Microbiology and Tumorbiology Center
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DNA VACCINES AND BACTERIAL DNA IN IMMUNITY

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Doctoral thesis: DNA vaccines and bacterial DNA in immunity

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ABSTRACT

This thesis describes DNA-based vaccination and the importance of bacterial DNA in different immunological perspectives.

Intranasal (i.n.) DNA vaccination utilizing a plasmid encoding the chlamydial heat shock protein 60 (p-hsp-60) generated lower bacterial burden and reduced pathology in the lungs of mice after subsequent infection with *C. pneumoniae*. This DNA vaccine-induced protection was dependent on T cells and induction of IFN-γ. Co-administration of a plasmid encoding IL-12 further enhanced the protective effect of the DNA vaccine. Interestingly, in the absence of CD8 T cells, CD4 T cells induced by the p-hsp-60 DNA vaccine were involved in deteriorating the outcome of infection. This detrimental response was linked to a shift towards a Th2 cytokine profile in the lungs.

I.n. DNA vaccination using the chlamydial outer membrane protein 2 (OMP-2) combined with a CTLA-4 expression system also generated reduction of bacterial load in mouse lung after *C. pneumoniae* infection compared to controls. In contrast, subcutaneous (s.c.) OMP-2 protein-based immunization in Freund's complete adjuvant (FCA) generated increased lung bacterial load, with increased disease severity. This pathological outcome of disease was dependent on T cells and associated with a Th2 immune response. When oligodeoxynucleotides containing CpG motifs mixed with Freund's incomplete adjuvant (FIA-CpG ODN) were used as adjuvant this detrimental response was shifted towards partial protection against infection. This partial protection was linked to a Th1 type of immune response.

In an approach to identify protein(s) that are included in CpG DNA stimulation, an isolation procedure was developed for a crude protein extract prepared from peripheral blood mononuclear cells (PBMC). The binding between the protein(s) and CpG ODN in the extract was followed by electromobility shift assay (EMSA) analysis. Using a series of different chromatographic purification steps and protein sequence analysis, heat shock protein 90 (hsp90) was identified as the most likely candidate. A direct binding of hsp90 to CpG ODN was confirmed by using commercially available hsp90 in EMSA analysis. Moreover, microscopic analysis revealed cellular redistribution of hsp90 from the cytoplasm into specific nuclear compartments upon CpG ODN treatment. Thus hsp90 may be a central protein in CpG DNA recognition and signaling.

Initially, the expression of the antimicrobial peptides LL-37 and HBD-1 were shown to be downregulated early during *Shigella* infection both *in vivo* and *in vitro*. The influence of different bacterial components involved in this downregulation was further investigated. Various bacterial components were utilized, including bacterial lysate, LPS and plasmid DNA prepared from different *Shigella spp*. Indeed, plasmid DNA was found to downregulate the expression of the antimicrobial peptide LL-37 in PBMC. Such a downregulation of antimicrobial peptides, in which plasmid DNA may act as one mediator, might represent an effective strategy in combating peptide attack during the initial phase of infection.

To my family…

LIST OF PUBLICATIONS

I. Svanholm, C., **Bandholtz**, **L.**, Castanos-Velez, E., Wigzell H. and Rottenberg ME. (2000). Protective DNA immunization against *Chlamydia pneumoniae*. Scand J Immunol, 51(4):345-53

II. **Bandholtz**, **L.**, Kreuger, MR., Svanholm, C., Wigzell, H. and Rottenberg ME. (2002). Adjuvant modulation of the immune responses and the outcome of infection with *Chlamydia pneumoniae*. Clinical and Experimental Immunology. In press.

III. **Bandholtz**, **L.**, Guo, Y., Palmberg, C., Mattsson, K., Ohlsson, B., High, A., Shabanowitz, J., Hunt, D., Jörnwall, H., Wigzell, H., Agerberth, B. and Gudmundsson, GH. Hsp90 binds CpG oligonucleotides directly, implications for hsp90 as a missing link in CpG signaling and recognition. Submitted.

IV. Islam, D., **Bandholtz**, **L.**, Nilsson, J., Wigzell, H., Christensson, B., Agerberth, B. and Gudmundsson GH. (2001). Down-regulation of bactericidal peptides of innate immunity: A novel immune escape mechanism in enteropathogenic infections mediated by bacterial DNA. Nat Med, 7(2):180-85

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LIST OF ABBREVIATIONS

APC	Antigen presenting cell
CpG ODN	Oligodeoxynucleotide-containing CpG motif
C/M	Cytoplasmic/membrane
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EMSA	Electromobility shift assay
ERK	Extracellular receptor kinase
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
HBD-1	Human beta defensin-1
Hsp	Heat shock protein
I.d.	Intradermal
IFN	Interferon
Ig	Immunoglobulin
I.n.	Intranasal
I.v	Intravenous
IL	Interleukin
IRAK	IL-1 receptor-associated kinase
I.m.	Intramuscular
JNK	C-jun NH_2-terminal kinase
MALT	Mucosal-associated lymphoid tissues
MAPK	Mitogen-activated protein kinase
MyD88	Myeloid differentiation marker 88
MHC	Major histocompatibility complex
MOMP	Major outer membrane protein
N	Nuclear
NK cell	Natural killer cell
ODN	Oligodeoxynucleotide
OMP	Outer membrane protein
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PRR	Pattern recognition receptor
PO	Phosphodiester
PS	Phosphorothioate
S.c.	Subcutaneous
TNF	Tumor necrosis factor
TCR	T-cell receptor
Th cell	T helper cell
TLR	Toll-like receptor
TRAF6	Tumor necrosis factor receptor-associated factor 6

1 THESIS BACKGROUND

In this work, the focus has been DNA vaccines and bacterial DNA in connection to immunity. DNA vaccination techniques were applied in the study of *Chlamydiae pneumoniae*. Bacterial DNA has been investigated as an adjuvant and a regulator for expression of antimicrobial peptides. Moreover, we have searched for the existence of protein(s) that bind oligodeoxynucleotides containing a CpG motif (CpG ODN) directly and that could be involved in recognition and signaling of CpG ODN. As an introduction, some general aspects of the immune system and thesis-specific areas are reviewed.

1.1 IMPORTANCE OF IMMUNE RESPONSES

The ability of a host to sense invasion of pathogenic microorganisms and respond with an appropriate immune response is necesssary for limiting the infection spread and survival of the host. The immune system of vertebrates has traditionally been divided into two major parts: the fast innate immunity and the slower developing adaptive immunity. The former is present in all multicellular organisms and comprises physical barriers (e.g. skin, mucous surfaces) and cells, including macrophages, granulocytes, dendritic cells (DC) and natural killer cells (NK). Activation of these cells may result in the release of a complex network of molecules such as antimicrobial peptides, chemokines and cytokines and usually leads to inflammation at the infected site. Innate immunity represents the first line of host defense with the prime goal to destroy intruders “on the spot”. Another central function mediated by innate immune defenses is, if necessary, to initiate and direct the adaptive immune response. This becomes important when pathogens survive or avoid the early immune defense reactions in a host. The adaptive immune system evolved later and only occurs in vertebrates, with B and T lymphocytes as key mediators. Activation of B and/or T cells provides long-lasting immune responses, which might result in long-lived protective immunity. Innate and adaptive immunity should not be categorized into separate strategies to combat infections. These are two arms of the immune system with constant cross-talk and the ability to interact with eachother in different ways. For example, different kinds of infections will induce the

production of different innate cytokines, determing which parts of the adaptive immune system become activated.

1.1.1 Innate immune recognition

As a response to a microbial infection, an innate immune response rapidly develops, resulting in a wide range of fast-acting effector functions. To sense and respond against pathogenic intruders, cells of the innate immune system appear to have evolved a set of germ-line encoded innate receptor molecules. These are named pattern recognition receptors (PRRs). PRRs are able to recognize unique conserved pathogen-associated molecular patterns (PAMPs) that are derived from bacterial, fungal, viral or protozoan structures. Several different PRRs have been described in the literature and are connected to this initial recognition. CD14 (1), the mannose receptor (2), the peptidoglycan recognition protein (3), the scavenger receptors (4) and the members of the Toll-like receptor (TLR) family (5) are examples of PRRs. Engagement of PRR to PAMPs may create different signals in the cell, e.g trigger them to engulf bacteria or induce the release of functional effectors of innate immunity (6). TLR recognition of PAMPs is a novel example of interaction that results in the release of cytokines and chemokines that within minutes to hours alarm that "danger" is close, and this can subsequently lead to effective adaptive immune responses (Fig 1.).

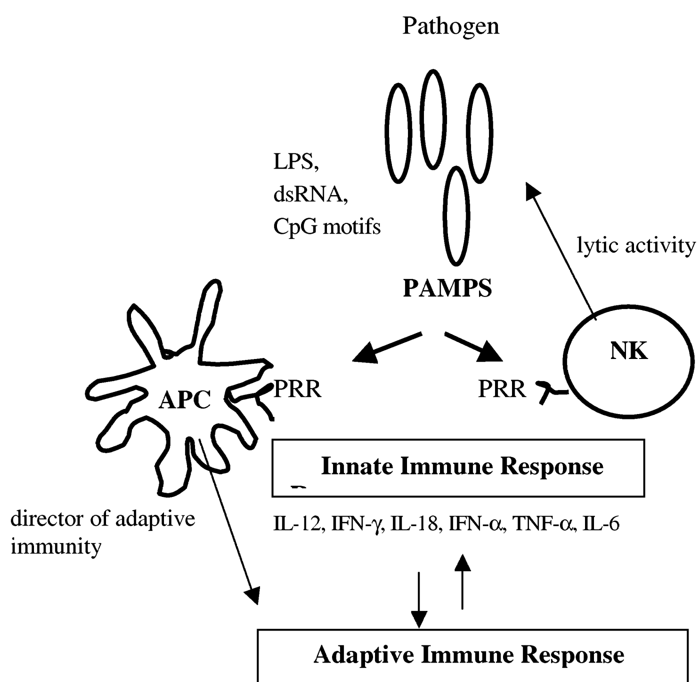

Fig 1. PAMP and PRR interaction. Invading microorganisms expressing pathogen-associated molecular pattern (PAMPs) are recognized by innate immune cells (including macrophages, DC and NK cells) via pattern recognition receptors (PRR). This process signals to innate cells the presence of foreign organisms and activates the secretion of a myriad of effector cytokines which play a variety of roles in directing immune response (adapted from (6)).

In mammals, TLRs are transmembrane receptors containing an extracellular leucine-rich domain, and an intracellular TIR (Toll/interleukin-1 receptor) signaling domain. More downstream signaling pathways include recruitment of the myeloid differentiation marker 88 (MyD88), the IL-1 receptor-associated kinases (IRAKs) and tumor necrosis factor receptor-associated factor 6 (TRAF6), resulting in mitogen-activated protein kinases (MAPKs) and NF-κB activation (7). In humans, ten different TLRs have been characterized and so far the association are: TLR-2 to peptidoglycan and lipopeptide recognition, TLR-3 to dsRNA, TLR4 to LPS, TLR-5 to bacterial flagellin and TLR-9 to unmethylated CpG (reviewed in (7)).

1.1.2 Adaptive immune recognition and initiation

The adaptive immune responses are initiated in peripheral lymphoid tissues, (i.e. spleen, lymph nodes and mucosal-associated lymphoid tissues, MALT) where antigens are trapped or transported from different body sites e.g. by dendritic cells. This concentration of foreign material to certain areas enables recognition of intruders by B and T lymphocytes that are present in high numbers within peripheral lymphoid tissues. Lymphocytes, in contrast to the general recognition of common structures by PRRs, possess highly specific antigen receptors able to distinguish variable structures between microbes, known as the B-cell receptor (BCR) (surface immunogloblin, Ig) and the T-cell receptor (TCR). B-cells recognize soluble antigen through their surface Ig and once activated they will differentiate into antibody- synthesizing cells and are essential for the control of extracellular pathogens and their toxins. Naive T cells recognize antigen by their highly variable TCR in the context of self-major histocompability complex (MHC) on the surface of cells. There are two different MHC molecules, class I or class II. Depending on the MHC:peptide complex presented, two different type of T cells are activated.

CD4 and CD8 T cells

Endogenous peptides originating from intracellular microbes are generally presented to the immune system by MHC class I molecules to CD8 T cells (cytotoxic T cells, CTLs). The mechanism for the presentation of endogenous peptides has evolved as a way to screen the body for intracellular microbes. In addition, exogenous peptides that gain access to the cytosol may also be presented by MHC class I molecules. CD8 T cells mediate their effector functions through the production of cytokines, for example IFN-γ and TNF-α, and through a direct cytolytic effect by releasing perforin and granzymes or by Fas-mediated lysis. In contrast, MHC class II molecules, generally present antigens from the extracellular milieu contained in vesicular compartments to CD4 T cells (T helper cells, Th). Stringently, cells that can take up exogenous antigens and further present them via MHC class II molecules are specified as professional antigen presenting cells (APCs), and these include dendritic cells, macrophages and B cells. These cells have more in common than MHC class II molecule expression; they also express co-stimulatory molecules required for activation of naive T cells. The requirement for a second signal provided by the APCs

ensures that immune responses are not triggered unless infection is present. The DC are the most potent APC for Th cells (8). T cells are co-stimulated when their surface molecule CD28 binds to B7-1 (CD80) or B7-2 (CD86) on the surface of APC. The high affinity receptor for B7 molecules is CTLA-4, which is expressed on activated T cells and which upon binding will downregulate the proliferative T cell response (9).

Th1 and Th2 subsets

The CD4 T cell can be divided into two main functional subsets; Th1 and Th2 (Fig 2.). These subsets are largely based on their secretion of different cytokines. IFN-γ is the main signature cytokine produced by Th1 cells, while IL-4, IL-5 and IL-10 cytokines are characteristic for Th2 cells. However, it is important to remember that Th1 and Th2 responses are not separated as black or white. In fact, most immune reactions will induce both subtypes. Development of an appropriate Th type of immune response is essential for successful immunization and may determine the outcome of a number of diseases. Multiple factors influence the differentiation into Th1 or Th2 immune responses. These include the innate cytokine environment in which priming occurs (e.g. IL-4 and IL-12), the nature and the concentration of antigens and the type of APC, i.e. the presence of co-stimulatory molecules. In addition, the overall strength of TCR ligation and addition of adjuvants are also important parameters influencing Th subset development.

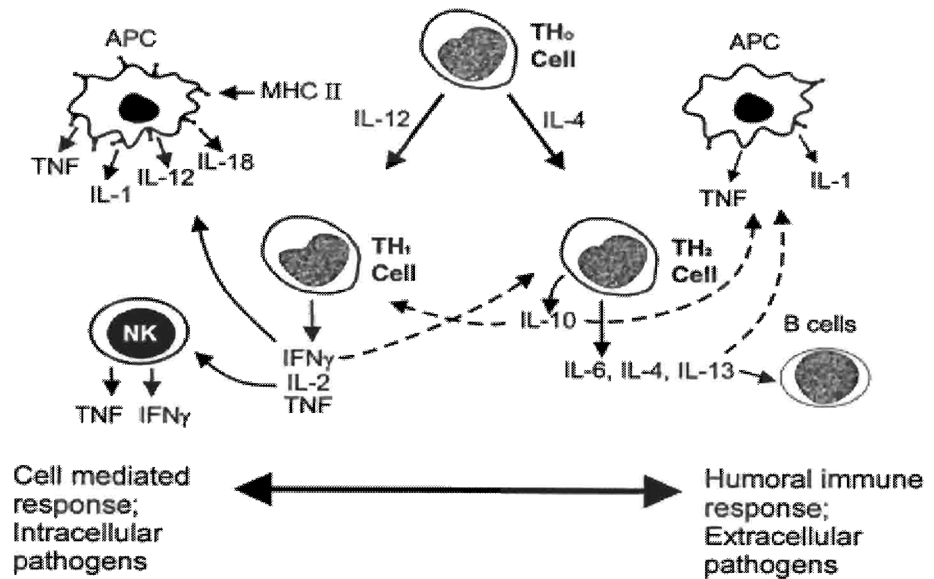

Fig 2. The polarization of Th1 and Th2 responses by CD4 Th cells and the role of the anti-inflammatory cytokines in T-cell differentiating. Solid lines indicate stimulatory pathways, and dotted lines indicate inhibitory pathways. Th_0 = uncommitted CD4 Th cell precursor (adapted from ref (10)).

A Th1 profile participates in cell- mediated inflammatory reactions, involving macrophage and CTL activation and will help B cells isotype switch to IgG2a and IgG2b in mice. Activation of a Th1 immune response is important for immunity against viral infections, tumors and a range of parasites and bacteria adapted to life within the cell. A Th2 profile mainly provides humoral immune responses, associated with IgG1 and IgE in mice, and will mediate eosinophil and mast cell degranulation (11). Activation of a Th2 type of immune response is essential during extracellular parasitic infections but is also implicated in allergic responses.

1.2 DISCOVERY OF IMMUNOSTIMULATORY MOTIFS

In the late nineteenth century William B. Coley found that injection of crude bacterial preparations was effective in the treatment of cancer patients. The mixture used has been referred to as Coley's toxin. After this finding many groups tried to explain the success behind Coley's experiment. In the 1980s, a Japanese research group working with Bacillus calmette-Guerin (BCG), found that the antimumor activity of BCG was dependent on its own DNA. Furthermore, this was explained by particular DNA sequences which harbor a central 5'-CG'-3 in a palindrome sequence (12, 13). A decade later Krieg *et al*., working with antisense ODN, described that immune activation of mouse B cells was due to C followed by G in a certain base context (14). In addition, Krieg *et al.* also demonstrated that methylation of the cytosine residue abolished the immune activation. Thus the secret message in Coley's toxin was beginning to be solved and a novel function was added to DNA other than being the source of genetic material. The danger code in DNA is today thought to rely partially on CpG motifs, which consist of an unmethylated CpG dinucleotide in a particular base context.

It is clear that except from bacterial DNA the genomes of many insects, viruses, nematodes and molluscs all exert immunostimulatory properties on our immune system, while vertebrate DNA is immunologically inert despite containing CpG in their genomes. This biological difference regarding DNA from different sources can be explained by structural diversity that lies within their genomes. In contrast to bacterial DNA, the frequency of CpG dinucleotides in vertebrate genomes is much lower, as would be predicted if base utilization was random (CpG suppression), and the cytosine nucleotides in these are often methylated (14). Moreover, the CpG dinucleotide in vertebrate DNA is not commonly found in the context to fulfil the criteria of a CpG motif (14). These structural DNA differences between species are one basis for danger recognition. Interestingly, CpG suppression is not only a phenomenon occuring in vertebrate DNA. The genomes of some small DNA viruses, retroviruses and certain intracellular parasites also have lower CpG content than expected (15-17). This CpG suppression may have evolved as an effective strategy to avoid host immune stimulation by those pathogens.

Different CpG motifs activate mouse and human cells

The immunostimulatory CpG motifs to mouse immune cells have the general structure of 5'-Pu-Pu-CpG-Pyr-Pyr-3' but the four most potent stimulatory hexamers described have the sequence of GACGTC, GACGTT, AACGTC and AACGTT (14). Interestingly, CpG motifs activating mouse immune cells might not be the most optimal for stimulation of human cells. Sequence motifs optimal for human immune stimulation are GTCGTT or TTCGTT (18, 19). Thus both mouse and human cells recognize unmethylated CpG dinucleotides in their respective motifs but the flanking regions as well as the number of CpG dinucleotides seems to differ.

1.2.1 Effects of CpG DNA

Concerning immune activation, a number of studies using bacterial DNA and/or synthetic oligonucleotides containing CpG motifs (CpG ODNs) have been utilized to trigger strong Th1-like immune responses on various mice or human cells. However, studies of the mouse have dominated. Monocytes/macrophages, DC and B cells are directly activated by the presence of foreign DNA. In contrast, NK and T cell activation in response to CpG DNA appears to be largely indirect, occurring through the action of cytokines produced by other cells (e.g. IL-12, IFN-α/ß). Immune effects on host cells are summarized in Figure 3. CpG DNA is a potent inducer of DC of mice or human origin, triggering them to produce high levels of Th1-like cytokines (20-22). In addition, increased antigen presenting capability, upregulation of co-stimulatory molecules (e.g. CD40, B7-1 and B7-2) and adhesion molecules (e.g. ICAM-I) are further major effects due to stimulation by CpG DNA (23, 24). The importance of CpG motifs for efficient DNA vaccination has been thoroughly confirmed in several studies (25-27).

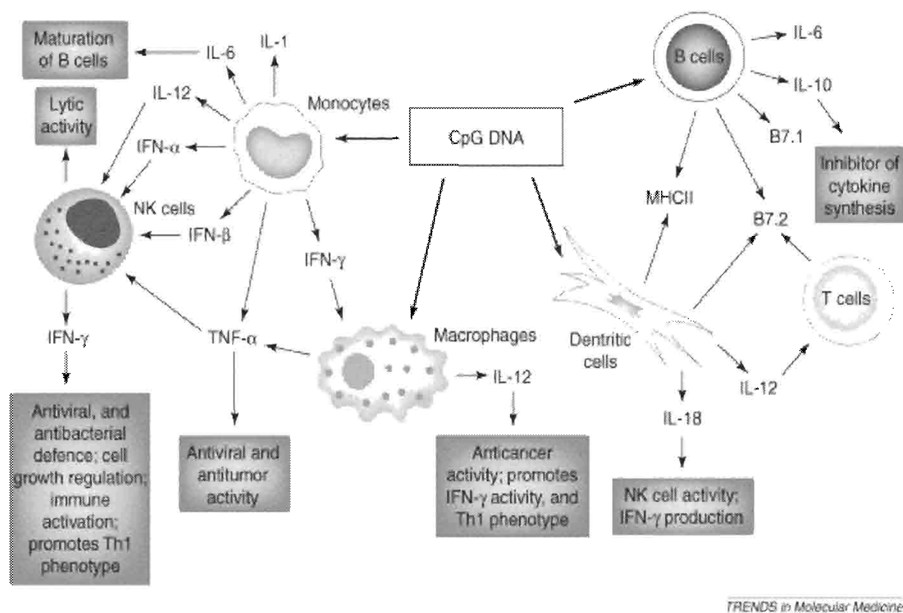

Fig 3. Effects of CpG DNA on host immune cells. Several cytokines that have therapeutic effects are produced in response to CpG DNA activation of host immune cells (adapted from ref (28)).

1.2.2 Role of CpG ODN as adjuvant

In animal models, synthetic CpG ODNs, generally made with a nuclease-resistant phosphorothioate (PS) backbone, have resulted in antigen-specific humoral and cellular immune responses against a range of bacterial, viral and parasitic antigens (28, 29). The adjuvant activity of CpG DNA does not appear to be dependent on any particular route of administration. CpG DNA has been successfully applied using intramuscular (i.m.), subcutaneous (s.c.), intransal (i.n.) and oral routes of delivery (30-32). Moreover, pretreatment of a single dose of CpG ODN protects mice for at least 2-4 weeks against lethal challenge with infectious organisms causing listeria, malaria, and leishmaniasis (33-35). In another study, CpG ODN reverted an already established Th2 response and prevented a lethal Th2-dominated leishmaniasis (36). Thus the ability of CpG ODN to alter and redirect an already established Th2 response highlights the therapeutic potential of CpG ODNs and supports the importance of CpG DNA in instructing the adaptive immune responses. CpG DNA is currently under preclinical and clinical evaluation against a wide range of infectious

diseases, allergy and cancer (28). We have investigated the effect of CpG ODNs as adjuvant for chlamydial antigens in a *Chlamydiae pneumoniae* mouse model.

Toxicology

However, in vaccine design it is of importance to consider that CpG DNA may also induce harmful side-effects. For example, in experimental mice CpG DNA induces a burst of cytokine production (TNF-α, IL-1) that results in a lethal shock (37). Mice injected with CpG DNA into knee joints developed arthritis due to TNF-α production by macrophages (38). It is a great challenge to define adjuvants, maybe other CpG motifs that promote an optimal Th1 response while minimizing induction of toxic cytokines.

1.2.3 Mechanism of action for CpG DNA

Although the immune effects of CpG DNA are well established, efforts are now focused on understanding the mechanisms of induction of the immune responses. However, recent progress during the last few years has revealed three major events in the CpG DNA signaling mechanism. These include (1) a sequence-independent uptake of CpG DNA, (2) endosomal maturation, and (3) involvement of TLR-9 and/or the DNA protein kinase (DNA-PK) in the recognition of CpG DNA (23, 39, 40). More downstream effects include recruitment of the adapter protein MyD88, IRAK and TRAF6. This results in a rapid increase in intracellular production of reactive oxygen species (ROS), activation of NF-κB, as well as of the MAPKs. TLR-9 recruitment of MyD88 is essential in CpG DNA signaling, while TLR-4 also utilizes MyD88-independent pathways (41, 42). A simplified mechanism of action is summarized in figure 4.

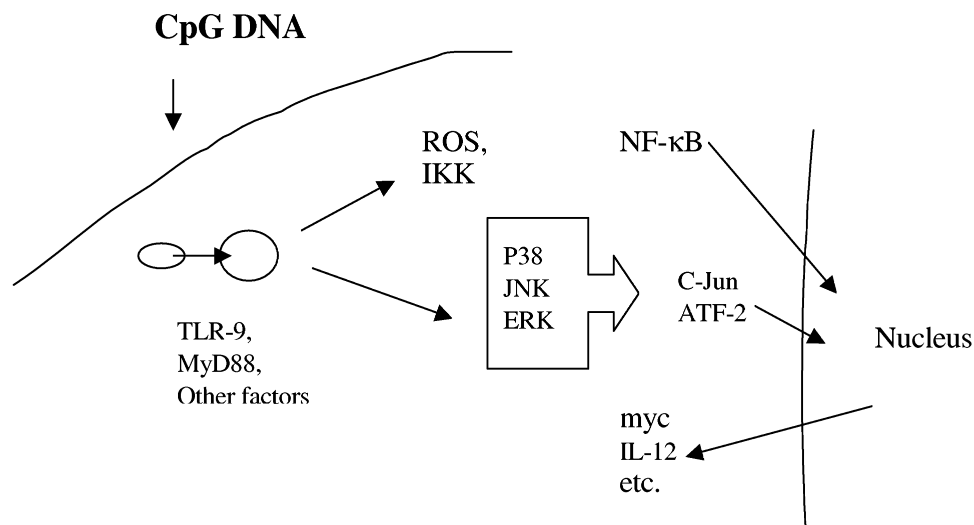

Fig 4. Molecular mechanisms of CpG-mediated immune activation. CpG oligodeoxynucleotides (ODN) or bacterial DNA is internalized in a sequence-independent fashion into an endosomal compartment. It is possible that the recognition of CpG motifs, which requires TLR-9 and MyD88, occurs in this endosomal compartment, but this has not yet been fully clarified. In any case, a signaling complex appears to be formed leading to the rapid generation of reactive oxygen species (ROS) and the activation of the inhibitor of NF-κB kinase (IKK). A second set of signaling factors that are activated by CpG DNA are the mitogen-activated protein kinases, p38, JNK, and ERK. These separate signaling pathways lead to the activation of specific transcription factors, including NF-κB, c-jun, and ATF-2, which induce gene transcription, such as the cell cycle regulatory gene *c-myc* and Th1-like cytokine genes, such as IL-12 (adapted from ref (43)).

Agents known to interfere with endosomal acidification such as chloroquine and bafilomycin inhibit the signaling pathways induced by CpG DNA (44-46). Thus endosomal acidification and/or maturation are prerequisites for the activation of the signaling induced by CpG DNA. The fate of CpG DNA therefore most likely depends on intracellular receptors at a very early stage. The only receptor so far connected to recognition of CpG DNA is TLR-9 (47). TLR-9-deficient ($TLR9^{-/-}$) mice failed to become immune activated in response to CpG DNA, as measured by splenocyte proliferation, inflammatory cytokine production from macrophages and dendritic cell maturation. Importance of TLR-9 has also been demonstrated using the non-CpG responsive cell line 293 that upon TLR-9 transfection responded to stimulation by CpG DNA (48). More recently, using immunohistochemical analyses, CpG DNA has been shown to co-localize with TLR-9 and/or MyD88 in endosomal vesicles (49, 50).

Interestingly, 293 cells transfected with either mouse or human TLR-9 responded optimally to mouse (GACGTT) or human (GTCGTT) CpG motifs, respectively (48). These data suggest TLR-9 as the main receptor that confers species selectivity.

Except from TLR-9, the catalytic subunit of DNA-dependent protein kinase (DNA-PK) has also been described as a recognition molecule involved in CpG DNA-mediated signaling (51). A more recent report highlighted the importance of other members of the phosphatidylinositol 3-kinase (PI3)-kinase family in CpG DNA signaling (52). In addition, a report by Zhu *et al.* suggested that heat shock protein 90 (Hsp90) may be a central motif in the CpG-mediated effects (53). Presently, a clear picture of the subsequent intracellular pathways following CpG stimulation is not completely understood. We have searched for direct CpG ODN binding protein(s) that may be involved in recognition and signaling of CpG ODN.

1.3 VACCINES

The protection obtained after vaccination lies in the induction of memory responses. Upon reinfection with the same pathogen, the B and/or T cell responses are vigorous, faster and more efficient. Thus, they guard for the reappearance of their specific antigen and eventually result in long-lived or even life-long immunity. When a vaccine is given to an individual the immune system will react like this is the first encounter with an infection and a memory pool of T and/or B cells subsequently develop. If these vaccinated individuals later become exposed to the pathogen to which the vaccine was directed against, the immune system will react in a more sensitive and rapid manner. Vaccines are prophylactic, which means that they are given to healthy people in order to prevent eventual future disease. Some of the most effective vaccines are those against tetanus, diphtheria, poliomyelitis, rubella, mumps and measles, whereas many serious infectious diseases, e.g. acquired immunodeficiency syndrome (AIDS), tuberculosis and malaria, still lack effective and safe vaccines. Moreover, in several cases, such as cancer and some persistent infections, therapeutic vaccines, i.e. given during disease, are required. Traditional vaccine design has utilized; (1) live attenuated microorganisms, (2) whole inactivated microorganisms and, (3) subunit preparations. However, due to modern molecular techniques, a new generation of vaccines is rapidly growing, namely the genetically engineered vaccines. Among these, recombinant protein subunits, recombinant live

vectors and DNA-based vaccines are some examples. In this thesis, a DNA-based vaccine approach against the human pathogen *C. pneumoniae* has been evaluated.

Chlamydia pnemoniae

The obligate bacterium gram negative *C. pneumoniae* is a significant human pathogen. Infection by *C. pneumoniae* causes a range of respiratory tract diseases (54). The bacterium infects alveolar macrophages, smooth muscle cells and epithelial cells and the unique developmental cycle of *Chlamydia spp* is described in Figure 5. The nature of the inclusion is common within the genus, and unique relative to all other intracellular pathogens. The completion of the chlamydial genome revealed that it encodes over 800 proteins (55).

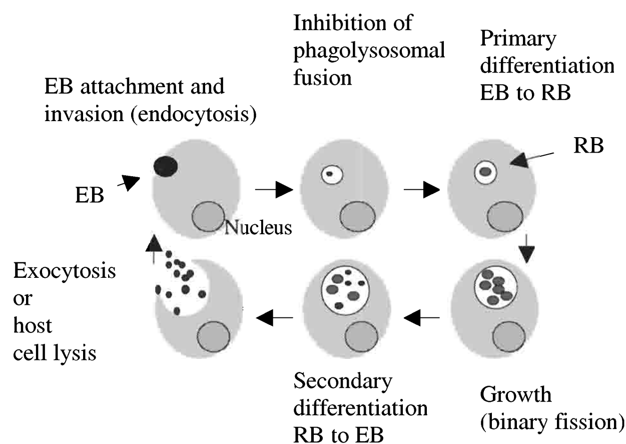

Fig 5. Chlamydia development cycle. Chlamydia are characterized by a developmental cycle involving a metabolically inactive infectious developmental form called the elementary body (EB) that, after entry into the target host cell, differentiates into a metabolically active but extremely fragile developmental form called the reticulate body (RB). The RBs grow within an intracellular vacuole, called an inclusion, that does not fuse with lysosomes. At approximately 20 hours post-infection and after multiple divisions by binary fission, the RBs differentiate into the EB developmental stage and infectious EBs are released to initiate new rounds of infection.

1.3.1 Immunization with DNA

In 1984 Dubesky *et al.* (56) and 1990 Wolff *et al.* (57) demonstrated that administration of plasmid DNA into an animal resulted in the expression of the protein encoded by the plasmid. Since then, DNA vaccines have within a short period progressed from theory into clinical trials, as against hepatitis B (58), HIV (59) and malaria (60). Some of the advantages of using a DNA-based vaccine include the ability to produce a combined humoral and cell-mediated immune response, without the risk associated with live attenuated vaccines, i.e. reversion to a more virulent form. Moreover, DNA vaccines can be manufactured, distributed and stored at relatively low cost.

Conceptually, a DNA vector aimed for gene vaccination can be divided into two major units: (1) a transcription unit coding for an appropriate vaccine candidate and (2) an adjuvant unit in the DNA backbone containing CpG motifs. Two other prerequisites of a DNA vaccine is to harbor a strong promoter (e.g. CMV) and a polyadenylation region at the 3'end of the insert for the stability and proper translation of mRNA. In Figure 6 a description of a DNA vaccine vector is presented. The plasmid DNA vector, coding for a foreign gene product, is introduced into living cells, that allows expression of encoded antigen in a way that resembles naturally produced antigens. However, the process that allows plasmid DNA uptake and the transport to the nucleus of the cells is not well understood.

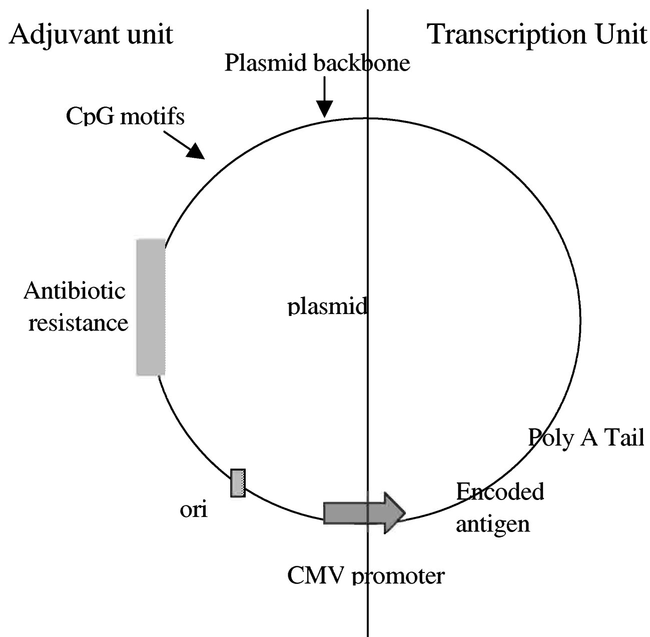

Fig 6. Essential elements of a DNA vaccine. A plasmid DNA vector harbour a transcriptional unit, which consist of a viral promoter gene encoding (in most cases a CMV-promoter), an insert containing the antigen, and transcription/terminal sequences (Poly A). The plasmid is replicated in bacteria using a procaryotic origin of replication (ori). Bacteria carrying the plasmid are selected based on antibiotic resistance, which is mediated by the gene carrying the resistance marker. The adjuvant properties of a plasmid vector are highly influenced by the number of CpG motifs within the plasmid backbone.

Delivery of a DNA vaccine

Since their discovery, DNA vaccines have been administered using several different routes, but the most common have been intramuscular and intradermal. Other ways of administration include intravenous (i.v.) and mucosal (oral, intranasal, vaginal). The plasmid DNA is taken up by different cells depending on the route of administration. For example, within the dermis of the skin, there are a variety of APCs such as Langerhan's cells and tissue macrophages, which could be transfected by the injected plasmid DNA (61). Conversely, for immunization within the muscle tissue, where APCs are sparse, myocytes are the main cells transfected (62). However, the principal cells which initiate an immune response after DNA vaccination are the dendritic APCs (63). The plasmid DNA can be introduced into an animal simply by needle injection and no specific formulations are necessary to render plasmids applicable for their purpose. However, DNA vaccines are often administered using different

formulations or delivery systems (64). The route and form of presentation is of importance in a DNA vaccination protocol against a particular pathogen.

1.3.2 Immune responses to DNA vaccines

DNA- based immunization induces both humoral and cell-mediated immune responses, as has been demonstrated in numerous animal models for viral, bacterial, and parasitic infections (reviewed in (65-67). In chlamydial vaccine design, DNA as a delivery vehicles for chlamydial antigens was shown to be successful against infection with both *C. pneumoniae* and *C. trachomatis* (68-71). In addition, DNA vaccination has also been successfully used against autoimmune diseases (72), allergies (73) and cancer (74). A mechanism of action underlying this vaccination strategy, i.e. how antigen is processed, presented and recognized of immune cells, is summarized in Figure 7. CTLs induced against a particular antigen may result from either (1) direct transfection of APCs (not shown in the figure) or (2) be based on cross- presentation. The latter is a process in which specialized APCs take up proteins produced and released by other cell(s) initially transfected with the DNA vaccine (e.g. myocytes and keratinocytes). This provides the two important signals needed for T-cell priming, namely signal 1 (peptides on MHC class I molecules) and signal 2 (co-stimulatory molecules). Muscle cells which are transfected upon i.m. DNA immunization do not express co-stimulatory molecules, like most somatic cells, but in this way may serve as antigen producers. In experiments in which myocytes harboring plasmid DNA were transplanted into mice, a CTL response was still induced against encoded antigen (75). In addition, if the injected muscle is removed within a few minutes after DNA vaccination, an immune response will still occur against the encoded antigen (76). Since a limited number of APC are directly transfected upon DNA vaccination, cross-presentation is necessary for generation of maximal immunity (77). However, the complete mechanism of action behind the process of cross-presentation is still not fully understood.

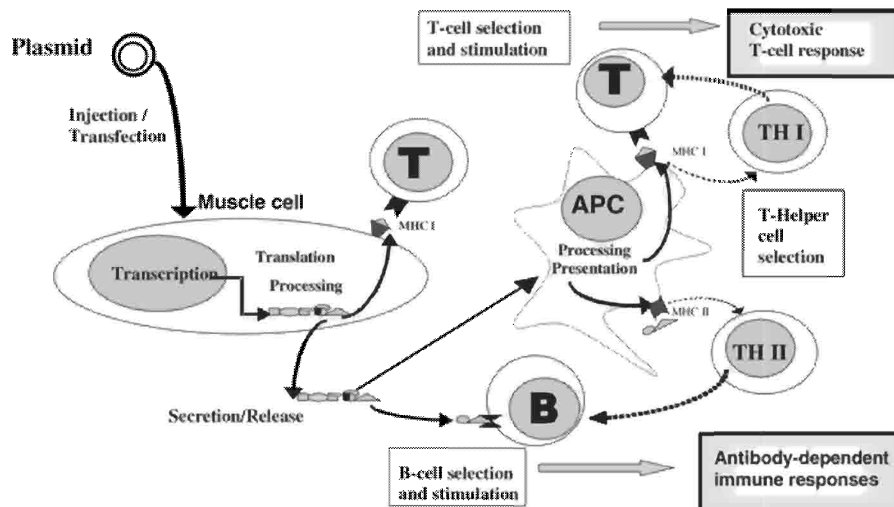

Fig 7. Immune mechanisms induced by inoculation with naked DNA. The example shows a myocyte which is transfected by the inoculated DNA, produces the antigen encoded by the plasmid, and exposes processed peptides of the antigen together with MHC-I molecules to stimulate a T-cell response after cross-linking with a T-cell receptor. Antigen released from the cell stimulates B-cells by binding to their immunoglobulin receptors and induces a humoral immune response. Alternatively, released antigen - but also expression plasmids - may be taken up by antigen-presenting cells (APC). These are able to present their antigens to the immune system via MHC-I and MHC-II pathways, thus stimulating and regulating both arms of the immune system (adapted from ref (78)).

1.3.3 Optimizing DNA vaccines

Since the era of “naked” DNA vaccines (i.e., plasmids dissolved in saline solution), several strategies have evolved to increase their effectiveness. In fact, a very low percent of the cells at the injected site upon DNA vaccination express the introduced genes (57). Some strategies to achieve better effects of DNA vaccines have involved co-delivery of genes or adjuvant molecules with regulatory and/or stimulatory properties. These include molecules such as IL-12, GM-CSF, IFN-γ, CD80, CD86, CTLA-4, CD40L, ICAM-1, LFA-3 and CpG motifs present in synthetic ODNs or bacterial DNA (reviewed in (64, 79)). The use of various delivery systems such as liposomes and attenuated live vector systems, e.g. *Salmonella* and *Vaccinia* (64, 80) are further examples of enhancing DNA vaccination efficacy. These strategies mainly function to target associated antigens into APC. Other attempts include prime- and boost strategies, in which immune responses are primed with a DNA vaccine and then boosted with recombinant proteins or recombinant viral vectors encoding the

same antigen as the DNA vaccine (81). Futhermore, manipulating the DNA vector backbone or expression of multi-epitopic vaccine(s) are novel strategies to increase the efficacy of DNA vaccines. We have utilized some of these mentioned strategies in order to optimize our DNA-based vaccine against *C. pneumoniae*.

1.4 ANTIMICROBIAL PEPTIDES AND *SHIGELLA* INFECTION

Antimicrobial peptides

Antimicrobial peptides constitute an important part of the first line of defense against microorganisms. Defensins and cathelicidins are two major classes of antimicrobial peptides present in mammals. The defensins are classified into two groups; those localized in granules of phagocytes (the α-defensins) and those expressed by epithelial cells (ß-defensins) (82). In humans, 4 different ß-defensins are produced, where HBD-1 is constitutively expressed at epithelial surfaces, while HBD-2 is inducible and is found at higher levels in tissues that are constantly exposed to and colonized by microorganisms (83). Cathilicidins are a family of diverse antimicrobial peptides in mammals, of which LL-37 is the only member in humans. LL-37 is present both in blood cells and epithelial surfaces such as lung, gut and skin (84). Utilizing knockout mouse models, the importance of defensins and cathelicidins have been demonstrated against orally administered bacteria (85) and Group A Streptococcus (GAS) skin infection (86), respectively. Moreover, upregulation of the expression of LL-37 was observed in human inflammatory skin disorder, whereas in normal skin no expression was detected (87). Besides the role of being antimicrobial, these peptides also serve several immunomodulatory functions in inflammation and infection, for example exhibiting chemotactic activity for monocytes and T cells (88, 89). Thus antimicrobial peptides are important components of innate immunity and constitute a link to the adaptive immune system.

Shigella infections

Shigella is a highly contagious gram negative bacteria that causes shigellosis or bacillary dysentery. There are four different species of *Shigella*; *S. sonni, S. flexneri*, *S. dysenteriae* and *S. boydii*. Clinically, *Shigella* infections appear as bloody diarrhoea (90). *Shigella* enters the colonic mucosa through M cells and infects resident tissue macrophages. From the phagolysosome of macrophages, the bacteria escape to the cytoplasm from where cell apoptosis is induced. Macrophage apoptosis is an essential event in *Shigella* pathogenesis, which results in IL-1ß cytokine secretion (91). IL-1-ß initiates a massive inflammatory response, which leads to destruction of the colonic mucosa. The next step in *Shigella* invasion is basolateral spread of the bacteria into non-professional phagocytes, the colonic epithelial cells. The invasive process is triggered by proteins encoded by the 200-kD virulent plasmid that is present in all *Shigella spp* (90). Once within the epithelial cells some plasmid encoded proteins allow vacuole destruction, which permit rapid cytoplasmic growth of the bacteria followed by spread into neighboring cells by an actin-dependent process. The mechanism of *S. flexneri* is known is some detail (reviewed in (92)) that are outside the scope of this thesis.

Shigella infections are most serious when affecting infants, killing 600 000 children each year (90). At present, no vaccine is available against *Shigella* infections and occurrence of multiresistant bacteria to antibiotics makes prevention and treatment of shigellosis a difficult task. However, understanding more of the mechanisms of action for *Shigella spp* will probably enable future vaccine design against this bacterium.

In this thesis work, we have found that the expression of LL-37 and HBD-1 is downregulated during *Shigella* infection and we have investigated bacterial components that may be involved and hence could unravel the mechanism behind this immune escape strategy.

1.5 AIMS OF THIS THESIS

The aim of the present study:

(1) To investigate the efficacy of DNA vaccination against *Chlamydia pneumoniae* utilizing an expression vector coding for the chlamydial heat shock protein 60 (p-hsp-60)

(2) To investigate different vaccine approaches and adjuvants in relation to the outcome of infection with *C. pneumoniae*

(3) To isolate and identify protein(s) that directly bind(s) CpG ODNs in a protein extract which may be involved in recognition and/or signaling by CpG DNA.

(4) To investigate the mechanism behind the downregulation of antimicrobial peptides during *Shigella* infections, starting to determine which bacterial components mediate this downregulation.

1.6 RESULTS AND DISCUSSION

1.6.1 Vaccination studies against *Chlamydiae pneumoniae* (Paper I, II)

Do we need a vaccine against C. pneumoniae?

Infections caused by *C. pneumoniae* are responsible for approximately 10% of pneumonia in industrialized countries and causes moderate to severe upper and lower respiratory diseases (54). The acute phase is in most cases associated with mild respiratory distress. However, persistence and/or re-infection of *C. pneumoniae* has been suggested to be a risk factor in the development of atherosclerosis (93). Infection with the bacteria may promote the initial endothelial injury that is the first step in the development of vascular disease, which will later result in the formation of a fibrous plaque. A fibrous plaque consists of proliferating smooth muscle cells, foamy macrophages and T-cells that become embedded in a collagenous matrix of connective tissue (94). Of interest in the context of this work, anti chlamydial-OMP-2 and HSP-60 immune responses have also been suggested to participate in the pathogenesis of heart disease, probably by cross-reaction with host molecules (95, 96). However, the mechanism by which *Chlamydia* might causes or accelerates cardiovascular disease is speculative (97). Finally, *C. pneumoniae* has also been associated with asthma (98), Alzheimer's disease (99) and multiple sclerosis (100).

Diagnosed *C. pneumoniae* infections are treatable with antibacterial therapy. However, antibiotic treatment does not affect established pathology. A vaccine therefore represents an attractive approach to protect the greatest number of people, with the primary aim of preventing persistent infection (101).

Present study (I, II)

In this thesis work, two *C. pneumoniae* antigens were used as candidates for a DNA- and protein-based vaccines: the chlamydial heat shock protein 60 (HSP-60) and the outer membrane protein 2 (OMP-2). In the case of protein based immunization, two different adjuvants were used. Protein was immunized in the presence of Freund's incomplete adjuvant containing immunostimulatory CpG (FIA-IS-CpG) or in Freund's complete adjuvant (FCA). FCA and FIA are oil-based adjuvants, but FCA

also contains killed mycobacteria. Hsps are known to be major targets of immune responses against infection, and have been successfully employed as vaccines in several experimental bacterial and fungal diseases (102-105). OMP-2 is the second most abundant protein in *C. trachomatis* EBs and homologues are present in all *Chlamydia* species. In addition, OMP-2 is expressed when reticulate bodies differentiate into EBs (106) and the conservation of the OMP-2 sequence among different *C. trachomatis* serovars makes it an attractive candidate.

The influence of the route of immunization (I, II)

In the initial series of experiments, two different routes of immunization were analyzed with plasmids encoding hsp-60 (p-hsp-60) or omp-2 (p-omp-2), intradermal (i.d.) or intranasal (i.n.). The i.n. route of immunization with p-hsp-60, but not p-omp-2, increased protection against infection with *C. pneumoniae*. However, the i.d route of immunization did not protect mice against challenge with *C. pneumoniae* after immunization with any of the DNA constructs, p-hsp-60 and p-omp-2 (unpublished data). Thus the route of administration and choice of antigen clearly affects the outcome of protection as measured by pathology and/or bacterial lung burden. An i.n. route of administration probably favors a more effective local immune response in the respiratory mucosal inductive site where bacteria will be encountered. However, the i.m. route of DNA vaccine administration has also been proved to induce a partial protection against *C. pneumoniae* (70).

Cytokine profile and the outcome of infection (I, II)

Protective i.n. DNA p-hsp-60 - or s.c. protein FIA-IS-CpG-OMP-2 - based immunization strategies against infection with *C. pneumoniae* were linked with a Th1 type of cytokine response. Mice lacking the IFN-γ gene were not protected after p-hsp-60 immunization, indicating that IFN-γ is necessary for DNA-vaccine induced protection. In addition, increased specific IFN-γ cytokine production was observed *in vitro* after co-culturing spleen cells from i.n. p-hsp-60 or s.c. FIA-IS-CpG-OMP-2 immunized and challenged mice with recombinant HSP-60 or OMP-2, respectively. In contrast, s.c. inoculation with FCA mixed with the chlamydial HSP-60 and OMP-2 proteins increased bacterial load in lungs after infection, which coincided with increased pathological outcome of infection. This worsening of infection was

dependent on both $CD4^+$ and $CD8^+$ cells. S.c. FCA-OMP-2 immunization favored the induction of a Th2 cytokine immune response, dominated by higher specific IL-10/IFN-γ ratio than controls. FCA is known to be a strong Th1 cytokine inducer (107). However, it has also been reported by others that FCA induces a Th2 or a non-polarized Th1/Th2 response (108-113). Thus the type of cytokine response (Th1/Th2) does correlate with the outcome of infection with *C. pneumoniae.*

In addition, different antibody isotype patterns were measured in sera from mice immunized with different adjuvants together with the OMP-2 protein: A Th1 type of antibody response (IgG2a) was detected in sera from FIA-IS-CpG-OMP-2 immunized and challenged mice, whereas a Th2 type of antibody-response, dominated by IgG1, was observed in sera from FCA-OMP-2 immunized and challenged mice. However, since OMP-2 is not exposed on the surface of the extracellular form of chlamydia, represented by the EB, we suggest low relevance of antibody in the protected effects induced.

Interestingly, i.n. immunization of $CD8^{-/-}$ mice with p-hsp-60, but not with plasmid backbone alone, resulted in enhanced susceptibility to infection. This worsening was correlated with increased IL-10 and diminished IFN-γ mRNA in the lungs compared to controls. This DNA-vaccine induced harmful response in $CD8^{-/-}$ mice further supports the relevance of a vaccine to prime the relevant immune responses for protection. A harmful role of $CD4^+$ cells in the absence of CD8 has also been reported in primary infection against *C. pneumoniae* (114).

IL-10 is likely to mediate the harmful effect (II)

In our investigations, increased levels of IL-10 production correlated positively with increased bacterial load and/or pathology in mice. We wanted therefore to test if IL-10 causes the detrimental effect of FCA-OMP-2 immunization by using IL-10 knockout mice. Indeed, IL-10 knockout mice did not show increased susceptibility to infection after FCA-OMP-2 immunization compared to controls. Furthermore, we wanted to test whether FCA-OMP-2 immunization could cause a direct pathological effect in the absence of infection, since OMP-2 has been associated with a pathological outcome of infection due to cross-reactivity or antigenic mimicry (95). However, we observed no direct pathogenic effect in hearts or lungs of FCA-OMP-2

immunized mice compared to controls. In summary, the exact mechanism of Th2-induced severity of disease has not been addressed in this study, but IL-10 is likely to mediate the harmful effect induced in FCA-OMP-2 immunized and challenged mice. In line with our results, IL-10 has previously been indicated to mediate susceptibility in humans and experimental models against primary and secondary chlamydial infections (115-118).

Importance of CpG ODNs as adjuvants (II)

Any candidate protein for use in vaccine design should be presented to the immune system in a safe and effective formulation. In this work, we concluded that FCA was not a suitable adjuvant for chlamydial antigens. We wanted therefore to analyze the role of adjuvant in this adverse reaction. For this purpose, the effect of FCA was compared with that of immunostimulatory CpG motif mixed with FIA (FIA-IS-CpG). Mice immunized s.c. with OMP-2 emulsified in FIA-IS-CpG showed lower bacterial burden than control mice immunized with adjuvant alone (Fig 1.). In accordance with our results, a recent report showed that s.c. immunization using major outer membrane protein (MOMP) protein with CpG ODNs as adjuvant gave protective immune responses against *C. trachomatis* (119). Finally, CpG DNA is an extremely potent adjuvant for mucosal immunization using oral, intrarectal or i.n. immunization routes (30-32, 120, 121). It would therefore be of great interest in the future to test IS-CpG-OMP-2 using an i.n. route of immunization.

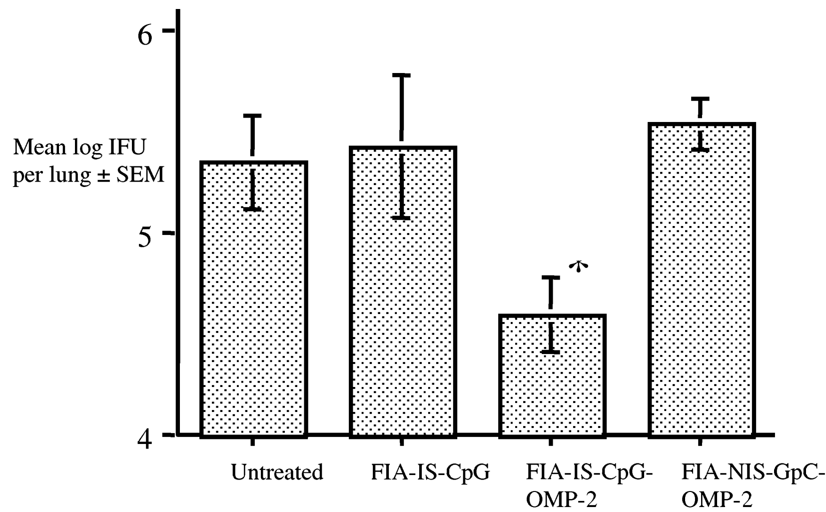

Fig 1. Immunization with OMP-2 using FIA-IS-CpG as adjuvant results in partial protection against infection with *C. pneumoniae*. Mice (8 animals per group except FIA-IS-CpG-OMP-2 which comprised 15 animals) were immunized with OMP-2 in the presence of immunostimulatory CpG or non-stimulatory GpC oligonucleotides. Control animals were administered with FIA-IS-CpG or left untreated. The mean of the $\log_{10}$ IFU per lung +/- SEM is depicted. Cumulative data from two independent experiments are shown. * Differences vs FIA-IS-CpG, FIA-NIS-GpC-OMP-2 and untreated, infected mice are significant ($p<0.05$ Students t-test).

Optimizing DNA vaccines (I, II)

We have examined two successful strategies to improve the efficacy of our DNA-based vaccines. Firstly, co-administration of plasmid encoding cytokine genes, IL-12 or IFN-γ, together with p-hsp-60 further increased the protective effect of the vaccine compared to mice immunized with p-hsp-60 alone. These cytokines might function to increase innate immune activities to create a cytokine environment for Th1 development. Secondly, using chlamydial OMP-2 protein combined with a CTLA-4 expression system, gave reduced bacterial load in the lung against infection compared to controls immunized with a mixture of p-CTLA-4 and p-omp-2. CTLA-4 would target the antigen to APC, to direct the antigen to the site of immune induction. Indeed, cell culture supernatant containing CTLA-4-OMP-2 fusion protein from cells transfected with p-CTLA-4-omp-2 was proven to bind to B7-1 molecules on the surface of B7 expressing cells. Increased immunogenicity of p-CTLA4-omp-2 was measured by augmented antibody levels, probably related to increased uptake and efficient targeting of the fusion protein to APC. In accordance with our results, fusion

constructs with CTLA-4 and/or L-selectin speeded up immune responses against encoded antigen (122, 123). Thus, antigen targeting to APC via CTLA-4 may improve DNA vaccination efficacy.

Conclusion

We have demonstrated two novel antigens for use in chlamydial vaccine design. However, it is almost certain that vaccines designed to stimulate protective immunity against *C. pneumoniae* and other intracellular bacterial pathogens will require the incorporation of several antigens. An effective mix of antigens would include those able to stimulate multiple arms of the immune system, including CD4 T cells, CD8 T cells and antibody production. However, we have not demonstrated importance of antibodies but do not formally exclude any role of antibodies for protective immune responses. Recent progress in chlamydial genomics and proteomics will enable the search for possible candidate antigens responsible for inducing long-lived protective immunity. Promising delivery vehicles of such proteins include DNA and virus vectors as well as cellular, dendritic-based vaccines. Among these, vector-mediated immunization with DNA has received the most attention (63, 68, 124-126). Most adjuvants used in animal models are not safe in humans, e.g. FCA. CpG ODNs are strong candidate molecules in human vaccine design that could turn out to be useful in chlamydial vaccine design.

1.6.2 Hsp90 binds CpG oligonucleotides directly, implications for hsp90 as a missing link in CpG signaling and recognition (Paper III)

In this study we investigated the presence of protein(s) that may be involved in the signaling pathway for immune stimulation by CpG DNA. The approach was to identify protein(s) that bind ODNs containing a CpG motif in a crude protein extract.

In an initial series of experiments, we performed electromobility shift assay (EMSA) analyses using P^{32} end-labeled CpG ODN and crude protein extracts from PBMC. EMSA analysis revealed a shifted band using both nuclear (N) and cytoplasmic/membrane (C/M) protein fractions from PBMC. These results could also be reproduced in a variety of cell lines. Moreover, this DNA-protein interaction was not sensitive to EDTA or EGTA, indicating that neither Mg^{2+} nor Ca^{2+} ions were necessary for the binding. In addition, the reducing agents DTT and ß-mercaptoethanol or high pH did not effect the binding. The conditions that weakened the interaction were low pH or high concentrations of NaCl. By including the detergent SDS in the running buffer, the binding between the protein(s) and the CpG ODN was completely abolished. These early experiments gave us information about the character of the DNA-protein interaction, which was valuable in designing our approach to isolate the binding protein(s).

The EMSA analyses demonstrated that the ODNs required special flanking bases to CpG for binding of protein(s) in the extract. However, the binding was independent of cytosine methylation. After several efforts to isolate the binding protein(s) with dynabeads and 2D-gels, which were not successful, we decided to try traditional chromatography for the isolation.

Isolation procedure

In order to characterize the CpG DNA binding protein(s) we designed an isolation procedure. Due to the presence of potential DNA-binding transcription factors in the N fraction, C/M protein extracts were chosen as starting material.

After several different chromatographic approaches, following the binding with EMSA, we ended up with four different purification steps, which included reverse phase, cation exchange, size exclusion and anion exchange chromatographies. In order to investigate the purity of the final fraction, in which binding was detected, SDS/PAGE was utilized. This revealed three visible protein bands after silver staining. Hence, the situation could be due to (1) several proteins with direct CpG ODN binding capacity, (2) co-operation between proteins necessary to establish the CpG ODN binding, or (3) only one protein exhibiting CpG ODN binding. To evaluate these possibilities we decided to identify the three proteins in order to find out if one or all of them could be candidates in our concept.

Characterization of hsp90 as a CpG binding protein

A preparative SDS/PAGE stained with Coomassie was performed and the protein bands were cut out. By digestion of the three proteins with trypsin, tryptic fragments were obtained which were further identified by nano-HPLC microelectrospray ionization mass spectrometry. The sequenced peptides identified hsp90 in all three protein bands. In the 80kDa band, the peptides covered 40% of the hsp90-alpha sequence and 26% of the hsp90-beta sequence, suggesting hsp90 as a strong candidate as a binding protein in our preparation. To confirm this finding, commercially available cytoplasmic forms of human hsp90 were used in EMSA and indeed, direct binding to CpG ODN was detected. Since hsps have recently been found to be involved in antigen presentation (127) and several hsps have been demonstrated to signal via TLRs (96, 128, 129), our result was further strengthened. Furthermore, hsp90 has been suggested to be involved in CpG-DNA stimulation (53) and was recently postulated to be involved in LPS signaling as a part of a signal transducing multimeric complex together with hsp70 (130).

Visualization of hsp90 upon CpG ODN stimulation

To date, uptake of CpG ODN seems to be sequence-independent and in order to exert its biological effects CpG DNA needs to be internalized into endosomal compartments (44-46). However, in our study we observed discrimination in the uptake of unmetylated and methylated CpG ODN. Such a difference at the level of uptake was recently reported by Takeshita *et al.*, demonstrating that TLR-9

expression enhances cellular uptake of CpG DNA, but not non-CpG DNA (50). Furthermore, we demonstrated that after 20 min CpG ODNs were localized in nuclear compartments. Such a rapid transition of CpG ODNs to nuclear compartments has also been demonstrated by others (49, 50). The reason for the nuclear localization of CpG ODNs is poorly understood and has not been addressed in this study. However, the backbone composition of the ODNs has been reported to influence the uptake rate and the distribution in the cell (131, 132). In our microscopic analyses, hsp90 (but not hsp70) showed redistribution from the cytoplasm to nuclear compartments upon CpG ODN stimulation, i.e. following the pattern of CpG ODN distribution (Fig 1.). In addition, a CpG non-responding cell line did not show such a redistribution of hsp90. In conclusion, we have characterized hsp90 as a binding protein for CpG ODN and upon stimulation hsp90 translocated from the cytoplasm to the nucleus.

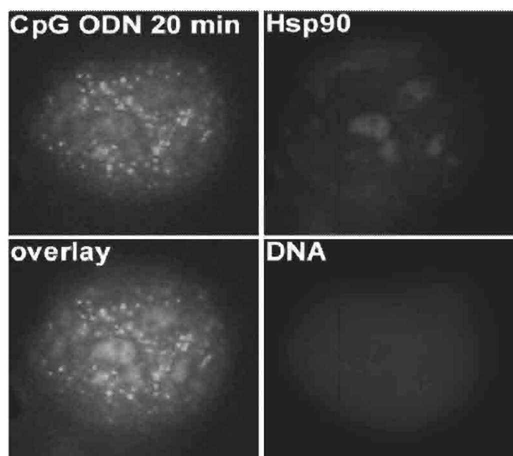

Fig 1. Redistribution of hsp90 from cytoplasm to nuclear compartments upon CpG ODN stimulation. Hp90 redistributed from cytoplasm to nuclear compartments after 20 min treatment with AACGTT ODN, i.e. followed the pattern of CpG ODN distribution.

Role of Hsp90

At present, we can only speculate on the function of hsp90 in CpG signaling. The function of hsp90 may be at different levels. Firstly, hsp90 may act as a transfer molecule allowing CpG ODN to ligate TLR-9 and subsequently trigger the initiation of signaling cascade, i.e. by inducing conformational changes of TLR-9 and thus enableling recognition of unmethylated CpG DNA. Secondly, hsp90 may also be the target of intracellular signaling leading to recruitment of hsp90 to the nucleus. The latter hypothesis is supported by a finding that hsp90 actually has been detected in the assembly of transcriptional complexes (133). Taken together, more research is needed for a more complete picture concerning molecule(s) involved in CpG-mediated signaling. For example, TLR-9 has never been shown to directly bind to CpG ODN and signaling induction via TLR-9, as for DNA-PK, is still unknown. In this perspective and based on our experiments, we suggest that the binding of CpG ODN to hsp90 may be a missing link in CpG DNA signaling and recognition.

1.6.3 Down-regulation of bactericidal peptides of innate immunity: A novel immune escape mechanism in enteropathogenic infections mediated by bacterial DNA (Paper IV)

Immune escape mechanisms of Shigella spp

In order to escape the host immune system *Shigella spp* utilize at least two documented immune escape mechanisms. Firstly, *Shigella* bacteria escape from the phagosome of macrophages by inducing apoptosis in these cells. Secondly, the bacteria also invade non-specialized phagocytic cells such as the epithelial cells of the colonic mucosa (90, 91). By invading the epithelial surfaces, pathogens have evolved different strategies to avoid or resist different antimicrobial peptides. Such strategies include modifications of bacterial membranes rendering them less sensitive to an attack of antimicrobial peptides, and production of proteinases that can degrade or inactivate these membrane active peptides (reviewed in (134)). In the case of *Shigella* bacteria, no known mechanism of action to avoid or resist antimicrobial peptides has been reported. The present study was aimed to investigate if the expression of antimicrobial peptides is effected during *Shigella* infections.

Shigella downregulate the expression of LL-37 and HBD-1

Down-regulation at the transcriptional level of both HBD-1 and LL-37 was first observed in biopsies from patients with bacillary dysentery. Moreover, in biopsies from patients with watery diarrhoea not associated with *Shigella* or *Salmonella* infections, the expression of these gene products was also found to be downregulated. This implies that the suppression of these effectors is not unique to *Shigella* infections and may be shared by several pathogens infecting the colonic mucosa. In addition, LL-37 was also downregulated at the protein level in the surface epithelium of infected patients, as shown by immunohistochemical studies. However, this downregulation could not be confirmed by Western-blot analysis of protein extracts from *Shigella* infected biopsies. Instead, the amount of LL-37 in these samples increased, which may be due to recruited neutrophils harboring LL-37. This finding is supported by detection of a large number of positively stained neutrophils in the biopsies, as evident by double immunohistochemical staining. To investigate if this downregulation is a direct effect from the bacteria two different cell lines of epithelial

and monocytic origin, HT-29 and U937, were infected with live *S. dysenteriae* bacteria, and downregulation of LL-37 transcript was indeed observed. Western-blot analyses of the supernatants from *Shigella*-infected U937 cells confirmed downregulation also at the protein level. However, this was not the case with the HT-29 cell line. Instead, the amount of LL-37 in these samples was increased, most likely due to higher proteinase activity in the U937 cells.

This is the first report indicating that pathogens have evolved strategies to combat host innate immune defenses by downregulating the production of endogenous antimicrobial peptides such as HBD-1 and LL-37.

Bacterial DNA as one mediator of downregulation

To investigate the mechanism underlying the downregulation of these peptides, we started to analyze which bacterial components may be involved. The U937 cell-line was therefore infected with sonicated lysates of *S. dysenteriae* or LPS from *E.coli* and *Shigella*, respectively, and the expression of LL-37 was analyzed by RT-PCR together with Southern blot analysis. In addition, sonicated lysates of *S. dysenteriae* were used directly or treated with Dnase I or proteinase K before addition to the cell-line. This set of experiments revealed that downregulation of LL-37 could be mediated by disrupted bacteria (i.e. the lysate) but upon treatment with Dnase I the downregulation was abolished, indicating that bacterial DNA could be a mediator for the downregulation. Interestingly, in the present study we demonstrated that plasmid DNA prepared from *Shigella spp* (*S. dysenteriae*, *S. flexneri* and *S. boydii*) can suppress the transcription of LL-37 in PBMC. We also showed that LL-37 kills these *Shigella spp* with a MIC value of 5-10μM. A possible scenario of the downregulation of LL-37 in the colonic mucosa is illustrated in Fig 1. Plasmid DNA is liberated upon infection due to early lysis of some *Shigella* bacteria. Released plasmid DNA may then act as one mediator to promote local suppression of LL-37 expression that further enables bacterial invasion into host epithelium.

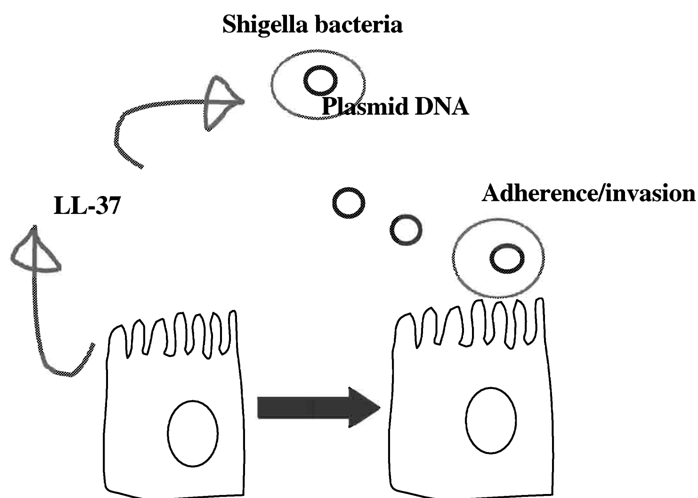

Fig 1. Downregulation of LL-37 at mucosal surfaces during *Shigella* infection. In a first phase LL-37 kills *Shigella* bacteria and the disrupted bacteria release plasmid DNA. This DNA is able to downregulate the expression of LL-37 and hence enables further bacterial invasion into host epithelium.

1.7 CONCLUDING REMARKS

This thesis highlights DNA vaccines and bacterial DNA in connection to immunity in different aspects. Major conclusions in the thesis are described below.

1. A DNA vaccine approach can be utilized to induce protective immune responses against infection with *C. pneumoniae*. Furthermore, depending on the type of adjuvant used with the antigen in question the outcome of the responses may be affected. We have shown that CpG ODNs are novel adjuvants in chlamydial vaccine design. Increased knowledge of how the bacteria circumvent the immune system and identification of protective antigens will provide better approaches to develop new effective vaccines against *C. pneumoniae*.

2. We have identified hsp90 as a binding protein to CpG ODN and demonstrated intracellular redistribution of hsp90 upon CpG ODN stimulation. Hsp90 may therefore act as a ligand transfer molecule and/or be a target of intracellular actions of CpG ODN stimulation. A more complete picture of molecules and/or signaling events involved in CpG DNA-induced effects will allow better approaches to manipulate Th1 immune responses in novel vaccine strategies.

3. The expression of the antimicrobial peptides, HBD-1 and/or LL-37 was shown to be downregulated in *Shigella*-infected colonic biposies and cell lines. Downregulation of these innate effectors at an early stage of disease may be an effective strategy utilized by pathogens to establish infection at mucosal surfaces. In an immune escape strategy, combating an attack from these peptides, bacterial components such as plasmid DNA may serve as one mediator.

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