Mucosal adjuvants and their mode of action in the female genital tract

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♡*Till de underbara människorna i mitt liv som jag är lycklig nog att kalla min familj.*

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Abstract:
Sexually transmitted infections (STIs) cause a socioeconomic burden, morbidity and even mortality in a large part of the human population all over the world today. One of the most common genital ulcerative diseases is caused by herpes simplex virus type 2 with over 536 million people infected world-wide. Despite tremendous efforts, there are only vaccines against sexually transmitted human papillomavirus available today. The lack of success in vaccine development against STIs has partly been due to insufficient knowledge about how to induce protective immunity in the female genital tract.

Development of new vaccines is largely based on the use of highly purified or recombinant antigens, with limited immunogenicity. This has generated a need for development of potent vaccine adjuvants. Although a few adjuvants are included in the licensed vaccines, they are all administered systemically and their mode of action is poorly defined. In this thesis we have identified two new potent mucosal adjuvants for induction of immunity against genital HSV-2 infection, the glycosphingolipid alpha-galactosylceramide (α-GalCer), which is a potent agonist of invariant natural killer T (iNKT) cells and AFCo1, a cochelate structure of proteoliposome derived from *Neisseria Meningitides* serogroup B, with a combined immunostimulatory and delivery system function.

By employment of genome-wide gene expression microarray analysis combined with a bioinformatics approach we assessed the molecular signatures of two classes of immunostimulatory mucosal adjuvants, namely α-GalCer and the Toll-like receptor 9 agonist CpG ODN, both of which have been shown by our group to induce comparable immune protection against genital herpes infection in mice. Local administration of the adjuvants elicited expression of a number of core genes among which several were cytokines and chemokines as well as inflammasome associated genes. “Inflammatory response” was identified as the common main bio-function with Tnf as the common key regulator of gene expression. An adjuvant-induced enhancement in the frequency of vaginal dendritic cells and macrophages was also observed.

In summary, results presented in this thesis could identify two new mucosal adjuvants with the ability to confer protective immunity against genital herpes, as well as the molecular signature of mucosal adjuvants in the mouse female genital tract. These results may contribute to the future development of safe and potent mucosal adjuvants to be included in novel vaccines against STIs.

Keywords: mucosal adjuvants, genital tract, HSV-2, alpha-galactosylceramid, CpG ODN, proteoliposome-derived cochleate, inflammation, bioinformatics, mouse

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Original papers

This thesis is built upon the following papers, which are herein referred to by their Roman numerals:

I. Lindqvist M, Persson J, Thörn K, Harandi AM.
The mucosal adjuvant effect of alpha-galactosylceramide for induction of protective immunity to sexually transmitted viral infection.
J Immunol. 2009 May 15;182(10):6435-43.

II. Lindqvist M*, Navabi N*, Jansson M, Samuelson E, Sjöling A, Orndal C, Harandi AM.
Local cytokine and inflammatory responses to candidate vaginal adjuvants in mice.
Vaccine. 2009 Dec 10;28(1):270-8.

III. Del Campo J, Lindqvist M, Cuello M, Bäckström M, Cabrerra O, Persson J, Perez O, Harandi AM.
Intranasal immunization with a proteoliposome-derived cochleate containing recombinant gD protein confers protective immunity against genital herpes in mice.
Vaccine. 2010 Feb 3;28(5):1193-200.

IV. Lindqvist M, Brinkenberg I, Samuelson E, Thörn K, Harandi AM.
A genome-wide transcriptome profiling unravels molecular correlates of mucosal adjuvants in the female genital tract
Manuscript

**Both authors contributed equally to the work*

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Abbreviations

AFCo	Adjuvant Finlay Cochleate
AFPL	Adjuvant Finlay Proteoliposome
α-GalCer	Alpha-galactosylceramide
APC	Antigen presenting cell
CBA	Cytometric bead array
cLN	Cervial lymph nodes
CpG ODN	Cytidine phosphate guanosine oligodeoxynucleotide
CTL	Cytotoxic T lymphocyte
DAMP	Danger associated molecular pattern
DC	Dendritic cell
ELISA	Enzyme linked immunosorbent assay
FGT	Female genital tract
g	Glycoprotein
gLN	genital lymph nodes
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
i.d.	Intradermal
i.m.	Intramuscular
i.n.	Intranasal
i.vag.	Intravaginal
Ig	Immunoglobulin
IL	Interleukin
IPA	Ingenuity pathway analysis
MdLN	Mediestinal lymph node
MHC	Major histocompatibility complex
MPL	Monophosphorolipid A
MyD88	Myeloid differentiation factor 88
N-9	Nanoxynol-9
NALT	Nasal associated lymphoid tissue
NF-κB	Nuclear factor κ B
NK	Natural killer
NKT	Natural killer T
NLR	Nod like receptor
OD	Optical density
NLRP	Nod like receptor protein
PAMP	Pathogen associated molecular pattern
Pfu	Plack forming units
PL	Proteoliposome
PRR	Pattern recognition receptors
RT-PCR	Reverse transcription polymerase chain reaction
SAM	Significant analysis of microarray
SEM	Standard error of mean
SLPI	Secretory leukocyte protease inhibitor
STAT	Signal transducers and activators of transcription
STI	Sexually transmitted infection
TGF	Transforming growth factor
Th	T-helper
TIR	Toll/interelukin 1 receptor domain
Tk	Thymidine kinase
TLR	Toll like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRAF	TNF-receptor associated factor
Treg	T regulatory
TRIF	Toll-receptor associated activator of interferon
WT	Wild type

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1. Introduction

Vaccines have probably saved more human lives than any other medical discovery. Traditional vaccines have been based on whole inactivated or killed pathogens and have mainly aimed at inducing a protective antibody response. These vaccines could potentially constitute a safety risk by inducing serious adverse effects in immunocompromised individuals. The challenges we are facing today with millions of people all over the world infected with sexually transmitted infections (STIs) like human immunodeficiency virus (HIV) and herpes simplex virus type 2 (HSV-2), causing morbidity and even mortality demands a more rational vaccine development. To combat these viral infections a systemic as well as local mucosal defence is necessary and although antibodies are important, induction of a strong cellular response is crucial. The trend towards including safer, albeit less immunogenic antigens in vaccine formulations has created a need to develop potent adjuvants to direct and boost immune responses. Few adjuvants are included in the licensed vaccines today, none of them are mucosally administered and the mode of action of adjuvants is still not well known.

Today there are only licensed vaccines against a single STI, human papillomavirus. A contributing factor to the lack of vaccines against STIs is, at least in part, due to the scarce knowledge about induction of protective immunity in the female genital tract (FGT).

1.1 Female genital tract

The FGT can be divided into an upper sterile part consisting of the ovaries, fallopian tubes, uterus and endocervix, and a lower part that includes the ectocervix and the vagina. Although the FGT shares many common features with other mucosal sites, it also contains some unique features, due to being a reproductive organ [1]. Although the different parts of the FGT have distinct characteristics in terms of immunity, this thesis will mainly be focused on immunity in the vagina.

1.1.1 Innate immunity

Innate immunity is the first line of defence against foreign molecules, consisting of inherent, non-specific, physical and chemical barriers as well as a cellular response. As part of innate immunity, the vagina contains mucins, soluble factors like mannose-binding lectin, complement factors, antimicrobial peptides and phagocytic cells. Anti-proteases, such as secretory leukocyte protease inhibitor (SLPI) and elafin, both detected in the vagina, can besides from protecting against harmful proteases secreted during inflammation, function as antimicrobial agents [2-6]. Other protective agents detected in the vagina include the defensins [7], which have been shown to have antimicrobial properties, by permeabilizing microbial membranes [8]. The vagina contains a commensal bacterial flora, predominantly inhabited by *Lactobacillus*, which has virucidal effects through the secretion of lactic-acid, contributing to a hostile environment with low pH [9]. Soluble mediators, secreted during an innate immune response, are important in preventing infection. The chemokines, CCL3, CCL4 and CCL5 bind to the HIV co-receptor CCR5 and thereby interfere with infection *in vitro* [10]. Their importance has been further strengthened by a study detecting an elevation in the concentration of CCL5 in the genital mucosa of highly exposed, yet HIV-uninfected, African women [11].

1.1.1.1 Pattern recognition receptors

The discovery of Toll-like receptors (TLRs) in the 90´s resolved a long standing mystery of how pathogens are recognized by the innate immune system and how immunity to pathogens is triggered. TLRs belong to the broader class of pattern recognition receptors (PRRs), which exist as either membrane bound or soluble proteins, with the ability to recognize both endogenous danger associated molecular patterns (DAMPs) as well as pathogen associated molecular patterns (PAMPs) [12, 13]. TLRs are composed of a leucine-rich domain recognizing specific ligands, a transmembrane region and a Toll/interleukin 1 receptor domain (TIR), which mediate down-stream signalling upon cross-linking of homo- or hetero-dimer TLRs [14, 15]. To date there are 10 identified TLRs in humans [16] and 13 in mice [17], for which most of their ligands have been identified. Functional expression of all 10 human TLRs has been detected throughout FGT, with the exception of TLR10 which was detected only in the fallopian tubes [18]. TLRs are mainly expressed by professional antigen presenting cells (APCs), like dendritic cells (DCs) and macrophages, but also on other cells involved in initial recognition of pathogens, such as epithelial cells [19, 20]. The cellular localization of TLRs has been shown to be of great importance for the recognition and distinction of foreign molecules (Fig.1). TLR 1, 2, 4-6 and 10-13 are membrane expressed and recognize bacterial and fungal components. In contrast, TLR 3, 7, 8 and 9 are localized to the membrane of endosomal compartments, responding to microbial RNA and DNA [21]. All TLRs mediate signalling via adaptor protein myeloid differentiation factor 88 (MyD88), with the exception of TLR3, which instead utilizes toll-receptor associated activator of interferon (TRIF), a pathway that also can be used by TLR4. MyD88 binds IL-1R-associated kinase (IRAK), which upon activation phosphorylates and activates TNF-receptor associated factor 6 (TRAF6), leading to production of pro-inflammatory mediators via activation of transcription factor nuclear factor κB (NF-κB), activator protein 1 (AP-1) and interferon regulatory factor (IRF).

A more recently discovered group of PRRs are the soluble Nod-like receptor (NLR) proteins, located in the cytoplasm, of which NOD1 and NOD2 are extensively studied. NLRs are characterized by three structural domains, one leucine-rich domain similar to the one in TLRs, one nucleotide binding domain called NOD/NACHT and the effector signalling domain which can be a pyrin domain (PYD), caspase recruitment domain (CARD) or baculovirus inhibitor of apoptosis protein repeat (BIR) domain (Fig. 1) [22]. Expression of NOD1 and NOD2 has been detected in all parts of the FGT [18]. NOD proteins recognize bacterial derivatives and initiate a signalling cascade, leading to the activation of NF-κB. Recent studies have shown the ability of NOD-like receptor proteins, NLRP1, NLRP3 and NLRC4 to oligomerize upon stimulation, and together with adaptor proteins containing additional CARD domains, form complexes, named inflammasomes, which can recruit and activate caspases. While NLRP1 and NLRC4 recognize bacterial derivatives and flagellin respectively, NLRP3 responds to a wide range of microbial stimuli as well as danger signals such as uric acid and necrotic cell components. Recently it was also shown that particulate adjuvants, including the widely used alum, induce the activation of NLRP3 inflammasome [23]. Activated caspase-1 cleaves the proforms of the cytokine interleukin 1 (IL-1) and IL-18, leading to secretion of active IL-1β and IL-18 [24, 25]. IL-1β and IL-18 can in turn induce expression of additional inflammatory mediators via the shared intracellular signalling domain, TIR, of TLRs.

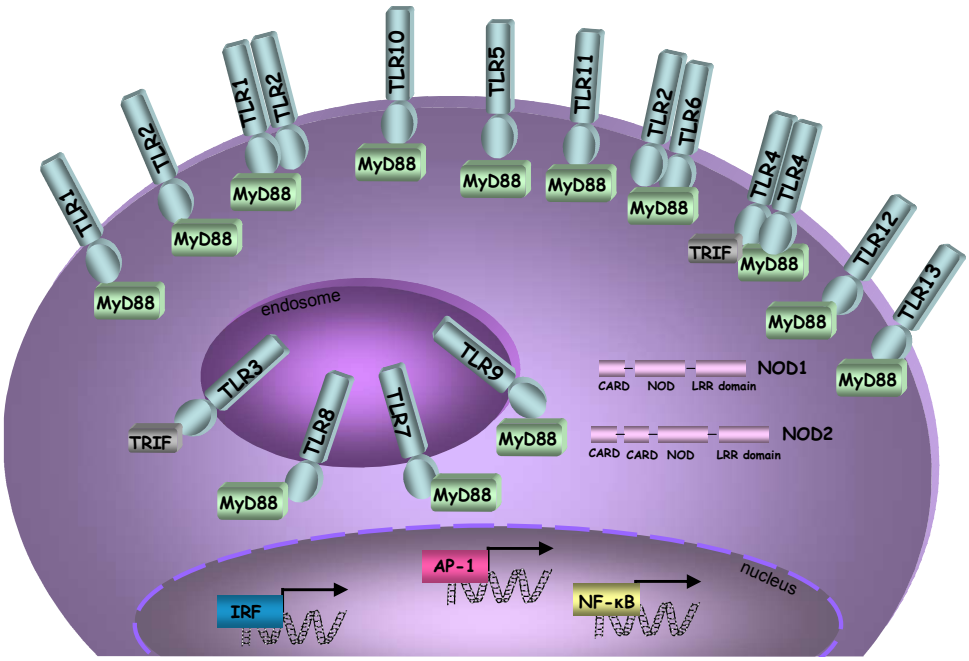

Figure 1. Schematic drawing of the cellular localization of pattern recognition receptors.

1.1.2 Adaptive immunity

Adaptive immunity, unlike inherent innate immunity, evolves throughout the lifetime of an organism. The FGT lacks local lymphoid structures, like Peyer's patches in the GI tract, as primary inductive sites. However, one group has reported vagina-associated lymphoid tissue in mice following HSV-2 infection under the influence of progesterone and estrogen treatment [26]. It is believed that activated APCs carry the antigen to the draining caudal and lumbar lymph nodes, where they present antigen to T-lymphocytes [27]. T-lymphocytes originate from bone marrow and develop in thymus. The T-lymphocytes are broadly divided, based on their respective expression markers, into CD4 cells, which are characterized by helper and delayed type hypersensitivity activity, and CD8 cells that are cytotoxic [28]. CD4 T cells, also referred to as T helper cells, are further classified as being Th1 or Th2 type due to their counteracting cytokine profiles, secreting, IFN-γ, TNF and IL-2 or IL-4 and IL-5, respectively [29, 30]. More recently we have learned that there are more T helper subtypes e.g. Th17, Th9 and regulatory T cells (Tregs) with different cytokine signatures than the classical Th1 and Th2 cells and additional subsets will certainly be discovered [31]. Differentiation of Th17, Th9 and Tregs are all driven by TGF-β and additional cytokines determine their fate of becoming regulatory or effector cells. Besides being characterised by their cytokine expression profiles, the different T helper subsets require unique transcription factors. Th17 cells, which secrete IL-17, have been ascribed a role in creating a pro-inflammatory milieu and contribute to the pathology of a number of autoimmune diseases. A regulatory relationship between the Th1 and Th17 subsets has been described and although both subsets induce an inflammatory response, the responses are divergent in the characteristic cell recruitment and cytokine production [32]. Th9 cells can diverge from the Th2 subset under the influence of TGF-β, and although these cells have a unique cytokine profile with IL-9 secretion, a Th9-specific transcription factor is yet to be identified and it is debated if they represent a separate lineage. Similar to the Th2 subtype, Th9 cells may be involved in allergic responses [33]. Tregs, identified by the transcription factor Forkhead box P3 (FoxP3), were originally referred to as suppressor cells, due to their secretion of TGF-β and IL-10. This

subset of helper T cells is important for the control of autoimmune responses, maintaining a tolerogenic environment in the gut as well as suppressing inflammatory responses [34, 35].

A unique feature of vaginal immunity compared to other mucosal sites is the dominance in frequency and function of IgG antibodies as opposed to IgA, which is the most prevalent and functionally important isotype at other mucosal sites [36]. The antibody profile differs somewhat throughout FGT, with a higher IgA concentration in the upper FGT. IgA is produced in the cervix, where secretion is mediated by epithelial poly immunoglobulin receptors (pIgRs), whose expression is under the influence of hormones, albeit no such antibody receptors have been detected in the vagina [37]. Whereas it is believed that IgA detected in vaginal secretions originates from the upper genital tract, the question whether IgG is locally produced or if it is transudation from the sera is still open for discussion. There have been studies indicating local production in humans and non-human primates [38, 39], although most studies concur that a large portion of the IgG detected in the genital tract is systemically derived [40].

1.1.3 Cells of the vagina

The first cells a pathogen entering the vagina comes into contact with are the stratified squamous epithelial cells, which not only form a physical barrier, but also secrete glycocalyx in addition to exhibiting several immune effector mechanisms (Fig. 2) [41]. Vaginal epithelial cells are polarized, giving them distinct features on the apical and basal side, which plays a role in their effector functions. PRRs are expressed not only on macrophages and DCs of the vagina, but also on epithelial cells, enabling them to directly respond to pathogens and secrete cytokines [18]. Macrophages found in the vagina express different surface markers compared to those found in the GI-tract, and similarly to DCs, macrophages may facilitate HIV-1 transmission [42, 43]. One type of DCs can be found in lamina propria, while a distinct subset, referred to as Langerhans cells, due to their expression similarities to skin Langerhans cells, can be found interspersed in the epithelial cell layers [27]. Natural killer (NK) cells as well as neutrophils, both part of the innate response, can be found in the lamina propria [44]. Cells linking innate and adaptive immunity that can be found in the vagina are NKT cells, which express features of both NK- and T-cells and gammadelta T cells (γδ T cells), often located in or in close vicinity to the epithelial layer [45, 46]. γδ T cells recognize conserved non-peptide antigens, up-regulated on stressed cells [47].

A low number of lymphocytes can be detected in the lamina propria and in the epithelial cell layer of the vagina, the more profound are CD4 and CD8 positive T cells. The presence of B cells in the vagina has not been well documented, unlike for the upper FGT where data seem to consistently report the presence of B cells. There are those claiming that no B cells can be detected in the epithelia or lamina propria of the vagina [27], while others detect plasma cells in the lamina propria [48]. Tregs have also been detected in the vagina of naïve mice, indicating their possible role in regulation of inflammatory response [49].

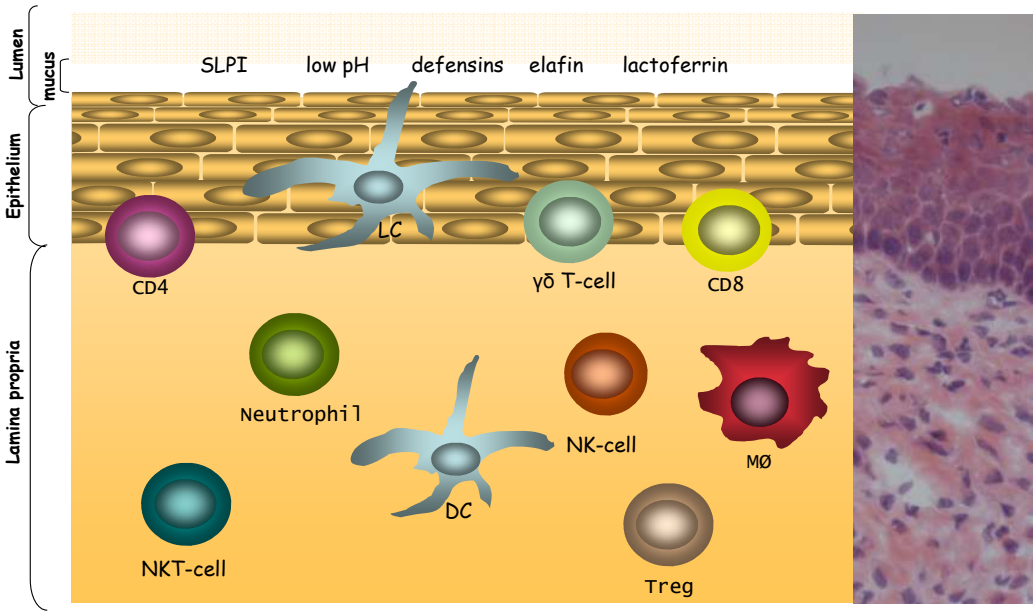

Figure 2. Schematic drawing (left) and microscopic photograph with hematoxilin/eosin staining (right), showing the structure and cellular content of mouse vaginal tissue.

1.1.4 Hormonal regulation

Due to its function as a reproductive organ, the FGT is under strict control of sex hormones, leading to fluctuations in immunity throughout the hormone cycle. Progesterone and estrogens control the mucosal- and epithelial barrier, cytokine production, antigen presentation, cell composition and antibody secretion in the FGT. The estrous cycle consists of four phases; proestrous, estrous, metestrous and diestrus. During diestrus, immunity is under the influence of progesterone. Progesterone influences the epithelial barrier by reducing the thickness of the cell layer, which is microscopically visible in mice, but for humans the difference is, although significant, very small and may be biologically irrelevant [50, 51]. Another innate feature under the control of sex hormones is the concentration of lactoferrin in the vagina, which varies throughout the cycle, and peaks during the estrous phase [52, 53].

Differences in number and localization of cells have also been observed in the murine FGT during the estrous cycle, with more DCs at metestrus and diestrus than proestrus and estrus [27, 50]. The antigen presenting capacity of DCs is also reduced by estrogen treatment, speculated to be due to higher expression of transforming growth factor β (TGF-β) (reviewed in [54]). The number of neutrophils detected in the vagina varies substantially during the cycle and are more abundant during metestrus and diestrus in mice, correlating with an increase in the chemoattractant cytokine macrophage inflammatory protein (MIP) -2 (CXCL2/3) [50, 55]. Although there are great fluctuations in the cell populations in mice, no significant differences in DCs, macrophages or T lymphocyte populations could be detected in the vagina of healthy women throughout the menstrual cycle [51]. However, dramatic differences in location and number of T lymphocytes and APCs have been detected in women during inflammation [56].

The antibody response is also greatly influenced by sex hormones. IgA levels in the vagina are highest during estrus, while for IgG the pattern is reversed, showing highest levels during diestrus in mice [57]. In humans, a study has shown that the levels of IgG in vaginal wash samples fluctuated during hormone treatment, whereas IgA levels were constant [40].

Generally it can be said that estrogen gives rise to a more anti-inflammatory state in the FGT. Studies in both humans and mice have shown an increased risk of infections during

treatment with estrogen and progesterone (i.e. in contraceptives), although it is unclear whether normal levels of these hormones can impact on the risk of infections [58-61]. The great impact of sex hormones on all aspects of immunity in the FGT needs to be taken into consideration when designing vaccines against STIs.

1.1.5 Induction of immunity

The term "common mucosal immune system" has been used to refer to the oral, respiratory-, urogenital- and GI–tract [62]. Today we know that although there is cross-talk between the mucosal sites, they are distinct areas with differences in structure and function. The mucosal immune system can be divided into induction and effector sites. Induction sites are mucosa associated lymphoid tissue (MALT) as well as local and regional draining lymph nodes where sampled antigens are presented to naïve immune cells. The lamina propria and epithelial layer can be referred to as effector sites where the activated immune cells exert their effector functions [63]. One interesting cross-talk of the mucosal compartments exists between nasal associated lymphoid tissue (NALT) and FGT. Nasal immunization has been proven to be efficient in inducing immunity in the FGT [64, 65]. This is most likely due to cellular homing mechanisms, further discussed below [66, 67].

NALT is one of the main components of MALT, the other being Peyer's patches (PPs) in the GI-tract. NALT has structurally only been described in rodents, consisting of paired lymphoid structures, situated above the soft palate at the entrance of the pharyngeal duct. No corresponding structure has been detected in humans, although it has been suggested that Waldeyer's ring may be equivalent, functioning as a primary lymphoid structure to the respiratory tract. NALT has a complete repertoire of immunocompetent cells needed to successfully induce an immune response and consists of aggregates of follicular B cells and intrafollicular T cells, overlayed with respiratory epithelial cells interspersed with antigen sampling M cells, much like PPs [68]. An immune response in NALT can also be initiated by draining of antigens via afferent lymph to cervical lymph nodes (cLN) and mediestinal lymph node (MdLN). A feature that distinguishes NALT from PPs is the difference in their development. Thus, while PPs develop during embryogenesis, NALT development requires antigen exposure and is not detected in rodents until after birth [69]. An attractive feature of nasal immunization is the low dose of antigen required due to lack of digestive enzymes, although the close proximity of NALT to the brain via the olfactory bulb raises potential safety concerns for development of nasally delivered vaccines. It has been shown that cholera toxin, when used as a nasal adjuvant in mice, redirected antigen into the central nervous system [70]. Further a strong association between usage of a nasal influenza vaccine, containing *Escherichia coli* heat labile toxin (LT), and Bell´s palsy (facial nerve paralysis) led to withdrawal of the vaccine from the market [71]. Two cases of transient Bells´s palsy was also observed in a phase I study following nasal delivery of a subunit vaccine against HIV and tuberculosis consisting of antigen and a mutant form of LT as adjuvant [72]. This strengthens the need to extensively evaluate safety of novel vaccine candidates for delivery through the nose.

1.2 Inflammation

The host's initial response to infections is usually an inflammatory response, involving both innate and adaptive immunity. The endeavour with an inflammatory response is to clear the infection. However the inflammatory immune response may itself become more harmful to the host than the damage caused by the invading pathogen. Inflammation is initiated by pathogen recognition through PPRs followed by secretion of pro-inflammatory cytokines and

chemokines, which further activates and recruits inflammatory cells to the site of infection. Macrophages and neutrophils are the first cells to be recruited, followed by NK cells. Upon activation these cells secrete molecules, including cytotoxic cytokines and nitric oxide that cause tissue damage. To limit the tissue damage, anti-inflammatory mechanisms are initiated, through production of cytokines such as IL-10 and TGF-β mainly by DCs, macrophages and NK cells. Once adaptive immunity is in play, CD4 T cells, especially Th1 type, secrete additional pro-inflammatory cytokines, while Th2 type are known to be more active in an anti-inflammatory milieu during persistent infection. A more recently discovered subset of CD4 T cells is the Th17, secreting IL-17, which have been shown to be involved in inflammation [73, 74]. IL-17 can also be secreted by γδT cells in the mucosa contributing to inflammation, although γδT cells can also secrete anti-inflammatory mediators such as IL-10 and TGF-β, suppressing T cell dependent inflammation [47]. A fine balance between pro-inflammatory mechanisms and anti-inflammatory mechanisms is kept during homeostasis (Fig. 3) and inflammation is also regulated by Tregs.

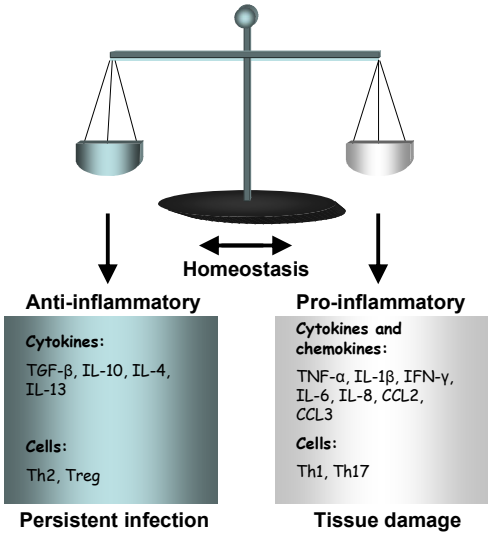

Figure 3. Illustration of important mediators in the balance of an immune response during an infection.

1.2.1 Cytokines

Originally the terms lymphokine and monokine were used to describe factors produced by lymphocytes and monocytes respectively but later they were commonly named cytokines. Cytokines are small proteins functioning as paracrine or autocrine soluble mediators of cells involved in immune responses. Cytokines can be divided into different superfamilies based on sequence and receptor binding homology. Major superfamilies include; interferons, tumour necrosis factor (TNF), interleukins and chemokines [75].

1.2.1.1 Interferons

Interferons (IFN) type I and II were the first cytokines to be discovered when they were shown to protect cells from viral infection and recently another, type III has been described [76, 77]. There are 7 different type I IFNs, of which the most famous ones are IFN-α and IFN-β, while there is to date only one IFN belonging to type II, namely IFN-γ. Type I IFN plays a major role in innate immunity and is secreted following TLR signalling during viral infections. Upon receptor binding, type I IFN mediates signalling via Jak/STAT (Janus kinase/Signal transducers and activators of transcription) which leads to secretion of interferon stimulated genes (ISGs). ISGs directly inhibit viral replication and trigger apoptosis

in infected cells [78]. Another interferon, which is important not only in the host response against viral infection but also in the majority of immune responses, is IFN-γ. IFN-γ is essential for initiation, expansion and sustaining a Th1 type of immune response. Further, IFN-γ activates a variety of innate immune cells, e.g. APCs, to enhance antigen presentation via up-regulation of MHC molecules and co-stimulatory molecules. In addition, IFN-γ activates NK cells and neutrophils to enhance their cytotoxic effects. Several clinical trials have been conducted using IFN-γ as therapeutic agent against cancers and infections with varying results [79]. The newly discovered third class consists of λ IFNs (IL-28 and IL-29) and resembles type I IFNs in terms of function. An important difference between type I and type III IFNs is the expression of receptors, while type I receptors are widely expressed, type III receptors are mainly expressed on DCs and epithelial cells, rendering them more specific in their response [80].

1.2.1.2 Tumour-necrosis factor

The TNF superfamily consists of over 20 ligands and 30 receptors [81]. Members of the family have a wide range of effector mechanisms, and are involved in signalling pathways during both development and host defence. They can induce inflammation and differentiation (e.g. TNF), mediate costimulatory signals (e.g. CD40L) and survival signals (e.g. BAFF) as well as cell death signals (e.g. TRAIL and LIGHT) [82, 83]. TNF ligands are expressed by immune cells and the two first discovered TNF proteins were lymphotoxin (LT, later re-named TNF-β) and TNF-α, which although sharing sequence homology, perform distinct effector functions. TNF-α is a major player in acute inflammation, responsible for local (e.g. stimulate expression of adhesion molecules and chemokines) and systemic effects (e.g. induce fever and secretion of acute phase proteins), depending on the level of concentration. Dysregulation of TNF and other members have been shown to contribute to a wide range of diseases, e.g. diabetes, MS and cancer, making them attractive therapeutic targets. However, caution needs to be taken when addressing TNF as a therapeutic agent as its receptors TNFR1, which is expressed on virtually all cell types, and TNFR2, mainly expressed by immune cells and endothelial cells, may explain the non-specific systemic toxicity that can be caused by TNF. This limits its usefulness in therapeutics. The toxic effects of TNF are believed to be mediated via NFκβ signalling [83].

1.2.1.3 Interleukins

The interleukin family can be further divided into subfamilies of IL-1, IL-6-like, IL-10, interferon type III, common γ-chain and IL-12. These signalling molecules have diverse functions and besides being involved in homeostasis, they initiate a wide range of responses, both pro-inflammatory and anti-inflammatory [84]. Examples of pro-inflammatory cytokines are IL-1β, which has similar biological effects as TNF-α, and IL-6, a multifunctional cytokine involved in acute phase inflammatory response [85, 86]. IL-12 is an important mediator in the early innate response against intracellular pathogens and stimulates a Th1 type of response and production of IFN-γ [87]. Interleukins are also involved in limiting the magnitude of an immune response. One regulatory cytokine is IL-10, which function by e.g. inhibiting the expression of co-stimulatory molecules and IL-12 production by activated macrophages [88].

1.2.1.4 Chemokines and homing

Chemokines are small chemotactic cytokines, which signal through seven-transmembrane G-coupled receptors and are involved in cell trafficking during development, homeostasis as well as pathological conditions. They can be broadly divided into homeostatic and inflammatory chemokines, based on their expression profiles, which may not always be exclusive [89]. Originally chemokines were designated names by the scientists who discovered them, this however led to confusion when new chemokines were rapidly identified and the

same molecule was reported under different names. A new nomenclature has been applied in which chemokines are divided into 4 different groups, based on their amino acid sequences; C, CC, CXC and CX3C, where C is a cystein residue and X any amino acid. The CXC chemokines can be further divided into ELR-CXC or non-ELR-CXC, where ELR refers to an amino acid sequence prior to CXC motif. ELR-CXC chemokines have been shown to bind receptors mainly expressed on neutrophils. In the same sense the receptors for the respective chemokines have been named XCR1 (for C), CCR1-9 (for CC), CXCR1-5 (for CXC) and CX3CR1 (for CX3C) [89]. Although the nomenclature for chemokines and their receptors is based on human data the great majority of chemokines or homologues are also found in mouse, especially chemokines coded by evolutionary conserved gene clusters. There is promiscuity among the binding of chemokines and receptors, meaning that one chemokine can bind several receptors and one receptor can respond to several chemokines, although to a lesser extent among the conserved ones [90]. Due to their multifunctional effects on immune cells chemokines have been used, often together with cytokines, to evoke an immune response against tumours. In contrast, antagonists, eg. monoclonal antibodies, against chemokines have also been investigated for the prevention of chronic immune responses.

To recruit leukocytes into the tissue, endothelial cells in the vessels need to express adhesion molecules called addressins. The best known mucosal addressin is the cell adhesion molecule-1 (MAdCAM-1), expressed in the GI-tract, which binds the integrin $\alpha_4\beta_7$ that is expressed on T and B cells induced in gut-associated lymphoid tissue (GALT). However, this molecule has not been shown to be expressed in the vagina. Expression of VCAM-1 and ICAM-1, which binds the integrins $\alpha4\beta1$ and LFA-1 respectively, have been detected in the vagina suggesting their involvement of lymphocyte homing there [48, 91]. Following recruitment of effector cells into the tissue, chemokines are important in directing the cells to specific anatomical locations within the tissue. Several chemoattractants and their receptors have been identified in the GI-tract, whereas the chemokines important for homing in the genital tract are less well defined and can differ between upper and lower genital tract in response to infection [92].

1.3 Herpes simplex virus type 2

Since the discovery of HIV in the 1980's STIs have gained much attention. Despite an increased general knowledge about preventative measures, the incidence of STIs is increasing worldwide. One of the most prevalent STIs is caused by herpes simplex viruses (HSVs).

HSV type I and 2 belong to the family of *Herpesviridae*, subfamily *Alphaherpesvirinae* and genera *Simplexvirus* [93]. The two subtypes are closely related with 83% similarity in nucleotide alignment and share many common proteins, enabling induction of cross-reactive immunity [94]. HSV-1 infection is most prevalent in the orofacial area, whereas HSV-2 infection has been regarded as the cause of genital HSV infection [95]. However, this difference in anatomic distribution is now less apparent, most likely due to a change in sexual behaviour.

1.3.1 Structure and infection

The HSV-2 virion has a size of approximately 120-150 nm [96]. Like all herpesviruses, HSV-2 has a double stranded DNA core, making up the genome which consists of a long (U_L) and a short (U_S) coding region, which generate in total 74 proteins [94]. The expression of HSV-2 genes is sequential in gene clusters, called immediate early, early, early late and late genes, due to their respective involvement in replication [97]. Surrounding the DNA is an icosahedral capsid followed by a tegument, containing viral proteins needed for initial processes of infection and replication, and an outer cell-derived lipid envelope containing membrane proteins (Fig. 4) [98]. The envelope contains at least 10 different glycoproteins (g), named gB, gC, gD, gE, gG, gH, gI, gK, gL and gM, of which several are crucial for attachment of the virus and subsequent infection of target cells. The only natural host of HSV are humans and

the virus primarily infects epithelial cells of the skin and mucosa, as well as neurons but has also been shown to infect immune cells. HSV-2 infection is most prevalent in the genital tract in the vaginal epithelial cells. Infection is initiated by binding of gC and gB to heparan sulphate, a glycosaminoglycan chain of cell surface proteoglycans [99]. For the initiation of entry, gD needs to bind one of it receptors, herpesvirus entry mediator (HVEM, member of the TNF family) or nectin-1 and -2 (member of the immunoglobulin superfamily). It is believed that binding of gD to one of its receptor causes conformational changes in gD that enables gB as well as gH-gL to be recruited, leading to activation of their membrane-fusing activity (reviewed in [100]).

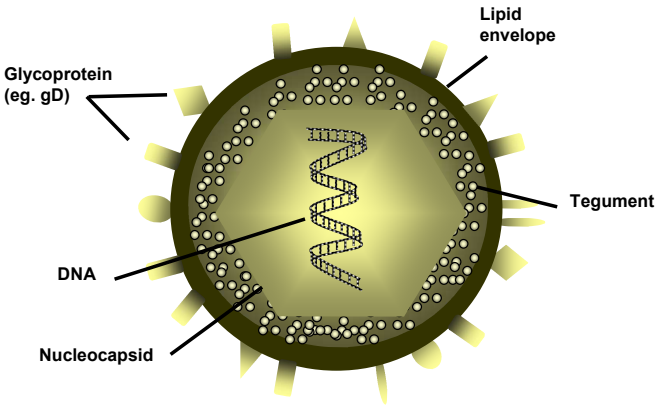

Figure 4. Cross-sectional schematic drawing of a HSV virion.

1.3.2 Replication and latency

Following infection, HSV-2 replicates locally and virions are shed from epithelial cells, whereby the epithelial cells die. It takes approximately 7-10 days for the epithelium to recover from infection [50]. Infection is spread by cell-to-cell contact, dependent on gE/gI, and the newly produced virions move by retrograde transport along sensory neurons to ganglia where they establish life-long latency [101, 102]. Recurrent disease may occur due to emotional and physical stress and is caused by ascending reactivated virus from the ganglia.

1.3.3 Symptoms and complications

Viral shedding may occur during both symptomatic and asymptomatic phases, leading to an increase of HSV transmission [103]. It was been estimated that 536 million people worldwide were infected with HSV-2 in 2003, with the highest prevalence in parts of Africa with up to 70% being infected. The prevalence was shown to increase with age and was higher among women compared to men [104]. Although the trend worldwide is towards an increase in the incidence of STIs, there was a study showing a decline in HSV-2 infections among young Swedish women during the 90's [105]. The majority of HSV-2 seroconversions are asyptomatic [106]. Genital HSV-2 infection, when symptomatic, most commonly gives rise to ulcers, being more severe during initial infection than recurrent activations. Complications, such as cystitis, meningitis, urethritis and cervicitis due to HSV-2 infection can be detected in around 13% of symptomatic patients [106]. HSV can transmit from mother to neonate with the great majority of infections occurring during birth, and give rise to neonatal infection in visceral organs or potentially lethal encephalitis [107, 108].

1.3.4 Correlation with HIV infection

Numerous studies have shown a correlation between HSV-2 and HIV infection, and a recent meta-analysis pointed to an overall three fold higher risk of acquiring HIV for HSV-2 positive individuals [109]. Being an ulcerative disease it is not hard to imagine that the risk of viral transmission of HIV is greater in HSV-2 infected individuals. Further, one could imagine that an ongoing HSV-2 reactivation could induce inflammation and recruit immune cells, being major target cells for HIV infection, and thereby increasing the risk of acquisition. Inflammatory cytokines, IL-1 and TNF have been shown to enhance HIV replication and HSV-2 induced recruitment of HIV-1 target cells, CD4 T cells expressing co-receptors as well as DCs expressing DC-SIGN [110, 111]. Studies have shown that women with STIs have lower concentrations of antimicrobial peptides than uninfected individuals, which could be a contributing factor for increased susceptibility to other infections [2]. Moreover, studies have shown that by suppressing recurrences of HSV-2 shedding with drugs, viral load of HIV is reduced [112, 113]. Clinical studies assessing the anti-HIV effect of HSV-2 suppressive drugs have however been disappointing, showing no reduction in HIV transmission among individuals undertaking acyclovir treatment compared to a placebo group [114-116]. This could possibly be explained, at least partly, by a study showing that HIV-1 target cells persistently stay at the site of HSV-2 reactivation in the skin despite the use of HSV suppressive drugs [110]. A recent study also showed that HSV-2 infection increased susceptibility to HIV-1 infection in the absence of HSV-2 clinical symptoms. By infecting Langerhans cells, HSV-2 interfered with expression of langerin and its binding to HIV-1, which normally functions as an innate barrier [117].

1.3.5 Immunity against HSV-2

Studies have shown that innate soluble factors, such as human defensins and SLPI, can directly inactivate HSV-2, possibly by interfering with binding to target cells [118, 119]. The vaginal flora is important in protection against STIs. Recently it was shown that *Lactobacilli* has a virucidal effect, not only by the low pH caused by secreted lactic acid, but also by hydrogen peroxide, which impairs the infection capacity of HSV-2 [120].

The first cells to respond to HSV are macrophages, secreting type I IFNs and TNF, which can have a direct antiviral effect [121]. HSV-2 can be recognized by TLRs as well as cytoplasmic PPRs, mainly expressed in DCs and B cells and further induce an innate response [122]. Signalling through TLR2 and TLR9, via MyD88 adaptor protein, leads to production of pro-inflammatory cytokines (IL-6, IL-8 and CCL2) and type I/III interferons respectively [123, 124]. Production of IFN-α in response to HSV-1 has been attributed to gD binding to chemokine receptors CCR3 and CXCR4 [125]. The importance of type III IFNs has been shown in murine models, where they blocked HSV-2 replication in the vaginal mucosa and prevented development of disease, in contrast to IFN-α which had a more modest antiviral activity [126]. Other innate cells involved in protection against genital HSV-2 infection are neutrophils, since higher viral titers were detected in mice lacking neutrophils [127]. The importance of IL-15 secretion by NK and NKT cells in immunity to genital herpes has been demonstrated [128, 129]. Intraepithelial γδ-T-lymphocytes have also been shown to be involved in immunity to HSV-2 [130].

Previously it was believed that intraepithelial Langerhans DCs captured pathogens from the vaginal lumen, due to their proximity. However, it has later been shown that it is the submucosal ($CD11b^+$ / $CD11c^+$) DCs that pick up HSV-2 antigen, transport it to draining lymph nodes for presentation to CD4 T cells [50]. CD4 T cells have been shown to be important in the resolution of primary genital infection as well as protection against reinfection with HSV-2, though it may be indirectly via secreted IFN-γ rather than a role as pure effector cells. Although a recent study suggests that FasL-mediated cytotoxicity of CD4 T cells is an

important effector mechanism in the defense against primary infection with HSV-2 [131-133]. IFN-γ secretion can be detected from lymph node DCs after an HSV-2 infection and low levels of IL-10 and IL-4 have also been detected [50]. Further, IFN-γ has been shown to have a crucial role in HSV-2 neurovirulence in mice [134]. The importance of Tregs has also been implicated in vaginal HSV-2 infection, where more severe symptoms of disease and death were seen in mice lacking Tregs, suggesting a role for these suppressor cells in controlling the inflammatory response [49].

The role of antibodies in protective immunity against HSV-2 infection is debated. IgA antibodies have been shown not to be critically important for protection against HSV-2 [135]. A study has shown a correlation between maternal type-specific IgG antibodies and protection of neonatals against HSV-2 infection, indicative of their importance [136]. Protective immunity against HSV-2 could also successfully be induced by passive immunization with HSV-2 specific IgG antibodies in a mouse model [36].

1.3.6 Evasion mechanisms

One of the reasons to why HSV successfully establishes latency is its envolvement of several immune evasion mechanisms. HSV-2 can down-regulate the expression of SLPI, one important innate factor that can inhibit HSV infection of epithelial cells [137]. The intermediate expressed HSV gene ICP47 inhibits translocation of human transporter associated with antigen processing (TAP) protein into ER, which is needed for peptide presentation on MHC I [138]. ICP47 has shown to bind murine tap with lower avidity than human TAP protein, and it is unclear if murine TAP is inhibited under physiological conditions [139]. HSV blocks IFN I synthesis and interferes with phophorylation needed for activation of Janus kinases, STAT and eIF-2α [140, 141]. HSV also blocks DC maturation, inhibits up-regulation of costimulatory molecules, cell migration and secretion of cytokines [142]. Further, HSV can also interfere with the alternative complement pathway via binding of gC to C3 and its activation products [143]. Herpesvirus genomes encode homologues to several important immunoregulatory genes, such as members of TNF superfamily, thereby regulating killing of infected cells, one example being the utilization of the HVEM receptor (TNFRSF14) for viral entry [144].

1.3.7 Antiviral treatment and resistance

There is to date no cure against HSV infection and the current treatment aims rather to suppress symptoms and reduce recurrences [145]. Current drugs on the market are nucleoside analogues and derivates, one of which is acyclovir, a guanosine analogue, which upon activation through phosphorylation by viral thymidine kinase inhibits viral replication [146]. Besides from being an expensive and demanding life-time treatment, prolonged and harsh treatment with acyclovir can lead to acyclovir resistance, especially among immunodeficient HIV infected patients. The majority of acyclovir resistance is due to thymidine kinase deficient HSV strains [147].

1.3.8 Mouse model of genital herpes

Even though humans are the natural host of HSV, mice are also susceptible to HSV infection and the mouse has for a long time been the model organism for studying immunological processes. Although phenotypically distant, mouse and man both have roughly 30 000 genes and 99% of these genes are homologues with great synteny [148]. There are however differences, and given the complexity of immunology, work with model organisms does not always reflect the physiological state in humans.

In 1994 Parr *et. al* established a mouse model for studying mucosal immunity to genital HSV-2 infection. Previous problems with the mouse model with age dependent resistance to vaginal HSV-2 infection could be overcome by treatment with progesterone prior to viral challenge. The increased susceptibility to viral infection during diestrus phase is believed to

be due to the hormonal thinning of the epithelial layer. Infection with HSV-2 in mice gives rise to local inflammation followed by infection of CNS leading to neurological symptoms and eventually death due to encephalitis. Protection can be achieved, using live attenuated thymidine kinase deficient (Tk^-) HSV-2 strains, which induce local, self limiting inflammation, followed by protective immunity against challenge with fully virulent HSV-2. These characteristics of HSV-2 Tk^- has made it a gold standard for studying immunity against HSV-2 [60]. The mouse model is however limited to study short term immunity and acute symptoms. Another animal model for genital herpes infection is a guinea pig model, which more resembles human disease with primary as well as spontaneous recurrent symptoms. The guinea pig model enables the study of acute, latent and recurrent herpes, making it a valuable complement to mouse studies [149].

1.4 Vaccine adjuvants

In 1796 Edward Jenner, a medical doctor from Great Britain, performed a set of experiments that would revolutionise medical science. Jenner immunized a healthy boy with cowpox from blisters of an infected woman and subsequently showed that the boy was protected against a smallpox challenge. These experiments led to coining the word "vaccine" from the latin word *vacca* (meaning cow). The goal with prophylactic vaccination is to mount strong, protective and long-lasting immune responses against pathogen encounter. Therapeutic immunization approach has also been considered to elicit immunity in those already infected with pathogens.

Traditional vaccines are based on whole inactivated or live attenuated pathogens given systemically to induce protective immunity mainly by humoral immune responses. However, due to potential safety concerns, the current trend is towards development of safer, albeit less immunogenic, highly purified or recombinant antigens in vaccine formulations. This in turn has led to a need to develop adjuvants to enhance or prolong the immunogenicity of the antigen [150].

The word adjuvant comes from the latin word *adjuvare* which means "to help". Adjuvants can be classified in different ways, one being based on their effector functions, that can be broadly divided into delivery systems and immunomodulators, and the other being particulate and non-particulate adjuvants based on molecular structure. By functioning as a delivery system, adjuvants enhance the antigen accessibility, which however requires that the adjuvant is physically associated with the antigen. The accessibility of the antigen can be achieved by targeting the antigen to APCs or depot generation, where the antigen is kept localized in a high concentration. Adjuvants having immunomodulatory effects can stimulate an immune response and/or modulate the nature of the immune response. [151]. The discovery of TLRs as innate pathogen receptors generated a rising wave of studies on the use of different TLR ligands as potential adjuvants, among which several have been identified. When including immunostimulatory adjuvants in vaccine candidates, this, however, raises potential safety concerns, owing to induction of excessive inflammatory responses. Therefore the aspects of safety need to be carefully evaluated for adjuvant containing vaccines [152].

1.4.1 Particulate adjuvants/ delivery systems

By particulate formation, these adjuvants mainly augment immune responses by functioning as delivery systems, enhancing uptake of the antigen.

Included in a great number of vaccines on the market today, aluminium adjuvants have successfully been given to millions of people for over half a century. The adjuvant effect of aluminium was discovered when antigen was precipitated in the presence of different anions,

but these preparations were highly heterogeneous making them unsuitable for vaccine formulations. By using preformed aluminium-hydroxide gels it was possible to better control the formulations, which together with the later introduced aluminium phosphate became known as aluminium-adsorbed vaccines or simply "alum". The adsorbing quality of aluminium compounds particulates the antigen, creates a depot effect and can enhance antigen uptake. [153, 154]. Although alum proved to be the adjuvant of choice for several life-threatening diseases, it fails to elicit adequate antibody responses to small size peptides as well as certain vaccines such as typhoid fever and influenza vaccines. In addition, alum is not able to mount CTL responses [155]. Even though the effectiveness of aluminium compounds have been known for decades and the type of responses induced, the mode of action of alum has only recently been studied. Aluminium adjuvants have been shown to be pore inducers of CTL response and induce a Th2 type of response with expression of IL-4, IL-5 and production of IgG1 and IgE antibodies, indicative of Th2 response in mice [156]. Alum induces NLRP3 inflammasome activation as well as uric acid production, leading to activation of inflammatory monocytes and DCs followed by secretion of IL-1β [23, 157]. The ability of aluminium compounds to induce a Th2 response is in some cases disadvantageous in their adjuvant application, not only because allergy-promoting IgE antibodies are produced, but also for the ineffectiveness against certain intracellular pathogens, e.g. viral infections and cancers, where a Th1 biased response is preferable to induce protective immunity. Further, alum is not suitable for mucosal route of immunization.

Emulsions consisting of either oil-in-water or water-in-oil micro droplets are used as adjuvants. These formulations function by enhancing uptake or antigen presentation. Water-in-oil emulsions introduce a depot effect and have given rise to antibody responses, of which the widely experimentally used Freund's adjuvant is an example [151]. MF59 is an oil-in-water emulsion, containing squalene extracted from fish oil and surfactants. MF59 functions as a delivery system, enhancing the uptake of antigen by DCs and is now included in a licensed vaccine against influenza (Table 1) [158].

Liposomes are lipid vesicles most commonly constituting a bilayer of phospholipids surrounding an aqueous core. Liposomes are used as delivery systems, enhancing uptake of the antigen which can be trapped inside the liposome or be adsorbed onto it, depending on the nature of antigen [159].

Other particulate adjuvants resembling liposomes are micro- and nanoparticles made of biodegradable polyester, polyactide-co-glycolide (PLG) or poly lactic acid (PLA). These particles enhance uptake by APCs of adsorbed antigen and can induce potent antibody as well as CTL responses. These particles also allow inclusion of additional adjuvants, e.g. TLR ligands, into their structure to form combined immunostimulatory and delivery systems [160].

1.4.2 Particulate adjuvants/ combined delivery systems and immunomodulators

The distinction between delivery systems and immunomodulators is not complete, and several adjuvants being developed utilize combined effector functions to further boost the immune response.

Immune stimulating complexes (ISCOMs) are open cage-like structures of cholesterol, saponins, phospholipids and an immunogen. ISCOMs induce a wide range of effector responses consisting of antibody production and strong T cell responses, including CTL responses [161].

Adjuvant Systems 04 (AS04) developed by GlaxoSmithKline (GSK) Biologicals is a mixture of aluminium salts and monophosphorolipid A (MPL), and is included in hepatitis B virus and human papillomavirus vaccines presently on the market (see Table 1). Besides the

enhanced uptake and depot effect created by aluminium salt, MPL, stimulates cytokine production via TLR2, TLR4 and NF-κB signalling [162].

Another adjuvant that is included in licensed vaccines is virosomes (Table 1), which are hollow virus-like particles consisting of membrane associated proteins, but lacking the genetic material. The mechanism of action of influenza-derived virosomes is similar to the virus itself, only lacking the ability to replicate. Two surface proteins, hemagglutinin and neuraminidase are the most important antigens for the induction of neutralizing antibody response against influenza. Recombinant hemagglutinin and neuraminidase are included together with adjuvants in the licensed subunit vaccines in Europe (Table 1). Depending on the placement of the antigen in virosomes, in the membrane or inside the vesicle, different pathways can be targeted, i.e. MHC II and MHC I respectively, making it possible to direct the immune response [163].

1.4.2.1 Proteoliposome and cochleate

Liposomes function as delivery systems and lack the ability to efficiently induce a strong innate and adaptive immune response needed to induce immune protection. By introducing additional immunomodulators into the lipid bilayer, the very same APC can be targeted by both immunomodulators and antigen [159]. When adding proteins to a liposome, the term proteoliposome is used. Bacterially derived proteoliposome from *Neisseria meningitides B*, (PL) consist of major outer membrane proteins and phospholipids in addition to several TLR ligands, including LPS, porins and small amounts of bacterial DNA containing CpG motifs functioning as immunostimulatory adjuvants. PL has been used as an antigen in the meningococcal vaccine VAMENGOC-BC™, produced in Cuba [164]. PL induces Th1 response and can function as an adjuvant together with OVA. Although a problem with these proteoliposomes has been the low stability [165]. The stability of liposomes can however be increased by the transformation of liposomes into a cohcleate structure. By adding Ca^{2+} to the aqueous liposome solution the liposomes fuse into larger unilamellar structures, which roll-up and form spiral cylinders (see Fig 7) [166]. *Neisseria meningitides B*-derived cochelates called Adjuvant Finlay Cochelate 1 (AFCo1) has been developed by Pérez et. al at Finlay Institute, Cuba [165]. AFCo1 has been proven to function as a potent adjuvant for various incorporated antigens [167].

1.4.3 Non-particulate/immunomodulatory adjuvants

The effects of the molecules belonging to this class of adjuvants rely on their immunomodulatory properties.

Since the discovery of TLRs, several TLR ligands and agonists have been explored as adjuvants. Cytidine phosphate guanosine oligodeoxynucleotide (CpG ODN), a TLR9 agonist, mimicking bacterial DNA has successfully been used as adjuvant in different experimental settings. The first TLR ligand to be included in licensed vaccines (together with aluminium salt) is MPL, a less toxic derivative of lipid A of lipopolysaccharide (LPS) from a *Salmonella* strain, which signals through TLR4 and TLR2 via NF-κB, giving rise to a Th1 response with cytokines and antibodies [168]. It is believed that while LPS activates TLR4 and signals via both MyD88 and TRIF and can induce septic shock, MPL mainly utilizes the TLR4/TRIF pathway and thereby induces less inflammation and is considered safe. Further MPL is inefficient in activating caspase-1, needed to secrete mature IL-1β [169].

Saponin purified from the bark of *Quillaja saponaria* (QS)-21 has shown promising results as adjuvant in vaccines against a variety of infections. Saponins induce humoral as

well as cellular responses when used as adjuvant, although its mechanisms of action are not well defined [170].

Toxins, such as enterotoxins derived from *Vibrio cholera,* cholera toxin (CT) and heat-labile toxin (LT) from enterotoxogenic *E.coli* (*ETEC)*, function as potent adjuvants. Both CT and LT bind to the ganglioside GM1, expressed on eukaryotic cells, and induce ADP-ribosylating enzymatic activity. When used as adjuvants, these molecules are believed to function by increasing permeability of the epithelium, enhancing antigen presentation and promoting isotype switch in B cells. The enzymatic activity is caused by the A-subunit and render these molecules too toxic to be used in humans. However, less toxic derivatives of these e.g. CTB (included in the licensed vaccine Ducoral), CTB-CpG and CTA1-DD are currently under investigation [171].

1.4.3.1 CpG ODN

One way for the innate immune system to detect bacteria is by recognition of their high content of CpG islands, in the genome, present at a higher frequency in microorganisms compared to mammals. CpG islands are unmethylated cytidine phosphate guanosine motifs, being recognized by TLR9, highly expressed on plasmacytoid DCs and B cells in humans and additionally myeloid DCs in mice. Activation of TLR9 leads to NFκβ and mitogen-activated protein kinase (MAP) signalling via the MyD88 adaptor molecule, resulting in a pro-inflammatory response. Upon CpG-induced activation, DCs and B cells up-regulate activation markers, co-stimulatory molecules such as MHC molecules and secrete pro-inflammatory cytokines e.g. IL-6, TNF-α and IL-8. CpG also indirectly activates NK cells by triggering DCs to secrete cytokines (Fig. 5) [172]. Cellular uptake and endosomal processing of CpG is necessary to activate TLR9 [173]. The uptake of CpG by B cells is hampered by lactoferrin (present in the FGT) and hence varies throughout the hormone cycle [174]. The innate immune stimulation mediated by CpG results in initiation and activation of the adaptive immunity including efficient T cell activation and antibody production.

The immunostimulatory capacity of bacterial CpG has been mimicked in synthesized oligodeoxynucleotide (ODN) containing CpG motifs, utilized to induce protective innate immunity against infections in mice [175-177]. A single vaginal delivery of CpG ODN has proven efficient to stimulate innate immunity, inhibit HSV-2 replication and protect mice against lethal HSV-2 challenge [176, 178, 179]. Further, CpG ODN has been used as an adjuvant in numerous successful experimental and clinical studies against infections, tumours and Th2 driven diseases i.e. allergy. Synthesized CpG ODN, containing species specific flanking regions and CpG motifs typically about 20 bp long can be divided into CpG-A ODN, containing a largely natural phosphodiester backbone, CpG-B ODN, with a nuclease-resistant phosphorotioate bakbone and CpG-C ODN with a complete phosphorotioate backbone. The flanking regions of these three types of CpG ODN are optimal for humans, while there is only one stimulatory mouse CpG ODN. CpG-A ODN efficiently activates NK cells, induces a strong CTL response and stimulates DCs to produce IFN-α. CpG-B ODN is instead a strong B cell stimulant and gives rise to a robust antibody response [172]. The third class, CpG-C ODN combines the DC activation property of class A with the strong B cell stimuli of class B [180].

The adjuvant effect by CpG ODN, detected following systemic as well as mucosal immunization with a wide range of antigens, is due to enhancement of APC function by up-regulation of receptors and co-stimulatotory molecules as well as secretion of cytokines and chemokines. This contributes to an inflammatory response and an antigen specific microenvironment leading to a boost of both cellular and humoral immune responses [181, 182].

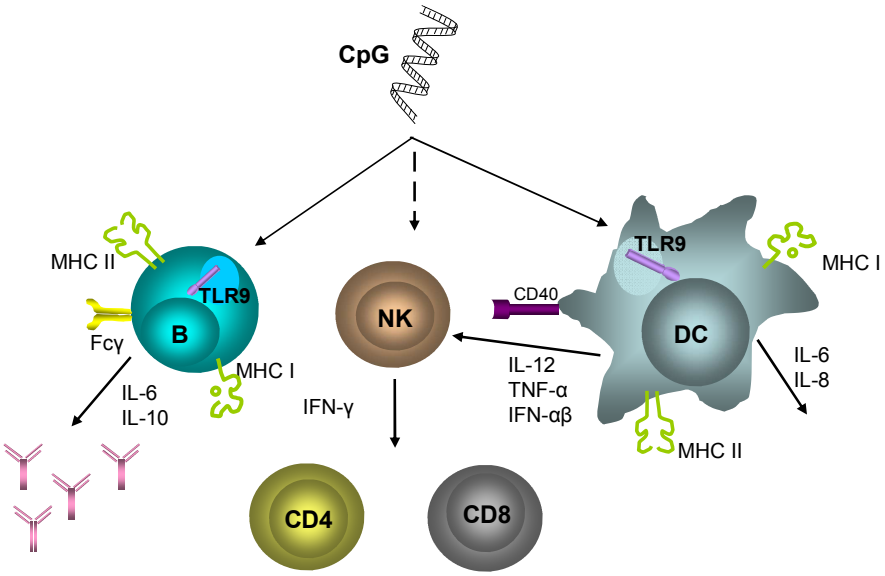

Figure 5. Activation of innate and adaptive immune responses by CpG. CpG is recognized by B cells and DCs expressing TLR9, and induces activation and secretion of pro-inflammatory cytokines as well as maturation of APCs by up-regulation of co-stimulatory molecules and receptors. CpG indirectly activates NK cells to secrete IFN-γ via cytokines, secreted by activated B cells and DCs, leading to activation of the adaptive arm of immune response accompanied by the maturation of B cells into antibody secreting plasma cells.

1.4.3.2 Alpha-galactosylceramide

Alpha-galactosylceramide (α-GalCer) is a glycosphingolipid, originally isolated from the marine sponge *Agelas mauritianus* has been shown to possess an antitumor effect in mice. Later a modified version of α-GalCer became produced under the name KRN7000 by Kirin Brewery Co, Japan [183]. KRN7000 was selected among the agelasphins based on its property to induce lymphocytic proliferation and DC activation in mice [184]. α-GalCer is presented on the MHC I-like molecule CD1d on APCs and is recognized by invariant NKT (iNKT) cells, expressing the TCRs Vα14-Jα18 combined with Vβ8, Vβ7 or Vβ2 in mice and the homologous Vα24- Jα18/Vβ11 in humans (Fig. 6). Due to a high homology between the five different CD1 molecules in humans and CD1d in mice, as well as the limited variation in TCR of NKT cells, α-GalCer can be presented on mouse CD1d and activate human iNKT cells and vice versa [185]. It was puzzling, after the discovery of α-GalCer, why humans respond so strongly to an antigen derived from marine sponge. Later it became clear that iNKT cells recognize glycosphingolipids from sphingomonas bacteria and it was speculated that the originally isolated agelasphins may be bacterially derived [186-188]. α-galactosylglycerol, which is expressed by *Borelia Burgdorferi*, structurally resembles α-GalCer and has been shown to stimulate iNKT cells [189]. Besides responding to exogenous ligands, an endogenous ligand to iNKT cells, Isoglobotrihexosylceramide (iGb3) has been identified in mice [190]. iNKT cells can be activated by iGb3 ligated to the CD1d molecule in the presence of secreted IL-12 from DCs activated through TLR signalling e.g. by gram negative bacteria containing LPS [188].

Upon activation, iNKT cells secrete large amounts of cytokines, eg. IFN-γ and IL-4. Different cytokine profiles expressed upon activation of iNKT cells is believed to depend on the experimental setting and the type of APCs involved [191]. Clinical trials with α-GalCer have been performed against tumours and results showed that α-GalCer treatment is safe, with no severe adverse effects. α-GalCer treatment led to increase in IFN-γ and IL-12 as well as NK cell activity in some patients. The anti-tumour effect seen following treatment varied, probably depending on the number of NKT cells, which is lower in cancer patients compared

to healthy controls [192-194]. Vα14 NKT cells constitute around 0.5-2.5% of the T cell population in blood and lymph nodes in mice, while in humans the frequency is 10 times lower [195]. Although iNKT cells constitutively express FasL and can secrete perforin, it is speculated if iNKT cells function as effector cells or merely as regulatory cells via secretion of cytokines [195]. There has been a study claiming that α-GalCer activated iNKT cells function mainly as regulatory cells *in vivo,* by activating cytotoxic NK cells [196]. The potential use of α-GalCer as an adjuvant has recently been explored with promising results in experimental mouse settings e.g. against influenza and malaria [197-199].

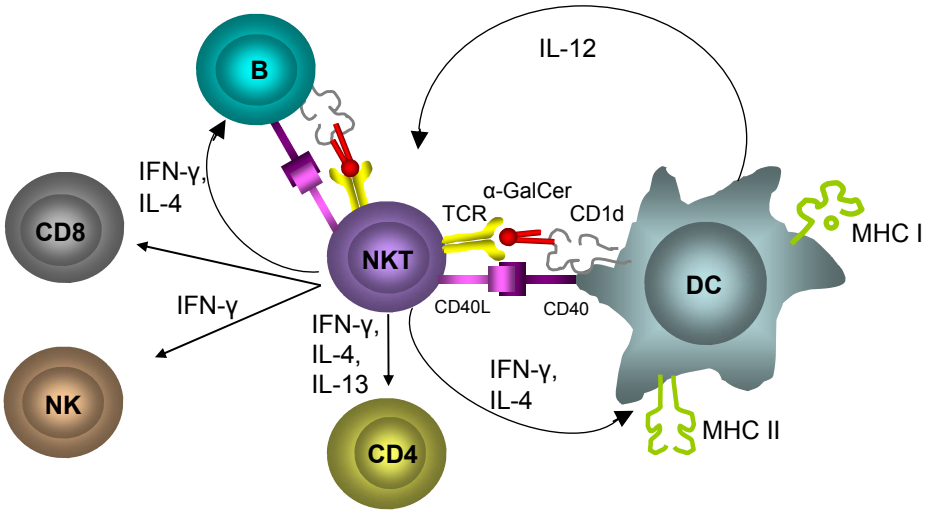

Figure 6. Immune activation by α-GalCer. α-GalCer is presented on CD1d molecule on DCs and B cells to iNKT cells expressing Vα14 TCR (in mice). Activated DCs, in addition to up-regulation of co-stimulatory molecules and receptors, secrete IL-12 which further activates iNKT cells to secrete IFN-γ and IL-4 thereby stimulating T cells and NK cells.

1.4.4 Adjuvants in licensed vaccines

Adjuvants can not be licensed on their own, but solely as components of vaccines. Numerous adjuvants show excellent adjuvant efficacy in experimental settings, as alluded to above, but some lack an acceptable safety profile or sufficient safety records. Besides alum, which is included in vaccines against diphteria, tetanus, pertussis, HPV and hepatitis A and B, only a few adjuvants are included in vaccines on the market today and they are all administered systemically (Table 1) [152, 200].

Table 1: Licensed vaccines containing non-alum adjuvants

Adjuvant	Infection	Generic name	Route	Manufacturer
MF59 (o/w)	Influenza	Fluad	i.m.	Novartis Vaccines
AS03 (o/w)	Influenza	Prepandrix	i.m.	GSK
AS04 (MPL+alum)	Hepatitis B, HPV	Fendrix, Cervarix	i.m.	GSK
Virosomes	Hepatitis A, Influenza	Epaxal, Infexal V	i.m.	Crucell

1.4.5 Mucosal vaccines

Although most infections occur at mucosal sites and the knowledge that local immunization is advantageous in inducing protective immunity at the site of infection, there are only a handfull of vaccines on the market today that are given via mucosal route. All of these are based on live attenuated or killed pathogens (Table 2) [201]. Practical limitations for studying mucosal immunity in humans, including access to mucosal samples and standardized read outs as well as the lack of mucosal adjuvants are at least in part responsible for this. However, other issues such as long-standing tradition in industry in producing vaccines for injection use also need to be taken into account [202]. Although mucosal delivery is preferable to avoid the use of needles, formulations of the mucosal vaccine is important to enhance the uptake of vaccine across the mucosa and to control the vaccine doses. Another challenge with the development of mucosal vaccines is to identify safe and potent adjuvants for this route of vaccination. The most potent mucosal adjuvants are CT and LT, although they are both toxic and can not be used in humans. TLR ligands have shown promising results in experimental settings as mucosal adjuvants. Previous work, by us and others, have shown CpG ODN to be a potent vaginal adjuvant enhancing immunity against HSV-2 antigen gD and gB, resulting in protective immunity in mice [203, 204].

Table 2: Licensed mucosal vaccines (modified from [205])

Infection	Generic name	Type of vaccine	Route	Manufacturer
Polio	Oral polio vaccine	Live attenuated	oral	Many
Typhoid	Vivotif	Live attenuated	oral	Crucell
Rotavirus	Rotarix, Rota Teq	Live attenuated	oral	GSK, Sanofi pasteur MSD
Cholera	Dukoral	Killed whole cell bacteria+CTB	oral	Crucell
Cholera	Shanchol, ORC-VAX	Killed whole cell bacteria	oral	Shantha Biotechnics, VaBiotech
Influenza	Flumist	Live attenuated	i.n.	Medimmune

1.4.6 Vaccines against genital herpes

The life-long recurrences of symptoms, the increase of resistant strains, the correlation to HIV susceptibility together with asymptomatic shedding of the virus, leading to unawareness of transmission, make it highly desirable to develop a vaccine against HSV-2. The ideal vaccine against HSV would be a prophylactic vaccine giving rise to long-lasting sterilizing immunity against both HSV-1 and HSV-2 at the infections site. A vaccine that does not prevent establishment of latency but reduces recurrent symptoms, without reducing viral shedding may instead be detrimental for transmission of the virus [206].

Table 3: Prophylactic HSV-2 peptide/subunit vaccines in clinical trials

Phase	Type of vaccine	Route	Outcome	Sponsor	Reference
I	gD2+alum	i.m.	no protection	GSK	[209]
I	gB2 CD8 epitope +HSP70	i.d.	no immunogenicity	Antigenics	[211]
III	gB2+gD2+MF59	i.m.	partial, transient protection	Chiron	[210]
III	gD + AS04 (alum+MPL)	i.m.	70% protection in HSV-1/HSV-2 seronegative women only	GSK	[208]
III	gD + AS04 (alum+MPL)	i.m.	Follow-up study on HSV-1, HSV-2 seronegative women, no significant protection	GSK	NIAID press release 30/09/10

Attempts to find a potent vaccine against HSV-2 has been ongoing for decades and everything from recombinant protein to live attenuated virus has been examined both in prophylactic and in therapeutic settings (reviewed in [207]). Several vaccines, including more recent subunit based vaccines, have reached clinical trials but so far none of them have been a complete success and there is yet no licensed vaccine against HSV-1 or HSV-2 (Table 3). One problem that became evident in a recent clinical trial is the cross-reactive immune responses found against HSV-1 and HSV-2. While it may be desirable to produce a vaccine that induces protective immunity against both types of HSV, it may also cause concerns due to the high frequency of HSV-1 positive individuals, which may not respond well to vaccination [208]. Due to practical limitations, clinical trials are mainly focused on antibody response as a read-out of the immunogenicity, which may be misleading. One thing that the clinical studies have taught us is the importance of the type of adjuvant being used. Although the same antigen and route of vaccination is being used, the outcome of the studies varies, most likely due to different adjuvants being included [208-210]. The mechanisms of action of adjuvants are poorly understood and increased knowledge in this field would greatly improve the rational for designing potent vaccines to combat various infections.

Specific aims

The overall objectives of this thesis were to identify new potent mucosal adjuvants, for induction of protection against genital HSV-2 infection in mice, and to study the mode of action of mucosal adjuvants in the female genital tract.

Specific aims of this thesis were to;

- Study the potential mucosal adjuvanticity of α-GalCer for induction of immunity against genital HSV-2 infection in mice.

- Evaluate the potency of *Neisseria meningitides B* derived proteoliposome and cochelate as intranasal adjuvants to evoke a protective immune response against genital HSV-2 infection in mice.

- Explore molecular and cellular correlates of the experimental mucosal adjuvants, CpG ODN and α-GalCer in the murine female genital tract.

3. Key methodologies

3.1 Mice

C57Bl/6 mouse strain was used throughout the studies, unless otherwise stated. KO mouse strains used here were CD1d$^{-/-}$, MyD88$^{-/-}$ and μMT, all on C57Bl/6 background and originated from MIVAC in-house breeding. Mice with unlimited access to food and water were kept under specific-pathogen free conditions in individually ventilated cages at the Experimental Biomedicine Animal facility, Sahlgrenska Academy at the University of Gothenburg. Daily caretaking of the mice was done by animal technicians. All experiments were approved by the Ethical Committee for Animal experimentation in Gothenburg, Sweden.

3.2 Reagents

Recombinant HSV-2 glycoprotein D (gD) was used as antigen throughout the genital herpes studies. gD protein is one of the crucial envelope HSV-2 glycoproteins highly conserved between HSV-1 and HSV-2 with an essential role in binding to and infection of target cells [212]. gD is also known to be weakly immunogenic by itself and has been shown to be able to induce neutralizing antibodies and antibody-dependent cellular toxicity response [213]. gD was produced in Chinese hamster ovary cells by the Mammalian protein expression core facility of Sahlgrenska Academy, the University of Gothenburg (for a more explicit description of production see Paper II). As adjuvant in Paper I we used α-GalCer, which was stored in chloroform methanol 2:1 and was evaporated and reconstituted in phosphate buffer saline (PBS)/Tween 0.5% upon use. Tween had to be added to the buffer to solubilize the glycosphingolipid. CpG ODN 1826 used in Paper II and Paper IV constituts two copies of mouse reactive CpG motifs with flanking regions (TCC ATG ACG TTC CTG ACG TT) and complete phosphorothioate backbone. CpG ODN was reconstituted in phosphate buffer saline (PBS).

3.2.1 Proteoliposome and cochleate

Proteoliposomes used in Paper III were produced by the vaccine production unit at Finlay Institute, Havana, Cuba, under good manufacturing practice (GMP) conditions. Briefly, PLs were obtained from the outer membranes of *N. meningitidis* serogroup B Cu 385-83 strain (B:4:P1.19.15;L3,7,9) by gentle extraction. PLs are membrane vesicles that contain major outer membrane proteins (PorA and PorB), a complex of proteins from 65 to 95 kDa and TLR ligands; LPS, phospholipids and traces of the bacterial DNA. Incorporation of gD protein within adjuvant Finlay proteoliposome 1 (AFPL1) was performed by detergent disruption of PLs followed by re-assembly in the presence of gD protein. To introduce cochelate structure, PLs were prepared by detergent elimination and Ca^{2+} incorporation by a rotational dialysis methodology (Fig. 7). The formation of cochleate was visualized by the appearance of a white suspension in the preparation and light microscopy analysis. The efficiency of incorporation was estimated by comparing the protein quantities in precipitate and supernatant, and was shown to be more than 85% in all cases.

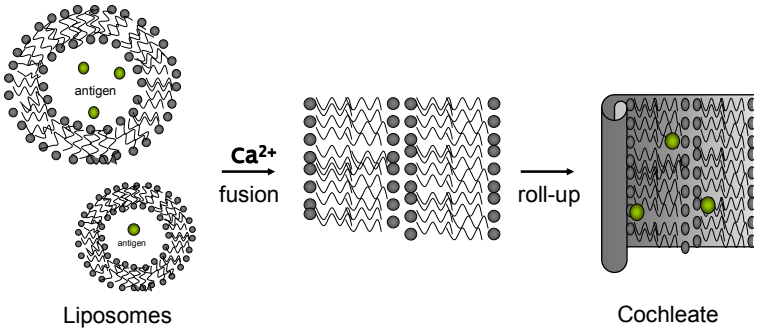

Figure 7. Cochleate formation by Ca^{2+} method.

3.3 Immunizations and administration regimes

Mucosal immunization was chosen for all studies. Mucosal vaccination has previously been shown in mice to induce superior long-term protection against HSV-2 compared to systemic immunizations [201]. Female mice were immunized either intranasally (i.n.) or intravaginally (i.vag.) with three doses, one week apart. This dose regimen has previously been successfully performed in our group and was shown superior to one or two immunizations (data not shown). Prior to i.vag. immunization, mice were given a long lasting progesterone (Depo-provera) to synchronize estrous cycles and increase vaginal susceptibility to the immunization. Mice were given a fixed dose of 5µg gD in all immunizations, while the doses of adjuvants varied with type of adjuvant as well as route of administration. Different doses of CpG ODN and α-GalCer have previously been tested in our laboratory. The volumes administrated to mice were kept to a minimum to avoid leakage. The dose of α-GalCer for i.n. immunization was 2µg and for i.vag. 5µg, while 12.5µg was given of AFPL1 and AFCo1. The dose of OVA used for *in vivo* CTL assay in Appendix I was 50µg and CpG ODN 30µg.

In the comparative studies on the adjuvanticity of α-GalCer and CpG ODN, in Paper II and IV, doses of adjuvants used, i.e. 5µg α-GalCer and 30µg CpG ODN corresponded to those found to be optimal in previous immunization studies where gD was shown to elicit protective immunity against HSV-2 in mice [204, 214]. Six days following progesterone treatment, mice were administered i.vag. a single dose of adjuvant or respective buffer diluents in equal volumes.

3.4 Cellular proliferation and cytokine assays

For analysis of T cell response, cell suspensions were prepared from lymphoid organs, e.g. spleen, caudal and lumbar lymph nodes, (herein referred to as genital lymph nodes (gLN)), following vaginal immunization. Suspensions of cervical lymph nodes (cLN) and mediestinal lymph nodes (MdLN) were additionally prepared following nasal immunization. Cells were re-stimulated with gD followed by 96h incubation (for [^{3}H]-thymidine incorporation proliferation assay) during which supernatants were collected for cytokine measurements using an enzyme-linked immunosorbent assay (ELISA) and a cytometric bead array (CBA). In Paper III the antigen presenting capacity was enhanced by using antigen-stimulated CD11c cells which were co-cultured with purified $CD4^+$ cells from immunized mice.

3.5 Antibody assay

The gD-specific antibody response was measured three weeks following the last immunization in sera and vaginal wash samples. Vaginal wash samples were screened by a sandwich ELISA coated with gD for both IgG and IgA isotypes specific for gD. Detected IgG antibodies were further run for subtypes IgG1 and IgG2c, indicative of a Th2 and Th1 response in C57Bl/6 mice respectively. Bound antibodies were detected using horseradish peroxidase conjugated goat anti-mouse IgG or IgA, IgG1 or IgG2c. Antibody titer was defined as the highest dilution giving an optical density (OD) value of 0.4 above the OD background.

A neutralization assay was performed on sera samples collected from nasally immunized mice. Briefly, serum samples serially diluted were mixed with a constant amount of HSV-2 (300 pfu) in each well. Plates were incubated at 37^0C, 5% CO_2 for 2h prior to transfer of serum/virus mixtures to 96-well plates, with a monolayer of GMK cells. Following three days incubation, cells were stained and neutralization was assessed blindly, by three colleagues, as the highest dilution still showing neutralizing capacity, based on confluence of the cells compared to virus control.

3.6 Mouse model of HSV-2 infection

To evaluate the protective effect of the different immunization approaches, mice were treated with progesterone and subsequently vaginally challenged with a lethal dose ($9*10^4$ pfu) of the 333 HSV-2 strain. Following challenge, mice were monitored daily for 20 days for symptoms of disease. Typically, non-immunized mice started to display mild signs of inflammation with redness around the genital tract, scored as 1 around day four. Subsequently, symptoms of moderate genital inflammation visualized as swelling and scratch sores were scored as (2) and severe and purulent genital lesions were scored as (3). At the final stage, when HSV-2 caused neurological symptoms with hind limb paralysis, scored as (4), mice were euthanized by cervical dislocation.

Local viral replication in the vagina was measured on day three following infection. Vaginal washes were performed and the samples were subjected to a standard viral plaque assay. Briefly, samples were diluted and added in duplicates to a monolayer of GMK cells in a 6-well plate. Plates were incubated at RT for 1h to facilitate viral infection, and viscous cell medium was thereafter added and following a 72h incubation the cells were stained, and plaque forming units (pfu) were counted in a light microscope.

3.7 Tissue extraction

Whole vaginas, cut right below the cervix, and gLNs were excised at different time-points following administration of adjuvants. RNA was extracted and reversly transcribed using RNeasy mini kit followed by QuantiTect Reverse Transcription kit. cDNA samples were subsequently used for real-time reverse transcriptase (RT)-PCR or microarray. Proteins were extracted using saponin treatment of the tissue, and supernatants were analyzed for cytokines and chemokines, and correlated to the weight of the organ. In Paper IV, local cell recruitment was studied by flow cytometry. Few studies have looked at cell populations in the vagina, due

to technical difficulties in retrieving enough quantities of cells. It was therefore necessary to pool vaginas from four mice per group, collected at 12h, 48h and 72h following local administration of CpG ODN or α-GalCer. Vaginas were cut into small pieces and disrupted by two rounds of enzymatic digestion followed by filtering through a 70µm cell strainer, to exclude epithelial cells. Live cells were visually distinguished by trypan blue staining and counted in light microscope.

3.8 Real-time RT-PCR

In Paper II, gene expression in the vagina and gLN were studied using Real-time RT-PCR. To study the inflammatory genes, array plates and supplemented software were used. To study the expression of genes in the vagina associated with the inflammasome in Paper IV, primer assays were used and the expression was normalized against three housekeeping genes, Gadph, Gusb and Actb. Fold changes of transcript expression in immunized mice were calculated in relation to transcription found in mice given respective diluent using the $\Delta\Delta C^t$-method.

3.9 Microarray and Ingenuity pathway analysis

RNA was extracted from the whole murine vagina at three different time-points, 4h, 24h and 48h, following adjuvant delivery. The time-points selected for microarray analysis in Paper IV were based on real-time RT-PCR study in Paper II and a later time-point (48h) was added in order to analyze the duration of the expression. Microarray expression analysis was performed using Affymetrix Mouse Gene 1.0 ST arrays at the SCIBLU Genomics core facility (Swegene Centre for Integrative Biology at Lund University), Sweden. Hierarcial clustering based on microarray expression raw data of PBS and CpG ODN groups is shown in Figure 8 to the right. Data were analysed and statistics were performed by significant analysis of microarray (SAM), to identify genes that were differently expressed between the groups treated with CpG ODN or α-GalCer and their respective controls. SAM identifies genes with statistically significant changes in expression by assimilating a set of gene-specific *t* tests. By performing permutation of the data, parametric assumptions can be avoided. Each gene is assigned a score on the basis of its change in gene expression relative to the standard deviation of repeated measurements for that gene [215]. The threshold was stringently set to a q-value of 0 and genes meeting this criterion and showing a positive or negative d-score (indicative of up-regulation and down-regulation, respectively), were chosen for further analysis. Genes with scores greater than the set threshold are deemed significant.

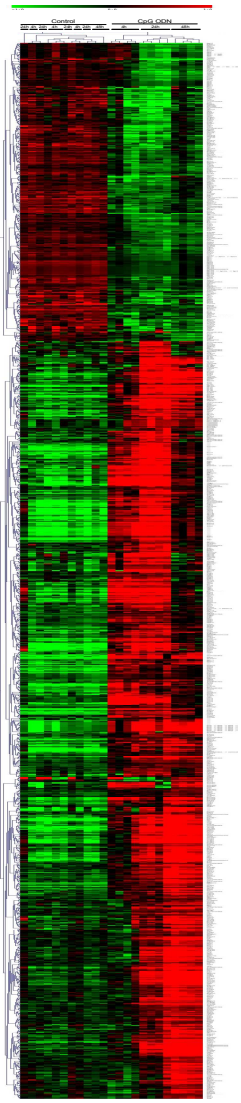

Figure 8. Heat map showing hierarchical clustering of gene expression in the vagina at 4h, 24h and 48h following administration of PBS (left hand side of columns) or CpG ODN (right hand side of columns) detected by Affymetrix Mouse Gene 1.0 ST array.

Ingenuity pathway analysis software (IPA, www.ingenuity.com) was used to analyze and model the complex microarray data to make it more comprehensive. IPA is based on the Ingenuity® knowledge base, which is built upon information from four different sources; expert findings, which is manually extracted information from articles, expert assist findings, which is manually reviewed automatically extracted findings from abstracts, expert knowledge, which is in-house created models of pathways and interactions, and supported third party information, manually reviewed content from selected sources and databases e.g. GO annotations. Ingenuity® knowledge is updated on a weekly basis and the manual review of the information added makes it a reliable analysis tool.

3.10 Histology of the vagina

Excised vaginas were frozen in OCT-compound in liquid nitrogen. Cross-sectional slides of the vaginas were prepared using a cryostat and were subsequently stained with hematoxilin/eosine, staining nucleus and cytoplasm respectively. Severity of inflammation was visually scored by a pathologist, under blind conditions, and inflammatory cells were identified by their round, dense nucleus. For the toxicology study of the epithelial barrier in Paper II, YOYO-1 was instilled into the vagina, followed by excision and washing of the tissue. The tissue was cut longitudinally and examined immediately for fluorescent staining. YOYO-1 has successfully been used in previous study to detect toxicity of microbicides [216].

3.11 Flow cytometry

Local cell recruitment following i.vag. administration of CpG ODN or α-GalCer was examined by flow cytometry. Vaginal cell suspensions were prepared according to above and 100 000 cells/well were used for staining. Cells were stained with monoclonal antibodies from eBioscience; Goat α-mouse Gr1-PE (RB6-8C5), MHC-II-FITC (M5/114.15.2), CD11c-PE (N418) and Cd11b-APC (M1/70). Samples were run on BD FACSCalibur, using Cellquest Pro software. Analysis was performed with FlowJo 7.5 and gates were set at live leukocytes, identified by forward, side scatter (FSC and SSC respectively) and negative 7AAD staining.

3.12 In vivo CTL assay

The cytolytic response following i.vag. immunization with OVA + α-GalCer or OVA + CpG ODN was studied using *in vivo* CTL assay. OVA, with the known CTL epitope SIINFEKL, was used as a model antigen since no CTL epitope has yet been identified in gD. Three weeks following the last immunization, mice were i.v. injected with a 50:50 mixture of SIINFEKL pulsed, $CFSE^{high}$ and unpulsed, $CFSE_{low}$ labelled naïve splenocytes. The following day spleen and gLN were collected and run in flow cytometry. Specific lysis was calculated accordingly: [1 – (ratio unprimed/ratio primed) × 100], where ratio = (percentage $CFSE_{low}$/percentage $CFSE^{high}$).

3.13 Statistical analysis

GraphPad Prism 4 software was used for statistical analysis. Results were shown as mean + standard error of mean (SEM) in all graphs and tables. Statistical significance between two groups was calculated with Student's *t*-test with 95% confidence interval. Statistical significance of the variance between multiple groups was calculated with One-way ANOVA followed by Tukey's multiple comparison test with 95% confidence interval. Data shown are representative or pooled from at least two separately conducted experiments (indicated in each experiment). Significance in the graphs and tables is illustrated by asterix (*$p<0.05$, **$p<0.01$, ***$p<0.001$). For statistical analysis of the microarray experiment, please see paragraph above named "microarray and ingenuity pathway analysis".

4. Results and discussion

4.1 Induction of protective immunity against HSV-2

Numerous studies have been performed on possible vaccine candidates against HSV-2 in mice and some candidates have also, as previously mentioned, reached clinical trials, although, up until today, none of them have been completely successful. Our group and others have previously shown that CpG ODN functions as a mucosal adjuvant giving rise to protective immunity in mice against HSV-2 when immunised together with gD or gB [203, 204]. It has also recently been shown that humoral immune responses can be induced by adjuvants in the absence of MyD88 signalling and our group showed that protective immunity against HSV-2 could be induced independently of MyD88 [217, 218]. In light of this we set out to examine the potential of a non-TLR dependent molecule, the glycosphingolipid α-galactosylceramide to function as a mucosal adjuvant to gD in inducing immunity against HSV-2.

4.1.1 The mucosal adjuvant effect of α-galactosylceramide

To evaluate the potency of α-GalCer as a mucosal adjuvant to gD, mice were immunized three times, by the nasal or vaginal route. The cellular immune response was studied four weeks after the last immunizations by re-stimulation with gD *in vitro*. The proliferative response in the spleen and draining lymph nodes displayed a significantly higher response in mice immunized with gD + α-GalCer compared to mice immunized with gD alone following both nasal and vaginal immunization (Fig. 9A and B). Although cLNs are known to be the draining lymph nodes following nasal immunization, low or no response could be detected there, while MdLN cells from gD + α-GalCer immunized mice showed greater proliferative responses. This is consistent with a study showing that MdLN is the major site of T-cell activation by $CD8^-$ DCs following nasal immunization with OVA + α-GalCer [219]. The fact that DCs and not B cells were responsible for activation of T cells in the mentioned study is interesting, since several studies have shown anergy in NKT cells following more than one dose of α-GalCer. In this study we gave three doses of α-GalCer and could not see any signs of anergy. It is speculated that it is the type of APC that determines the fate of NKT cell activation, B cells being more prone to inducing an anergic state. One could therefore speculate that mucosal immunization with α-GalCer mainly targets DCs. We could also detect secreted Th1/Th2 cytokines from re-stimulated cells, from the spleen, gLN and MdLN of gD + α-GalCer immunized mice, measured by CBA. High levels of IFN-γ were detected, moderate to high levels of IL-2 and IL-5 respectively as well as low levels of IL-4, while none or low levels were detected in gD immunized mice (Paper I, Table 1 and II). The importance of IFN-γ in the generation of a protective immunity against HSV-2 infection has previously been well documented [131, 220, 221]. The cytokine profile detected here indicates a mixed Th1/Th2 response both following nasal and vaginal immunization, which supports a previous study showing production of high levels of both IFN-γ and IL-4 after α-GalCer administration [197].

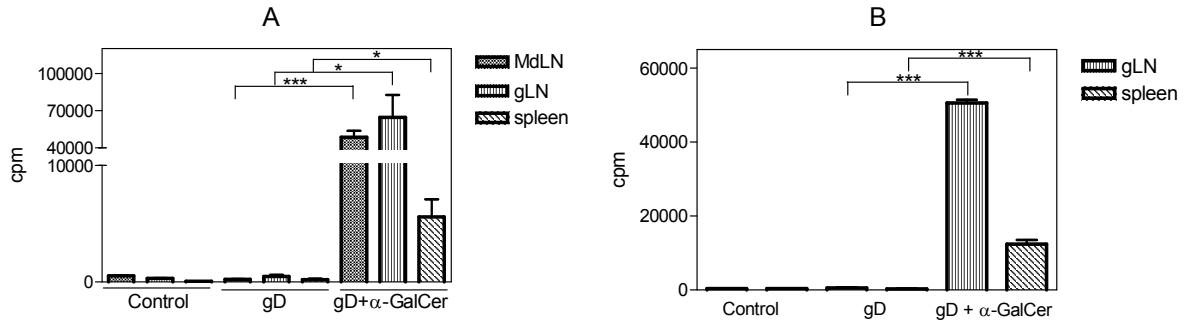

Figure 9. Lymphoproliferative response to gD restimulation *in vitro* in mice immunized with gD + α-GalCer i.n. **(A)** or i.vag. **(B)**.

The antibody response was examined in sera samples collected four weeks after the last immunization. Significantly higher IgG antibody titers could be detected in the sera of mice immunized with gD + α-GalCer compared to gD immunized mice, both i.n. and i.vag. (Fig. 10A and Paper I; Fig. 8A and B). Indicative of a Th2 and Th1 response respectively in C57Bl/6 mice, the IgG isotype subtypes confirmed cytokine data, showing a mixture of IgG1 and IgG2c in the sera following i.n. and i.vag. immunizations (Paper I; Fig. 2B and 8B). We could also verify that the humoral response following nasal immunization with gD + α-GalCer was dependent on the presence of CD1d molecule as CD1d deficient mice could not mount any detectable levels of antibody following immunization (Fig. 10B). We could also show that the induced antibody response was independent of the usage of the TLR adaptor molecule MyD88 (Fig. 10C).

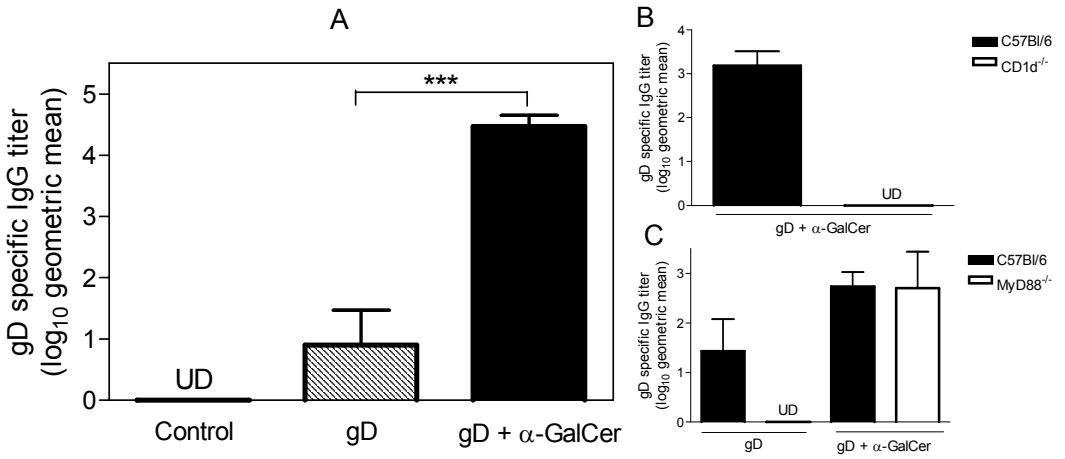

Figure 10. Antigen specific IgG response in sera of nasally immunized C57Bl/6 **(A)**, CD1d$^{-/-}$ **(B)** and MyD88$^{-/-}$ **(C)** mice.

To elucidate the protective effect of the immune responses induced by mucosal gD + α-GalCer immunization, the immunized as well as control mice were challenged vaginally with a lethal dose of HSV-2. While all control mice showed severe symptoms of disease and the majority had to be sacrificed, no or mild symptoms of disease occurred in the group i.n. immunized with gD + α-GalCer and in this group all animals survived (Fig. 11A). Further, we could show that the protective immunity induced by i.n. immunization with gD + α-GalCer was dependent on CD1d (Fig. 11B), but not critically dependent on MyD88 (Fig. 11C). Following i.vag. immunization, all control mice succumbed to infection, while gD + α-GalCer immunized mice showed no or mild symptoms of disease and 80% of the mice survived (Fig. 11D). We could also demonstrate that the protective immunity induced by i.n. gD + α-GalCer

immunization was independent of mouse strain. It is well known that C57Bl/6 mice are Th1 biased, and to exclude a bystander effect by the strain used we immunized Balb/c, which is a Th2 biased strain, as well as an outbred mouse strain, NMRI [222]. The i.n. immunized Balb/c and NMRI mice showed no or mild symptoms of disease and all survived the lethal vaginal HSV-2 challenge (Paper I; Fig. 4B and C).

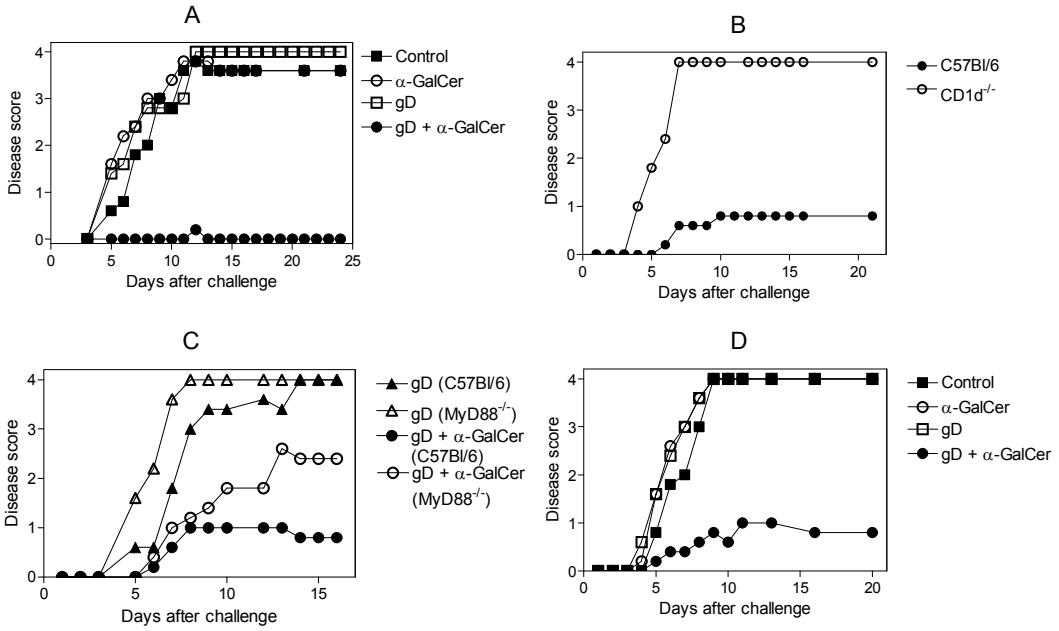

Figure 11. HSV-2 disease progression following challenge of i.n. immunized C57Bl/6 **(A),** CD1d$^{-/-}$ **(B)** and MyD88$^{-/-}$ mice **(C)** or i.vag. **(D)** immunized C57Bl/6 mice.

The potential of α-GalCer to enhance immunity to soluble antigens, and hence function as an adjuvant has previously been seen following systemic and i.n. immunization with promising results [197-199]. However, our results are the first showing that α-GalCer can function as a mucosal adjuvant giving rise to immunity against a sexually transmitted infection.

4.1.2 Proteoliposome and cochleate

PLs derived from *Neisseria meningitides B*, have been used as antigen in the meningococcal vaccine VAMENGOC-BC^{TM}, and successfully administrated in millions of doses to humans [165]. Finlay Institute, Havana, Cuba, has adapted this idea and demonstrated the potential of PLs to function as adjuvant, named Adjuvant Finlay Proteoliposome 1 (AFPL1), in several experimental studies. In a collaborative project, we set out to examine the potential of AFPL1 and the cochleate structure termed AFCo1 in the induction of immunity against HSV-2. Mice were i.n. immunized three times with gD incorporated into AFPL1 or AFCo1. A significantly higher gD specific IgG antibody response could be detected in the sera as well as in vaginal washes following immunization of AFPL1gD and AFCo1gD compared to control groups, although the level was higher with the cochleate formulation (Fig. 12A and Paper III; Fig. 1). The antibody response was significantly lower in the sera of immunized MyD88 deficient mice compared to immunized C57Bl/6 mice, and completely absent in the vaginal wash samples from MyD88$^{-/-}$ mice (Fig. 12B).

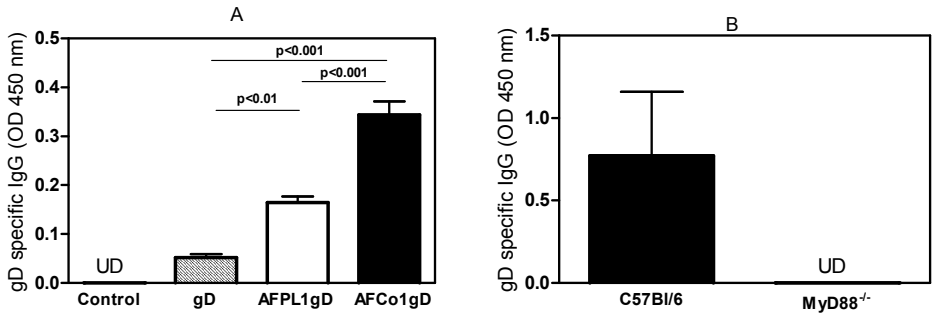

Figure 12. gD specific antibody response in vaginal washes following i.n. immunization of C57Bl/6 mice **(A)** and AFCO1gD immunized MyD88 deficient mice **(B)**.

The superiority of AFCo1gD over AFPL1gD was also seen for cellular response, where higher levels of proliferation were detected in mice immunized with AFCo1gD (Fig 13A). A clear difference was also seen in the production of IFN-γ which was undetectable in mice immunized with AFPL1gD, while high levels were seen for AFCo1gD immunized mice (Fig 13B). These responses were shown to be highly dependent on the MyD88 molecule as shown for AFCo1gD immunization in Fig. 13C and D.

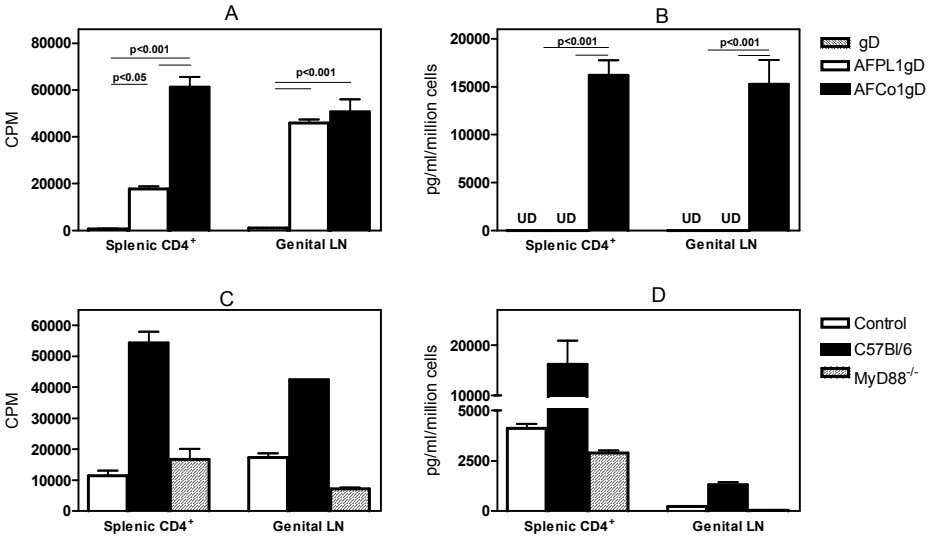

Figure 13. gD specific proliferative **(A and C)** and IFN-γ **(B and D)** responses in lymphoid organs of immunized C57Bl/6 **(A and B)** and MyD88$^{-/-}$ **(C and D)** mice *in vitro*.

A similar pattern was also seen for protection against genital HSV-2 infection following immunization. All control mice showed neurological symptoms and succumbed to infection. AFCo1gD immunized mice showed no or mild symptoms of disease and 100% survival. Mice immunized with APL1gD however showed moderate symptoms of disease and 60% survival (Fig. 14A). The difference in protection against HSV-2 infection observed between proteoliposome and cochleate formulation was speculated to be due to lack of IFN-γ production in the AFPL1gD immunized mice, since IFN-γ is critically important for immunity against HSV-2 [131, 221, 223]. The MyD88 deficient mice immunized with AFCo1gD showed earlier and more severe symptoms of disease than the control group and had to be sacrificed four days earlier than the controls (Fig. 14B), implicating the importance of MyD88 in the adjuvant effect of AFCo1 in this nasal immunization setting. It however needs to be taken into consideration that a previous study from our group has shown that MyD88$^{-/-}$ mice are more

susceptible to genital HSV-2 infection, probably due to inability to initiate a TLR mediated innate response, which otherwise is mounted through engagement of TLR2 and TLR9 by HSV [122, 224].

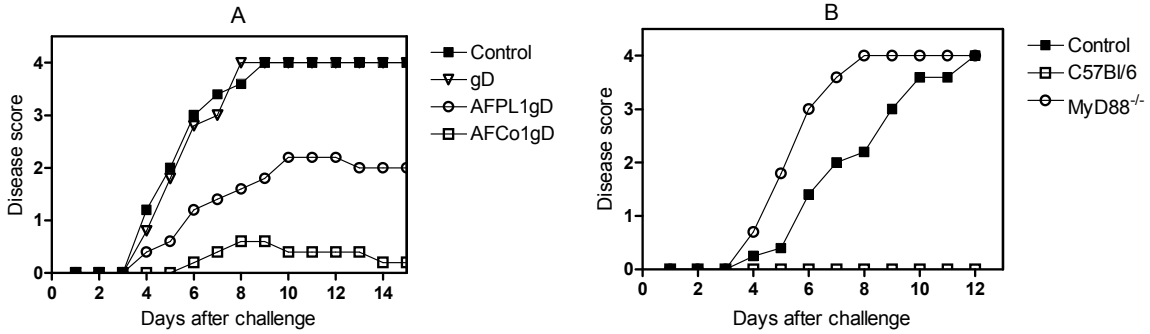

Figure 14. Disease progression in the immunized mice challenged i.vag. with HSV-2. **A)** Comparison of mice immunized with AFPL1gD or AFCo1gD. **B)** Score of symptoms following a vaginal HSV-2 challenge in C57Bl/6 wt and MyD88$^{-/-}$ mice immunized with AFCo1gD.

Taken together these data demonstrated that both AFPL1 and AFCo1 can function as nasal adjuvants when used together with gD for induction of protective immunity against HSV-2 in mice. Although AFCo1gD was superior to the proteoliposome formulation. It has previously been demonstrated that AFCo1 can induce a stronger Th1 immune response compared to AFPL1 when immunized with heterologous antigens [165].

The antibody response, as well as the cellular proliferative response with IFN-γ secretion were drastically reduced in MyD88 deficient mice, indicating that these responses were dependent on TLR signalling. The MyD88 dependence can be explained by the fact that the proteoliposomes derived from *Neisseria meningitides B,* besides working as a delivery system, contain several TLR ligands incorporated into the lipid bilayer. The TLR ligands include LPS, porins and small amounts of bacterial DNA containing CpG motifs functioning as immunostimulatory adjuvants. Although porins (TLR2) and CpG (TLR9), exclusively signal via MyD88 adaptor molecule, LPS can upon binding to TLR 4 induce signalling via both MyD88- dependent and -independent pathways via TRIF, which could result in the weak immune response detected in these mice [225]. Further, although TLR signalling is hampered in MyD88 deficient mice, AFCo1 still retains its adjuvant function as a delivery system. The antibody responses generated in AFCo1gD immunized C57Bl/6 mice was a mixture of IgG1 and IgG2c subtypes (Paper III; Fig. 1C), while MyD88$^{-/-}$ mice did not show any IgG2c (Paper III; Fig. 4C), which indicates a Th2 biased response. This is in agreement with a recent study showing the importance of MyD88 signalling in B cells for class switch to IgG2c and IFN-γ production, hallmarks of Th1 response [226]. The poor Th1 response in addition to a lack of vaginal gD specific IgG response induced by AFCo1gD in MyD88 deficient mice could explain the absence of protection against HSV-2 infection observed in these mice.

All in all, we could show that AFCo1 can function as a potent nasal adjuvant to elicit a potent MyD88 dependent protective immunity against genital HSV-2 infection.

4.2 Correlates of adjuvanticity

Vaginal immunization with non-replicating antigens generates weak local humoral responses with little dissemination to systemic compartments [227]. However, our group has previously shown that CpG ODN can function as a vaginal adjuvant to gD in a genital herpes mouse model, giving rise to a Th1 biased response with 80 % protection against HSV-2 challenge [204]. Within this thesis we have identified another potent vaginal adjuvant, α-GalCer, that when administered with gD, can give rise to a mixed Th1/Th2 response and a protective immunity to genital herpes, comparable to that of CpG ODN i.e., 80% survival following genital HSV-2 infection.

CpG ODN and α-GalCer can be classified as immunostimulatory adjuvants and the use of such adjuvants raises potential safety concerns, due to the possibility of inducing overt inflammation. In Paper II we examined the local inflammatory response in mice following vaginal delivery of CpG ODN and α-GalCer. We could detect, by real-time RT PCR, a group of genes commonly up-regulated by both adjuvants, but also differences in gene expression profiles between the two adjuvants and α-GalCer induced less inflammation compared to CpG ODN. In light of these results, we decided to elucidate the mechanism of action of these two classes of experimental vaginal adjuvants. In Paper IV, we conducted a genome-wide expression experiment on the vaginas of mice administered CpG ODN or α-GalCer followed by a bioinformatic approach. The results from both these two studies (Paper II and Paper IV) will be presented and discussed together below.

4.2.1 Gene expression

By using whole mouse genome microarray chip, we could study the expression of over 28000 genes in the vagina following local administration of CpG ODN or α-GalCer and their respective diluents, which were used as baselines, at 4h, 24h and 48h. To detect differently expressed genes between adjuvant treated and control groups, SAM was used and a stringent threshold for significance was set to q-value 0. The q-value is equivalent of p-value in t-tests and measures the false discovery rate. Of the significant genes, i.e. those with a q-value of 0, that had a positive d-score were deemed up-regulated and the ones with a negative d-score were considered down-regulated. The d-score is a statistics-based score of the relative difference in gene expression, which account for gene-specific fluctuations. A total of 913 genes were considered significantly up-regulated in the vagina, of which the majority (642) was solely up-regulated by CpG ODN, while 61 genes were exclusively up-regulated by α-GalCer. However, 210 genes were commonly up-regulated by both adjuvants (Fig 15A and Paper IV; Table S.1). Interestingly only CpG ODN induced significant down-regulation of genes in the vagina (Paper IV; Table S. 2).

The kinetics of the response induced by the two adjuvants was distinct. CpG ODN up-regulated over 300 genes at 4h and increased over time to 509 at 48h, while α-GalCer only up-regulated a few genes at 4h followed by a peak with 247 genes at 24h and a dramatic decrease to 32 genes at 48h (Fig 15B).

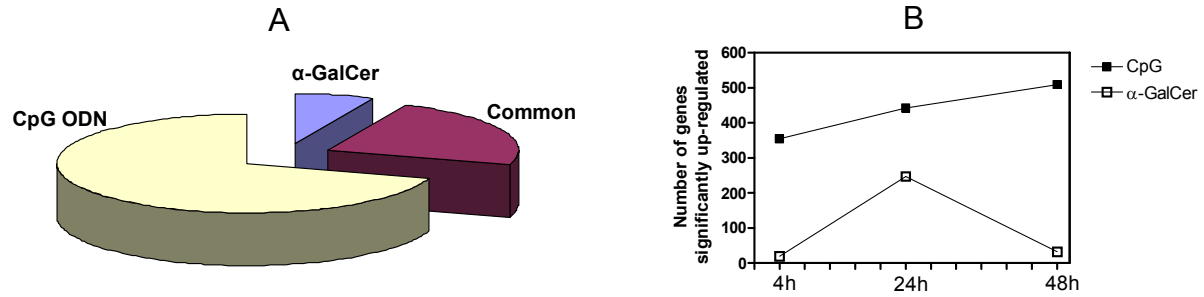

Figure 15. Total number of genes significantly up-regulated in the vagina following local administration of CpG ODN or α-GalCer irrespective of time-point **(A)** and at different time-points **(B)**.

Within 48h after vaginal adjuvant delivery, 43 genes belonging to the family of cytokines, chemokines and their receptors (as identified by GO numbers) were up-regulated. CpG ODN exclusively up-regulated 24 of these genes, α-GalCer solely 6 genes and 13 of them were in common for the two adjuvants. A majority of these genes were chemokines and were confirmed by real-time RT-PCR (Fig 16 and Paper II; Fig. 5a). Among the commonly up-regulated chemokines were Ccl2, Ccl7 and Ccl12 (known to recruit monocytes and macrophages), Ccl22 (chemoattractant for DCs, monocytes and NK cells) and Cxcl2 (known to attract polymorphonuclear leukocytes). Additionally identified core genes included the mature DC attractant chemokine, Ccl19, and chemokines attracting activated T cells, namely Cxcl9, Cxcl10 and Cxcl11. Of note, the majority of common core genes were significantly up-regulated by using microarray and RT-PCR. The exact function of these common genes in the mucosal adjuvanticity however needs to be further studied.

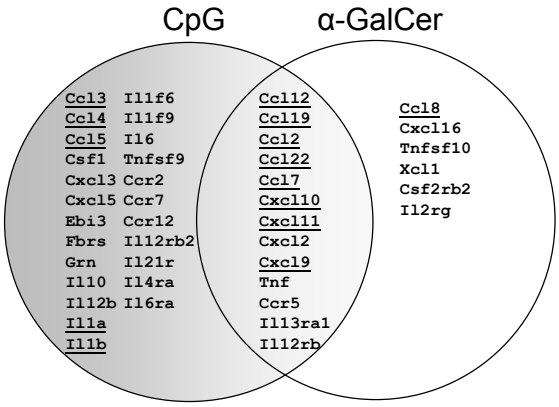

Figure 16. Venn diagram of cytokines, chemokines and receptors exclusively or commonly up-regulated in the vagina within 48h following local delivery of CpG ODN or α-GalCer, detected by microarray (Paper IV). Genes that are underlined were confirmed with real-time RT-PCR (Paper II).

4.2.2 IPA analysis

To analyse the massive amount of data generated by the whole mouse genome microarray, we employed an *in silico* program, called ingenuity pathway analysis (IPA), that is based on reviewed information collected from several databases. IPA has previously been used in other studies, to elucidate relationships and connections between differently expressed genes, to understand the mechanisms of action of CpG ODN in murine spleen and to predict immunogenicity of the human yellow fever vaccine [228, 229]. The main biological function of the genes down-regulated by CpG ODN are involved in organ and tissue development. IPA identified "inflammatory response" as the main bio function of the up-regulated genes induced at all time-points by both adjuvants, based on number of molecules involved and the p-value. The majority of top functional categories identified among up-regulated genes by

CpG ODN and α-GalCer were in common. These categories included cellular development, proliferation and movement, although it is important to point out that these categories are not mutual exclusive, but overlap in many instances. The p-value was lower and the number of genes up-regulated in each category was higher in CpG ODN treated group compared to the α-GalCer group (Table 4).

Table 4. Commonly identified top functional categories of genes up-regulated by CpG ODN or α-GalCer among "molecular and cellular functions" as well as "physiological system development and function" by IPA.

Functional category	**CpG ODN**		**α-GalCer**	
	p-value	# genes	p-value	# genes
Cellular development	$\leq 3.13*10^{-6}$	170	$\leq 1.96*10^{-2}$	39
Cellular growth and proliferation	$\leq 3.17*10^{-6}$	175	$\leq 1.03*10^{-2}$	48
Cellular movement	$\leq 2.26*10^{-6}$	136	$\leq 1.19*10^{-2}$	31
Cell-to-cell signalling and interaction	$\leq 4.05*10^{-6}$	151	$\leq 1.30*10^{-2}$	42
Cell-mediated immune response	$\leq 2.26*10^{-6}$	106	$\leq 1.02*10^{-2}$	35
Hematological system development and function	$\leq 4.05*10^{-6}$	213	$\leq 1.30*10^{-2}$	64
Hematopoiesis	$\leq 3.13*10^{-6}$	125	$\leq 1.14*10^{-2}$	34
Immune cell trafficking	$\leq 4.05*10^{-6}$	145	$\leq 1.30*10^{-2}$	45

4.2.3 Inflammatory response

After identifying "inflammatory response" as the main bio function, we were interested in looking at the importance and interaction of genes within the inflammatory response. Based on data stored in the Ingenuity knowledge base, graphical networks were created in IPA, indicating biological interactions of genes, where each line represents an interaction. At all time-points studied, CpG ODN induced large complicated network structures involving over 100 genes. α-GalCer did not induce any structured networks at 4h or 48h, while an inflammatory network containing some 50 genes could be created at 24h (Fig 17). These results indicate that even though inflammatory response is identified as main bio function of both adjuvants, the response is stronger in CpG ODN treated mice. In these network structures we could identify a few genes that functioned as major inducers of the inflammatory response. Among the major induces Tnf was detected in all networks created and was found to be significantly higher expressed in the vagina of CpG ODN administered mice at protein level compared to the control group (Paper II; Fig. 3c and Paper IV; Fig. 3). An important discrepancy that was noted in the results obtained in the studies performed in paper II and IV, on gene expression in the vagina, was the expression of IFN-γ, which is known to play a key role in host defense against viral infections. While a significant up-regulation could be detected by real-time RT-PCR, the up-regulation detected by microarray was not significant, likely due to the stringent threshold set. Expression of IFN-γ could however be detected on protein level in both studies (Paper II; Fig. 3c and Paper IV; Fig. S.1). TNF and IFN-γ have previously been identified as the major inducer genes, responsible for 90% of gene activation in murine spleen cells following intraperitoneal injection of CpG ODN [228].

Several other inflammatory cytokines and chemokines were detected on protein level in the vagina of CpG ODN administered mice, among them CCL2, CCL3, IL-6 and IL-12 as well as the anti-inflammatory cytokine IL-10 (Paper II; Fig. 3a and c). From these proteins, only IL-10 showed increased level compared to control following α-GalCer administration. The majority of inflammatory genes being targeted by CpG ODN are likely to be induced by NF-κB signalling via TLR9 activation. However, several genes in IL-1-, TNF-α- and CD40-induced pathways involved in NF-κB signalling were up-regulated by CpG ODN, of which

the majority were detected at an early time-point (Paper IV; Fig. 4A). In addition, we could detect activation of inflammasome-associated genes, Nlrp1b and Nlrp3 as well as IL-1β in the vagina by microarray and real-time RT-PCR (Paper IV; Fig. 6A and B). Expression of IL-1β was confirmed on protein level (Paper IV; Fig. 6C), indicating yet another pathway involved in the inflammatory response initiated by CpG ODN. To date it has only been reported that particulate adjuvants can induce inflammasome activation [230]. However, we failed to detect Nlrp1 and Nlrp3 at protein level by Western blot in the murine vagina following administration of adjuvants (data not shown).

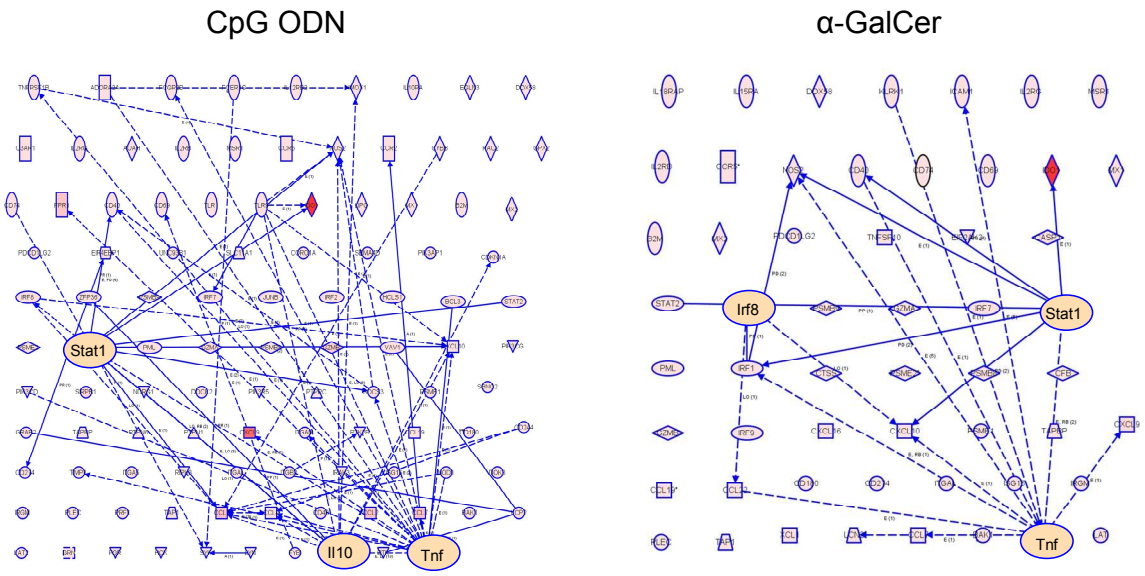

Figure 17. Inflammatory network based on *in silico* IPA of genes identified by microarray analysis as significantly up-regulated in the vagina 24h after adjuvant delivery.

4.2.4 Cell recruitment

The high expression of chemokines in the vagina of mice receiving CpG ODN or α-GalCer administration lead us to study the pattern of immune cell recruitment in the vagina following vaginal administration of adjuvants. Mice were given an i.vag. dose of either CpG ODN or α-GalCer, and after 8h, 24h and 48h the vaginas were excised and frozen in liquid nitrogen. By staining with hematoxilin/eosin, which stains nucleus and cytoplasm respectively, we examined the histology of the vagina following adjuvant treatment compared to control. We noted a general increase in cells in the CpG ODN group compared to the control at 8h, which culminated at 24h but was comparable to control at 48h. Inflammatory cells were identified by their round dense nucleus, and could be found both in the epithelial layer and in the lamina propria at 8h and 24h post CpG ODN treatment. α-GalCer gave rise to a more modest increase in infiltrating cells, with a few inflammatory cells detected at 8h. At 24h and 48h the α-GalCer treated vagina did not differ from that of controls (Fig. 18 and Paper II; Fig. 4). Taken together, these results indicate a more inflammatory state in the vagina of CpG ODN administered mice compared to mice receiving α-GalCer.

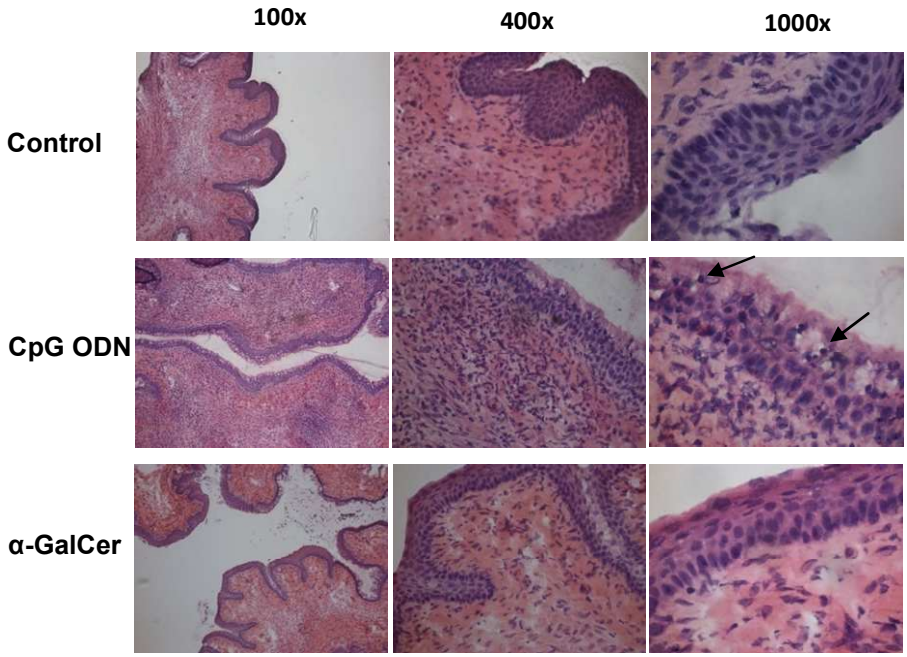

Figure 18. Histological pictures of murine vaginal tissue stained with hematoxilin/eosin 24h following vaginal administration of CpG ODN or α-GalCer. Arrows indicate inflammatory cells, identified by their dense round nucleus.

In an attempt to broadly characterize the immune cells being recruited to the vagina by CpG ODN and α-GalCer, we performed flow cytometric analysis on cells obtained from mouse vagina after vaginal administration of adjuvants. For this purpose, vaginas were excised at 12h, 48h and 72h following administration of CpG ODN, α-GalCer or their respective diluents. Cell suspensions were prepared by enzymatic digestion and enumerated. By simply counting live cells in a microscope we could see that both CpG ODN and α-GalCer caused an influx of cells at 12h, with roughly twice the cell number compared to controls. The cell number went down to control levels in the α-GalCer group at later time-points, while the CpG ODN group continued to have elevated cell numbers (Paper IV; Fig. 7A). This is in consistency with our histological results, which showed an increase in infiltrating cells early on by both adjuvants and a quicker resolution of inflammation in the α-GalCer treated vagina (Paper II; Fig.4). Looking at the frequency of cells at 12h, we could detect an increase in the frequency of macrophages, identified as being $CD11b^{+}$, MHC II^{+}, $CD11c^{-}$ cells, in both CpG ODN and α-GalCer treated mice compared to their respective controls. No increase in the frequency of live leukocytes of $CD11b^{+}$, MHC II^{+}, $CD11c^{+}$ cells, identified as DCs, was seen (Fig. 19). At 48h and 72h, the picture was different since an elevated frequency of DCs following both CpG ODN and α-GalCer treatment was observed as compared to that seen in control mice (Paper IV; Fig.7C and D). This indicates that both adjuvants can trigger an early influx of macrophages, followed by an increase of DC frequency. Our CpG ODN results are consistent with a previous study showing enhanced levels of macrophages in the vagina at 12h post local CpG ODN delivery followed by an increase of DCs at 48h [231].

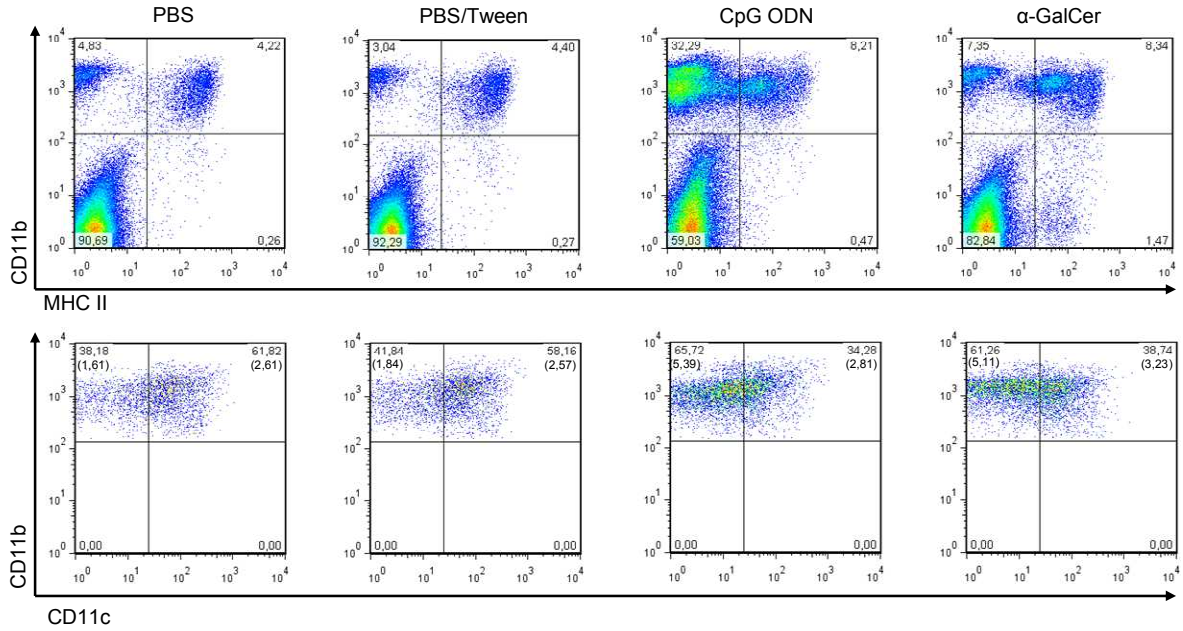

Figure 19. Flow cytometry analysis of vaginal cells 12h following adjuvant delivery. Live leukocytes were identified by forward and side scatter and negative 7AAD staining. Frequency of DCs were gated as being $CD11b^+$, $MHCII^+$, $CD11c^+$ and macrophages expressing $CD11b^+$, $MHCII^+$, $CD11c^-$. The lower row is gated on the $CD11b^+$, MHC II^+ population in the upper row. Numbers in paranteses show frequency of live leukocytes. Plots illustrate one representative experiment out of three independently perfomed experiments.

At 12h, we could also detect a large increase in the frequency of $CD11b^+$, Gr-1^+, MHC II^+ cells in the vagina following administration of CpG ODN or α-GalCer, although to a higher extent by CpG ODN (Fig. 20). We called these cells DC-like cells, due to their expression of surface markers, although they are more similar to macrophages in the kinetics of their response. We speculate that these cells may be inflammatory monocytes, precursors to DCs, previously detected in several studies capable of priming T cells and produce iNOS and TNF [157, 232]. Interestingly, we could detect a significant up-regulation in the gene expression of TNF and iNOS by both adjuvants (Paper IV; Fig. 4B and Table 1)

Another cell type recruited by CpG ODN was neutrophils, identified as being $CD11b^+$, Gr-1^+, MHC II^-. A small increase in the frequency of neutrophils was detected in the α-GalCer administered mice, while a dramatic increase was seen in mice receiving CpG ODN at 12h, which was restored to control levels at 72h (Fig. 20 and Paper IV; Fig.7B). Although neutrophils represent a hallmark of inflammation, they have also been ascribed a role in antigen presentation under inflammatory conditions and during cross-presentation, which may presumably contribute to initiation of an adaptive immune response [233, 234].

The increase in total cell numbers as well as change in the frequency of different cell populations in the vagina following adjuvant delivery suggests that the changes in gene expression are not solely due to up-regulation of the genes in the resident vaginal cells. It is hence reasonable to speculate that the detected up-regulation of genes by CpG ODN and α-GalCer is due to activation of resident cells as well as recruitment of additional immune cells.

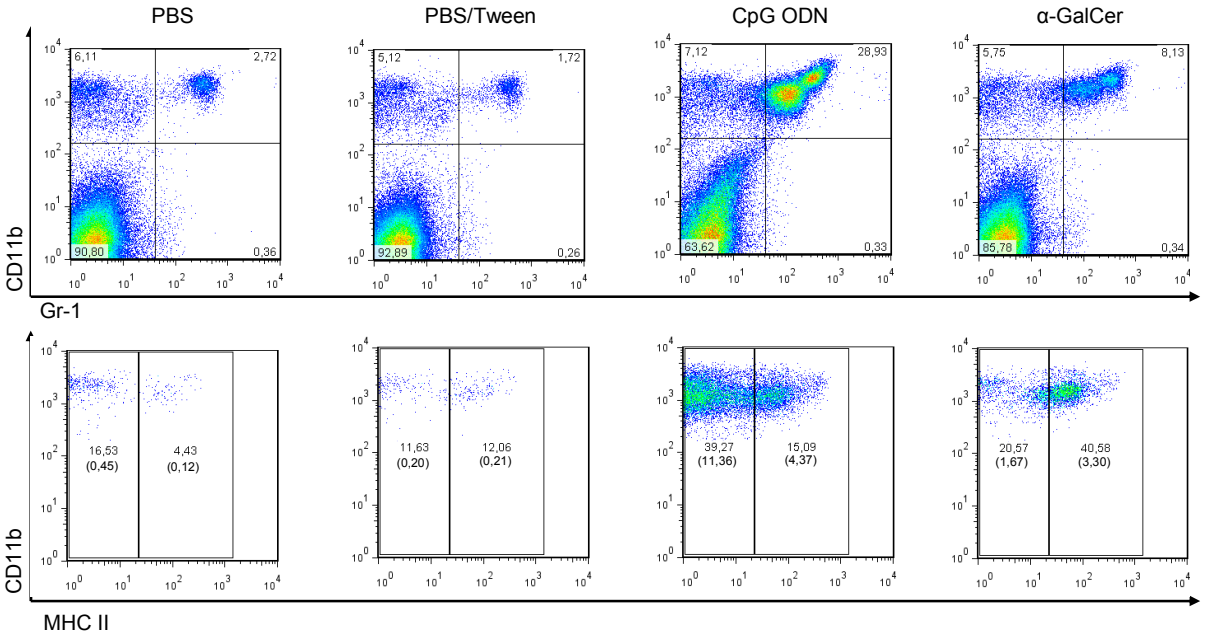

Figure 20. Flow cytometry analysis of vaginal cells 12h following adjuvant delivery. Live leukocytes were identified by forward and side scatter and negative 7AAD staining. The frequency of DC-like cells were gated as being $CD11b^+$, $Gr\text{-}1^+$, MHC II^+ and neutrophils as $CD11b^+$, $Gr\text{-}1^+$, MHC II^-. The lower row is gated on the $CD11b^+$, $Gr\text{-}1^+$ population in the upper row. Numbers in paranteses show frequency of live leukocytes. Plots are representative of three independently perfomed experiments.

4.2.5 Toxicity assessment

The safety of new vaccines is an important issue that needs to be addressed in human clinical trials. Pre-clinical animal models can help to identify starting doses to be used in human clinical trials, assess potential toxicity and target organs. It is common procedure to test adjuvant and antigen together as a vaccine formulation, unless it is a new synthetic adjuvant, which is then considered a new chemical [235]. By including immunostimulatory adjuvants, such as CpG ODN or α-GalCer in vaccine candidates there is always a potential safety risk. We have shown here that both these experimental adjuvants by themselves generate an inflammatory response. Although an inflammatory response most likely is necessary for adjuvanticity, excessive inflammation may be toxic and hence can cause tissue damage. By IPA we could identify toxic molecules among up-regulated genes by CpG ODN and α-GalCer. With the exception of NF-κB signalling pathway, which was on the top toxicity list for CpG ODN, all of the toxic functions were related to liver damage for both adjuvants. It has previously been shown that systemic administration of CpG ODN and α-GalCer caused liver toxicity in mice [236, 237]. However, our microarray analysis was performed on vaginal tissue and vaginal delivery usually induces only weak systemic responses.

Several safety concerns have been raised with the use of CpG ODN as adjuvant in human vaccines, including its possibility of enhancing immunogenicity of self proteins, leading to autoimmunity and the induction of pathologic inflammatory response [181]. Although the toxicology results obtained in rodent models for CpG ODN may be exaggerated due to higher TLR9 expression in rodents compared to in humans, clinical studies have reported toxic effects by CpG ODN [235]. A recent phase III clinical trial including CpG ODN combined with hepatitis B antigen was halted due to one patient being diagnosed with Wegener's granulomatosis, which is an inflammation of blood vessels [238]. Furthermore, vaginal treatment

of rhesus monkeys with CpG ODN did not induce any protection against SIV infection, but rather increased the viral RNA levels in plasma compared to control monkeys [239]. Studies evaluating the potential use of a detergent based spermicide nanoxynol-9 (N-9) as a microbicide, was shown in humans to increase, rather than decrease, the risk of HIV infection. It was later shown in mice that the compound disrupted vaginal epithelial barrier, induced inflammatory response and recruited inflammatory cells, leading to an increase in HSV infection [216, 240, 241].

We sought to examine the impact of CpG ODN and α-GalCer on the mucosal epithelial barrier, which serves as an important innate protection mechanism in the vagina. Even though α-GalCer has not shown to induce a strong inflammatory response in our studies, the trace amount of detergent, Tween, used as buffer may itself induce damage to the epithelial barrier. Further, safety concerns about systemic α-GalCer treatment have been raised in mice models, where iNKT cells have shown to be involved in abortion following systemic i.p. injection [242]. α-GalCer has however been proven to be a safe nasal adjuvant in mice, since no antigen was detected in the brain of nasally immunized mice [243].

By instilling a fluorescent dye, called YOYO-1, into the vaginas following adjuvant delivery, it was possible to detect acute toxicity to the vaginal epithelial cells. YOYO-I is a membrane impermeable dye, which becomes fluorescent once inside nuclei, thereby indicating disrupted cell membranes, and has successfully been used in another study showing the toxic effects of N-9 [216]. We therefore included N-9 as a positive control in our study.

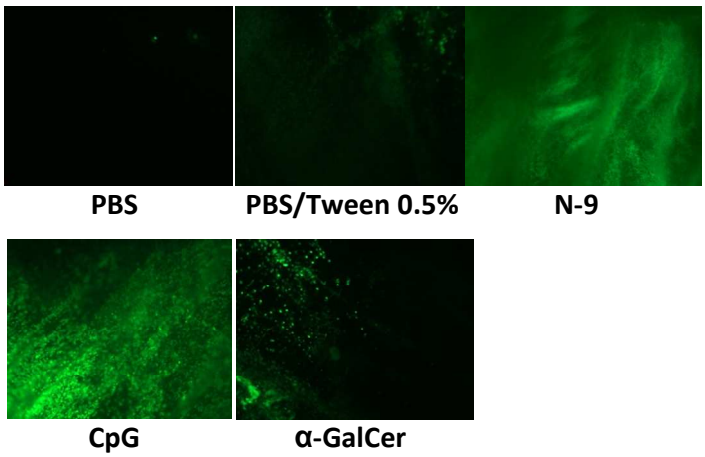

Figure 21. YOYO-1 staining of vaginal tissue, indicating toxicity by disrupted cell membranes following CpG ODN delivery.

Administration of PBS did not induce any fluorescence, indicative of an intact cell layer, PBS/Tween 0.5% showed weak fluorescent response, which was not increased by the addition of α-GalCer. However the vaginas of mice given CpG ODN showed high fluorescent staining throughout the tissue, comparable to N-9, indicative of breeched epithelial cell membranes (Fig. 21).

It is however needless to mention that this work was not intended to pinpoint toxicity profile of these adjuvants. Further systematic toxicity studies are thus required to identify their full toxicity profile in order to inform further development of these two classes of immunostimulatory mucosal adjuvants.

5. Appendix

5.1 Appendix I

To further examine the mode of action of CpG ODN and α-GalCer as vaginal adjuvants, we studied the CTL response *in vivo* following vaginal immunization. Due to lack of a known CTL epitope in gD, the model antigen OVA was used. Following vaginal immunization with OVA together with CpG ODN or α-GalCer, strong, and comparable CTL responses, with comparable levels of specific cell lysis in spleen and gLN were detected by flow cytometry (Fig. 22). To induce a CTL response by exogenous antigens, cross-priming by DCs is necessary, usually with the aid of T cells. It was recently shown that cross presentation of exogenous antigen, following systemic injection, by DCs could occur by distinct pathways. It was shown that while CpG ODN is dependent on T cell help, α-GalCer mediated aid by activated NKT cells, and the chemokines and receptors involved in the recruitment of CTLs differed. It was also demonstrated, that by immunizing with a combination of CpG ODN and α-GalCer it is possible to induce a synergistic effect, probably due to involvement of different pathways [244]. Hence, a combined use of CpG ODN and α-GalCer as adjuvants for immunization deserves further exploration.

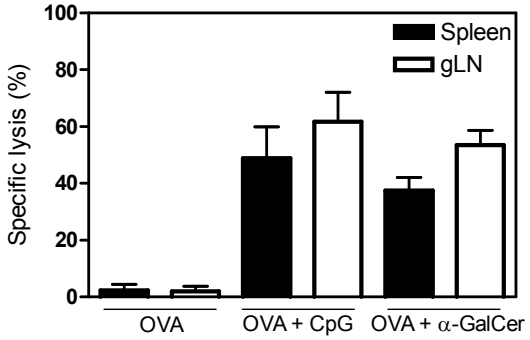

Figure 22. CTL response *in vivo* following vaginal immunization with OVA + CpG ODN or OVA + α-GalCer. Data shown are pooled from two independently performed experiments (n = 5). Bars show mean + SEM.

5.2 Appendix II

The importance of antibodies for protection against HSV-2 is debated. However, functional importance of IgG antibodies against genital HSV-2 infection has previously been shown [36]. Here, we could show significant HSV-2 neutralizing capacity of sera containing gD specific IgG from mice nasally immunized with gD + α-GalCer (Fig. 23).

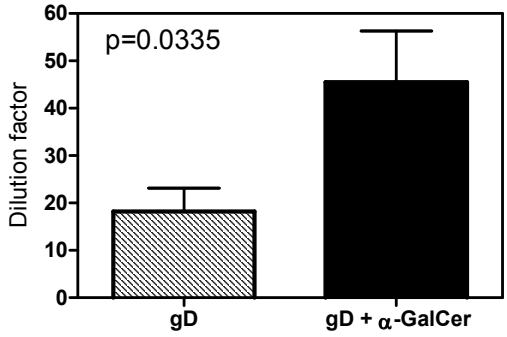

Figure 23. HSV-2 neutralizing capacity of sera from nasally immunized mice. Data shown are pooled from individually performed experiments (total n=12). Bars show mean+ SEM and *t*-test was used for statistical analysis.

To elucidate the importance of the detected IgG antibodies in the protective immunity induced by gD + α-GalCer, we nasally immunized B cell deficient (μMT) mice. μMT mice lack the μ-chain due to a disrupted exon and hence, the development of B cells is arrested in pre-B cell stage [245]. It has previously been shown that IgG antibodies can up-regulate the expression of human CD1d on DCs [246]. Thus, we first examined the level of CD1d expression in naive WT and μMT mice. Comparable levels of CD1d expression were detected in all lymph nodes studied (cLN, MdLN and gLN), while lower levels were displayed in the spleen of μMT mice compared to WT mice (Fig. 24A).

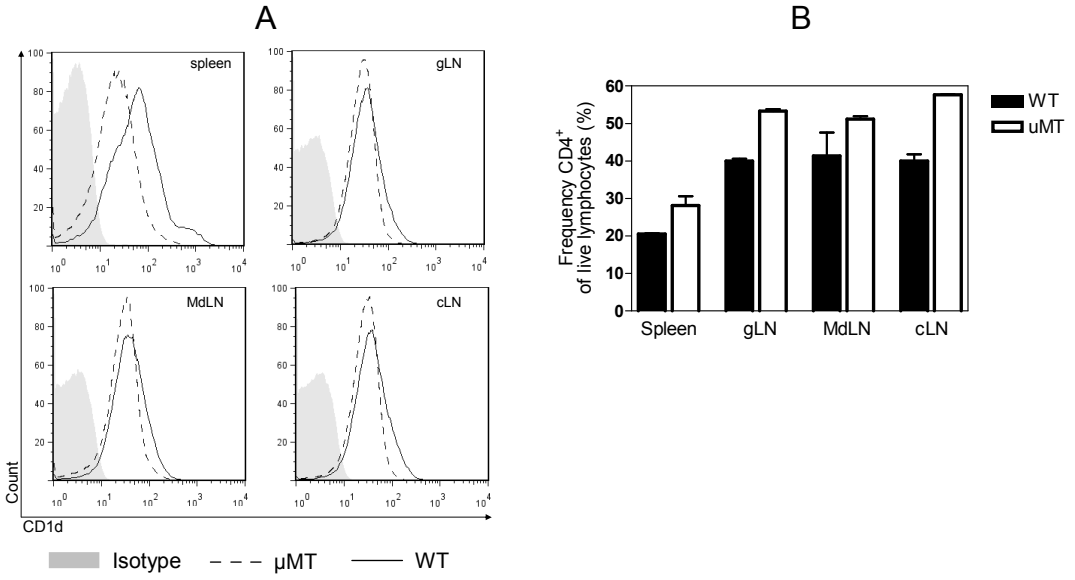

Figure 24. CD1d expression **(A)** and frequency of $CD4^+$ cells **(B)** in lymphoid organs from naïve WT and μMT mice. Gates were set on live leukocytes identified by forward, side scatter and negative 7AAD staining. Histogram plots shown in (A) are representative of two experiments with pooled samples from two mice per group. Bars in (B) show mean + SEM and data are pooled from two experiments (n=4).

The induction of a cellular immune response was studied three weeks following the last nasal immunization with gD + α-GalCer by testing proliferative response in lymphoid organs. To consider the observed difference in the frequencies of $CD4^+$ cells in naïve WT versus μMT mice (Fig. 24B), $CD4^+$ cells were purified from spleen prior to re-stimulation *in vitro* with gD protein and the frequency of $CD4^+$, $TCR\beta^+$ cells were measured in the lymphoid organs of immunized mice. The frequency of $CD4^+$, $TCR\beta^+$ cells was comparable in the spleen of immunized WT and μMT mice, while μMT mice had roughly twice the frequency of that seen in WT mice in all lymph nodes studied (Fig. 25A). A moderate to high proliferative response was detected in all lymphoid organs studied in WT mice immunized with gD + α-GalCer. While no or low proliferation was detected in the spleen and cLN of the immunized μMT mice, a low response was seen in the purified $CD4^+$ cells as well as gLN and MdLN. The proliferative response in immunized μMT mice was lower compared to immunized WT mice, irrespective of the correlation of $CD4^+$ cells in the spleen (Fig. 25B).

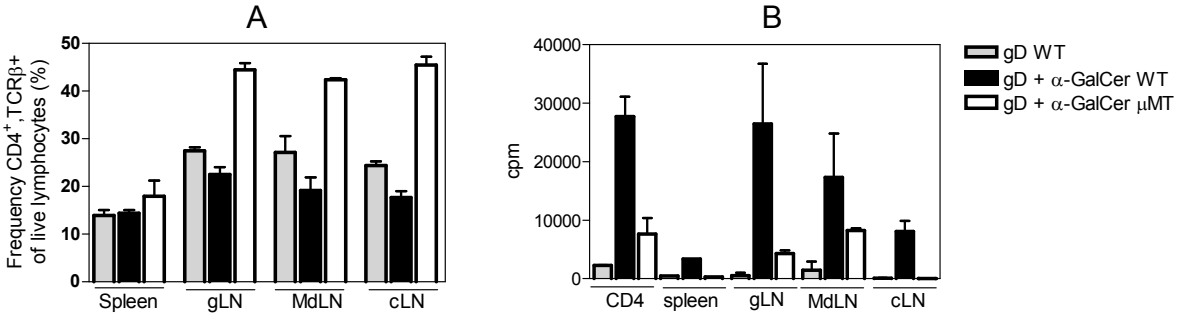

Figure 25. Cellular immune response of lymphoid organs detected *in vitro* three weeks following i.n. immunization of WT and μMT mice with gD + α-GalCer. The frequency of $CD4^+$, $TCR\beta^+$ cells was measured by flow cytometry **(A)** and the proliferative response of *in vitro* re-stimulated cells was measured in counts per minute, with the subtraction of background from un-stimulated cells **(B)**. Data shown are pooled from two experiments. Bars show mean + SEM.

The cytokine responses by gD re-stimulated lymphoid cells were measured in supernatants with ELISA after 96h. No or low cytokine production was detected in gD immunized group and by splenocytes from gD + α-GalCer immunized WT and μMT mice. In gLN and cLN, the cytokine production was higher in immunized WT mice compared to μMT. However the immunized WT and μMT mice showed comparable high levels of IFN-γ (Fig. 26A) and IL-5 (Fig. 26B) by purified $CD4^+$ cells and cells from MdLN.

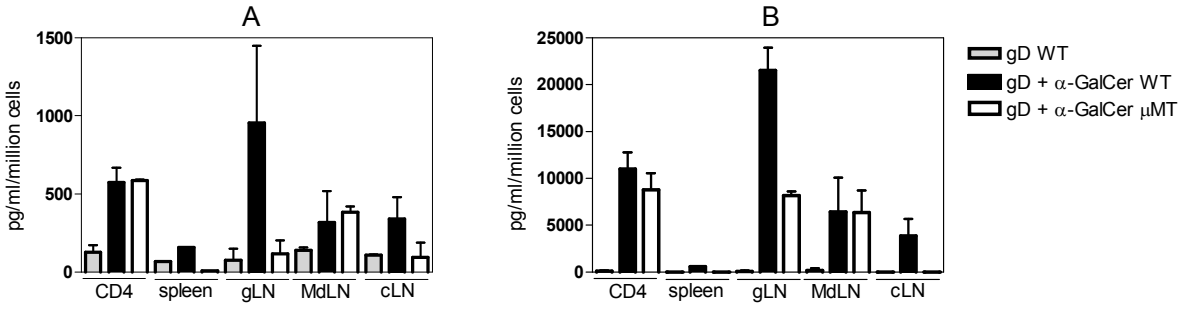

Figure 26. IFN-γ **(A)** and IL-5 **(B)** responses detected *in vitro* 96h after antigen re-stimulation of lymphoid organs three weeks following nasal immunization of wt and μMT mice with gD + α-GalCer. Data shown are pooled from two experiments. Bars show mean + SEM.

Following immunization, the mice were challenged with a lethal dose of HSV-2. Despite the reduction in HSV-2 replication detected in the vagina of immunized μMT mice (Fig 27A), the protective immunity induced by i.n. gD + α-GalCer immunization was critically dependent on B cells/antibodies. The gD + α-GalCer immunized μMT mice showed comparable kinetics of the symptoms of disease as that of naive WT control group and they all had to be sacrificed (Fig. 27B).

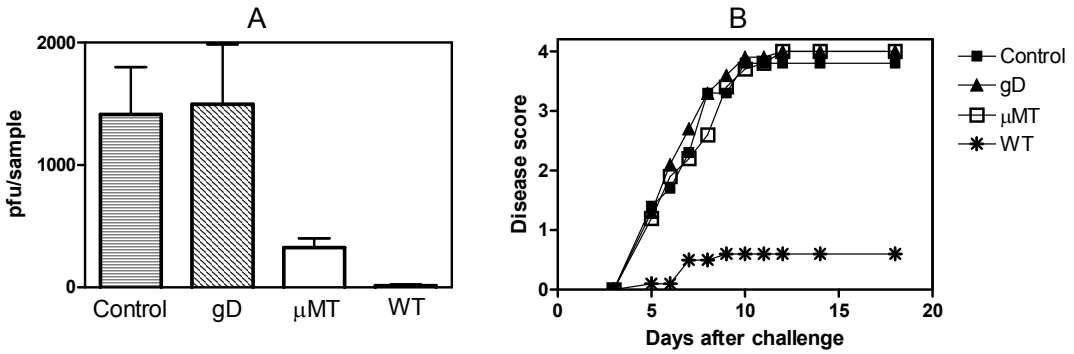

Figure 27. Viral replication **(A)** and symptoms of vaginal HSV-2 infection **(B)** following a vaginal HSV-2 challenge of WT and μMT mice i.n. immunized with gD + α-GalCer. The mice were observed on a daily basis for disease symptoms and scored according to; 0= healthy, 1= genital erythema, 2= moderate genital inflammation, 3= purulent genital lesions and 4= sacrificed due to hind limp paralysis. Results shown are pooled from two independently performed experiments (n=10). Bars in (A) show mean + SEM.

Previous studies where live attenuated HSV-2 lacking thymidine kinase gene (Tk^-), was administered to B cell deficient mice showed complete protection to a subsequent challenge with a fully virulent HSV-2 strain, while following nasal immunization, B-cells were important. This discrepancy could be due to detection of a higher number of HSV-2 specific T-cells in the vagina following i.vag. administration compared to i.n. Both studies agree on the importance of IgG antibodies early on in the response against infection, since B cell deficient mice show earlier and more severe symptoms of primary HSV-2 infection compared to WT mice, whereas protection against spread of the virus to sensory ganglia is dependent on a strong $CD4^+$ T response involving IFN-γ production [223, 247]. This is consistent with our findings where a low $CD4^+$ T response and IFN-γ production were detected in μMT mice, which appeared to correlate with no protection.

In summary these results suggest the importance of B cells/ antibodies in the protective immunity elicited by i.n. immunization with gD + α-GalCer, however, the current results do not allow us to conclude if the effect by B cells/antibodies is direct or indirect via B cell help for the development of memory CD4 cells. Thus, further studies are needed to elucidate the importance of the humoral arm of the protective immunity against genital HSV-2 infection.

6. Concluding remarks

STIs are a major health problem with a great socioeconomic burden worldwide today [248]. Development of vaccines against STIs would greatly improve quality of life, and in some cases, save lives. However up until now two vaccines against human papillomavirus are the only licensed vaccines developed against STIs. Within the framework of this thesis, we have identified two new potent nasal adjuvants for induction of protective immunity against genital HSV-2 infection, namely iNKT cell stimulator, the glycosphingolipid α-GalCer and a cochleate structure of proteoliposome derived from *Neisseria Meningitides B* termed AFCo1 with combined immunostimulatory and delivery properties. PL derived from *Neisseria Meningitides B* is included in meningococcal vaccine administrated in millions of doses to humans and has a good safety record.

We could further show that α-GalCer can function as a vaginal adjuvant, giving rise to 80% protection against genital HSV-2 infection in mice. Our group has previously demonstrated that CpG ODN, a TLR9 agonist, could function as a potent vaginal adjuvant to gD, inducing a similar level of protection (80%) as α-GalCer [204]. In an attempt to understand the mechanism of action of these two classes of adjuvants, we performed a genome-wide expression microarray analysis on vaginal tissue samples following vaginal administration of CpG ODN or α-GalCer combined with an *in silico* biofunction analysis. Based on the *in silico* analysis of gene expression, we identified "inflammatory response" as the main bio function induced by both adjuvants, although the expression profiles differed between the two adjuvants in terms of genes and kinetics. CpG ODN elicited a rapid response that sustained throughout the time points tested, while α-GalCer gave rise to a more modest and transient gene expression. Even though the two adjuvants increased expression of distinct genes, a number of genes were commonly up-regulated by both adjuvants, among which several were cytokines and chemokines.

Based on gene expression, detected by microarray (Paper IV) and real-time RT PCR (Paper II and IV) as well as complementary flow cytometry experiments (Paper IV), I postulate the following model for the mode of action of CpG ODN and α-GalCer as adjuvants in the murine female genital tract (Fig. 28).

Following administration into the vaginal lumen, the two adjuvants are likely to activate epithelial cells before entering lamina propria. Epithelial cells in the vagina are known to express TLR9 as well as CD1d and have effector functions, e.g. secrete cytokines [20, 249]. Both adjuvants may induce activation of macrophages and DCs, although by distinct pathways. CpG ODN induces APC activation (most likely via TLR9) at earlier time-point than α-GalCer, as detected by up-regulation of e.g. Cd40 and various pro-inflammatory cytokines. α-GalCer, presented on DCs or epithelial cells, may activate NKT cells, present at low frequency in the vagina, to express Il4 and Ifng, which further activates DCs. The expression of inflammasome-associated genes, including Nlrp3, by both adjuvants can lead to secretion of the active form of IL-1β. In addition, CpG ODN up-regulates several other pathways involved in NF-κB signalling, further contributing to the strong inflammatory milieu induced by CpG ODN. The inflammatory response initiated by CpG ODN is presumably controlled by the expression of regulatory cytokines e.g. Il10 and Tgfb, possibly secreted by activated macrophages.

Upon activation, macrophages and DCs secrete several chemokines, hence attracting other effector cells to the site. Up-regulation of the endothelial adhesion molecules Icam-1 and Vcam was also indcued. These adressins have previously been detected in the vagina and are known to bind the integrins LFA-1 and $\alpha_4\beta_1$, respectively, expressed on lymphocytes [48]. Following binding to the adhesion molecules, immune cells migrate into the tissue, directed by secreted chemokines. Early Cxcl2 expression by CpG ODN may lead to a large influx of

neutrophils to the induction site in the lamina propria. Other cells being recruited by induced secretion of Ccl chemokines by both adjuvants are macrophages, DCs and DC-like cells. Recruited DCs expressed $CD11b^+$ and $CD11c^+$, which are markers for the cells known to pick up and transport HSV-2 antigens to the draining lymph nodes and present it to CD4 T cells.

CpG ODN also up-regulates Ccr7, which together with its ligand Ccl19, induce migration of mature DCs to the draining lymph nodes and thereby enhance antigen presentation to T cells.

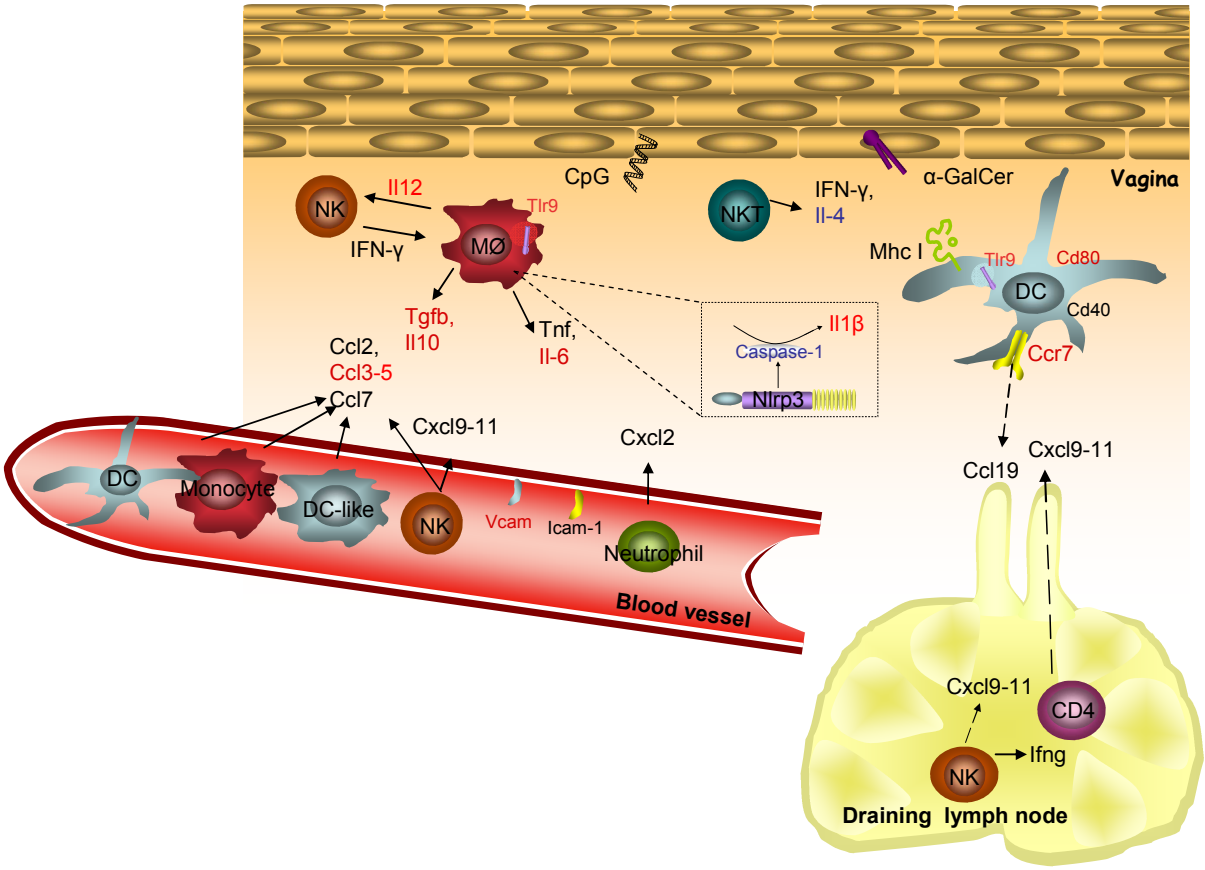

Figure 28. Hypothetic diagram illustrating the mechanism of action of CpG ODN and α-GalCer in the vagina following local administration. Genes expressed and cells recruited to the vagina by either or both adjuvants according to the following colour code; ■ = common, ■= CpG ODN exclusive and ■=α-GalCer exclusive. Illustrated cytokines, genes and cells in the figure have been detected either by microarray, real-time RT PCR or flow cytometry in Paper II or IV.

At later time, NK cells are recruited by both CpG ODN and α-GalCer, probably by expression of Ccl and Cxcl chemokines. Cxcl chemokines, namely 9, 10 and 11 could probably in the presence of antigen enhance recruitment of effector T cells from the lymph nodes to the tissue. Cxcl9, 10 and 11 might also be responsible for recruitment of NK cells to the lymph nodes, which could be detected after CpG ODN administration (unpublished observation). Such recruited NK cells may together with NKT cells be the source of Ifng, contributing to development of a Th1 skewed response [251].

While CpG ODN induces an early and strong pro-inflammatory innate response with a large influx of neutrophils, α-GalCer, induces a delayed and more modest response. This in

fact has been reflected in the excessive inflammation and vaginal tissue damage observed following vaginal delivery of CpG ODN.

In the presence of HSV-2 antigen (gD), both CpG ODN and α-GalCer have been shown to be potent vaginal adjuvants. We can not however predict the outcome of a mechanistic study including the antigen together with the adjuvants. Further studies are hence needed to pinpoint the mode of action of adjuvants in the female genital tract when given together with an antigen.

The knowledge gained here will hopefully inform development of potent and safe mucosal adjuvants to be included in vaccines against sexually transmitted infections for humans.

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