

**Development of novel immunization approaches to generate
immunity in the female genital tract with special
reference to genital herpes**

Sara Tengvall



Department of Microbiology and Immunology
Institute of Biomedicine
The Sahlgrenska Academy at Göteborg University
Sweden
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Till mamma och pappa

ABSTRACT

Development of mucosal vaccines for inducing immunity in the female reproductive tract would have profound implications for the prevention of sexually transmitted diseases. Despite numerous efforts, no such vaccines are currently available for human use. The main objective of this doctoral thesis was to develop novel immunization approaches to generate immunity in the female genital tract with special emphasis on immunity against genital herpes.

Mammalian innate immune systems sense and respond to pathogens through a series of pattern recognition receptors such as Toll-like receptors (TLRs). Detection of pathogen associated molecular patterns by TLRs triggers a signalling pathway mainly through adaptor protein MyD88, which results in a coordinated set of immune responses that includes both innate and acquired immunity. By using a well-established mouse model of genital HSV-2 infection, it was shown in this thesis that the efficacy of intramuscular immunization with a DNA vaccine encoding glycoprotein D (gD) from HSV-2 can be improved with a timely administration of synthetic oligodeoxynucleotide (ODN) containing immunostimulatory CpG motifs, a TLR9 ligand. Another important finding in this thesis work was introduction of CpG ODN as a potent vaginal adjuvant for induction of acquired immunity in the female genital tract as well as for systemic immune response. Thus, vaginal immunization with HSV-2 gD in combination with CpG ODN induced potent gD specific antibody as well as cellular immunity, and conferred protection against subsequent vaginal challenge with a lethal dose of HSV-2.

The potential of rectal immunization route to induce protective immunity in the female genital tract was also investigated. Thus, rectal immunization with a live attenuated HSV-2 TK⁻ was shown to confer antibody and cellular response as well as protection against an otherwise lethal vaginal challenge with a virulent HSV-2 strain. Importantly, unlike intravaginal route, rectal route was shown to be independent of sex hormonal influence. It was also documented that TLR/MyD88 signalling pathway is important for innate immune protection against primary genital herpes. By contrast, the usage of MyD88 was shown to be dispensable for induction of acquired immune protection induced by vaginal or rectal immunization with HSV-2 TK⁻. In addition, while rectal immunization with the TLR/MyD88 targeting adjuvant CpG ODN in combination with gD failed to elicit protective immunity, rectal immunization with cholera toxin and gD conferred a potent antibody and cellular immune responses as well as protection against genital herpes. These results have implications for the development of vaccines to generate immunity in the female genital tract against sexually transmitted infections.

Keywords: female genital tract immunity, genital herpes, mucosal adjuvant, TLR, MyD88 and CpG ODN

ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-IV) and additional results presented in appendices:

- I **Tengvall S**, Josefsson A, Holmgren J, Harandi AM.
CpG Oligodeoxynucleotide augments HSV-2 glycoprotein D DNA vaccine efficacy to generate T helper 1 response and subsequent protection against primary genital herpes infection in mice.
Journal of Reproductive Immunology 2005 Dec;68(1-2):53-69.
- II **Tengvall S**, Lundqvist A, Eisenberg RJ, Cohen GH, Harandi AM
Mucosal administration of CpG Oligodeoxynucleotide elicits strong CC and CXC chemokine responses in the vagina and serves as a potent Th1-tilting adjuvant for recombinant gD2 protein vaccination against genital herpes.
Journal of Virology 2006 Jun;80(11):5283-91.
- III **Tengvall S**, O'Hagan D, Harandi AM
Rectal immunization confers a sex hormonal- and MyD88- independent protective immunity against genital herpes simplex virus type 2 infection in mice.
Submitted for publication
- IV **Tengvall S**, Harandi AM
Importance of the adaptor molecule MyD88 in innate and adaptive immune protection against genital herpes infection in mice.
Manuscript

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ABBREVIATIONS

HSV-2	Herpes simplex virus type 2
APC	Antigen presenting cell
gD	glycoprotein D
TK	Thymidine kinase
FGT	Female genital tract
CpG ODN	Cytidine-phosphate-Guanosine oligodeoxynucleotide
CT	Cholera toxin
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
IFN- γ	Interferon- γ
Ig	Immunoglobulin
IL	Interleukin
Im	Intramuscular
Ivag	Intravaginal
Ir	Intrarectal
IP-10	Gamma interferon-inducible protein 10
MHC	Major histocompatibility complex
MIP-1 α/β	Macrophage inflammatory protein 1 α/β
MIP-2	Macrophage inflammatory protein 2
RANTES	Regulated on activation, normal T cell expressed and secreted
gLN	Genital lymph node
Th1	T helper 1
CTL	Cytotoxic T lymphocytes
TLR	Toll like receptor
PAMP	Pathogen-associated molecular pattern

INTRODUCTION

History of herpes

The old greek Hippocrates once described lesions spreading in the skin, and so the name herpes from Greek herpein, meaning creep or crawl, came in use. In 1736 the disease was recognized as a clinical entity by Jean Astruc, and the causative agent, herpes simplex virus (HSV) was isolated in 1964 by Slavin and Gavett from clinical specimens of genital herpes patients [1].

THE FAMILY OF HERPES VIRUSES

The family *herpesviridae* consists of nearly 100 members with specificity for many animal species. All herpes viruses are so called DNA-viruses containing linear double-stranded DNA. The virion structure has four components; an electron-dense core containing the viral DNA, an icosahedral capsid enclosing the core, an amorphous structure named the tegument containing many viral proteins critical for the initiation of infection and finally a lipid bilayer envelope that encloses the viral particle. There are about a dozen different glycoproteins inserted into the envelope which enables viral binding and entry into target cells. So far eight different herpesviruses have been isolated from humans. Based on their biological characteristics, these can be classified into one of three subclasses (known members infecting humans inside brackets); *alfaherpesvirinae* [herpes simplex virus (HSV) 1 and 2, and varicella zoster virus (VZV)], *betaherpesvirinae* [human herpes virus (HHV) 6 and 7, and human cytomegalovirus (HCMV)] and *gammaherpesvirinae* [Epstein-Barr virus (EBV) and HHV-8].

Herpes simplex virus

HSVs from the *alfaherpesvirinae* are divided into two subfamilies, HSV-1 and HSV-2. These are closely related viruses sharing many pathogenic properties. HSV-1 is primarily responsible for the common cold sore and HSV-2 is the main causative agent of genital herpes. They possess an efficient and rapid cytolitic reproductive cycle (18-24h), the ability to establish latency in the sensory ganglia and a wide host cell range; mainly explained by their binding and entry receptors [2]. The most common target cells are epithelial cells, fibroblasts, neurons and lymphocytes. To initiate infection the virus attaches to the target cell surface (illustrated in Figure 1) by interactions between the viral glycoprotein C (gC) and cell surface heparan sulphate. In this primary interaction redundancy exists since other glycoproteins such as gB can substitute for gC to mediate binding [3].

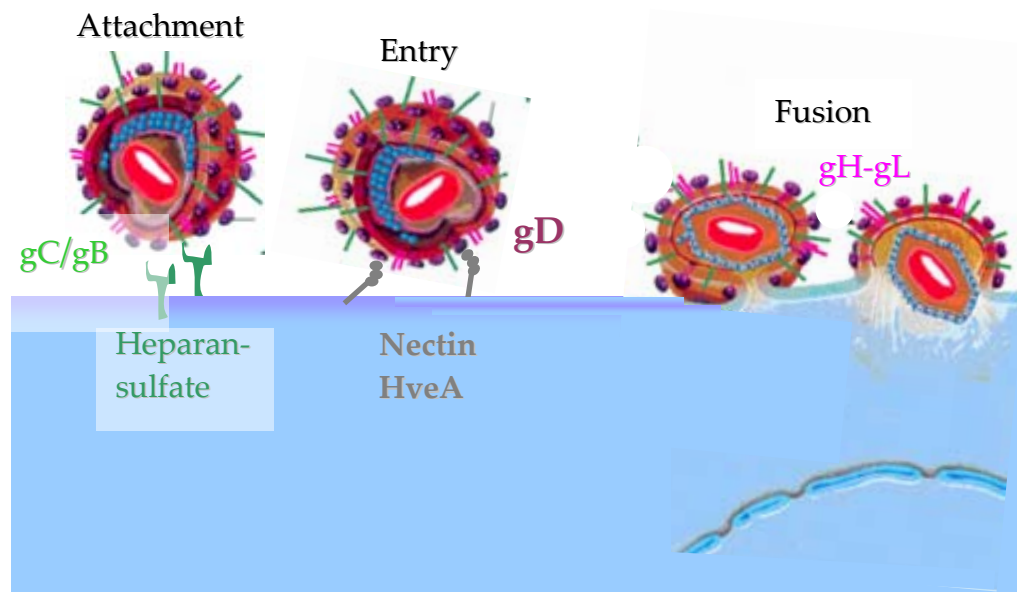


Figure 1. Illustration of attachment and entry of HSV into target cell. Adopted from [4] with modifications.

Once the initial attachment of a virus to gC or gB has occurred, co-binding to a co-receptor is required for a successful cell entry. This involves the high affinity binding of viral gD to one of several candidate cellular molecules that belong to one of two structurally unrelated molecule families [3]. One belongs to the immunoglobulin superfamily whose members are nectin-1 α (HveC, Prr1) and nectin-1 β (HigR), which mediate cellular entry of both HSV-1 and HSV-2. In addition, Nectin-2 α and nectin-2 δ mediates entry of HSV-2 but not wild type HSV-1 [2]. These receptors are expressed in a variety of tissues, including herpes infection target cells in skin, brain and spinal ganglia [5]. The second co-receptor is a member of the tumour necrosis factor (TNF) receptor family, and is called herpes virus entry mediator A (HveA). HveA is found primarily on lymphoid cells and serves as receptor for the entry of HSV-2 and some HSV-1 strains.

Following viral attachment, the viral envelope fuse with the host cell membrane in a process that is believed to require the participation of gB, gD and gH-gL heterodimer [5]. After fusion, the virus becomes uncoated and release proteins from the tegument and the nucleocapsid into the cytoplasm. The nucleocapsid associates with microtubules, and is transported to the nucleus where the capsid docks at a nuclear pore. The HSV-genome is thereby inserted into the cell nucleus. Some proteins from the tegument necessary for viral genome transcription, such as VP16, are also transported into the nucleus where they stimulate the expression of "immediate early" (α) genes by host cell RNA polymerase II. These immediate early transcripts are transported to the cytoplasm for translation, after which they become imported

back to the nucleus in order to regulate transcription of “early” (β) genes. β proteins participate primarily in DNA replication and are present either in the cytoplasm or the nucleus. Viral DNA replication produces long concatemeric DNA molecules that are the source for “late” (γ) gene transcripts. The γ proteins are HSV structural proteins such as envelope glycoproteins, tegument proteins and proteins needed for viral assembly and egress [5].

GENITAL HERPES INFECTION AND DISEASE

Genital herpes is a genital ulcerative disease (GUD) mainly caused by HSV-2, but also to a lesser extent HSV-1 [6]. The prevalence of HSV-2 varies. Higher rates are generally observed in developing than developed countries and in urban than in rural areas. In Sweden about one fourth of the adult population is affected by HSV-2 [7]. Studies in many sub-Saharan African and the Caribbean countries show, however, prevalence of around 50% in adults [8]. Overall, the prevalence is higher in women than men [8]. The acquisition of HSV-2 is usually a consequence of transmission by genital contact and the main factors attributed to the spreading of the infection include asymptomatic viral shedding and under-diagnosis of the disease [9]. Following primary infection and replication in the genital tract mucosal epithelial cells, HSV-2 can be transmitted via peripheral nerve axons to the sacral ganglia innervating the site of infection. After transport, viral replication occurs for several days in the infected sensory ganglia and latency is established. The primary infection displays an incubation time of about one week, whereupon lesions start to appear and usually remains over a period of 10 days. In the majority of patients with primary genital HSV-2 infection, reactivation of latent virus in the sensory ganglia may give rise to 3-4 recurrences per year. Recurrent symptoms are usually less severe than symptoms during the primary infection [10]. Although the activation of latent virus is spontaneous, there is an association to immune suppression, fever, menstruation, emotional stress, UV-irradiation or tissue damage [10]. Genital ulcers are common but also disseminating disease and life-threatening complications (such as meningitis) may occur in immunocompromized individuals or newborns (neonatal herpes). HSV-2 can also cross the placental barrier and affect the foetus during early pregnancy. This can lead to a spontaneous abortion or serious damage to foetus, including mental retardation. However these medically serious complications are rare, they constitute a significant burden given the high rates of HSV seropositivity in the population [11]. Genital herpes is thus an incurable disease that by its self constitutes a major health problem. In addition, several epidemiological studies have demonstrated that prevalent HSV-2 is associated with a 2- to 4-fold increased risk of HIV-1 acquisition [12].

Antiviral treatment

Available antiviral agents, such as acyclovir and valacyclovir, are based on guanosine analogues and work in a two-step process; after transport into the cell by a

guanosine-transporter they become phosphorylated by the virus-encoded thymidine kinase (TK) into monophosphates (such as acyclovir-monophosphate) which competes with thymidine. The monophosphate-form of the drug is then converted by intracellular kinases to a triphosphate (acyclovir-triphosphate), a metabolically active form that compete with deoxyguanosine triphosphate for the viral DNA polymerase. Incorporation of the triphosphate-form of the drug into the viral DNA chain interrupts its DNA transcription, and inhibits viral replication [13]. Although antiviral treatments are important for limiting disease severity, especially for recurrent HSV infections, they can not cure the disease. In addition, they are expensive and HSV strains resistant to acyclovir are emerging [14] [15].

IMMUNE EVASION MECHANISMS BY HSV

In order to infect the genital tract mucosa and establish latency in the sensory nerve ganglia, herpes viruses have evolved a number of immune evasion mechanisms. For example gC interferes with C3b unit of the complement system, and gE mimics IgG Fc-receptor and binds up anti-HSV antibodies, thus complement- and antibody-dependent virus neutralization and lysis of infected cells are avoided [16]. One important tactic used by HSV-2 is inhibition of presentation of antigenic peptides by major histocompatibility complex type I (MHC I) by binding of ICP47 to TAP (the transporter associated with antigen processing) and thus blocking the transport of peptides into the ER. ICP47 appears to possess high affinity for human and porcine TAP but not murine homologues, and this may explain why the anti-HSV response in mice is more effective than in humans [17]. Furthermore, a recent study showed that HSV-1 interrupts MHC II processing by reduction of Ii (invariant chain) expression and the residual activity of the MCH II processing pathway is down regulated by gB that form complexes with DR and DM. These are in turn restrained from cell surface expression [18].

THE FEMALE GENITAL TRACT IMMUNITY

The female genital tract (FGT) is part of the common mucosal immune system, but is also unique in several aspects. The genital tract needs to tolerate sperm and developing foetus, whilst being able to respond to sexually transmitted bacterial and viral pathogens. Importantly, the mucosal immune system of the FGT is under a strong hormonal control that regulates the presence/transport of immunoglobulins, the levels of cytokines and the distribution of various immune cell populations [19]. Unlike other mucosal surfaces where S-IgA is the dominant antibody isotype, the lower genital tract secretions are dominated by IgG antibodies. The human female genital tract can be divided into three major compartments: 1) the non-sterile vagina and ectocervix, which are lined with stratified squamous epithelium and; 2) the sterile endometrium and fallopian tubes that are lined with columnar epithelium; and 3)

the endocervix, also being lined with columnar epithelium and where sterility may be related to the temporal phase of the menstrual cycle.

Another difference between the FGT mucosa and most of the other mucosal surfaces is the commensal flora. The commensals that normally colonize the vaginal mucosa play a significant role in vaginal defence; these include *Lactobacillus* spp., *Gardnerella vaginalis*, coagulase-negative Staphylococci, *Enterococcus* spp., *Ureaplasma urealyticum* and *E. coli* [20]. The lactobacilli are especially important as they metabolize glycogen released by vaginal epithelial cells to lactic acid, thus lowering the vaginal pH to 3.5-5, thereby limiting the number of bacterial strains that can survive and grow.

The FGT also differs from the gastrointestinal tract in that it lacks distinct lymphoid tissues. However, lymphoid aggregates similar to Peyer's patches (composing of CD11c⁺ dendritic cells and CD3⁺ and CD4⁺ T cells) have been shown to transiently form in the lamina propria of mice in response to vaginal inoculation with an attenuated HSV-2 strain, and further to correlate with protective immunity against a subsequent wild-type challenge [21]. In addition, the epithelial cells of the genital tract may play a key role in antigen presentation and immune response. Similar to gastrointestinal epithelial cells, vaginal epithelial cells are shown to constitutively express MCH II and function as APC [22]. The stratified epithelial cells of the vagina and ectocervix also express a more restricted pattern of cytokine and chemokine release, whereas the columnar epithelium of the endocervix expresses a cytokine profile similar to that reported for intestinal mucosal epithelium in response to pathogens (including IL-6, IL-7, RANTES) [23] [24]. Such differences probably reflect the fact that while the vagina and ectocervix must tolerate endogenous microflora, the endocervix should protect the sterile upper genital tract from pathogenic invasion.

The murine FGT immunity

Mice and humans differ somewhat in the genital tract build-up. In mice, both the cervix and the vagina are lined with keratinized stratified squamous epithelial cells [25]. Another difference is that in mice there is a predominance of CD4⁺ T cells in the vagina, while in humans there is a predominance of CD8⁺ T cells [19]. Mice have a short menstruation cycle of about 5 days and this can further be divided into four separate phases with specific characteristics; proestrus, estrus, metestrus and diestrus. The ovarian folliculi develop during proestrus, ovulation occurs in estrus, the corpora lutea form at the time of metestrus and the unfertilized ova become eliminated in diestrus. Experimental progesterone treatment of mice results in a state where the vaginal epithelium becomes extremely thin and histologically similar to the diestrus phase [26].

Interaction between other mucosal surfaces and the FGT

Many studies in experimental animals have shown that the nasal mucosa could serve as an induction site for immunity in the FGT. Experiments performed in rodents and primates have demonstrated that intranasal immunization with viral or bacterial antigens, admixed with cholera toxin-B subunit (CTB) as a mucosal carrier, elicits considerable antigen-specific IgA and IgG antibody responses in the vaginal mucosa. [19] Also, intranasal immunization with an attenuated live HSV-2 strain (TK-) was demonstrated to result in both HSV-specific antibody and HSV-specific T cell responses in the vagina of mice [27]. However, the protection afforded in the FGT after nasal immunization was also demonstrated to be dependent primarily on antibodies, as B-cell deficient mice are not protected against vaginal challenge after intranasal HSV-2 TK- immunization [27].

Also, a link between the adjacent rectal mucosa and genital tract mucosa has been suggested [28]. Targeted iliac-lymph node immunization and in vivo labelling experiments in non-human primates has shown that T and B cells from the iliac lymph node preferentially home to both the genital and rectal mucosa [29]. In addition, long-term presence of vaginal influenza-specific IgG antibodies in humans has been demonstrated in women after rectal administration of influenza vaccine [30].

ROLE OF SEX HORMONES IN THE FGT IMMUNITY

One very important aspect in the FGT immunity is the effect of the sex hormones estrogen and progesterone on mucosal immune regulation in the FGT. Generally, the upper female genital tract (fallopian tubes and uterus) and the lower (cervix and vagina) respond differently to the female sex hormones. In the following, the immune responses in the vagina will be described. Intraepithelial Langerhans cells that sample the lumen of the vagina for antigens are present in the vaginal mucosa in all stages of the estrus cycle, but most numerous in the metestrus-diestrus phase [31]. Also, ovariectomized rodents that are treated with estrogen show a significant decrease in IgG- as well as IgA-levels [32]. In addition, estrogen-treated animals display significantly lower levels of antigen presentation by vaginal epithelial cells, macrophages, B cells and DCs, and furthermore decreased antigen-specific lymphocyte proliferation [33]. Recently, it was demonstrated that an important mediator in estrogen-reduced immune response in the vagina is TGF- β . Thus, anti-TGF β , but not anti- IL-10, IL-6 or TNF- α antibodies, restored antigen-presentation and proliferation responses of estrogen-dominated rats [34].

Progesterone-treatment of mice, on the other hand, was shown to induce inflammation [35], but also to increase the susceptibility of rodents and primates to primary genital HSV-2 [36] or SHIV infections [37]. In humans, the use of oral contraceptives among commercial sex workers are associated with an increased risk of HIV-acquisition [38]. With regard to vaginal immunization it has been shown that

vaginal inoculation of mice with live HSV-2 TK⁻ results in protective immunity in progesterone, but not estrogen-, dominated mice [26].

INNATE DEFENCE AGAINST HSV-2 IN THE FGT

Macrophages are involved in anti-herpes immunity during the first hours of primary herpes infection. During this early response, cytokines (primarily type I interferons (IFN) and TNF- α) are produced by the macrophages and exert a direct anti-viral effect and also activate macrophages themselves. In the next phase, interleukin (IL)-12 together with above mentioned cytokines induce production of IFN- γ , mainly in NK-cells [39].

Neutrophils are present throughout the female genital tract and are the most common leukocytes present in the vaginal epithelia of normal mice [40]. Studies in the murine genital herpes model suggest that neutrophils are involved in innate immune resistance against primary herpes infection, as mice depleted in neutrophils have significantly higher viral titers [41] [42].

NK cells, large granular non-T-cell lymphocytes, and NKT cells which express both T cell receptors and NK cell markers, respond rapidly to viral antigens and have the ability to kill virally infected cells without prior sensitization and/or rapidly releases IFN- γ . NK and NKT cells are regulated by IL-15 produced by monocytes/macrophages and DCs. Several recent studies emphasise the importance of IL-15, and NK and NKT cells for the innate immune response against primary HSV-2 infection. Thus, IL-15⁺ mice that lack NK and NKT cells and RAG-2⁺ γ ^{c+} mice that lack all lymphoid cells but produce IL-15 are 100-fold more susceptible to vaginal HSV-2 infection than control mice [43] [44].

Cytokines and chemokines

Cytokines are small proteins that act in paracrine or autocrine manner at picomolar concentrations. The first hours after viral infection, cytokines are produced by infected cells, or cells that come in contact with viral products, and activates the infected cell itself or the surrounding cells to interfere with viral replication (for example type 1 interferons) and/or activate immune cells to kill the intruder (for example IFN- γ) [45].

Chemokines are a family of growth factor-like proteins and can be secreted by a variety of cells. They function as cell activators and recruitment molecules; induces extravasation of leukocytes from blood out to the inflamed tissue. The specific chemokine effects are mediated via members of 7-transmembrane-spanning G-protein-coupled receptors. Chemokines and their receptors are redundant in their ability to bind to one another; thus one chemokine can bind to several chemokine receptors and several chemokines may bind to the same receptor type [46].

Chemokines are, according to presence and position of cysteine-residues near the NH₂-terminus of the protein divided into four subgroups; CC, CXC, C and CX₃C. The CC chemokines, including MIP-1 α , MIP-1 β and RANTES, have two cysteine-residues nearest to the N-termini and act primarily on monocytes/macrophages, eosinophils, basophils and Th1 cells [47]. The CXC chemokines, including MIP-2 and IP-10, have two cysteine-residues separated by one amino acid nearest to the N-termini, and act primarily on neutrophils, T cells, and NK-cells [47].

TOLL-LIKE RECEPTORS

The innate immune system has evolved to recognize pathogens that enter through a variety of mucosal surfaces and to induce a rapid cascade of events leading to antimicrobial defence. This is mediated through pattern recognition receptors (PRRs), which bind and respond to pathogen-associated molecular patterns (PAMPs) [48]. Among PRRs, Toll-like receptors (TLRs) have recently attracted much attention. So far 11 different TLRs have been identified and these recognize different PAMPs (Table 1).

Table 1. The Toll-like receptor (TLR) family and its ligands (Adopted from [49]) with modification.

TLR molecule	Ligands (organism of origin)
TLR1	Triacetylated lipopeptides (bacteria)
TLR2	Envelope protein (virus) Lipopeptide (virus) Lipoprotein (bacteria) Peptidoglycan (bacteria) Atypical LPS (bacteria) Porin (bacteria) Lipoarabinomannan (mycobacteria) Glycolipids (spirochetes) GPI anchor (parasite) Zymosan (yeast) Various heat-shock proteins (host)
TLR3	Double-stranded RNA (virus)
TLR4	LPS (Gram-negative bacteria) Envelope protein (virus) Taxol (plant) Various heat-shock proteins (host)
TLR5	Flagellin (bacteria)
TLR6	Diacetylated lipopeptides (bacteria)
TLR7	Single-stranded RNA (virus)
TLR8	Single-stranded RNA (virus) (in humans)
TLR9	Unmethylated CpG DNA (microbial DNA, host) Chromatin-IgG complexes (host)
TLR10	Unknown
TLR11	Profilin-like protein from <i>Toxoplasma gondii</i>

Upon ligand binding, TLRs activate signalling through the Toll/interleukin-1 domain present in the cytoplasmic tails of these proteins (Figure 2). The induced signalling pathway consists of two cascades: a MyD88-dependent pathway and a TRIF-dependent (MyD88-independent) pathway. TLR-2, -5, -7, -8, -9 and -11 signalling is MyD88-dependent, while TLR-3 and -4 signalling can be mediated via both MyD88 and TRIF [48]. The MyD88-dependent pathway mediates TLR-induced production of proinflammatory cytokines, including IL-12p40, whereas the TRIF-dependent pathway is indispensable for the induction of type I IFNs through TLR3 and TLR4.

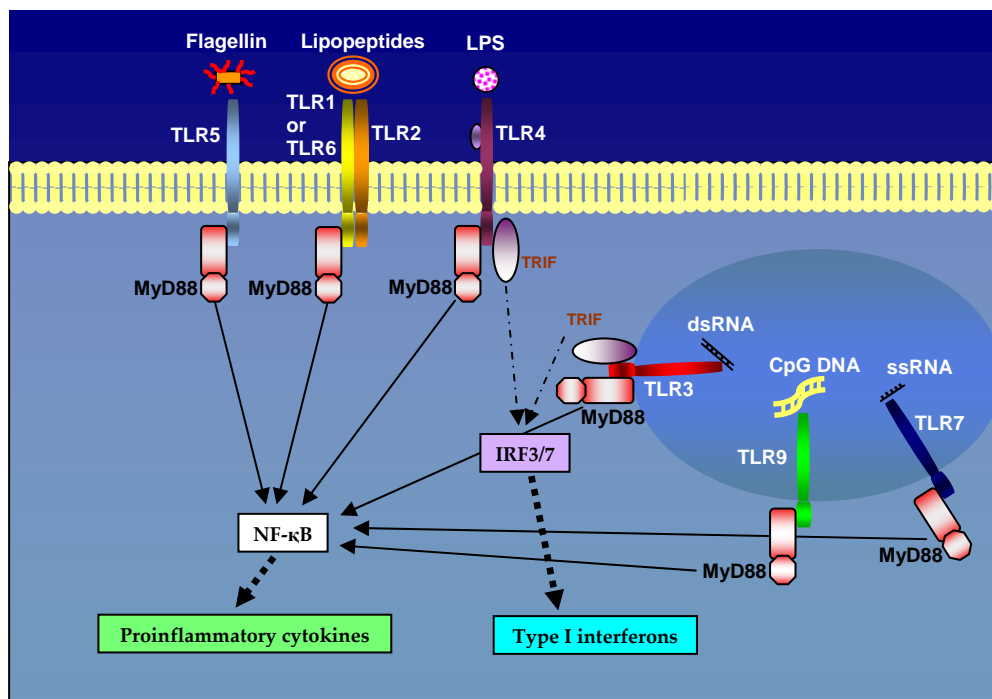


Figure 2. TLR signalling pathway.

Innate immune recognition of HSV-2

Herpes simplex viruses contain several putative PAMPs, such as TLR ligands, which are suggested to be involved in the primary viral clearance and subsequent induction of HSV specific adaptive immunity [50]. HSV glycoproteins have been shown to activate TLR-2 upon binding to target cells, followed by activation of TLR9 by viral nucleotides in the endosomal compartment [51].

ACQUIRED IMMUNITY AGAINST HSV-2

Natural genital HSV-2 infection is very effective for the induction of an acquired immune response, since exogenous re-infections are rare [52]. However, the acquired immunity is proposed to be, at least in part, site-specific as autologous auto-infections with genital HSV-2 strains may occur at cutaneous sites even after the establishment of systemic immune responses [53]. In addition, individuals who are seropositive for HSV-1 are as vulnerable as HSV-1 seronegative people to acquire

HSV-2. Thus, prior infection with HSV-1 does not confer protection against HSV-2 infection. However, HSV-2 infection in a HSV-1-seropositive individuals is more likely to be asymptomatic than symptomatic [54].

Dendritic cells - inducers of immunity against HSV-2

The vaginal mucosa is constantly exposed to non-infectious and infectious agents, and is under constant surveillance by resident dendritic cell populations, macrophages and B cells. After intravaginal inoculation HSV replication occurs primarily within the vaginal epithelium, whereas immune cells do not typically support viral replication [31] [55]. The antigen-presenting cells of the vaginal mucosa include intraepithelial CD1a⁺, MHC II⁺, CD11c⁺, CD123⁻, DC-SIGN⁻, CD4⁺ Langerhans cells (LC) and CD1a⁻, MHC II⁺, DC-SIGN⁺, and CD4⁺ dendritic cells (DCs). LCs are abundant in the epithelial layer of the vaginal and ectocervical mucosa of women, and DCs are predominant in submucosal layers [56] [57]. Several studies have demonstrated that submucosal DCs become quickly recruited to HSV-2 infected epithelium and emerge subsequently in the draining lymph nodes where they are responsible for the stimulation of IFN- γ secretion by CD4⁺ T cells [55] [58] [59]. Other antigen-presenting cells, such as LCs, B cells, and macrophages were shown not to migrate to the draining lymph nodes after HSV-2 infection and not to stimulate T cell proliferation and IFN- γ secretion [55].

Humoral response against HSV-2

Genital HSV-2 infection elicits HSV-specific IgA and IgG antibodies in the genital tract mucosa of both humans and mice [60]. In mice it has been demonstrated that a passive transfer of HSV-specific serum IgG from immune animals to the vaginal lumen of naïve mice reduce the viral load and pathological signs of the disease following vaginal challenge with fully virulent strain of HSV-2 [61]. Furthermore, IgG appears to be the main protecting antibody against wild type HSV-2 in the vaginal tract following local immunization [61], and IgA is dispensable for the immune protection [62].

However, vaccinated animals lacking CD4⁺ T cells, or lacking the ability to produce IFN- γ , succumb to vaginal HSV-2 challenge despite high levels of HSV-specific IgG antibodies. Further, mice lacking B-cells are capable of mounting a protective immune response to HSV-2 [63]. In humans, a recent clinical trial using glycoprotein-based vaccine was shown to induce high levels of HSV-specific neutralizing antibodies – but did not influence either the duration of the first clinical HSV-2 episode or the frequency of subsequent recurrences [64]. Nonetheless, the importance and the role of antibodies in immunity against genital herpes in humans are not fully understood.

T cell response against HSV-2

Studies in mice have demonstrated that T cells, and in particular CD4⁺ T cells are required for acquired protective immunity against genital HSV-2 infection [65] [61] [63]. Thus, vaginal vaccination confers protection against a subsequent lethal vaginal HSV-2 challenge in mice lacking CD8⁺ T cells (CD8^{-/-}), but not CD4^{-/-} mice. Interestingly, exogenous IFN- γ treatment was shown to rescue vaccinated, but not unvaccinated, CD4^{-/-} mice from subsequent HSV-2 infection [63].

VACCINE DEVELOPMENT AGAINST GENITAL HERPES

Over the last decade numerous efforts have been made to develop a successful vaccine against genital herpes. Several candidate vaccines have been tested in experimental models of genital herpes, and some have also reached advanced human clinical trials (Table 2). However, despite encouraging results in animal models, results in human clinical trials have been disappointing conferring no protection or only protection in one subgroup; women that were seronegative for both HSV-1 and HSV-2 [66]. One possible reason for the low success may be that all trials have been focused on systemic, and not mucosal, immunization approaches.

Table 2. HSV-2 human clinical trials.

Type of vaccine	Antigen	Adjuvant	Route of administration	Protection	Ref
Autoinoculation of live HSV	HSV	-	Im	No	[53]
Whole inactivated HSV	HSV	-	Im	No	[67]
Inactivated subunit HSV (DNAse treated)	HSV	- Alum	Im	No	[68] [69]
Attenuated live HSV	HSV	-	Im	No	[70] [71]
Disabled infectious single cycle (DISC) HSV	HSV	-	Im	No	[72]
Recombinant protein	gD	Alum	Im	No	[73]
Recombinant protein	gD/gB	MF59	Im	No	[64]
Recombinant protein	gD	AS04	Im	Partial	[66]

A very useful experimental model that has substantially contributed to our understanding of protective immunity against genital herpes is a mouse model in which vaginal immunization with a live attenuated strain of HSV-2 lacking the gene of thymidine kinase (HSV-2 TK⁻) elicits immunity against a subsequent vaginal challenge with a fully virulent HSV-2 [26]. Thymidine kinase is an enzyme needed for viral replication within neural cells, and hence this virus causes a local mucosal infection that is cleared within approximately a week.

Although attenuated viruses such as HSV-2 TK⁻ are very useful for experimental purposes, they are precluded from human use due to safety concerns, e.g. reversion back to wild type. Thus, current candidate vaccines are based on recombinant HSV proteins, heterologous viral vectors expressing HSV proteins, DISC HSV or DNA vaccines.

Principles of DNA vaccines

DNA vaccination refers to direct immunization with the gene encoding the antigen of interest located on a suitable eukaryotic expression plasmid. After delivery the plasmid is taken up by host cells and travels to the nucleus, where it is expressed using the host machinery. The DNA immunogen passes through the host endogenous transcription, translation and post-translational machinery, generally resulting in a protein with the correct three-dimensional conformation, phosphorylation and glycosylation. Since the antigen is produced within the cell, the antigen is presented on MHC class I and preferentially induces a CTL response [74]. Also, the antigen can be secreted and taken up by other APCs and presented on MHC class II which leads to activation of CD4⁺ T cells [75]. The required elements of a DNA vaccine are 1) the plasmid backbone (a double stranded DNA ring), 2) an inserted viral gene, 3) two Ori-sequences (one for replication of the plasmid DNA within the host cells, one for allowing replication within transformed bacteria) and 4) an antibiotic resistance gene (for selection of bacterial cells transformed with the plasmid). The inserted viral gene must be under the control of a promoter, such as the immediate early CMV promoter, to be active in mammalian cells. In addition, a eukaryotic polyA signal (often derived from bovine growth hormone) is required to provide stability to mRNA and prevents rapid turn-over.

Adjuvants

Adjuvants can be defined as substances that enhance or modulate the immune response towards a co-administered antigen. The only approved adjuvants for human use are aluminium salts, or alum, (aluminium phosphate and aluminium hydroxide), AS04 (Alum + monophosphoryl lipid A) and MF59 adjuvant, a squalene oil-in-water emulsion [76]. It is believed that alum enhances the immune response by activating antigen presenting cells (APC) e.g. dendritic cells (DC), stimulate the complement system and induce chemokine production [77]. Although alum has a good safety record and is the adjuvant of choice for induction of potent antibody response, it is a weak adjuvant for induction of cell-mediated immunity, and in addition is mainly useful for parenteral, and not mucosal vaccines [78]. Given the fact that the vast majority of pathogens invade or cause disease in the mucosa, and that parenteral immunization generally induces only weak mucosal immunity, there is a great need to develop safe and effective mucosal adjuvants.

Cholera toxin (CT)

Cholera toxin (CT) from *Vibrio cholerae*, the pathogen responsible for severe cholera diarrhea in humans, has been extensively used as a potent mucosal adjuvant in animal studies. Mucosal administration of CT increases the permeability of epithelial cells [79], which can lead to increased uptake of co-administered antigen. Also, CT administration results in increased antigen presentation by DC, B cells and macrophages [80]. Thus, CT administration increases the induction of mucosal humoral as well as cell mediated immune responses to co-administered antigens. However, the inherent toxicity of CT precludes its use as a mucosal adjuvant in humans. Mice are by contrast less sensitive to the toxic effect of CT and may be given CT orally without any diarrhea.

Immunostimulatory CpG motifs

In 1893 a pioneering surgeon, William B Coley, reported that many cancer patients could be cured from advanced metastatic cancer by repeated injection of bacterial extracts. Almost a century later it was shown that the anti-tumor properties of the bacterial extracts could be reproduced with purified bacterial DNA, and that little or no anti-tumor activity was observed in bacterial fractions comprising proteins, RNA, lipids or carbohydrates [81]. Bacterial DNA, and viral DNA, differs from mammalian DNA in their methylation patterns and utilization of CpG dinucleotides. In a certain base context, these unmethylated CpG dinucleotides (CpG motifs) are present at much higher frequency in prokaryotic than eukaryotic genomes [82]. DNA containing CpG motifs appears to be taken up in a non specific manner by endocytosis and activates TLR9 [83], that is located in the endosomal compartment of cells. For activation of TLR9, endosomal maturation is required, and co-localization of CpG DNA, TLR9 and MyD88 in the late endosome has been shown to be dependent on phosphatidylinositol 3-kinase (PI3-kinase) [84]. Interaction of TLR9 with CpG motifs induces recruitment of MyD88 to the TIR-domain which then initiates a signalling cascade involving IRAK (IL-1R-associated protein kinase), TRAF6 (tumor necrosis factor receptor-activated factor 6) and TAK1 (TGF- β -activated kinase). This results in activation of either of two signalling pathways; one involving jnk (c-Jun NH₂-terminal kinase) and p38 MAPK-family (mitogen-activated protein kinase) that culminates in activation of the transcription factor AP-1 (activating protein 1); and one involving the IKK complex leading to activation of the transcription factor NF- κ B [85]. Depending on CpG ODN used and target cell type, different immunostimulatory effects can be elicited.

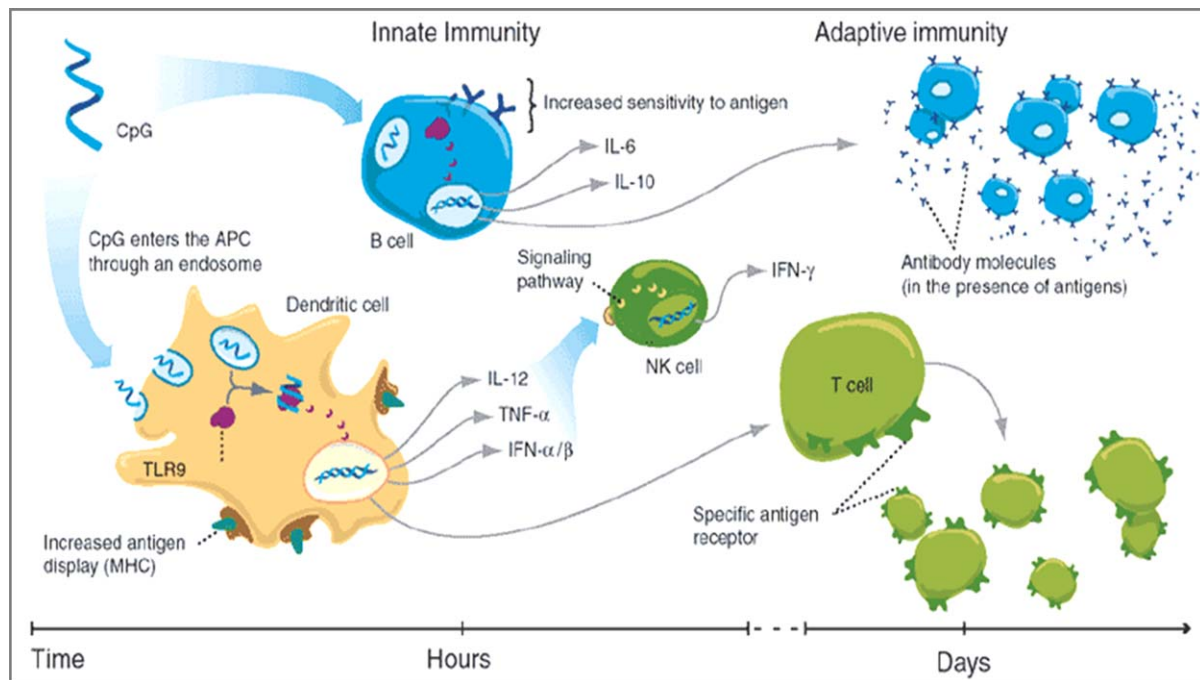


Figure 3. Effect of CpG on innate and adaptive immunity (adopted from [86])

CpG DNA is a potent activator of B cells, stimulating the expression of costimulatory molecules and secretion of IL-6 and immunoglobulins. CpG DNA can also stimulate antigen-presenting cells to secrete IL-12, which then costimulates NK cells to secrete IFN- γ . Dendritic cells stimulated by CpG DNA secrete IL-12, TNF- α , IL-6 and a variety of chemokines (especially those associated with Th1 immune responses), upregulate CD86 expression, and promote increased T-cell activation [87]. In addition, CD4⁺ T cells express TLR9 and CpG ODN has been shown to directly promote activated CD4⁺ T cell survival [88] [89]. A summary of the effect of CpG ODN on innate and adaptive immunity is depicted in figure 3.

The immunostimulatory features of bacterial DNA can be mimicked by synthetic oligodeoxynucleotide (ODN), 8-30 base length and containing one or more CpG motifs. Because the phosphodiester backbone of native DNA is rapidly degraded by serum and cellular nucleases, in vivo applications with CpG ODNs generally use the nuclease-resistant phosphorothioate-modified backbone, which improves their cellular uptake and prolongs the in vivo half-life [90].

The utility of CpG ODN as a Th1-tilting immunostimulator/adjuvant has been reported, either singly or in combination with various antigens, for the induction of both systemic and mucosal immune responses in experimental animal models [90, 91]. In addition, CpG are currently tested in phase I clinical trials against asthma/allergy, phase II clinical trials in combination with vaccine candidates and phase III clinical trials for cancer therapy [92] [93].

AIMS OF THE STUDY

The overall objectives of this thesis were to study the protection and related immune responses induced by prophylactic immunizations against *HSV-2*, and to identify candidate mucosal adjuvants for possible use in a prophylactic mucosal vaccine against genital herpes in mice.

The specific aims were:

- To evaluate the potential of CpG ODN as a Th1-tilting adjuvant in DNA vaccination against genital herpes.
- To study the potential of CpG ODN as a vaginal adjuvant for HSV-2 protein immunization in inducing immune protection against genital herpes.
- To investigate the efficacy of rectal immunization route for live attenuated HSV-2 and recombinant HSV-2 protein immunization in induction of protective immunity against genital herpes.
- To examine the importance of the adaptor molecule MyD88 in innate and adaptive immunity in the female genital tract against HSV-2.

MATERIALS AND METHODS

gD DNA vaccine and HSV-2 strains (Paper I-IV)

The DNA vaccine construct was prepared by first isolating HSV-2 DNA from infected GMK-AH1 cells by using the NucleoSpin virus kit (Clontech laboratories). The gD-gene was PCR-amplified according to a standard protocol using primers: forward 5' CTCTAAGCTTACTAGTCGCCGTTTTTCGTG 3' and reverse 3'CTCTCTCGAGTTTATGCGGAGTCCAAGTCC 5'. This generated a fragment with 1303 bp that, after further purification using the QIAquick PCR purification kit (QUIAGEN, Germany), was inserted into the commercial expression vector PVAX.1 (Invitrogen Corp, CA). Restriction enzymes HindIII and XhoI were used for the cutting of gene and vector, and the gene was subsequently ligated into the vector. After complete sequencing of the new DNA construct using Big dye 3.1 sequencing kit and ABI prism 310 automated sequencer (Applied Biosystems, CA), the PVAX-1 gD was transformed into a chemically competent *E. coli* (Invitrogen Corp, CA) according to the manufacturer's protocol. Plasmid purification was then performed by use of Endofree plasmid Giga kit (Qiagen, Germany), dissolved in endotoxin-free TE-buffer and kept in -20°C until used.

The virus used for vaginal challenge in paper I-IV was HSV-2 strain 333 and the virus used for immunization in papers III and IV was HSV-2 TK⁻ (lacks the gene for thymidine kinase, hence referred to as HSV-2 TK⁻). Briefly, viral stock was made by infecting a monolayer of African green monkey kidney cells (GMK-AH1) with the respective virus strain and was subsequently prepared by one cycle of freezing and thawing followed by removal of cellular debris by centrifugation. In order to ensure that the virus was lacking expression of TK, a test was performed whereby acyclovir was added to the cell culture. In contrast to HSV-2 (333), the HSV-2 TK⁻ were unable to replicate in the presence of acyclovir, thus was truly lacking TK.

For vaginal challenge with virulent HSV-2 (333) in paper I-IV, as well as for immunization studies with HSV-2 TK⁻ in paper III and IV, 9×10^4 PFU of the respective virus was administered in a volume of 15-20 μ l per mouse.

Mice and immunization regimes (Paper I-IV)

Various immunization routes were used. In paper I groups of female C57Bl/6 mice were administered gD DNA vaccine alone intramuscularly (*im*), three times, with up to 10 days intervals, CpG ODN 24h before gD DNA or gD DNA 48h before CpG ODN. Both DNA vaccine and CpG ODN were injected in the same anatomical sites of the mouse hind limb quadriceps muscle. In some experiments, groups of mice were immunized three times via intranasal (*in*) or intravaginal (*ivag*) routes with gD DNA 48h before CpG ODN.

In paper II groups of female C57Bl/6 and outbred NMRI mice were *ivag* vaccinated, once or twice (with 10 days interval) with recombinant HSV-2 gD protein alone or in mixture with CpG ODN.

The same immunization procedure was kept for intrarectal (*ir*) protein immunization in paper III, with the addition of one group administered gD in mixture with cholera toxin (CT). Female C57Bl/6 mice immunized with live/inactivated HSV-2 TK⁻ *ir* were immunized only once.

CpG ODNs (Paper I-III)

The CpG ODN used (paper I-III) was 1826 (TCC ATG ACG TTC CTG ACG TT), a 20-mer containing two copies of a CpG motif with potent immunostimulatory effects on the murine immune system. The control ODN used was (TCC ATG AGC TTC CTG AGC TT), a 20-mer containing no CpG motif. All ODNs were used with complete phosphorothioate backbone. The ODNs were purchased from Operon Biotechnologies GMBH (Germany).

Saponin extraction of tissues (Paper II)

In order to analyze for local cytokine and chemokine response after vaginal administration of CpG ODN, saponin-extracted tissue samples were used. During the extraction procedure, vagina and genital lymph nodes were collected, washed in PBS buffer, weighed and frozen in a PBS containing protease inhibitors, i.e. soy trypsin inhibitor and phenylmethylsulfonyl fluoride, and EDTA. Saponin was added to the solution to permeabilize the cell membranes. The samples were stored at 4°C overnight. The organs were centrifuged down and the supernatant analyzed for their chemokine contents by ELISA. The same method was applied during the analysis of mucosal antibody responses (paper I-III).

Antibody determination (Paper I-IV)

Serum IgG, IgG1, IgG2a and IgG2c

ELISA was used for the determination of HSV-specific antibodies in both sera and vaginal extracts. PVAX.1-gD-transfected CHO-K1 cell lysate (paper I) and recombinant protein gD (paper II and III) were used as coating antigens in gD specific ELISA. For detection of HSV-specific antibodies after HSV-2 TK⁻ immunization, ELISA was done in plates coated with irradiated HSV (paper III and IV).

Proliferation and cytokine assays (Paper II-III)

To enable the study of both the mucosal and systemic antigen-specific T cell responses, we used a proliferation assay in which purified CD11c⁺ cells were co-cultured with cells from either genital lymph node cells or purified splenic CD4⁺ T

cells. Briefly, CD11c⁺ cells were purified using microbeads (Miltenyi Biotec, Germany) and pulsed with HSV-2 antigens over night. After the unused antigens were washed off, the CD11c⁺ cells were co-cultured with purified CD4⁺ T cells. Supernatants were taken after 96 h for the determination of their cytokine or chemokine contents by ELISA or a cytometric bead array (CBA). The proliferation of cells was determined after four days of culturing and measured by ³H labelled thymidine incorporation technique.

Mouse model of HSV-2 infection (Paper I-IV)

In this thesis, a well established and widely used mouse model of genital herpes infection has been used [26]. Briefly, female C57BL/6 mice were first injected subcutaneously with 3.0 mg of Depo-Provera (Upjohn, Belgium) and then after 6 days, challenged intravaginally with 9×10⁴ PFU of HSV-2 strain 333. The progesterone treatment synchronizes the estrus-cycle of the animals and facilitates infection. Genital pathology following HSV-2 challenge was monitored daily and scored on a five-point scale: 0, no signs of infection; 1, slight redness of external vagina; 2, swelling and redness of external vagina; 3, severe swelling and redness of both vagina and surrounding tissue and hair loss in the genital area; 4, hind-limb paralysis ; and 5, death. Animals were sacrificed after they reached stage 4.

To assess viral shedding, vaginal washes from infected mice were analyzed by plaque assay. GMK-AH1 cells were then grown to confluence in 6-well plates. Samples were diluted and added to GMK-AH1 monolayers. Infected GMK-AH1 cells were incubated for 48 h at 37°C in medium containing methyl cellulose, newborn calf sera and penicillin streptomycin. Monolayers were subsequently fixed and stained with crystal violet. The viral plaques were counted under a light microscope. The number of plaque forming units (PFU) per millilitre was calculated by making a plaque duplicate count for every sample.

Immunohistochemistry (Paper III)

In paper III rectal and genital tissues were excised from intrarectally or intravaginally HSV-2 TK⁻ infected mice. Tissue samples were embedded in OCT, snap frozen and stored in -70°C. 8 µm cryo sections were stained with HSV-2 specific monoclonal antibodies, followed by incubation with a secondary antibody conjugated with horse-radish peroxidase and finally developed using 3,3-diaminobenzidine (DAB). The presence of HSV-2 infected cells in the rectal mucosa was controlled by staining with an irrelevant antibody (Rabbit IgG).

Statistics (Paper I-IV)

All statistical analysis was performed using GraphPad Prism software (Software Inc, San Diego, California). In paper I Student's t-test was used for comparison of significance between two groups, and for paper II and III one way ANOVA was used

to compare several groups. $P < 0.05$ was considered significant. In figures in paper I-IV * means $p < 0.05$, ** means $p < 0.01$ and *** means $p < 0.001$.

RESULTS AND DISCUSSION

CpG oligodeoxynucleotide augments gD DNA vaccine efficacy to generate T helper 1 response and elicit protection against primary genital herpes infection in mice (Paper I)

In this study the combined use of immunostimulatory CpG ODN and DNA vaccine encoding HSV-2 glycoprotein D (gD DNA) was investigated. Other research groups have demonstrated that immunity against genital herpes can be induced by intramuscular (*im*) DNA vaccination. However, the protection levels varied and none of them conferred complete protection [94, 95] [96]. Hence, we were interested in examining whether the efficacy of HSV-2 DNA vaccination could be improved by combined use of CpG ODN. Previous studies had indicated a decrease in antigen expression from a DNA vaccine when CpG ODN was administered in a mixture with the vector, possibly because of competition for cellular uptake [97]. Our working-hypothesis was that if CpG ODN is delivered prior to gD DNA, APCs is likely to be attracted to the injection site and thus leads to antigen uptake by APCs that would eventually initiates an antigen specific immune response. By contrast, if CpG ODN is instead administered after gD DNA an inflammatory milieu is induced while antigens were maximally expressed from the vector, leading to increased immune response. We found that gD is maximally expressed 48h after *in vitro* transfection of CHO.K1-cells, and the main bulk of gD protein was found in CHO.K1-cell lysates and not in the supernatants.

Im immunization of mice with gD DNA, three times with one week interval, resulted in a potent gD-specific IgG response in both serum and saponin-extracted vaginal tissues (I, Figure 2A). When CpG ODN was injected at the same anatomic site 24h before or 48h after gD DNA, no change in serum/vaginal gD-specific IgG levels was observed. However, the gD DNA→CpG ODN group showed a doubling of serum gD-specific IgG2c-to-IgG1 ratio as compared to those of other groups (I, Figure 2B). Since IgG2a gene is replaced by IgG2c gene in C57Bl/6 mice, IgG2c isotype is thought to be associated with a Th1 type immune response in C57Bl/6 mice [98].

Moreover, splenocytes, splenic purified CD4⁺ T cells and genital lymph node cells from gD DNA→CpG ODN immunized mice also displayed an enhancement in gD-specific proliferation as compared to the other groups (I, Figure 3A and 4A). This shows that the immunization in fact resulted in an amplified gD-specific immunity. The most important correlate for protection against genital herpes however, is the production of IFN- γ by proliferating antigen-specific cells. *In vitro* recall gD stimulation of spleen cells (I, Figure 3B), splenic CD4⁺ T cells (not shown) and gLN

cells (I, Figure 4B) from the gD DNA→CpG ODN immunized mice also showed increased gD specific IFN- γ response as compared to the other groups.

In agreement with an enhanced gD-specific cellular proliferative and IFN- γ response in the gD DNA→CpG ODN immunized mice, this group was the only group that had undetectable viral titers following a virulent intravaginal HSV-2 challenge and survived the challenge without showing any macroscopic signs of disease (I, Figure 5A and Table 1).

In contrast to the increased immune response seen in mice immunized with gD DNA→CpG ODN, administration of CpG ODN prior gD DNA reduced the observed immune responses. Hence, CpG ODN→gD DNA immunized group developed similar total serum gD-specific IgG, but not enhanced IgG2c-to-IgG1 ratio (I, Figure 2A and B), and actually showed significantly lower gD-specific proliferative responses by spleen/CD4⁺ T cells and gLN cells as compared to gD DNA→CpG ODN (I, Figure 3A and 4A). More so, spleen/CD4⁺ T cells and gLN cells from this group also produced less IFN- γ than the other immunized groups (I, Figure 3B and 4B). Following HSV-2 challenge, the same group displayed increased viral replication in vaginal fluids as compared to the other immunized groups and some mice also developed mild symptoms of the disease (I, Figure 5A and Table 1). The likely reason to why CpG ODN administration prior gD DNA was inferior to even gD DNA alone in inducing gD-specific immunity is discussed in the following. After CpG ODN injection, tissue resident antigen-presenting cells respond very quickly (within hours) to CpG motifs, undergo maturation and migrate to the regional lymph node in the absence of expressed gD. It is possible that for example 4 or 8 hours prior gD DNA would have been a better time point.

While gD DNA→CpG ODN immunization protocol was successful for intramuscular vaccination, mucosal intranasal or intravaginal immunization failed in conferring mice with any appreciable levels of protection. Others have reported substantial immunity afforded by intranasal vaccination with DNA vaccines [99] [100] [101], especially when adjuvants such as cholera toxin are co-administered, or liposomes used as delivery system for the plasmids. However, in our hands even when gD DNA is delivered in lipofectamine, no antigen-specific immunity was elicited (not shown). In order to ensure that plasmids are not entangled in mucus, we have also performed extensive vaginal washing with PBS prior administration and did not achieve any better results (not shown). One completely different explanation could be promoter attenuation. Our gD gene is as previously described under control of the CMV promoter. In vitro studies of transfected cells has demonstrated that this promoter, as well as a wide range of other commonly used promoters in plasmids, are inhibited in the presence of IFN- γ and TNF- α [102]. The exact mechanism for promoter attenuation is not fully understood, but it has been shown that inhibition is

at mRNA level and that the cytokines do not cause plasmid DNA degradation, inhibit total cellular protein synthesis or kill transfected cells. In addition, neutralizing antibodies against these cytokines were shown to enhance transgene expression in vivo [102]. The DNA vector, being of microbial origin, naturally contains CpG motifs. Since intravaginal administration of CpG ODN had previously been shown to induce a rapid and very high production of the CC chemokine RANTES [103], in a smaller follow up study we compared the gD expression of transfected CHO-cells in the presence of RANTES. In agreement with previous studies, IFN- γ and TNF- α , especially in combination with each other, significantly reduced the expression of gD from the vector (Figure 1 and appendix 1). Interestingly, RANTES alone resulted in a nearly complete abrogation of gene expression. This suggests that promoter attenuation is an important inhibitory mechanism in vaginal gD DNA vaccination and warrants further studies.

Mucosal administration of CpG DNA elicits robust CC and CXC chemokine responses in the vagina and serves as potent Th1-tilting adjuvant for gD protein vaccination against genital herpes (Paper II)

In this study, the impact of vaginal administration of immunostimulatory CpG ODN on local chemokine responses in mice was examined. Furthermore, the potential of using CpG ODN as a vaginal adjuvant in protein immunization protocol for induction of immunity against HSV-2 was investigated.

Chemokine production after vaginal CpG ODN administration

The CC and CXC chemokines studied in this paper are known to be important for recruitment of various immune cells (Table 1) to the site of infection/immunization. In addition, RANTES, together with MIP-1 α and MIP-1 β have been shown to be associated with development of a Th1 type of immune response [104]. Importantly, these CC chemokines are natural ligand for HIV-1 coreceptor CCR5 [105].

Table1 . Chemokines studied in Paper II.

Chemokine	Produced by	Recruitment of
MIP-1 α (CC)	Monocytes, macrophages, T cells, neutrophils, eosinophils	Monocytes, T cells, neutrophils, NK-cells, DC [47] [106]
MIP-1 β (CC)	Macrophages, T cells, B cells, neutrophils,	Monocytes, T cells, basophils [47]
RANTES (CC)	Monocytes, macrophages, T cells, epithelial cells	Monocytes, T cells, NK cells, DC [47]
IP-10 (CXC)	Monocytes, lymphocytes, endothelial cells	T cells [107]
MIP-2 (CXC)	Fibroblasts, endothelial cells, monocytes	Neutrophils [108] [109]

We found that administration of a single dose of CpG ODN to naïve C57Bl/6 mice resulted in a rapid response of all the chemokines studied in the saponin extracts of vagina, and genital lymph nodes. Except for a transient and low induction of IP-10, none of these chemokines could however be detected in the blood after intravaginal administration, suggesting that the response was local and not systemic.

CpG ODN as adjuvant for vaginal vaccination

As CpG ODN was efficient as inducer of chemokines associated with a Th1 type response, we next sought to investigate its potential as a mucosal adjuvant in vaginal protein gD immunization. Groups of female C57Bl/6 mice were twice, with 10 days interval, intravaginally immunized with CpG ODN, gD or gD in mixture with CpG ODN. Four weeks after the last immunization, gD-specific IgG antibody was found in sera and, to a lower extent, the vaginal extracts of only the gD+CpG ODN immunized mice (II, Figure 3A). The same group also had a higher production of IgG2c than IgG1 antibody (II, Figure 3B), indicating that the induced immune response was Th1-directed.

The impact of CpG ODN as an adjuvant for vaginal immunization with gD in induction of cell-mediated immune response was examined by in vitro gD recall stimulation of genital lymph node (gLN) cells or splenic CD4⁺ T cells from vaccinated mice. As expected, control, CpG ODN and gD groups displayed low gD-specific gLN and CD4⁺ T cell proliferative responses (II, Table 1, Figure 1A and B). By contrast, a potent gD-specific proliferative response was observed in gLN as well as CD4⁺ T cells from gD+CpG ODN immunized mice (II, Table 1; Figure 1A,B).

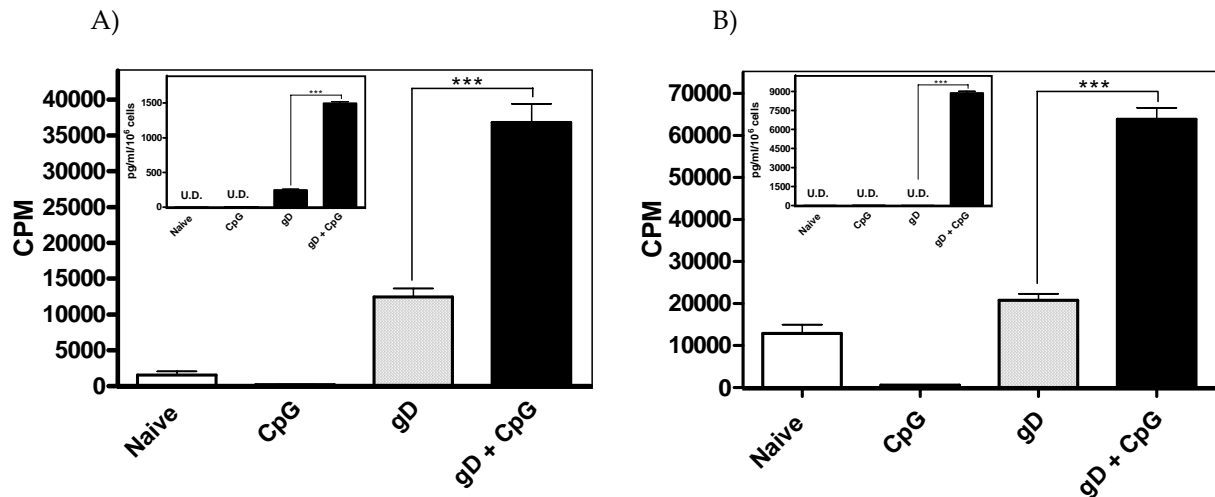


Figure 1. In vitro gD-specific proliferative and IFN- γ responses (inlay) by A) gLN cells and B) CD4⁺ T cells

As previously mentioned, the most important correlate of protection against genital herpes infection is the production of IFN- γ by especially CD4⁺ T cells. While the levels of IFN- γ were undetectable or very low in gLN/CD4⁺ T cell culture supernatants from control, CpG ODN and gD group, a high production was observed in gLN as well as CD4⁺ T cells from gD+CpG ODN immunized group (II, Table 1, Figure 1A,B). In addition, we also examined the levels of MIP-1 β , as a representative of CC chemokines associated with the development of Th1 response, and found that the levels of this chemokine is significantly higher in gLN/CD4⁺ T cell culture supernatants from the gD+CpG ODN immunized mice than other immunized groups (II, Table 1).

Somewhat unexpected observation was the trend seen in gLN and CD4⁺ T cell proliferation by CpG ODN immunized group. This group actually showed a diminished proliferative response as compared to control group, suggesting that CpG ODN when repeatedly administered without antigen can lead to exhaustion of cellular immunity. Several publications have demonstrated that CpG ODN acts as a survival factor for CD4⁺ T cells [88] [89]. Nonetheless, repeated systemic CpG ODN administration has recently been shown to induce lymphoid follicle destruction and immunosuppression in mice [110].

In order to determine the strength of immunity elicited by ivag immunization regimes, ivag immunized mice as well as control unimmunized mice were challenged four weeks after the last immunization with a lethal vaginal inoculation of virulent HSV-2. In agreement with the in vitro data, the gD+CpG ODN immunized mice showed significantly lower levels of HSV-2 replication in their vaginal fluids as compared to the other groups, and also 80% resisted the challenge with no or very low pathology-scores (II, Figure 4A, B, C). To ensure that the observed response was due to recognition of CpG motifs in the

oligodeoxynucleotide, a group was included that instead was immunized with gD in mixture with a control GpC ODN containing no CpG motif. This group showed high levels of viral replication and were unable to clear the infection (II, Figure 4 A, B, C). In addition, we could document that twice immunization is required for induction of immune protection as mice immunized ivag with gD+CpG ODN were not protected against a subsequent HSV-2 challenge (II, Figure 6).

Vaginal CpG ODN responses in outbred NMRI mice

We also investigated the impact of vaginal administration of CpG ODN on induction of chemokine response as well as the potential of CpG ODN as vaginal adjuvant for gD immunization in outbred NMRI mice. We found that the chemokines induced in the vagina of inbred C57Bl/6 mice following vaginal administration of CpG ODN could be induced in the vagina of outbred mice after CpG treatment, albeit to a lower degree and with delayed kinetics (II, Figure 2). In contrast to inbred mice however, we were not able to detect these chemokines in the gLN extracts from outbred mice. Following twice immunization with gD+CpG ODN, outbred NMRI mice developed adaptive immunity that protected 60% from lethal challenge, and the remaining showing mild symptoms of the disease (II, Figure 5A, B, C). This demonstrates that vaginal administration of outbred NMRI mice, with a mixed genetic background, could elicit a chemokine response, and that twice vaginal immunization of NMRI mice with gD+CpG ODN conferred substantial protection.

Rectal immunization confers a sex hormonal- and MyD88-independent protective immunity against genital herpes simplex virus type 2 infection in mice (paper III)

In this study, we sought to investigate if rectal route of immunization could be used for induction of immunity in the female genital tract. Since induction of immunity following vaginal immunization has been reported to be very much dependent on the sex hormones progesterone and estrogen [26] [36], we also examined if this could be overcome by using the rectal route of immunization. One time vaginal administration of a live attenuated strain of HSV-2 lacking the gene for thymidine kinase (TK⁻) is known to induce a strong and complete protective immunity against a subsequent virulent HSV-2 challenge [26]. Hence, mice were immunized once with HSV-2 TK⁻ by rectal or vaginal route for comparison. In order to examine if a productive infection is required to provoke an immune response, groups of mice were also immunized with the same dose of inactivated HSV-2 TK⁻.

Rectal immunization with HSV-2 TK⁻ confers protective immunity against subsequent virulent vaginal challenge, independently of sex hormonal status.

We first began by verifying that live HSV-2 TK⁻ is able to infect cells in the rectal

mucosa. Immunohistochemistry staining with HSV-2 specific antibodies were used to detect infected cells in rectal tissues after rectal administration of HSV-2 TK⁻. Numerous infected cells could be detected in rectal tissues 24h after viral inoculation, and even 7 days after initial infection some HSV-2 infected cells were present (III, Figure 1 A-F). However, we were not able to detect infected cells in vaginal tissues from rectally infected mice at any time point, excluding the possibility of a leakage of virus inoculum from the rectum to the vagina.

Next, we asked if rectal immunization with live HSV-2 TK⁻ resulted in HSV-2 specific mucosal and/or systemic immunity. Thus, one month after immunization, the HSV-specific proliferative response by gLN and splenic CD4⁺ T cells were examined. As expected, vaginally HSV-2 TK⁻ immunized mice displayed very high HSV-specific gLN/CD4⁺ T proliferative and IFN- γ responses (III, Figure 2 A-D). Control and mice immunized intravaginally or rectally with inactivated virus showed low levels of proliferation and IFN- γ (III, Figure 2 A-D). Interestingly, rectal immunization with HSV-2 TK⁻ resulted in significantly increased levels of gLN/CD4⁺ T cell proliferation as compared to rectal immunization with inactivated virus (III, Figure 2 A, B). Yet, the levels of IFN- γ for the same group were significantly increased in CD4⁺ T, and not in gLN, cell culture supernatants (III, Figure 2 C, D). Most importantly, the rectally HSV-2 TK⁻ immunized mice developed potent protective immunity against subsequent HSV-2 challenge, as very low levels of HSV-2 replication were detected in the vaginal fluids and 100% of these mice survived the challenge with no or mild vaginal inflammation (III, Figure 4 A-C).

It has been shown by other research groups that vaginal immunization of mice with HSV-2 TK⁻ that are under the influence of progesterone results in complete protection against subsequent challenge, whilst protection is inhibited if mice are under the influence of estrogen [26]. We therefore next wanted to investigate if induction of immunity following rectal immunization with HSV-2 TK⁻ is under the influence of the sex hormones progesterone and estrogen. Hence, groups of mice were treated with progesterone or estrogen 6 days before rectal immunization or left untreated. One month after immunization, the animals were given a lethal HSV-2 challenge. Intravaginal immunization with HSV-2 TK⁻ was, as previously reported, affected by sex hormonal treatment. However, neither progesterone nor estrogen treatment had any impact on immunity afforded by rectal immunization as all mice, irrespective of their hormonal status, survived the challenge with no symptoms of the disease (III, Table 1).

Induction of immunity following rectal HSV-2 TK⁻ immunization does not require MyD88 signalling

HSV-2 has been reported to engage both TLR2 and TLR9, both which signals via the adaptor protein MyD88 [51]. This prompted us to examine if rectal immunization

with HSV-2 TK⁻ requires MyD88 for induction of protective immunity against HSV-2. To our surprise; however, rectally HSV-2 TK⁻ immunized MyD88^{-/-} mice mounted an even higher HSV-specific IgG antibody response (III, Figure 5A) and an increased production of IgG2c in comparison to rectally immunized C57Bl/6 mice (III, Figure 5B). More so, rectal HSV-2 TK⁻ immunization provided MyD88^{-/-} mice with a complete protection against subsequent vaginal virulent HSV-2 challenge (III, Table 2). These results show that MyD88 is not required for induction of an adaptive immune protection against genital herpes elicited by rectal HSV-2 TK⁻ immunization.

Rectal immunization with gD in mixture with CT, but not CpG ODN, induces potent protective immunity against genital herpes

Next, we examined if rectal immunization with HSV-2 gD protein and a TLR-targeting adjuvant CpG ODN or a non TLR targeting adjuvant CT could elicit immunity in the female genital tract. Hence, mice were immunized rectally with glycoprotein D (gD) in mixture with CpG ODN or cholera toxin (CT) and the resulting immunity. In contrast to intravaginal gD+CpG ODN immunization (paper II), rectal immunization with gD+CpG ODN neither induced gD-specific humoral and cell-mediated responses (III, Figure 6A-E) nor protective immunity against vaginal HSV-2 challenge (III, Figure 7A-C). By contrast, rectal immunization with gD+CT induced high proliferative gLN/CD4⁺ T cell (III, Figure 6A,C) and IFN- γ responses (III, Figure 6B,D). Importantly, the gD+CT immunized mice showed substantial protection against subsequent vaginal challenge as low levels of viral replication were detected, and 80% of these animals survived the challenge without any pathology scores (III, Figure 7A-C).

Our finding that CT, but not CpG ODN, could serve as adjuvant in rectal gD protein immunization is in agreement with a recent report that rectal immunization of mice with rotavirus-like particles in mixture with CT or *E. coli*-derived heat-labile toxins, but not TLR ligands CpG ODN or resiquimod, could elicit protective immunity against enteric rotavirus challenge [111].

In a follow-up study (Appendix 2), we examined the importance of MyD88 in immunity afforded by rectal gD+CT immunization. While, the immunized C57Bl/6 mice were substantially protected, rectal gD+CT immunization failed to elicit a considerable level of protection in MyD88^{-/-} mice as only one third of the immunized MyD88^{-/-} mice survived the challenge dose. One explanation could be that live HSV-2 TK⁻ infection at the rectal mucosa results in necrosis, which leads to the release of various inflammatory stimuli such as heat-shock proteins. Although some heat-shock proteins have been demonstrated to activate the innate immune system via TLRs, others activate scavenger receptors [112]. Hence, necrosis as a result of live infection probably causes the release of other inflammatory stimuli that have a redundant function to TLR/MyD88. As for CT, there is no prior report indicating the

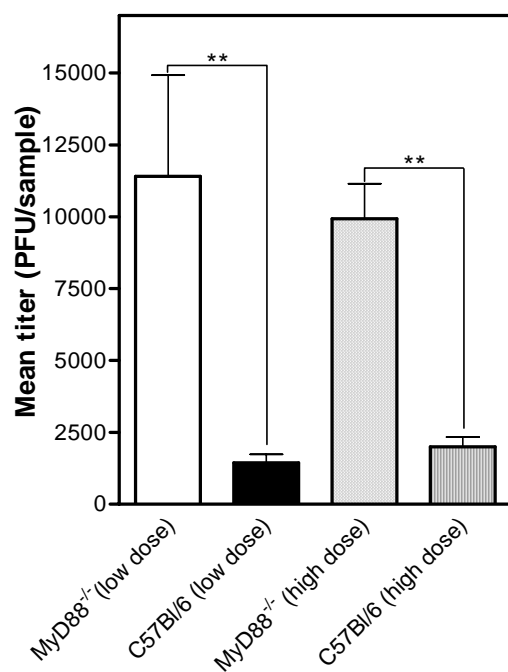
involvement of TLR in the adjuvant effect of CT. However, CT is known to induce IL-1 response in macrophages [113] and presumably other APCs. Since IL-1 receptor requires the usage of MyD88 [50], it is likely that IL-1 receptor/MyD88 signalling rather than TLR/MyD88 signalling is involved in the adjuvant effect of CT. Further studies are needed to clarify the role of MyD88 in induction of immunity via rectal immunization route.

Importance of MyD88 for innate and adaptive immune protection against genital herpes infection in mice (paper IV)

In this study we wanted to examine the importance of MyD88 signalling for innate immune protection against primary genital herpes infection. Furthermore, we wanted to investigate if MyD88 was essential for induction of acquired immunity in the female genital tract following vaginal HSV-2 TK⁻ immunization.

Innate immune protection against primary genital herpes is MyD88-dependent

Firstly, naïve C57Bl/6 and MyD88^{-/-} mice were subjected to a high or low vaginal HSV-2 challenge dose. Three days after challenge, the viral replication were significantly higher in MyD88^{-/-} than C57Bl/6 mice given a high challenge dose and furthermore MyD88^{-/-} mice developed signs of the disease faster and died as a result of neurological illness 2-3 days before C57Bl/6 mice (IV, Figure 1A-C). Still at a 10-fold lower dose, MyD88^{-/-} had the same level of replicating virus in vaginal washings as MyD88^{-/-} challenged with a high dose (IV, Figure 1A and below). In agreement



with the viral replication both low and high dose challenged MyD88^{-/-} also developed disease significantly earlier than C57Bl/6 mice (IV, Figure 1B) and all of them succumbed to the low dose infection, whereas 40% of C57Bl/6 mice resisted the low challenge dose (IV, Figure 1C). This result demonstrates that the usage of MyD88 is of fundamental importance for innate immune protection against genital herpes.

Figure 2. Mice lacking MyD88 are more susceptible to vaginal HSV-2 infection.

Acquired immunity against HSV-2 is not MyD88-dependent

In order to investigate if MyD88 signalling is required for induction of adaptive immunity following vaginal immunization, MyD88^{-/-} as well as C57Bl/6 mice were vaginally immunized with HSV-2 TK⁻. As could be seen in rectally HSV-2 TK⁻ immunized mice (Study III), a stronger HSV-specific IgG response was induced by vaginal immunization of MyD88^{-/-} than C57Bl/6, however the increase in IgG2c level seen following rectal vaccination was not observed after vaginal immunization of MyD88^{-/-} mice (IV, Figure 2A,B). Still, the levels of IgG1 were significantly increased in MyD88^{-/-} mice. Both C57Bl/6 and MyD88^{-/-} mice developed comparable levels of HSV-specific cellular response. Comparable levels of gLN/CD4⁺ T cell proliferation (IV, Figure 3A and 4A) as well as IFN- γ (IV, Figure 3B and 4B) were detected. Also, similar to rectal immunization (III), vaginal HSV-2 TK⁻ immunization resulted in complete protective immunity in MyD88 deficient mice (IV, Figure 5A-C).

The finding that MyD88 did not seem to be required for initiation of acquired immunity following either vaginal or rectal HSV-2 TK⁻ immunization was somewhat unanticipated as MyD88 has been recently shown to be critical for the adaptive immunity against systemic viral infections. MyD88^{-/-} mice were shown to display a defective CD8⁺ T cell response accompanied by persistent viral infection following lymphocytic choriomeningitis virus (LCMV) infection [114]. Also, MyD88^{-/-} mice was shown to display a diminished ability in activation of DCs and lower percentage of IFN- γ producing CD8⁺ T cells following vaccinia virus (VV) infection as compared with wild type mice [115]. The difference in requirement of MyD88 for the development of adaptive immunity following different viral infections could possibly be explained by the fact that while LCMV and VV rely on CD8⁺ T cells for protection, CD4⁺ T cells were shown to play a crucial role in protection against genital HSV-2 infection [63]. Another possible explanation could be the route of virus challenge. Thus, while HSV-2 challenge is at the genital tract (mucosal site), both LCMV and VV were used for systemic challenge. Perhaps MyD88 is critical for the development of systemic protection, but that redundancy exists at mucosal surfaces.

Appendix 1. RANTES blocks gD expression in gD DNA transfected CHO-cells *in vitro*.

Following the failure in targeting the genital tract mucosa for DNA immunization, we conducted a series of experiments in order to facilitate uptake of DNA vaccine and also understand the underlying mechanism. First, we formulated gD DNA vaccine in chitosan, known to facilitate uptake of antigens across the mucosal tissues, or lipofectamine, known to facilitate DNA uptake by mammalian cells, and used the formulated gD DNA in separate vaginal immunization settings. Results showed that such formulations did not help eliciting any better immune protection than naked gD DNA (not shown).

Since plasmids are of microbial origin, they naturally contain CpG motifs, and thus one possibility is that these motifs are activating an innate immune response that somehow interferes with gD DNA uptake and/or expression through promoter attenuation. Previous studies showed that expression from the CMV promoter, as well as other commonly used promoters, are inhibited by the cytokines IFN- γ and TNF- α [102] [116]. This attenuation was shown to be at mRNA level and these cytokines did not cause vector degradation, nor inhibited total cellular protein synthesis or killed transfected cells. The notion that there is a rapid and high release of the CC chemokine RANTES in the vaginal mucosa following local application of CpG ODN [103], led us to examine if RANTES had any impact on expression of gD from gD DNA transfected cells *in vitro*. Hence, CHO-cells were transfected with 2 μ g of gD DNA per 5×10^5 cells by 30 μ g lipofectamine plus reagent (GIBCO-BRL, US), and after 5h CHO-cells were washed in order to remove residual transfection reagents. Subsequently, medium alone, or medium supplemented with 5 ng/ml of recombinant (r) IFN- γ , 5 ng/ml of rTNF- α , a combination of the two or 5 ng/ml of rRANTES were added to the transfected cells. 30 hours after transfection, cells were lysed using Triton-X and lysates were examined for gD contents by a gD-specific ELISA. The results showed a decrease in gD expression by IFN- γ and TNF- α singly and a further decrease when combination of the two was used. Interestingly, RANTES abolished gD protein expression almost completely (Figure 3).

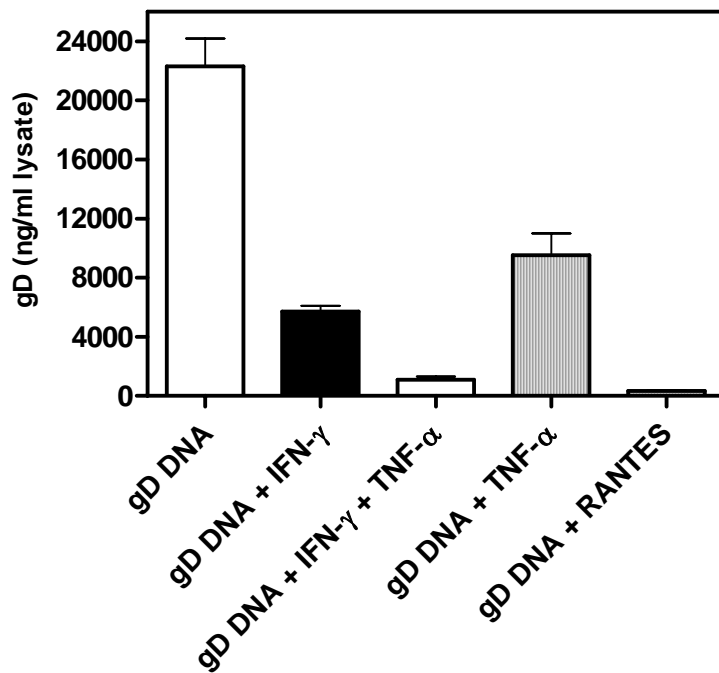


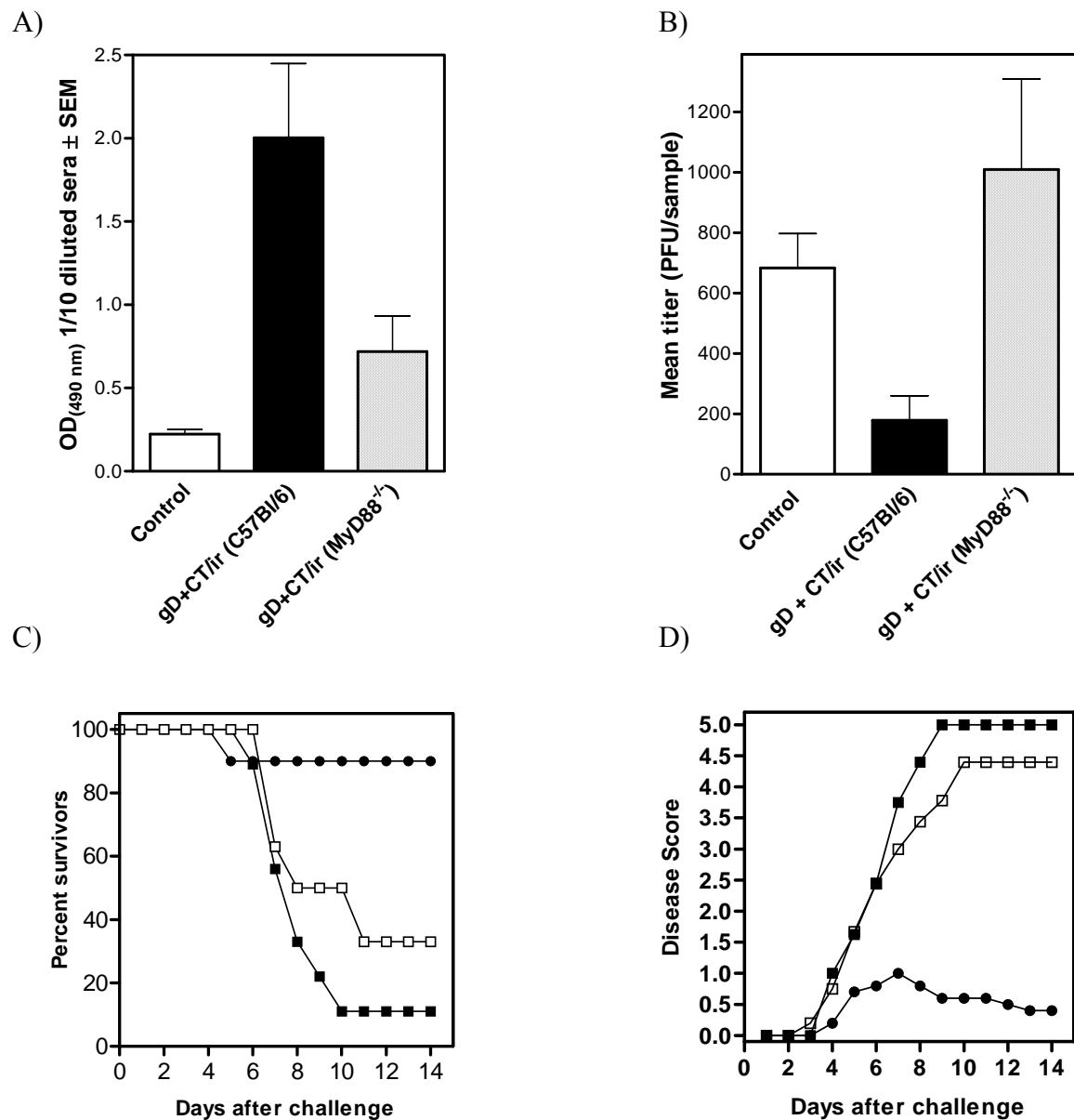
Figure 3. RANTES blocks expression of gD from gD DNA transfected cells in vitro.

These results suggest that RANTES produced in the female genital tract mucosa in response to CpG motifs present in the gD DNA vector, can inhibit gD gene expression in vitro that may explain the failure of vaginal immunization with gD DNA vaccine. However, further studies, including use of plasmid vector devoid of CpG motif are needed to further explore this issue.

Appendix 2. MyD88 is required for induction of immunity against genital herpes following rectal immunization with gD + CT

Since cholera toxin (CT) was more potent than CpG ODN in inducing protective immunity against genital herpes in rectal immunization with protein gD, we wanted to investigate if MyD88 signalling is required for induction of adaptive immunity following rectal immunization with gD + CT. To this end, C57Bl/6 and MyD88^{-/-} mice were rectally immunized twice, with 7-10 days interval, with 5 μ g gD + 10 μ g CT, and the resulting immunity was examined 3 weeks after the last immunization. To our surprise, although C57Bl/6 mice were protected against subsequent vaginal virulent HSV-2 challenge, MyD88^{-/-} mice failed to mount a protective immunity. As depicted in Figure 4A, lower levels of gD-specific IgG were detected in serum from immunized MyD88^{-/-} mice as compared to immunized C57Bl/6 mice. Also, high viral replication was detected in vaginal fluids from control as well as MyD88^{-/-} gD+CT/ir immunized mice, while C57Bl/6 gD+CT/ir mice had low levels of replicating virus (Fig.4B). Also, the immunized C57Bl/6 mice showed very low symptoms of the disease and 90% survived the challenge, while the majority of the gD+CT immunized MyD88^{-/-} mice developed rapidly progressive disease (Figure 4C,D).

Figure 4. MyD88 is required for induction of adaptive immunity following rectal immunization with gD in mixture with CT. A) gD-specific IgG response in serum following rectal immunization with gD+CT in C57Bl/6 and MyD88^{-/-} mice, B) Viral replication three days after a vaginal HSV-2 challenge, C) disease severity and D) survival following challenge. Control (■), gD+CT C57Bl/6 (●) and gD+CT MyD88^{-/-} (□).



These results suggest that acquired immunity following rectal immunization with protein gD in mixture with CT, in contrast to what was observed for live HSV-2 TK-rectal immunization, is dependent on MyD88.

CONCLUDING REMARKS

In this thesis, efforts have been made to develop novel immunization approaches to elicit immunity in the female genital tract with special emphasis on immunity against the sexually transmitted pathogen HSV-2. This virus is the causative agent of genital herpes, a disease that has increased dramatically in the trace of the HIV endemy. The disease burden of genital herpes is enormous, especially in sub-Saharan Africa, where prevalence of over 70% has been reported. In fact, many in the research community today believe that the first step towards fighting HIV-spread is to combat genital herpes.

The quest for an efficient prophylactic vaccine against HSV-2 has been ongoing since decades, and several vaccine-candidates have reached advanced clinical trials. Yet, the results so far have been disappointing. Hence, intramuscular immunization with gD2 and gB2 recombinant glycoproteins from herpes formulated in MF59 (emulsion adjuvant) or in AS04 (Alum + monphosphoryl lipid A) induced high antibody titers, but failed in conferring protection (MF59, [64]) or conferred protection only in females that were seronegative for both HSV-1 and HSV-2 (AS04, [66]). Moreover, using knockout mice, it has been demonstrated that although strong vaccination elicits both systemic and mucosal antibody responses, HSV-2 specific antibody response is dispensable for protection. By contrast, while a full protection was induced in animals lacking CD8⁺ T cells, animals lacking CD4⁺ T cells were unable to combat the infection. Specifically, protection has been shown to require IFN- γ producing CD4⁺ T cells. Thus, based on the very limited success of clinical trials that induced high specific antibody titers, in addition to studies in experimental models of genital herpes, it is reasonable to conclude that an efficient and protective vaccine against HSV-2 requires induction of a strong CD4⁺ T cell immunity (Th1 type immunity).

Microbial DNA and synthetic ODN containing immunostimulatory CpG motifs are recognized by TLR9. Upon ligand binding, TLR9 activates a signalling pathway that, via the adaptor protein MyD88, leads to activation of the transcription factor NF- κ B, which results in the production of pro-inflammatory cytokines and chemokines. When administered at mucosal surfaces CpG ODN has been shown to induce a production of Th1 polarizing cytokines and chemokines such as IL-12 and RANTES. Hence, due to the fact that protection against genital herpes is associated with aTh1 immunity, we examined the possibility of induction of potent immunity by including CpG ODN as an adjuvant in different immunization protocols. Thus, in our first study (I) we could demonstrate that CpG ODN administered intramuscularly after, but not prior, intramuscular immunization with a DNA vaccine encoding HSV-2 gD

resulted in an enhanced Th1 type immunity and sterilizing protection against a subsequent vaginal challenge. While other studies have demonstrated variable levels of protection against vaginal virulent HSV-2 challenge following intramuscular DNA vaccination, our study is actually the first to show complete protection by DNA vaccination. However, mucosal immunization with gD DNA vaccine failed in inducing any appreciable levels of protection. Therefore, we focused on the development of other mucosal immunization approaches involving recombinant HSV-2 gD protein in the following studies.

Previous work from our group demonstrated that CpG ODN administration at the vaginal mucosa one day prior challenge could provide mice with innate immune resistance against HSV-2 infection [103]. In the second study of this thesis, we show that the CC chemokines MIP-1 α and MIP-1 β as well as the CXC chemokines MIP-2 and IP-10 are rapidly induced following vaginal administration of CpG ODN. RANTES, MIP-1 α and MIP-1 β are associated with Th1 type immunity and attract various immune cells, such as monocytes, dendritic cells and Th1 cells to the inflamed tissue. In addition, MIP-2 attracts mainly neutrophils that have been shown to be very important for innate resistance against HSV-2, and IP-10 mainly activated T cells. Taken together, vaginal administration of CpG ODN alerts the immune system and attracts innate immune cells such as neutrophils for immediate resistance and also antigen-presenting cells such as macrophages and dendritic cells for initiation of an adaptive immunity, as well as activated T cells to exert their effector function.

Even though sexually transmitted pathogens are capable of eliciting immune response in the FGT, vaginal immunization with non-replicating antigens elicits little or no antigen specific local immune response. Given the fact that CpG ODN is a potent inducer of Th1 type immunity in the vaginal mucosa, we next addressed the question if CpG ODN could serve as a vaginal mucosal adjuvant for recombinant gD protein vaccination (II). Most interestingly, CpG ODN in mixture with gD turned out to be very efficient for eliciting gD-specific local as well as disseminating acquired immunity. Therefore, CpG ODN was shown to serve as a potent, and thus far the only, vaginal adjuvant capable of eliciting a strong acquired immune protection against a sexually transmitted pathogen.

While the genital tract is an attractive site for immunizations against sexually transmitted pathogens, genital tract immunity resulting from vaginal immunization appears to be greatly influenced by sex hormones, which in turn may limit the use of vaginal immunization in humans. Therefore, we further investigated if the rectal route of immunization could be used for induction of immunity in the female genital tract (III). By using a live attenuated HSV-2 TK⁻ strain we could show that rectal

immunization provides mice with potent protection independent of sex hormonal influence. In contrast to vaginal gD+CpG ODN immunization, CpG ODN failed to serve as an adjuvant for rectal gD immunization. However, rectal immunization with gD in mixture with CT induced high gD-specific immunity and conferred protection against vaginal challenge. Based on this finding, rectal mucosal immunization should be considered in future vaccine development studies for induction of immunity in the female genital tract against sexually transmitted pathogens.

In order to better understand the requirements for immunity afforded following mucosal vaccination, mice lacking the gene for MyD88 were immunized rectally or vaginally with HSV-2 TK⁻ (III and IV). Surprisingly, with either route of immunization, the immunized MyD88^{-/-} mice were able to mount a fully protective immunity against HSV-2. Nonetheless, MyD88 was shown to be important for innate immune resistance against primary HSV-2 infection (IV). Finally, in a follow up study (Appendix 2) we observed that rectal immunization with gD in mixture with CT, in contrast to rectal live HSV-2 TK⁻ immunization, failed in conferring any appreciable level of protection in MyD88^{-/-} mice. These observations illustrate that the requirement of MyD88 for induction of immunity in the female genital tract is dependent on the nature of vaccine/adjuvant, and that this information should be taken into account in designing a mucosal vaccine against genital herpes.

To summarize, in this thesis we have shown that CpG ODN is a potent inducer of the chemokines RANTES, MIP-1 α and MIP-1 β in the female genital tract mucosa. This finding may have important implications since these chemokines are natural inhibitors of HIV infection. Also, we show that CpG ODN is a potent adjuvant for vaginal immunization with recombinant protein, and elicits local as well as disseminating systemic immunity. This may be of fundamental importance for immunization against pathogens such as HIV, where both strong local and systemic immunity following vaccination is desirable. Finally, we show that rectal route of immunization elicits both systemic and vaginal immunity and has the advantage of being unaffected by the female sex hormones. These results have implications for the development of vaccines to generate immunity in the female genital tract against sexually transmitted infections.

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