assay was performed. Collection of the small intestinal villi was performed as described by Cox and Houvenaghel (1987;1993). The *in vitro* villous adhesion assay was based on the technique described by Girardeau (1980).

### 7.3.10. Statistical analysis

Differences between the groups in mean $log_2$ antibody serum titers (IgM, IgA and IgG) and $F4^+$-*E. coli* shedding in faeces were tested for statistical significance using General Linear Model (Repeated Measures Analysis of Variance, SPSS 7.5 for Windows). Differences in the frequency of $F4^+$-*E. coli* excreting animals were tested for statistical significance using a chi-square test (Pearson). Differences between groups in cell proliferation assays and faecal score were analysed for statistical significance using the Kurskal-Wallis test. Differences in the the ADWG between the groups was tested for statistical significance using a two-sample t test. $P < 0.05$ was considered as statistically significant.

## 7.4. Results

7.4.1. Faecal shedding of $F4^+$- E. coli

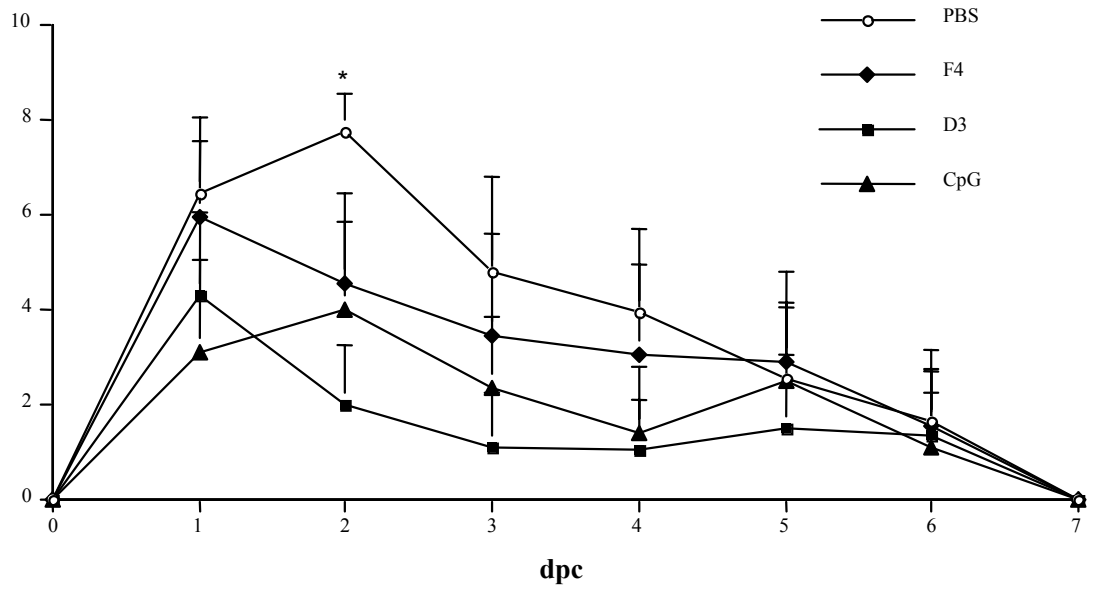


**FIGURE 7.1**: Mean faecal excretion of $F4^+$-*E. coli*/gram faeces ($log_{10}$± SEM)/group/day until 7 days post challenge (dpc). Pigs were IM injected two times (at the age of 8 and 25 days) with F4-fimbriae with or without different immunomodulators ($1\alpha,25(OH)_2D_3$ and CpG-ODN) and intragastrically infected with $1.10^{10}$ $F4^+$-*E. coli* one week later. Significant difference ($p<0.05$) between control group and $D_3$-group 2 dpc is indicated with an asterisk.

Before challenge (Day 0) all animals had no $F4^+$-*E. coli* excretion and showed no diarrhoea (faecal score = 0, not shown). In the control group, a peak in the faecal $F4^+$-*E. coli* excretion (mean $F4^+$-*E. coli*/gram faeces of $10^{7,8}$) was seen 2 dpc followed by a gradual decrease. Seven dpc no $F4^+$-*E. coli* could be isolated from the faeces of all pigs. All F4-vaccinated groups showed a reduced $F4^+$-*E. coli* excretion but this was only significant for the $D_3$-group 2 dpc (Figure 7.1). A significantly lower faecal score was observed in the CpG-group in comparison with the control group 2 dpc (Table 7.1.). At that moment a significantly higher ($P<0,05$) number of $F4^+$-*E. coli* excreting animals was observed in the control group in comparison with the $D_3$-group (Figure 7.2). No statistical difference was observed between the F4-group and the 'supplemented' groups. The duration of $F4^+$-*E. coli* excretion (6 days) was not significantly different between the groups.

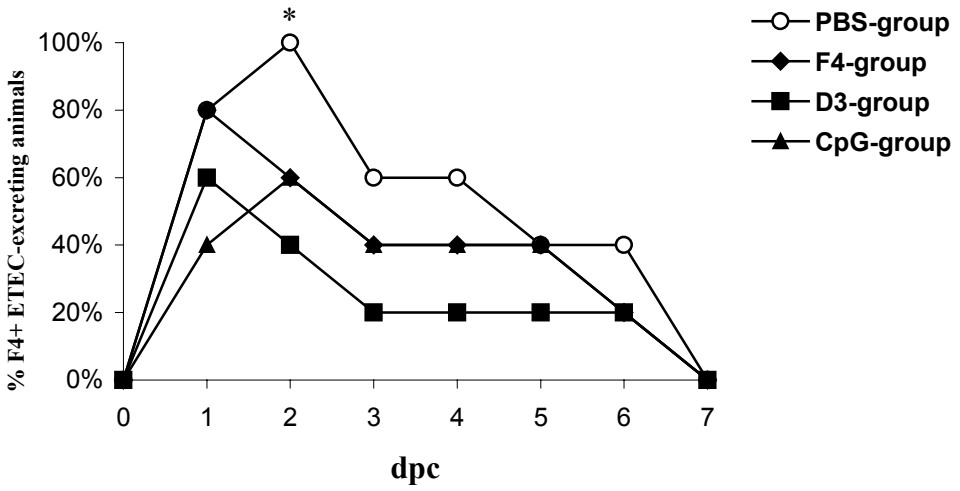


**FIGURE 7.2**: % of F4$^+$-*E. coli* excreting animals until 7 days post challenge (dpc). Significant difference (P<0.05, two-by-two table) was found between the $D_3$-group and the control group 2 dpc.

**TABLE 7.1. :** The daily faecal score for each individual animal in the different treatment groups. Pigs were intramuscularly (IM) injected at the age of 8 and 25 days old and infected 8 days later with $1.10^{10}$ F4$^+$-ETEC.

| | | Faecal score[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| groups | Pig | 1 dpc* | 2 dpc | 3 dpc | 4 dpc | 5 dpc | 6 dpc | 7 dpc | Total faecal score/group during observation period |
| PBS | 1 | 1[b] | 0[a] | 3 | 1 | 1 | 0 | 1 | |
| | 2 | 2[c] | 1 | 1 | 1 | 1 | 0 | 0 | |
| | 3 | 1 | 1 | 0 | 2 | 2 | 1 | 0 | |
| | 4 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | |
| | 5 | 3[d] | 1 | 0 | 1 | 0 | 0 | 0 | 33 |
| F4 | 6 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | |
| | 7 | 3 | 2 | 3 | 3 | 3 | 1 | 0 | |
| | 8 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | |
| | 9 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | |
| | 10 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 27 |
| $D_3$ | 11 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | |
| | 12 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | |
| | 13 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | |
| | 14 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | |
| | 15 | 1 | 1 | 1 | 1 | †[b] | | | 17 |
| CpG | 16 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | |
| | 17 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | |
| | 18 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | |
| | 19 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | |
| | 20 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 18 |

[a]Faecal score: 0: normal faeces ; 1: pasty faeces ; 2: semi liquid faeces; 3: watery faeces.
†[b]: pig died of bronchopneumonia. *:days post challenge.

7.4.2. Humoral immune responses

All pigs were F4-seronegative prior to the start of the experiment. The kinetics of the F4-specific serum antibody responses is shown in figure 7.3. The control group showed no antibody response before challenge infection, even though a slight increase in baseline values occurred just before challenge.

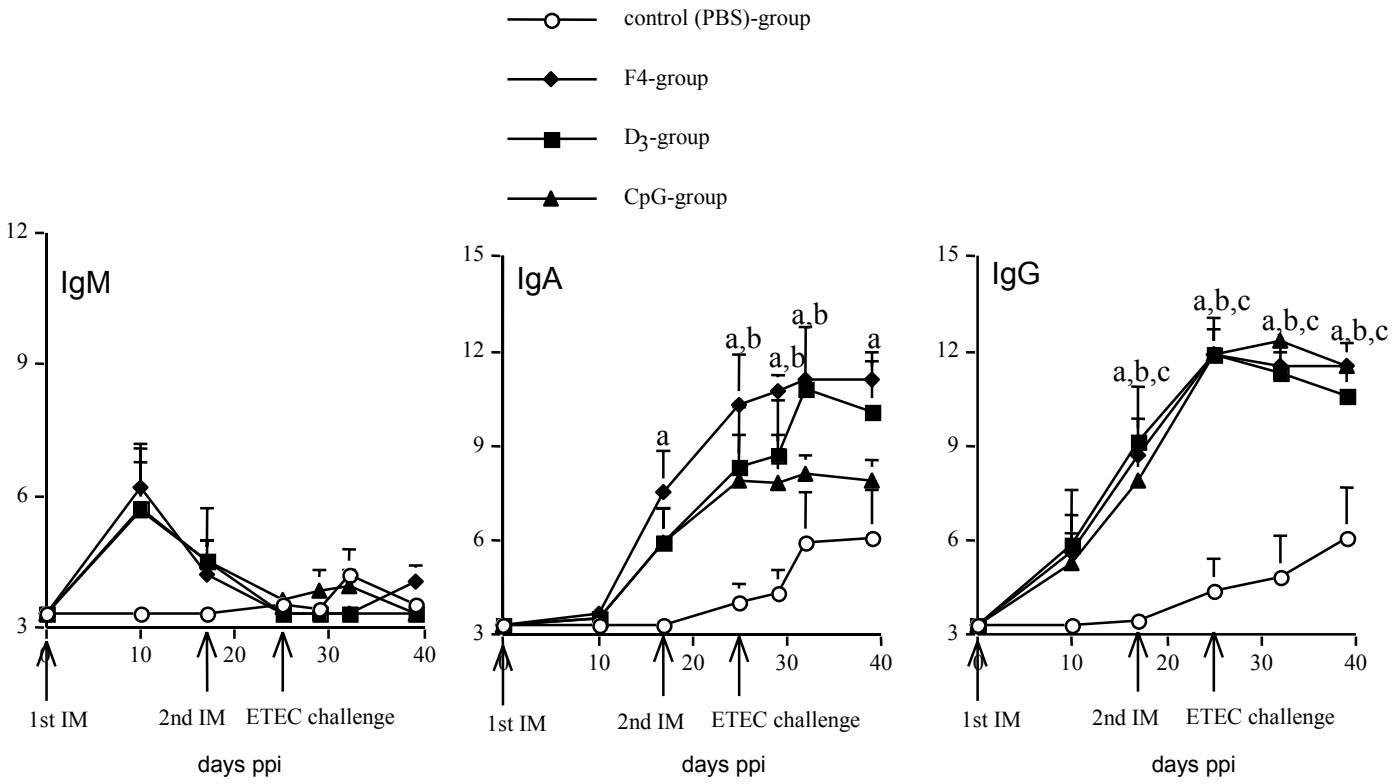


**FIGURE 7.3**: Kinetics of the F4-specific IgM, IgA and IgG serum response following 2 IM immunisations and challenge infection with $F4^+$-*E. coli*. Pigs in each group (n=5) were injected on two occasions with PBS in IFA (○) or 100 µg F4-fimbriae in IFA (♦) with or without supplementation of 2 µg of $1\alpha,25(OH)_2D_3$ (■) or 500 µg of CpG-ODN (▲). The antibody titers are plotted as mean $\log_2$ titers ± the SEM. and significant differences ($P<0,05$) are indicated with a, b and c.
a: statistical difference between the F4-group (♦) and the control group (○).
b: statistical difference between the $D_3$-group (■) and the control group (○).
c: statistical difference between the CpG-group (▲) and the control group (○).

Following vaccination the F4-specific IgM antibody titers peaked 10 days post primary immunisation (dppi) in all 3 F4-immunised groups, whereafter it decreased again to reach baseline values 25 dppi. Seventeen dppi, the IgG antibody titer in the F4-immunised groups was significantly higher ($P<0.05$) than the control group and remained significantly higher after the booster immunisation. After the $F4^+$-*E. coli* challenge, 3 out of 5 control animals showed an IgM and IgG antibody response, while low IgM and decreased IgG responses were seen in the F4-immunised groups (no apparent booster response).

Following the IM immunisations an increase of the F4-specific IgA-titers was also observed in the F4, $D_3$ and CpG-group but was only statistical significantly different from the control group in the F4-group (from 17 dppi onwards). The $F4^+$-*E. coli* challenge resulted in a strong increase of the IgA titer in the $D_3$-group with significantly higher ($P<0,05$) IgA titers than in the control group 0, 4 and 7 dpc. After the *E. coli* challenge, the control group showed a good increase of the of the IgA titer ($2^{4}$-$2^{6,12}$) to reach a plateau 7 dpc.

7.4.3. F4-specific proliferation.

The lymphocyte proliferation in response to F4-fimbriae and ConA were analysed 4 dpsi (Figure 7.4A) and 14 dpc (Figure 7.4B). Spontaneous proliferations (mean cpm of control medium cultures ± SEM) was 537 ± 70 cpm (4 dpsi) and 254 ± 52 cpm (14 dpc).

Four dpsi, the F4-specific proliferation was significantly higher ($P<0,05$) in the CpG-group than in all other groups and significantly higher in the F4 and D3 group than in the control group. ConA-induced stimulation resulted in high proliferation in all groups with the highest proliferation seen in the CpG-group that was significantly higher than in the control and $D_3$-group (Figure 7.4A).

Fourteen dpc the F4-specific proliferation in the control group was still significantly lower ($P<0,05$) than in the other vaccinated groups. There were no significant differences between the F4-vaccinated groups. Again, the ConA-induced proliferation was significantly higher ($P<0,05$) for the CpG-group than for the other groups, whereas the F4- and $D_3$-group had significantly higher ConA-induced proliferations than the control group (Figure 7.4B).

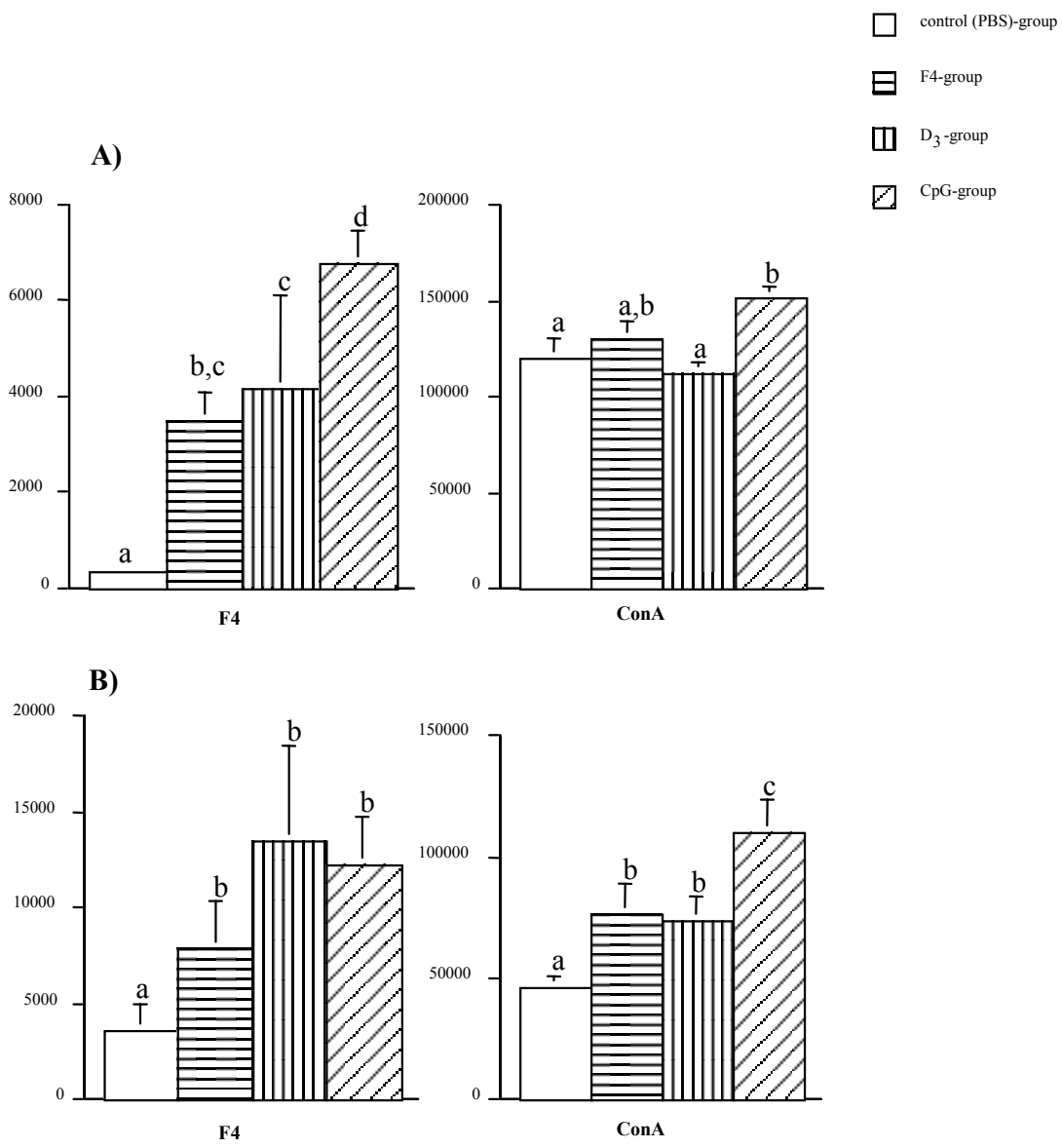


**FIGURE 7.4**: F4-specific (10 µg/ml final concentration) and ConA-induced proliferation (10 µg/ml final concentration) proliferation of PBMC 4 dpsi (A) and 14 dpc (B). Results are presented as mean cpm ± SEM. Bars with a different letter are significantly different ($p<0,05$).

7.4.4. Weight gain

The mean body weight increased from 3,45 kg ± 0,41 kg at the start of the experiment to 8,37 ± 1,0 kg at moment of challenge to 16,17 ± 1,48 kg 14 dpc. No significant differences (P>0.05) between the experimental groups were observed. The ADWG between the first immunisation and the challenge 25 days later was + 0.211 ± 0.05 kg for the control group, + 0.211 ± 0.04 kg for the CpG, + 0.200 ± 0.03 kg for the F4 and +0.193 ± 0.03 kg for the $D_3$-group. Fourteen dpc, the ADWG was + 0.612 ± 0.126 kg for the $D_3$-group, + 0.593 ± 0.08 kg for the F4-group, + 0.562 ± 0.09 kg for the CpG-group and + 0.519 ± 0.06 kg for the control group. There were no significant differences (P>0.05) between the groups. A decrease in the mean daily weight gain was seen in all groups following weaning with an overall increase of 227 ± 36 g the week before weaning to 100 ± 69 g the week after weaning.

## 7.5. Discussion

At weaning piglets become deprived of lactogenic antibodies and need an active mucosal immunity to protect them against enteropathogenic microorganisms. However to obtain protection immediately post-weaning, piglets have to be immunised during the suckling period. In the present study it was analysed whether IM immunisation of piglets with F4 in IFA during the suckling period could protect against oral challenge with $F4^+$-*E. coli* and whether addition of 1α,25$(OH)_2D_3$ or CpG-ODN could improve this protection.

Intramuscular immunisation with F4 fimbriae was able to reduce $F4^+$-*E. coli* excretion in faeces upon challenge with $F4^+$-*E. coli*. Addition of CpG-ODN and 1α,25$(OH)_2D_3$ reduced even more the *E. coli* excretion which was statistically significant for 1 α ,25$(OH)_2D_3$ on day 2 post challenge. All of the non-immunised (control) pigs excreted $F4^+$ *E. coli* and 60 % developed a transient and severe diarrhoea (semi liquid to watery diarrhoea) within 2 days post challenge, which is in agreement with previous observations (Cox et al., 1991). Pigs can excrete high numbers of $F4^+$-*E. coli* in the absence of diarrhoea: post-weaning diarrhoea is a multifactorial disease complex in which $F4^+$-*E. coli* is one of the factors. The number of excreting and diarrhoeic pigs in all F4-immunised groups was lower than in the control group. This was not due to the lack of specific receptors as all pigs in this study expressed the F4R. Best protection, as evaluated by the excretion of $F4^+$-*E. coli* and the degree of diarrhoea after challenge, was obtained by adding 1 α ,25$(OH)_2D_3$ to the antigen.

In order to evaluate the supplementation of 1α,25$(OH)_2D_3$ or CpG-ODN on the humoral immunity, the F4-specific serum antibody responses were measured. After IM immunisation similar F4-specific serum IgM and IgG responses were observed, whereas the animals that received only F4 displayed the highest serum IgA responses. Van den Broeck et al (1999) demonstrated that the F4-specific IgA serum titer is a useful measure for the mucosal immune response in piglets after an oral immunisation since they are induced at the gut-associated lymphoid tissue (GALT). The IgA response after an intramuscular immunisation of pigs is mainly induced in systemic lymphoid tissues so that the F4-specific IgA titer is less correlated with the IgA in the gut mucosa. However, some of the dimeric serum IgA will be secreted at the mucosal surfaces what could explain the protection observed in the F4-group. Furthermore, studies have shown that non-mucosal immunisations can induce antigen-specific IgA ASC which are capable of homing to mucosal tissues (Quiding-Jabrink et al., 1997; Kantele et al., 1997). Although, the number of antigen-specific IgA ASC in the GALT were not enumerated in the present study, an increased homing of antigen-specific IgA ASC to the gut-associated lymphoid tissue (Peyer's Patches) has been observed in previous studies following intramuscular immunisation with antigen and 1α,25$(OH)_2D_3$ (Chapter 3). Similar observations have been made in mice where subcutaneous injections with hepatitis B surface antigen together with 1α, 25$(OH)_2D_3$ induced increased numbers of IgA and IgG ASC in lamina propria of lungs and intestines (Daynes et al., 1994; 1996). This was at least partly due to the migration of antigen-pulsed dendritic cells from the local draining lymph nodes towards the Peyer's patches, where the activation and differentiation of antigen-specific B cells is initiated (Enioutina et al., 1999;2000). This might explain the protection as well as the booster IgA serum response using 1α, 25$(OH)_2D_3$ upon $F4^+$-*E. coli* challenge.

In the present study, neither 1α,25$(OH)_2D_3$ nor CpG-ODN enhanced the F4-specific serum antibody responses, whereas significant increases did occur with these immunomodulators when HSA (Van der Stede et al., 2001) or ovalbumin (Van der Stede et al., 2002) were used as immunizing antigens. This could be due to the nature of the F4-antigen that consists of repetitive epitopes of identical FaeG protein subunits (Oudega et al., 1989) and induces already potent immune antibody responses difficult to ameliorate with 1α,25$(OH)_2D_3$ or CpG-ODN.

Remarkably, the combination of F4-fimbriae in IFA even reduced the faecal $F4^+$-*E. coli* excretion although this reduction was not significant as compared to the control group. The mechanism for this reduction is not clear but the high serum IgA titers could be involved. F4 suspended in IFA is good for inducing high serum antibody titers. IFA and other water in oil adjuvants are excellent stimulators of the humoral as well as cellular immunity (Lindblad et al., 2000).

In order to analyse the effect on cellular (memory) immunity, lymphocyte proliferation assays were performed. All immunized groups showed F4-specific proliferations, which were however significantly higher in the CpG-group than in all other groups. Also in a previous study, with CpG-ODN primed animals, enhanced OVA-specific as well as mitogen-induced proliferation were observed (Van der Stede et al., 2002). So, it appears that CpG-ODN have the intrinsic capacity to trigger directly or indirectly via APC the lymphocytes for better proliferation. This is consistent with the observations of Lipford et al (1997) who demonstrated that CpG-ODN may influence the signal threshold of antigen-reactive T cells *in vivo* and that this effect was due to the CpG-mediated activation of antigen-presenting cells like dendritic cells and macrophages. CpG-ODN priming enhanced also the antigen non-specific proliferation as evidenced in the ConA stimulations. This is in accordance with the polyclonal activation of B- and T cells (in the presence of APC and cytokines) by Liang et al. (1996). As the effect of CpG-ODN on the cellular immunity was uniformly immunoenhancing this was not the case with $1\alpha,25(OH)_2D_3$ where a high variability in proliferation was observed among the animals. However, the mean proliferation of $1\alpha,25(OH)_2D_3$ injected animals was still higher than with F4 only. The enhanced cellular immunity perhaps contributed to protection. Further studies are required to fully characterize the role of cellular immunity in $F4^+$-*E. coli* infections. No indications of homing of F4-specific IgA ASC towards the GALT were obtained after IM immunisation with CpG-ODN, but secretion of F4-specific serum IgA could be involved in the decreased faecal excretion of $F4^+$-*E. coli*.

In conclusion, intramuscular immunisations of suckling piglets with F4-fimbriae reduced the $F4^+$ *E. coli* excretion upon challenge. In addition, supplementation of $1\alpha,25(OH)_2D_3$ improved significantly this reduction and was correlated with the presence of a secondary (mucosal) IgA response. Conversely, addition of CpG-ODN correlated with significantly higher T-cell proliferation. These results indicate that $1\alpha,25(OH)_2D_3$ and CpG-ODN have a potential to improve veterinary vaccines against enteropathogens.

# PART IV

## GENERAL DISCUSSION

# CHAPTER 8

# GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

Since most pathogens either replicate at a mucosa or at the mucosal barrier before entering the body, mucosal immune mechanisms are the first step in defense against these pathogens. Vaccines that activate mucosal immune mechanisms are therefore superior to other vaccines in affording protection against these pathogens. The best route for inducing protection in the intestinal mucosal tract is the oral route. However, only few oral vaccines are available. Soluble antigens mostly do not induce protection via this route even so inactivated bacteria. Protection can be obtained with attenuated enteropathogens but then these strains still induce clinical signs (Moon et al., 1993). A major hurdle in delivering antigen via mucosal surfaces is that most antigens are poorly immunogenic and induce a state of hypo- or unresponsiveness, called oral tolerance (Strobel and Mowat 1998).

Studies in mice have demonstrated that systemic injection of antigen in the presence of $1\alpha,25(OH)_2D_3$ as immunomodulator could be modulated towards a mucosal immune response with the appearance of IgA antibody secreting cells in the mucosa and of sIgA in intestinal mucosal secretions (Daynes et al., 1994, 1996; Enioutina et al., 1999, 2000).

The aim of the present PhD-thesis was to determine if an intramuscular immunisation in pigs, which normally induces a systemic immune response, could result in a mucosal immune response using $1\alpha,25(OH)_2D_3$ and CpG-oligodeoxynucleotides (CpG-ODN) and whether this was correlated with Th2-responses. Indeed, 1α,25-dihydroxyvitamin $D_3$ has been classified as a Th2-modulating adjuvant. Th2-like cytokines are considered to be major helpers for IgA responses. On the other hand, CpG-ODN are known as Th1-modulating adjuvants but are also able to stimulate directly B cells. 1α,25-Dihydroxyvitamin $D_3$ and CpG-ODN belong to two different classes of adjuvants: $1\alpha,25(OH)_2D_3$ belongs to the class of cytokines and hormones and the CpG-ODN belong to the 'bacterial products' such as bacillus Calmette-Guérin (BCG) and monophosphoryl lipid A (Edelman, 2000). Their immunomodulating effects and their possible role in the induction of active (mucosal) immunity are discussed in chapter 1.

Hereto, different immune parameters such as the antigen-specific isotype-specific serum antibody response, antigen-specific IgA titers in mucosal secretions, antigen-specific lymphocyte proliferation, phenotypic analysis of the lymphocyte subsets and cytokine-profiles (Th1/Th2) were evaluated after IM immunisation of antigens supplemented with or without $1\alpha,25(OH)_2D_3$ or CpG-ODN. Furthermore, in order to evaluate the protective capacity of an induced mucosal

immune response, a challenge experiment was performed with F4-fimbriae as immunogen and $F4^+$-enterotoxigenic *Escherichia coli* (ETEC) as challenge strain (Chapter 7). F4-fimbriae are proteinaceous surface antigens composed of repeating subunits and play a role in the pathogenesis of PWD in pigs as they allow the bacteria to adhere to specific receptors on the brush border of enterocytes with subsequent colonization of the small intestine (Jones and Rutter, 1972). Other antigens used in the present thesis were HSA and OVA (Chapter 2, 3 and 4).

## 8.1. The immunomodulating effects of 1α,25(OH)$_2$D$_3$ after IM immunisation of pigs

### 8.1.1. Humoral responses

To analyse the effect of 1α,25(OH)$_2$D$_3$ on the antibody response, IFA with or without 1α,25(OH)$_2$D$_3$ was injected intramuscularly in piglets. Analysis of the HSA-specific serum antibody response and HSA-specific antibody-secreting cells (ASC) demonstrated that the steroid hormone was able to enhance the HSA-specific serum IgA response as well as the number of HSA-specific IgA ASC in the local draining lymph node. In addition 1α,25(OH)$_2$D$_3$ also enhanced the number of antigen-specific IgG ASC in the local draining lymph node (Chapter 2). However, the effect of 1α,25(OH)$_2$D$_3$ on the antigen-specific IgM, IgG, IgG1 and IgG2 serum antibody response was not consistently higher in comparison with the control animals and varied between the studies. This could have been due to a different site of immunisation in the different studies. Indeed, it has been shown in pigs that injection at sites of which the draining lymph nodes also drain draining parts of the mucosal immune system increases IgA responses (Vanderpoorten et al., 1997). So, immunisation in the m. gluteobiceps (Chapter 2) and neck (Chapter 4 and 7) both draining parts of respectively the rectum and mammary glands may be beneficial for inducing IgA responses in comparison with IM immunisation in the m. gastrocnemius (Chapter 3 and 7). So the effect of 1α,25(OH)$_2$D$_3$ in modulating towards IgA responses is best studied after IM injection in the periphery (m. gastrocnemius). The adjuvant influence upon IM immunisation was consequently best demonstrated in chapter 3 in which the steroid hormone modulated towards IgA as it was the only antibody isotype that was enhanced after IM injection. That the number of IgA ASC was enhanced by 1α,25(OH)$_2$D$_3$ in pigs was similar to observations observed in mice (Daynes et al., 1994, 1996) but different from man (Kriezel et al., 1999). In sheep a slide increase of the serum IgA titer was observed (Scheerlinck et al., 2001). Due to its depot effect, IFA provides a good lipophylic ‘vehicle’ capable to store and transport the emulsified antigen and the steroid hormone with subsequent slow release of antigen (Cox et al., 1997). Besides inducing

significant antibody responses, IFA also stimulated the cellular immune response as we observed significant proliferation of the MC (Chapter 3 and 7).

Using $1\alpha,25(OH)_2D_3$, also higher antigen-specific IgA titers in mucosal secretions (saliva, faeces and nasal secretions) were obtained. This could be the result of diffusion of dimeric IgA out of the serum into the mucosal tissues and/or due to locally produced dimeric IgA by ASC originating from systemic lymphoid tissue. Subsequently, this dimeric IgA can be secreted as sIgA at the mucosal surfaces (McGhee et al., 1992). According to Coffin and co-workers (1997, 1998) there are a number of mechanisms by which intramuscular immunisation may induce production of antigen-specific IgA in the gut-associated lymphoid tissue (GALT and/ or MALT). Firstly, the antigen may reach the GALT via the by blood and stimulate an immune response after processing by intestinal antigen presenting cells. Secondly, antigen-specific IgA-ASC may be generated in the draining lymph nodes and home to the GALT. Thirdly, activated antigen-specific B-lymphocytes may be generated in draining lymph nodes, disseminate to the GALT and, in the presence of cytokines supportive for IgA production, differentiate into IgA ASC. Fourthly, antigen presenting cells capable of presenting antigen-specific peptides may be generated in draining lymph nodes and home to the GALT. Localization of the HSA-specific ASC in different lymphoid tissues revealed that the highest numbers of ASC were found in the systemic lymphoid tissues (local draining lymph node > spleen > bone marrow) indicating that lymphocytes activated in the 'periphery" preferentially migrate to systemic lymphoid tissues. The activation in the local draining lymph node is enhanced by $1\alpha,25(OH)_2D_3$ which is consistent with the enlarged local draining lymph node observed in some of the pigs. The presence of significant numbers of HSA-specific ASC in the Peyer's patches as well as antigen-specific IgA in mucosal secretions (saliva, nasal secrete and rectal content) using $1\alpha,25(OH)_2D_3$ suggests either that some antigen-specific B cells reach mucosa-associated lymphoid tissues or that either antigen or antigen-presenting cells carrying antigen reach these sites. That in pigs only few antigen-specific ASC were detected in the small intestinal lamina propria and significant numbers in the Peyer's patches is an argument in favor of one of both mechanisms. In mice, homing of antigen-presenting cells (DC's), due to $1\alpha,25(OH)_2D_3$, has been described (Enioutina et al., 1999,2000).

### 8.1.2. Cellular responses and Th1/Th2 bias.

The hallmark for the mucosal immune system is the production of sIgA. Historically, in mice Th2-cytokines are considered to be the best 'helpers' for IgA responses. However, sIgA release may also be induced in Th1-dominated responses, as observed with intracellular pathogens such as Salmonella in the gastrointestinal tract or influenza virus in the upper respiratory tract.

Furhtermore, TGF-β (secreted by Th3-cells) has been reported to be the most crucial sIgA "switch factor" (Lebman et al., 1990). Thus, either Th1, Th2 or Th3 cells or a combination of these cell types can support antigen-specific sIgA responses.

Therefore, in pigs *in vivo* mRNA expression of Th1-like (IFN-γ, IL-2), Th2-like (IL-4, IL-10), inflammatory (IL-6) and Th3-like (TGF-β) cytokines by MC of the local draining lymph nodes was measured to analyze the effect of $1\alpha,25(OH)_2D_3$ on the cytokine profile. After a first IM immunisation $1\alpha,25(OH)_2D_3$ treated piglets showed an increased IL-10 mRNA, traces of IL-4 and a significant lower IL-2 mRNA response than non-steroid treated piglets. Furthermore, low IFN-γ mRNA was seen in treated and non-treated piglets. This cytokine profile is indicative for a Th2-like response and is similar to what was observed in mice (Daynes et al., 1994, 1996). Such a Th2-microenvironment promotes IgA B-cell maturation and results in downregulation of the Th1-like cytokines (IL-2, IFN-γ) as described in mice (Adorini et al., 2001) and man (Lemire et al., 1995). TGF-β, the most crucial sIgA switch factor did not seem to be crucial for the effect of $1\alpha,25(OH)_2D_3$ since there was no change between treated and non-treated pigs the first two days after the IM immunisation. Ten days after the primary immunisation TGF- β mRNA could not be detected in the steroid-treated group. After the second immunisation, the cytokine profile seemed to be, in several aspects, the opposite of this after the first immunisation. IL-2 mRNA expression was higher in the $1\alpha,25(OH)_2D_3$ treated pigs and IL-10 mRNA was lower. However IFN-γ was also significantly lower in the treated pigs, so that this response is not indicative for a Th2-like cytokine response nor for a Th1 like cytokine response.

The Th1/Th2 paradigm stands for down regulation of Th2 cytokines by a Th1-cytokine profile whereas a Th2-cytokine profile down regulates Th1 cytokines. In the present study this is true for IL-2 (Th1-like) and IL-10 (Th2 like) since a high IL-2 mRNA expression was accompanied by a low IL-10 mRNA expression and vice versa. This is consistent with what is observed in mice and man (Mosman et al., 1996). However, the same is not true for IFN-γ (Th1-like) and IL-4 mRNA expression, since high IL-2 mRNA expression was accompanied with low IFN-γ mRNA-expression and high IFN-γ mRNA expression was accompanied with low IL-2 mRNA expression whereas IL-4 mRNA expression was consistently low. IL-10 and IL-2 are important cytokines for clonal expansion of activated B cells. Furthermore, IL-4, IL-5 and IL-6 are involved in the differentiation towards IgA producing B cells and the IgA secretion. Injection with $1\alpha,25(OH)_2D_3$ did not enhance IL-4 and IL-6 and there are no tools available for measuring porcine IL-5. From the results, we can conclude that $1\alpha,25(OH)_2D_3$ did not favor a Th2 microenvironment to the same extent as seen in mice. In fact only an increase of IL-10 was seen and then only after the first immunisation. That the increased expression of IL-10 mRNA is accompanied with a low IL-2 mRNA and vice versa could suggest that IL-10 may block the

transcription of IL-2 (Th1-like). In contrast to what is described by Blecha (2001), there was no evidence of a consistent down regulation of IFN-γ by IL-10 since high amounts of IL-10 mRNA in non-steroid treated pigs were accompanied by high IFN-γ mRNA expression.

That $1\alpha,25(OH)_2D_3$ induces a different cytokine-profile after a first immunisation than after the second suggests that the effect on cytokines depends on the differentiation and activation status of the cells (naïve versus memory cells). In pigs, it is not clear which lymphocyte populations are affected by $1\alpha,25(OH)_2D_3$. In the present study only $CD25^+$-cells ($IL\text{-}2R^+$-cells) were increased as a result of steroid treatment. In pigs, $CD25^+$-cells may be either monocytes, macrophages, T- and/or B cells (Denham et al., 1994; Lunney et al., 1987, 1994). No changes were observed in the percentage of $CD2^+$ (most T cells) $CD4^+$ (Th cells), $CD8^+$ (CTL-cells) and $IgM^+$-cells (B cells). Therefore it cannot be excluded that macrophages and/or dendritic cells are the primary target of $1\alpha,25(OH)_2D_3$ as observed in mice and man (Lemire et al., 1995, Enioutina et al., 1999, 2000, Adorini et al., 2001).

## 8.2. The immunomodulating effects of CpG-ODN after IM immunisation of pigs

### 8.2.1. Humoral responses

In chapter 4 it was shown that CpG-ODN sequence 5-GCT-AGA-CGT-TAG-CGT-3' as well as the reversed GpC-ODN sequence 5'-GCT-AGA-GCT-TAG-GCT-3' significantly enhanced the primary serum antibody responses, the OVA-specific IgA titers in saliva and nasal secretions as well as the antigen-specific and ConA-induced proliferation of MC in different lymphoid tissues. The enhanced IgA response might be interesting for protection against mucosal infections as discussed in 8.3. The CpG-ODN as well as the GpC-ODN had a phosphodiester backbone and were shown to be most stimulatory in mice (Krieg et al., 1995, Klinmann et al., 1999). The enhanced IgA response in pigs is a remarkable finding, as it has later been demonstrated *in vitro* by us (Chapter 5) as well as by others (Kamstrup et al., 2001) that this 'mice-specific CpG-ODN' did not had any effect on proliferation of porcine lymphocytes. Therefore, *in vitro* proliferation assays may not be as sensitive, comparing to *in vivo* studies, to evaluate the immunostimulatory effect of CpG-ODN. The dose of 500 µg used *in vivo* and the presence of other cell populations *in vivo* than *in vitro* might explain the immunostimulatory effects of both ODNs. Also species differences could be important. In chickens, a dose of 50 µg of the GpC-ODN caused a decreased antibody response in comparison with the CpG-sequence (Vleugels et al., 2002). All these results indicate that CpG-ODN are promising adjuvants for enhancing the antibody responses (including IgA responses) after IM immunisation. They could

even skip booster immunisations. Indeed, a single injection of OVA combined with CpG-ODN resulted in antibody titers, which were as high as the titers in the control group after two immunisations.

8.2.2. Cellular responses and Th1/Th2 bias

*In vitro* studies with porcine peripheral monomorphonuclear cells (Chapter 5) clearly showed that 5'-ggTGC-ATC-GAT-GCA-Gggggg-3' was the optimal sequence to induce proliferation. These CpG-ODN exerted effects on B-lymphocytes and enhanced the percentage of cells with MHC class II expression as well as the degree of expression following *in vitro* stimulation. A (polyclonal) B-cell proliferation is also observed in other species such as mice (Krieg et al., 1995), humans (Hartmann et al., 2000) and cattle (Brown et al., 1998 ; Pontarollo et al., 2002). In contrast, the CpG-ODN which was immunostimulatory in our *in vivo* study (Chapter 4) did not induce any proliferative responses. Following *in vivo* administration, these CpG-ODN significantly enhanced OVA-specific proliferation as well as the ConA-induced proliferation of MC from blood, spleen and the local draining lymph node. So, even though these non-optimal CpG-ODN had no direct effect on MC *in vitro* they had an effect when given *in vivo*. That ConA-induced proliferation was enhanced (Chapter 4) in the CpG-ODN injected pigs suggest that CpG-ODN also activate T cells as observed in mice (Lipford et al., 1997, Liang et al., 2000). This could occur directly or indirectly via activation of B cells or APC by upregulation of cell-surface molecules or enhanced secretion of cytokines (Lotz et al., 1996). Indeed, in mice (Roman et al., 1997) and man (Hartmann et al., 2000). CpG-ODN are known to be Th1-modulating adjuvants with increased secretion of IFN-γ and IL-12. CpG-ODN as well as GpC-ODN significantly decreased the mRNA expression of IL-6 (proinflammatory as well as Th2 (Mosmann and Sad., 1996)) while there was a tendency for a higher IFN-γ (Th1-like) mRNA-expression. Furthermore, CpG-ODN, but also GpC-ODN, preferentially lead to the secretion of IgG2 antibodies in comparison with pigs which did not receive these oligonucleotide sequences. Only the IgG2 response was significantly higher and not the IgG1 response. In mice, Th1-modulating adjuvants stimulate the secretion of IgG2a antibodies (Krieg et al., 1995). It should be stressed that the Th1/Th2 paradigm and the subsequent secretion of different Ig isotypes does not necessarily hold true for pigs as it is for mice. For example, IL-10 has been described in mice as a pivotal cytokine in the downregulation of murine Th2 responses. It inhibits Th1 cytokines, down-regulates MHC class II antigen expressing and inhibits the production of oxygen intermediates. This is not the case in cattle (Brown et al., 1994) indicating that findings in mice can not be blindly copied to other species.

## 8.3. The effect of 1α,25(OH)$_2$D$_3$ and CpG-ODN on protection at the intestinal mucosa after an IM immunisation

To test the effect of 1α,25(OH)$_2$D$_3$ and CpG-ODN on protection against mucosal infection, F4 fimbriae of enterotoxigenic *E. coli* (ETEC) were used as immunizing antigens and the F4$^+$-ETEC as a challenge strain. F4$^+$-ETEC are an important cause of neonatal and PWD in piglets. To protect against PWD, the induction of an active IgA response is required. Different factors such as cytokines, the route of immunisation and the antigen-dose can influence the induction of an IgA response. The effect of the dose of F4 on the IgA response was studied in chapter 6. Pigs were intramuscularly injected with 1, 0.1 or 0.01 mg of F4-fimbriae and tested for their F4-specific isotype-specific serum antibody response as well as for the number of antigen-specific isotype-specific ASC in the local draining lymph node. Intramuscular immunisation with 0.1 mg resulted in higher IgA serum antibodies and higher number of F4-specfic IgA ASC in the local draining lymph node than with the 1 mg or the 0.01 mg doses. The lowest IgA response was seen in the 1 mg group. Therefore, the effect of 1α,25(OH)$_2$D$_3$ and CpG-ODN on protection against and F4$^+$-ETEC infection was evaluated by intramuscularly immunizing F4-seronegative F4R$^+$ suckling piglets twice with 0.1 mg F4-fimbriae in incomplete Freund's adjuvant (IFA) supplemented with either 2 µg of 1α,25(OH)$_2$D$_3$ or 500 µg CpG-ODN or without supplement (Chapter 7). Following ETEC challenge all F4-vaccinated animals showed a reduced F4$^+$ ETEC excretion in comparison with animals injected with PBS in IFA. The best protection was seen using 1α,25(OH)$_2$D$_3$. Interestingly, IM injection of F4 in IFA also reduced the faecal ETEC excretion and induced even the highest F4-specific IgA serum antibody response. In chapter 6, it was already demonstrated that this dose was optimal for inducing IgA. 1α,25-Dihydroxyvitamin D$_3$ could not increase this IgA response. This in contrast with the HSA-studies in which the IgA response could be increased (Chapter 2 and 3) and shows that the nature of the antigen also influences the effect of 1α,25(OH)$_2$D$_3$ on the immune responses (Hughes et al., 1998). The F4-antigen, that has a polymeric nature, induces already potent immune antibody responses when given with IFA which are difficult to ameliorate.

In all IM immunisations in this thesis, IFA was used as adjuvant. IFA has been shown to stimulate the humoral immunity as well as the cellular immunity and has been used in experimentally veterinary vaccines such as equine influenza virus (Street 1967), hog cholera (Ott et al., 1962), Newcastle disease (Eidson 1982) and infectious canine hepatitis (Fastier et al., 1964). Remarkably, the combination of F4-fimbriae in IFA even reduced the faecal F4$^+$-E. *coli* excretion, although not significantly. The reason for this reduction could be due to the high serum IgA titers.

Of all F4-vaccinated groups, only the 1α,25$(OH)_2D_3$ injected animals displayed a secondary IgA serum response upon ETEC challenge (Chapter 7). This indicates that the vaccine, supplemented with 1α,25$(OH)_2D_3$, is able to prime the intestinal mucosal immune system. This is consistent with results in chapter 3, which demonstrated an increased number of antigen-specific IgA ASC in the PP as well as in the LP after an IM injection of HSA supplemented with 1,25(OH)2D3. However, in that study the number of antigen-specific ASC was not very high. There must have been enough $IgA^+$-memory B cells in the mucosa after the IM immunisation to give a booster response upon challenge that lead to a significant reduction in the faecal $F4^+$-E. coli excretion. Probably not enough F4-specific IgA ASC appeared to give complete protection. In mice studies, 1α,25$(OH)_2D_3$ changes the maturation and migratory properties of DC subsequent to antigen stimulation (Enioutina et al., 2000). The DC preferentially home towards the Peyer's patches and may be responsible for the good mucosal immune response and the appearance of $IgA^+$-B cells and $IgA^+$ memory cells. The mechanism of the mucosal priming of pigs intramuscularly immunized with antigen and 1α,25$(OH)_2D_3$ still has to be elucidated.

The protective effect of CpG-ODN could not be explained by enhanced antibody responses but rather by an enhanced cellular response as assessed by the proliferation assay. *In vivo* CpG-ODN enhance the mitogen-induced as well as the antigen-specific proliferation. Further studies are required to fully characterize the role of CpG-ODN in the protection against an $F4^+$-ETEC infections.

## 8.4. Main conclusions and future perspectives

In this thesis, the adjuvant-properties of 1α,25$(OH)_2D_3$ and CpG-ODN and more specifically their capacity to modulate towards an IgA response were analysed. The present work demonstrated, that IM immunisation of pigs with an antigen in IFA and supplemented with 1α,25$(OH)_2D_3$ or CpG-ODN enhanced the IgA serum antibody response as well as the IgA titers in different mucosal secretions. Especially, the steroid hormone seems to modulate towards the IgA isotype. The increased IgA response due to the steroid hormone seems to be transient and antigen-dependent. Whether or not a Th2-like response is involved in this modulation could not clearly be demonstrated. Most likely the classification of T-helper cells in Th1/Th2/Th3-cells is not that clear in pigs as it is in mice. CpG-ODN induced a more general activation of the immune system. It was demonstrated that CpG-ODN activate B cells directly whereas T cells are probably activated indirectly. An experiment in which newly-weaned piglets were challenged with $F4^+$-ETEC after they were IM immunized with F4-fimbriae during the suckling period showed that protection can be enhanced by addition of 1α,25$(OH)_2D_3$ and CpG-ODN to the fimbriae. Since

$F4^+$-ETEC but also other mucosal infections are an important cause of disease in young piglets with weight-loss, sometimes mortality but always important economical losses as a consequence, modulation of a systemic towards a mucosal immune response could be a major breakthrough in the prophylaxis against these diseases. Further studies are needed to examine whether in the presence of maternal antibodies a systemic (IM, ID, SC) or even an oral administration of vaccines supplemented with $1\alpha,25(OH)_2D_3$ still results in a priming of the intestinal mucosal immune system. CpG-ODN on the other hand may be used in vaccines by linking these CpG-ODN directly to antigens or by adding the motifs to the backbone of DNA-vaccines so enhancing the uptake and the immunogenicity of these vaccines.

# SUMMARY

Intestinal infections are an important cause of disease, weight-loss and mortality in young animals. Whether newborns and suckling animals can be protected by milk antibodies, this passive protection disappears after weaning. Weaned animals need an active intestinal immunity for protection against enteropathogens. This means that these young animals need to be immunized during the suckling period in the presence of lactogenic, often antigen-specific, antibodies. The presence of these antibodies mortgages the oral vaccination route. Parenteral vaccination in the presence of declining maternal serum antibodies is another option. However, parenteral vaccines in general stimulate the systemic rather than the mucosal immune system. Therefore the use of immunomodulating adjuvants, which could modulate from a systemic towards a mucosal immune response, in parenteral vaccines is an interesting approach to face this problem.

In the present thesis the immunomodulating capacity of 1α,25-Dihydroxyvitamin $D_3$ ($1\alpha,25(OH)_2D_3$) and synthetic cytidine-phosphate-guanosine-oligodeoxynuclotides (CpG-ODN) was studied on an intramuscular (IM)-induced immune response. 1α,25-Dihydroxyvitamin $D_3$, the active form of vitamin D, exerts its effects as a steroid hormone by activation of the nuclear vitamin D receptor and appears to modulate a systemic immune response towards a protective mucosal (IgA) immune response in mice. This IgA response was accompanied with the presence of a Th2-cytokine profile in the local draining lymph node after vaccination. CpG-ODN, which are DNA-sequences characteristic for many bacteria, have potent immunoenhancing properties and are classified as Th1-modulating adjuvants. Co-administration of CpG-ODN and an antigen showed induced antigen-specific humoral, cellular immune responses, and protection upon challenge.

Chapter 1 reviews the immunomodulating properties of $1\alpha,25(OH)_2D_3$ as well as CpG-ODN. Short definitions of these agents, their effects on the different immune cells, their toxicity as well as their applications are described. Most of these effects are determined for rodents and man but not for companion and farm animals. The Th1/Th2 paradigm and the role of the cytokines in modulating the immune responses are emphasized. In the final part of the review, the use of $1\alpha,25(OH)_2D_3$ and CpG-ODN as potent immunomodulating adjuvants in veterinary medicine are discussed.

Chapters 2 to 7 present the experimental work of the thesis. The objective was to examine the following questions:

Can 1α,25$(OH)_2D_3$ enhance the antigen-specific IgA response after IM immunisation?

Is the enhanced IgA response correlated with a Th2-like cytokine response ?

Can co-administration of an antigen with 1α,25$(OH)_2D_3$ prime for an antigen-specific mucosal intestinal immune response? Is there homing of antigen-specific antibody secreting cells (ASC) towards the gut associated lymphoid tissue (GALT)?

Can CpG-ODN be used as an immunomodulating/immunoenhancing adjuvant for IM immunisation? Which cell types are activated by CpG-ODN and which CpG-ODN sequences are immunostimulating?

Does intramuscular immunisation of F4-fimbriae supplemented with 1α,25$(OH)_2D_3$ or CpG-ODN result in protection against challenge with $F4^+$-ETEC ?

In chapter 2, the effect of 1α,25$(OH)_2D_3$ on the isotype-specific antibody response in serum as well as the IgA titer in mucosal secretions was determined after IM injection of human serum albumin (has) in incomplete Freund's adjuvant (IFA) with or without 1α,25$(OH)_2D_3$. The steroid hormone significantly enhanced transiently the antigen-specific IgA and IgM serum antibody responses and increased the IgA titers in saliva, feces and nasal secretions. 1α,25-Dihdyroxyvitamin $D_3$ also enhanced the antigen-specific IgA and IgG ASC in the local draining lymph nodes.

In chapter 3, ELIspot assays of systemic lymphoid tissues (local draining lymph node, spleen and bone marrow) as well as gut associated lymphoid tissue (GALT) showed slightly higher numbers of HSA-specific IgA ASC in the systemic lymphoid organs as well as in the Peyer's patches (PP) of the 1α,25$(OH)_2D_3$ injected pigs. This might suggest that IM administered antigen co-administered with 1α,25$(OH)_2D_3$ can prime the intestinal mucosal immune system. 1α,25-Dihydroxyvitamin $D_3$ increased the % of $CD25^+$ cells, an activation marker, in the local draining lymph node but did not affect the relative distribution of $CD4^+$, $CD8^+$, MHC $II^+$ and $IgM^+$ cells indicating more activated T- and B cells using the steroid hormone. Mixed cytokine profiles (Th1/Th2/Th3) of the monomorphonuclear cells of the local draining lymph node were detected by real time PCR. In pigs 1α,25$(OH)_2D_3$ did not induce a clear Th2 response as observed in mice. Although some trends in these profiles were observed: after the first immunisation a higher IL-10 (Th2) and a significantly lower IL-2 (Th1-like) mRNA expression were seen while after the booster immunisation even a significantly higher IL-2 (Th1-like) accompanied with lower IFN-γ (Th1-like) and TGF-β mRNA (Th3-like) expressions were observed.

In chapter 4, it was shown that CpG-ODN (5'GCT-AGA-**CG**T-TAG-**CG**T-3') can be used as adjuvant in pigs for intramuscular immunisations. Indeed, injection of 500 μg of these CpG-ODN together with ovalbumin (OVA) enhanced the serum IgM, IgA, IgG1, IgG2 as well as the

IgG antibody responses and induced showed significantly higher OVA-specific IgA antibodies in saliva and nasal secretions. This enhanced antibody response appeared CpG-dinucleotide independent as similar effects were seen after IM injection of antigen supplemented with 500 µg GpC-ODN (same sequence but CpG-dinucleotide is reversed in GpC). The OVA-specific antibody titers obtained after a single injection of antigen combined with either of both ODNs were as high as the titers in control OVA injected animals after two injections. CpG-ODN as well as GpC-ODN also stimulated the cellular immune responses since enhanced OVA and ConA-specific proliferation were obtained with MC of different lymphoid tissues. Furthermore, there was a tendency for a higher IFN-γ (Th1-like) mRNA-expression in the MC of the local draining lymph node while the IL-6 mRNA expression was significantly lower. No significant difference in TGF-β (Th3-like) and IL-10 (Th2-like) were observed. Since single immunisations containing ODN equals double immunisations without ODN, this adjuvant could make a second immunisation redundant.

In vitro proliferation assays of PBMC using two different CpG-ODN sequences (Chapter 5) showed that the swine-specific ODN sequence [$B_s$] = 5'-ggTGC**ATCGAT**GCAGggggg-3' is optimal for proliferation of porcine PBMC while the CpG-ODN used in chapter 4 did not induce any proliferation. Moreover, it was shown that addition of a phosphorothioate G-stretch at both ends ameliorates that proliferation. Results demonstrated that a 4-days culture with this motif increased B cell blasting as well as the B-cell percentage with 12%. On the other hand a similar decrease of $CD3^+$T cells was observed. Also MHC class $II^+$-cells were enhanced with 3% was as well as an enhanced expression of MHC II-molecules on these cells. On the other hand no difference in the percentage of macrophages was observed. Therefore, it is believed that B-cells are the primary target cells for this CpG-ODN. Whereas methylation of CpG-dinucleotide should reduce or eliminate immunostimulation of the CpG-ODN, this effect differed depending on the manufacturer of the oligodeoxynucleotides.

Chapter 6 demonstrated that also the dose of an antigen can modulate an IM induced immune response as shown for F4-fimbriae. Indeed, 1mg purified F4-fimbriae significantly decreased the primary F4-specific IgG and IgM responses in comparison with the lower doses of 0.1 and 0.01 mg F4. However, the high dose primed for an unusually high secondary IgM response. The polymeric nature of the fimbriae could make it act as a T-cell independent antigen. The dose of 0.1 mg induced the highest numbers of IgA ASC in the local draining lymph as well as the highest F4-specific IgA serum response, which was significantly higher than after injection with 0.01 and 1mg F4. The dose of 0.1 mg purified F4 seems to be optimal for inducing F4-

specific IgA responses after IM immunisation. It is conceivable that IM injection of different doses F4 induces distinct patterns of cytokines resulting in different ratios of F4-specific isotypes.

Chapter 7 analysed whether IM immunisation of suckling piglets with 0.1 mg F4-fimbriae could protect against oral challenge with $F4^+$-ETEC and whether addition of $1\alpha,25(OH)_2D_3$ or CpG-ODN could improve this protection. It was clearly shown that IM injection reduced the $F4^+$-ETEC faecal excretion upon challenge. The highest reduction and the only statistically significant reduction in comparison with the control group (no F4-immunisation) was observed in the $1\alpha,25(OH)_2D_3$ treated animals. Only the steroid-treated animals showed a secondary IgA response upon ETEC challenge indicating that F4-primed memory B cells might be present in the GALT at that moment. CpG-ODN did not enhance the secondary antibody response but significantly increased the F4-specific as well as mitogen-induced proliferation of pheripheral blood monomorphonuclear cells indicating a direct or indirect overall effect on T-lymphocytes. Nevertheless the CpG-ODN also seemed to reduce faecal excretion of $F4^+$-E. *coli.* Results of this chapter revealed the potential of $1\alpha,25(OH)_2D_3$ to be used in veterinary vaccines against enteropathogens.

The final chapter presents the general discussion and conclusions with respect to the obtained results. In this thesis it was demonstrated that $1\alpha,25(OH)_2D_3$ as well as CpG-ODN can be used as potential immunostimulating/-modulating adjuvants after IM immunisation in pigs. Both agents were able to enhance antigen-specific antibody responses and IgA antibody titers in mucosal secretions at some time points after IM injection. Moreover, results indicate that supplementation with $1\alpha,25(OH)_2D_3$ or CpG-ODN into the vaccine can prime mucosal surfaces in the gut. This priming resulted in a partial protection upon oral challenge with live bacteria. Despite strong adjuvanticity we have no evidence to consider CpG-ODN or $1\alpha,25(OH)_2D_3$ as typical Th1 or Th2-modulating adjuvants respectively. Moreover, there is no evidence at all that in pigs the Th1 or Th2-cytokine profiles are correlated with the subsequent secretion of specific Ig isotypes by differentiated B cells as clearly demonstrated in mice.

Nevertheless, the knowledge of the immunomodulating and -regulating mechanisms of CpG-ODN and $1\alpha,25(OH)_2D_3$ is of great importance to design new or improve current veterinary vaccines. The different techniques used in this thesis (*in vitro* proliferation, real time PCR for quantificantion of cytokines, ELIspot assays, etc) allow screening for other potential immunomodulators in different domestic animal species.

# SAMENVATTING

Intestinale infecties met o.a. enterotoxigene *Escherichia coli* (ETEC) vormen een belangrijke oorzaak van diarree met gewichtsverlies en mogelijks zelfs sterfte bij neonatale en pasgespeende biggen. Neonatale en zuigende biggen zijn beschermd tegen deze infecties door de aanwezigheid van een lactogene immuniteit. Deze passieve immuniteit kan geïnduceerd worden door vaccinatie van zeugen tijdens de dracht. Na het spenen verdwijnt deze lactogene immuniteit en worden de dieren opnieuw gevoelig voor intestinale infecties. Op dat ogenblik is een actieve intestinale mucosale immuniteit vereist voor protectie. Deze kan enkel bekomen worden indien de biggen gevaccineerd worden tijdens de zoogperiode, dus in de aanwezigheid van lactogene immuniteit. Melkcomponenten en antistoffen in de melk kunnen interfereren met een immunisatie via de orale weg. Parenterale immunisatie van zogende dieren is een ander alternatief. Parenterale vaccinatie stimuleert in de regel echter eerder het systemisch dan wel het mucosaal immuunsysteem. Om die reden moet er gebruik gemaakt worden van adjuvantia die in staat zijn een systemische immuunrespons te moduleren naar een mucosale respons.

Het in dit proefschrift beschreven onderzoek evalueert bij het varken de immunostimulerende en immunomodulerende eigenschappen van twee adjuvantia, namelijk 1α,25-dihydroxyvitamin $D_3$ ($1\alpha,25(OH)_2D_3$) en synthetische cytidine-fosfaat-guanosine oligodeoxynuclotide sequenties (CpG-motieven), op een intramusculaire immunisatie.
1α,25-Dihydroxyvitamin $D_3$, de actieve vorm van vitamine $D_3$, oefent zijn immunomodulerende werking uit door activatie van de nucleaire vitamine D receptor. Deze receptor vormt samen met de retinoïd X receptor een heterodimeer die de transcriptie regelt van bepaalde doelwitgenen. Deze doelwitgenen zijn onder andere genen voor cytokines zoals IL-2, IFN-γ, enz. Bij muizen werd aangetoond dat $1\alpha,25(OH)_2D_3$ in staat is een systemische immuunrespons te moduleren naar een mucosale IgA respons. Hierbij werden verhoogde IgA serumantistoffen alsook een stijging van het aantal IgA antistoffen-producerende cellen ter hoogte van het darm-geassocieerde lymfoïd weefsel (GALT) gevonden. Dit ging tevens gepaard met een stijging van Th2-cytokines ter hoogte van de lokaal drainerende lymfeklier. Daarom wordt $1\alpha,25(OH)_2D_3$ geklasseerd als een Th2-modulerend adjuvans.

CpG-ODN-motieven komen frequent voor in bacterieel DNA. Deze CpG-motieven behoren tot de klasse van ‘bacteriële’ adjuvantia. De immunostimulerende werking van bacterieel DNA en synthetische CpG-motieven is volledig te wijten aan de aanwezigheid van een CpG-dinucleotide in een welbepaalde context zijnde een “3’-purine-purine-CpG-pyrimidine-pyrimidine-5’ configuratie. Onderzoek bij muizen toonde aan dat CpG-motieven goede adjuvantia zijn wat zich vertaalt in een verhoging van de antigeen-specifieke humorale en cellulaire immuunrespons met

een verbeterde bescherming na een experimentele infectie. Bovendien moduleren de CpG-motieven de immunologische respons door activatie van B-cellen en antigeen-presenterende cellen en door Th1-cytokines, cytokines die een celgemedieerde respons in de hand werken.

De immunostimulerende eigenschappen van $1\alpha,25(OH)_2D_3$ en CpG-motieven alsook hun mogelijke toepassingen in de diergeneeskunde worden beschreven in deel 1 van deze thesis (hoofdstuk 1, overzicht van de literatuur). Verder wordt de recente kennis bij mens en muis over Th1-,Th2- en Th3-cytokines beschreven alsook hun rol in het moduleren van de antistoffenrespons naar bepaalde isotypes. Ook wat hierover gekend is bij het varken wordt bediscussieerd.

Deel III (hoofstukken 2-7) bevat het eigen onderzoek. Hierbij werden de volgende vragen onderzocht (Deel II, Aims of the study).

- Kan $1\alpha,25(OH)_2D_3$ de antigeen-specifieke IgA respons verhogen na intramusculaire (IM) immunisatie?
- Indien ja, is de verhoogde IgA respons gecorreleerd met een Th2-cytokinerespons?
- Is er een priming van de intestinale mucosale immuniteit na IM vaccinatie met antigeen plus $1\alpha,25(OH)_2D_3$ , m.a.w. vindt men antigeen-specifieke antistoffen-producerende cellen t.h.v. het GALT na deze vaccinatie?
- Kunnen CpG-motieven gebruikt worden als immunostimulerende en/of modulerende adjuvantia na IM immunisatie? Indien ja, welke sequentie en welke cellen worden door deze CpG-motieven geactiveerd?
- Leidt IM immunisatie van zuigende biggen met F4-fimbriae samen met $1\alpha,25(OH)_2D_3$ en/of CpG-motieven tot bescherming tegenover een infectie met $F4^+$-ETEC?

In hoofdstuk 2 werd aangetoond dat varkens na IM injectie met humaan serumalbumine (HSA) gesupplementeerd met 2 µg $1\alpha,25(OH)_2D_3$ tijdelijk een significant hogere serum IgA en IgM respons vertoonden dan varkens die geen $1\alpha,25(OH)_2D_3$ kregen toegediend. Verder werden bij deze varkens op sommige tijdstippen hogere HSA-specifieke IgA antistoffen gedetecteerd in de faeces, het speeksel en het neussekreet. Bovendien was de lokaal drainerende lymfeklier sterk vergroot en werden in de drainerende lymfeklieren ook een hoger aantal HSA-specifieke IgA en IgG antistoffen-producerende cellen gevonden. Deze resultaten tonen aan dat $1\alpha,25(OH)_2D_3$ in staat is de systemische maar ook de mucosale antigeen-specifieke IgA antistoffenrespons te verhogen.

Deze resultaten werden bevestigd in een tweede experiment (hoofdstuk 3) waarin werd aangetoond dat $1\alpha,25(OH)_2D_3$ moduleert naar een IgA respons. Immers in de groep geïnjecteerd

met HSA en 1α,25$(OH)_2D_3$ was enkel de HSA-specifieke serum IgA respons en niet de IgM of IgG respons verhoogd. Ook in de lokaal drainerende lymfeklier was er een niet significante toename van het aantal HSA-specifieke IgA antistoffen-producerende cellen, terwijl in de jejunale en ileale Peyerse platen (GALT) meer HSA-specifieke antistoffen-producerende cellen werden gedetecteerd bij dieren geïnjecteerd met 1α,25$(OH)_2D_3$. Op verschillende tijdstippen na IM injectie werd enerzijds de relatieve distributie van $CD2^+$-, $CD4^+$-,$CD8^+$-,$CD25^+$- en $IgM^+$ -cellen bepaald en anderzijds werd ook de cytokine mRNA expressie voor IL-2, IL-4, IL-6,IL-10, IFN-γ en TGF-β gekwantificeerd. Een significante stijging van $CD25^+$-cellen na de tweede IM injectie met antigeen plus 1α,25$(OH)_2D_3$ toont aan dat er een algemene activatie is van T-en/of B-cellen o.i.v. 1α,25$(OH)_2D_3$. Ondanks een duidelijk verhoogde IL-10 (Th2-like) en een verlaagde IL-2 mRNA (Th1-like) expressie na de eerste IM immunisatie, kon er geen duidelijke Th2-cytokinerepons worden aangetoond. Deze resultaten tonen aan dat intramusculaire toediening van een antigeen samen met 1α,25$(OH)_2D_3$ het mucosaal immuunsysteem bij biggen kan 'primen' met verhoogde aantallen IgA antistoffen-producerende cellen ter hoogte van de Peyerse platen.

In hoofdstuk 4 werd aangetoond dat muizen-specifieke CpG-motieven (5'GCT-AGA-**CG**T-TAG-**CG**T-3') alsook de omgekeerde "GpC-motieven" (=5'GCT-AGA-**GC**T-TAG-**GC**T-3'), kunnen gebruikt worden als immunostimulerend adjuvans bij varkens. Injectie van biggen met 1 mg ovalbumine (OVA) in incompleet Freund's adjuvant (IFA), gesupplementeerd met 500 µg van deze CpG-motieven of de GpC-motieven, verhoogden de OVA-specifieke antistoffenrespons. De antistoffenrespons, na 1 IM injectie van biggen met CpG-motieven, was even hoog als deze na 2 IM injecties in afwezigheid van deze motieven. Door CpG-en/of GpC-motieven te gebruiken, verhoogde tevens de OVA-specifieke IgA respons in speeksel en neussekreet. Beide motieven verhoogden tevens de OVA-specifieke evenals de ConA-geïnduceerde proliferatie van monomorfonucleaire cellen geïsoleerd uit het bloed, de milt en de lokaal drainerende lymfeklier. Dit ging gepaard met een significant lagere expressie van IL-6 mRNA. CpG-motieven induceerden tevens een lichte stijging van IFN-γ mRNA. Er kon echter geen duidelijke Th1-cytokine respons worden aangetoond in tegenstelling met wat volgens de literatuur zou kunnen verwacht worden.

Hoofdstuk 5 beschrijft de resultaten van de *in vitro* proliferatie van monomorfonucleaire cellen uit perifeer bloed na incubatie met 2 verschillende CpG-motieven motieven namelijk 5'-GCTAGACGTTAGCGT-3', dat gebruikt werd in de *in vivo* studie (hoofdstuk 4), en 5'-TGCATCGATGCAG-3', een varkensspecifiek CpG-motief. Er werden tevens wijzigingen in beide motieven aangebracht zoals onder andere methylatie van het C-nucleotide, toevoegen van poly-G staarten en fosfor-thioaatbindingen. Resultaten van deze studie toonden aan dat incubatie met het CpG-motief 5'-ggTGCATCGATGCAGggggg-3' (voorzien van een poly G

staart uit fosfor-thioaatbindingen) resulteerde in een toename van B- en MHC $II^+$-cellen met respektievelijk 12 en 3 % alsook in een kleine toename van 'B-cell blasting'. Ook de MHC II-expressie nam toe. Hieruit werd besloten dat dit dit CpG-motief de perifere bloed B-cellen van het varken stimuleert. Of deze stimulatie rechtstreeks of onrechtstreeks gebeurt kan hieruit niet geconcludeerd worden.

Het uiteindelijke doel van deze thesis was na te gaan of $1\alpha,25(OH)_2D_3$ en/of CpG-ODN na IM injectie ook protectie kon geven ter hoogte van de darm. Dit werd nagegaan in het F4-model waarbij dieren intramusculair worden geïnjecteerd met F4 antigeen en vervolgens aan een challenge met $F4^+$-enterotoxigene E. *coli* worden onderworpen. Hiertoe was het nodig de optimale dosis te bepalen voor een intramusculaire immunisatie. In hoofdstuk 6 werd de antigeen-specifieke isotype-specifieke antistoffenrespons nagegaan van IM injecties met verschillende dosissen F4-antigeen. Intramusculaire injectie met een hoge dosis (1 mg) F4 fimbriae onderdrukte significant de primaire F4-specifieke IgM en IgG respons in vergelijking met de lagere dosissen (0,1 en 0,01 mg F4 fimbriae) maar leidde tot een significant hogere secundaire IgM respons. De dosissen van 0,1 en 0,01 mg F4 fimbriae induceerden een hoger aantal F4-specifieke IgA en IgG antistoffen-producerende cellen in de lokaal drainerende lymfeklier. Daarenboven bleek uit deze studie dat voornamelijk de 0,1 mg dosis optimaal is voor de inductie van een F4-specifieke IgA respons. Dus niet alleen het adjuvans maar ook de dosis van het antigeen kan belangrijk zijn voor het moduleren van een immuunrespons.

In het finaal experiment (hoofdstuk 7) werd nagegaan of F4-receptor positieve biggen beschermd konden worden tegen een orale infectie met een virulente $F4^+$-ETEC stam door een IM immunisatie met 0,1 mg F4 fimbriae, al dan niet gesupplementeerd met $1\alpha,25(OH)_2D_3$ of CpG-motieven. Hiervoor werd het aantal $F4^+$-ETEC/gram faeces/dier bepaald na experimentele infectie. De resultaten tonen aan dat de IM immunisatie met 0,1 mg F4 fimbriae in IFA een daling van $F4^+$-ETEC in de faeces tot gevolg had in vergelijking met de controlegroep. Deze daling was meer uitgesproken en zelfs significant wanneer gelijktijdig respectievelijk CpG-motieven en $1\alpha,25(OH)_2D_3$ werden toegediend. Biggen, geïnjecteerd met $1\alpha,25(OH)_2D_3$, vertoonden een secundaire serum IgA respons na de experimentele infectie met ETEC. Dit in tegenstelling met de andere groepen. Daarom werd gesuggereerd dat IM injectie van antigeen samen met $1\alpha,25(OH)_2D_3$ resulteerde in een priming ter hoogte van de intestinale mucosa. De aanwezigheid van memory IgA B-cellen kan inderdaad een verklaring zijn voor de partiële bescherming bij de $1\alpha,25(OH)_2D_3$-behandelde biggen op het moment van de infectie. Tevens werd opnieuw aangetoond dat CpG-motieven de proliferatie van perifere bloed monomorfonucleaire cellen significant verhoogt. De rol van de cellulaire immuniteit en van de CpG-motieven in de bescherming tegenover intestinale infecties is onduidelijk.

Deel IV (hoofdstuk 8) bevat de algemene discussie en de belangrijkste conclusies. Dit werk heeft aangetoond dat systemische vaccinatie (IM immunisatie) kan leiden tot een partieel protectieve en tijdelijk verhoogde IgA respons mits toevoegen van 1α,25$(OH)_2D_3$ en/of CpG-motieven.

Vooral toevoegen van 1α,25$(OH)_2D_3$ verhoogt de antigeen-specifieke IgA respons in het serum en in de mucosale secreties. Er werd eveneens aangetoond dat CpG-motieven kunnen gebruikt worden als immunostimulerende en/of immunomodulerende adjuvantia. Of Th2- en/of Th1-cytokines betrokken zijn in deze modulatie kon niet duidelijk worden opgehelderd. De bekomen resultaten suggereren dat Th1 en Th2 niet zo uitgesproken zijn bij het varken dan bij mens en muizen. Het experiment waarbij biggen werden geïnfecteerd met een virulente $F4^+$-ETEC stam wees uit dat 1α,25$(OH)_2D_3$ kan gebruikt worden als potentiële adjuvantia in vaccins voor intestinale infecties na systemische (IM) vaccinatie. In de toekomst dient verder onderzocht te worden of beide adjuvantia en/of andere vaccinatieroutes de mucosale respons nog kunnen verbeteren.

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Yves

# CURRICULUM VITAE

Yves Van der Stede werd geboren op 26 oktober 1973 te Lokeren. Na het beëindigen van het secundair onderwijs, richting Wetenschappelijke B, aan het Sint-Lodewijkscollege te Lokeren, begon hij met de studies Diergeneeskunde aan de Universiteit Gent. In 1997 studeerde hij af als dierenarts met grote onderscheiding. Zijn interesse voor wetenschappelijk onderzoek, vaccinaties en immunologie bleek reeds uit zijn eindstudiewerk 'Inductie van een mucosale immuunrespons door middel van systemische immunisatie bij het varken' dat werd bekroond met de Vetoquinol prijs. Onmiddellijk daarna verkreeg hij een doctoraats- specialisatiebeurs, toegekend door het Vlaams instituut voor de bevordering van het wetenschappelijk-technologisch onderzoek in de industrie (IWT). Vanaf oktober 2001 werkte hij op een verkennend Europees Onderzoeksproject (VEO) toegekend door de Universiteit Gent. Zijn onderzoek aan het Laboratorium voor Immunologie der Huisdieren handelde over de immunomodulerende eigenschappen van $1\alpha,25(OH)_2D_3$ en CpG-ODN, en leidde tot dit proefschrift. In 1999 werd een Master of Science in the molecular Biology and Biotechnology gestart aan de VUB waarvan het diploma werd behaald in juni 2001 met grote onderscheiding. In juli 2002 behaalde hij het getuigschrift voor de doctoraatsopleiding in de diergeneeskundige wetenschappen. Sinds 1 augustus 2002 is hij voltijds terwerkgesteld als hoofd laboratorium te Lier (Dierengezondheidszorg Vlaanderen).

# PUBLICATIES

**Wetenschappelijke publicaties in tijdschriften met impact factor >0.3.**

**1)** Y. Van der Stede, E. Cox, W. Van den Broeck, B.M. Goddeeris. 2001. Enhanced induction of the IgA response in pigs by calcitriol after intramuscular immunisation. Vaccine, 19:1870-1878.

2) Y. Van der Stede, F. Verdonck, S. Vancaeneghem, E. Cox, B.M. Goddeeris. 2002. CpG-oligodeoxynucleotides as an effective adjuvant in pigs for intramuscular immunisations. Veterinary Immunology and Immunopathology, 86:31-41.

3) Y. Van der Stede, E. Cox, B.M. Goddeeris. 2002. Antigen dose modulate the immunoglobulin istoype responses of pigs against intramuscular injected F4-ETEC fimbriae. Veterinary Immunology and Immunopathology, 88: 209-216.

4) Y. Van der Stede, E. Cox, F. Verdonck, S. Vancaeneghem, B.M. Goddeeris. 2003. Reduced faecal excretion of F4+-E. coli by the intramuscular immunisation of suckling piglets by the addition of 1α,25-dihydroxyvitamin D3 or CpG-oligodeoxynucleotides. Vaccine, 21:1023-1032.

5) E. Cox, Y. Van der Stede, F. Verdonck, V. Snoeck, W. Van den Broeck, B.M. Goddeeris. 2002. Oral immunisation of pigs with fimbrial antigens of enterotoxigenic E. coli: an interesting model to study mucosal immune mechanisms. Veterinary Immunology and Immunopathology, 87:287-290.

**6)** F. Verdonck., E. Cox, K.Van Gog, Y. Van der Stede, L. Duchateau, P. Deprez, B.M. Goddeeris B.M. 2002. Different antibody responses following infection of newly weaned piglets with an F4 enterotoxigenic *Escherichia coli* strain or an F18 verotoxigenic *Escherichia coli* strain. Vaccine, 20: 2995-3004.

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**Lijst van abstracts, posters en actieve deelname (voordrachten) op Internationale en Nationale congressen (Symposia).**

1) Y. Van der Stede, W. Van den Broeck, E. Cox, B.M. Goddeeris. Modulation of the IgA antibody response in pigs following peripheral immunisation. Immunology Letters, 1997, p 288, Abstracts of the 13th European Immunology meeting 22-25 June 1997, Amsterdam, the Netherlands. Abstract + Poster
2) Y. Van der Stede, W. Van den Broeck, E. Cox, B.M. Goddeeris. Modulation by calcitriol of the IgA response in pigs against intramuscularly injected antigen. ID-DLO Symposium, 15-16 September 1997, Amsterdam, the Netherlands. Poster
3) Y. Van der Stede, T. Verfaillie, E. Cox, B.M. Goddeeris. Modulation by calcitriol of the IgA response in pigs against intramuscularly injected antigen. Immunology Letters 69,1:60, 11.21.10th International Congress of Mucosal Immunology 1999, June 27-1 july, Amsterdam, the Netherlands. Abstact + Poster + Oral communication.
4) Fifth Annual Meeting of the "Groupe de Contact du FNR: Vaccins Humains" 9 December 1999. Luik, Belgium. Oral presentation: modulation by calcitriol of an intramuscularly induced immune response in pigs.
5) Bouchout, H., Verdonck, F., Van den Broeck, W., Van der Stede, Y., Billau, A., Cox, E., Goddeeris, B.M. Induction of sytemic immuno-hyporesponsiveness in pigs by mucosal administration of antigen. 2000. Second World Congress on Vaccines and Immunisation, August 29-September 3, S 3-11.Abstract.
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9) Y. Van der Stede, T. Verfaillie, E. Cox, B.M. Goddeeris. The use of calcitriol to modulate an IM induced immune response in pigs. 2000. Vaccines: new concepts and strategies. Belgian Immunological society and the FRSM-contact group on human vaccines. (BIS-meeting 24th of November 2000, Charleroi, Begium). Poster.

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