

Immunization approaches and molecular signatures for mucosal immunity to primary and recurrent genital herpes

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Kompendiet

Till min familj



ABSTRACT

Genital herpes is most commonly caused by herpes simplex virus type 2 (HSV-2), and is a prevalent sexually transmitted infection worldwide. Despite numerous efforts, there is currently no licensed vaccine against the disease. This thesis evaluates the potential of different immunization strategies to engender protective immunity to genital herpes, using animal models of HSV-2 infection. Studying early molecular and cellular signatures of vaginal immunity to genital herpes represents the secondary objective of this thesis.

A well-established mouse model of genital herpes was used to investigate immunogenicity and protection against primary genital HSV-2 infection. A guinea pig model, which displays a HSV-2 infection that closely resembles the pathogenesis and symptoms of the disease in humans, was employed for studying the impact of immunization on the establishment of latency and recurrent genital herpes. Surface plasmon resonance technology was used to study the avidity and neutralizing epitope profile of IgG antibodies raised towards HSV-2 envelope glycoprotein D (gD) by immunization. Whole-genome microarray analysis combined with systems biology, protein array analysis and flow cytometry were used to identify early immune events in the murine vagina after delivery of a live attenuated HSV-2 strain, known as the gold standard for induction of protective immunity in mice.

Main results presented in this thesis include: I) Nasal and skin immunization with recombinant HSV-2 gD antigen in combination with the clinically tested adjuvant IC31[®] was highly efficient for induction of specific B and T cell responses and protection against primary genital herpes in mice; II) Nasal immunization elicited a high avidity, HSV-2 neutralizing IgG antibody response as well as protective immunity to both primary and recurrent genital herpes infection, with partial reduction of viral latency, in guinea pigs; and III) Identification of local inflammatory imprints connected to immune cell recruitment after vaginal immunization with live attenuated HSV-2 in mice.

The results presented in this thesis provide evidence on the potential of nasal and dermal immunization for induction of protective immunity to genital herpes as well as early molecular and cellular signatures of the protective immune response in the vaginal mucosa. These results may inform rational development of a vaccine to counter genital herpes infection in humans.

Keywords: Genital herpes, HSV-2, vaginal immunity, female reproductive tract, vaccine, adjuvant, systems biology.

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Genital herpes är en vanligt förekommande sexuellt överförbar infektion. Orsaken är vanligtvis herpes simplex virus typ 2 (HSV-2) och fler än 500 miljoner individer är smittade globalt. HSV-2 infekterar initialt den genitala slemhinnan och etablerar därefter en livslång infektion i nervsystemet (latent infektion). Efter primärinfektionen kan HSV-2 reaktivera och ge smärtsamma blåsor och sår i underlivet, men hos majoriteten smittas viruset utan symtom. Symtomfri infektion utgör det största hindret till att begränsa eller förhindra spridning av HSV-2. Infektionen kan också orsaka allvarlig sjukdom hos spädbarn om viruset överförs från mamman i samband med förlossning.

Vid besvärande symtom finns antivirala läkemedel tillgängliga, men infektionen går inte att bota och förblir livslång. Det skulle således vara ett stort framsteg om genital herpes gick att förhindra med hjälp av ett vaccin. Trots stora insatser har inget profylaktiskt vaccin lyckats förhindra spridning av viruset. De allra flesta vacciner ges som en injektion i muskeln, vilket fungerar utmärkt för flertalet andra virussjukdomar, såsom mässling och polio. Arbetet i den här avhandlingen har undersökt möjligheterna att inducera skydd mot genital herpes genom att injicera vaccin i huden eller ge det via näsans slemhinna. I djurmodeller kan vi påvisa att båda dessa strategier ger starkt skydd mot akut genital infektion samt delvis skydd mot latent infektion i nervsystemet.

Vaccinering baseras på att immunförsvarets celler aktiveras av hela eller delar av en sjukdomsalstrande mikroorganism (t.ex. bakterie eller virus), vilket gör att minnesceller och antikroppar bildas. Dessa delar av immunförsvaret kommer att reagera både specifikt och snabbt om kroppen utsätts för samma mikroorganism ytterligare en gång, vilket då oftast ger skydd mot sjukdom. När endast delar av en mikroorganism används krävs att så kallad adjuvans inkluderas i vaccinet. Adjuvans är molekyler som aktiverar immunsvaret och/eller förbättrar upptag av vaccinet. Vi har studerat två olika adjuvans som i kombination med ett protein från HSV-2 stimulerar immunförsvaret att producera minnesceller och antikroppar specifikt mot HSV-2. Båda dessa adjuvans har tidigare testats i människa och kan därför inkluderas i ett vaccin. Förhoppningen är att den information som presenteras i denna avhandling gällande immuniseringsvägar och vaccinkomponenter ska driva utvecklingen av ett vaccin mot genital herpes infektion framåt.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals (I-III).

- I. Wizel B, Persson J, Thörn K, Nagy E, Harandi AM.
Nasal and skin delivery of IC31[®]-adjuvanted recombinant HSV-2 gD protein confers protection against genital herpes.
Vaccine 2012; 30(29): 4361-8.
- II. Persson J, Zhang Y, Olafsdottir T, Thörn K, Cairns TM, Wegmann F, Sattentau Q, Eisenberg RJ, Cohen GH, Harandi AM.
Nasal immunization confers high-avidity neutralizing antibody response and immunity to primary and recurrent genital herpes in guinea pigs.
Submitted
- III. Persson J, Nookaew I, Mark L, Lindqvist M, Harandi AM.
Molecular and cellular imprints of live attenuated herpes simplex virus type 2 in the murine female reproductive tract.
In manuscript

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ABBREVIATIONS

APC	Antigen-presenting cell
CpG	Cytidine phosphate guanosine
DC	Dendritic cell
g	Glycoprotein
GO	Gene ontology
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSV	Herpes simplex virus
i.d.	Intradermal
i.m.	Intramuscular
i.n.	Intranasal
i.vag.	Intravaginal
ICAM-1	Intercellular adhesion molecule
ICP	Infected cell protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPA	Ingenuity pathway analysis
LAT	Latency-associated transcript
LC	Langerhans cell
MHC	Major histocompatibility complex
MPLA	Monophosphoryl lipid A
MyD88	Myeloid differentiation factor 88
NF- κ B	Nuclear factor- κ B
NK	Natural killer cell
NKT	Natural killer T lymphocyte
ODN	Oligodeoxynucleotide
pDC	Plasmacytoid dendritic cell
PAMP	Pathogen-associated molecular pattern
PRR	Pathogen recognizing receptor
s.c.	Subcutaneous
SPR	Surface plasmon resonance
STI	Sexually transmitted infection
T _{CM}	Central memory T cell
T _{EM}	Effector memory T cell
T _H	T helper cell
T _{Reg}	Regulatory T cell
T _{RM}	Tissue-resident memory T cell
TK ⁻	Thymidine kinase deficient
TLR	Toll-like receptor
TNF	Tumor necrosis factor

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Introduction to mucosa

In most mammals, the bone marrow and thymus are the primary lymphoid organs. Both support the development of leukocytes and other blood cells from common progenitor cells, and are sites for B and T lymphocyte maturation. The secondary lymphoid organs include lymph nodes, spleen and mucosa-associated lymphoid tissues. These organs are populated with immune cells that filter foreign particles from lymph, blood and mucosal surfaces ^{1,2}.

Mucosal membranes cover the respiratory, gastrointestinal and urogenital tracts as well as the eye conjunctiva, the inner ear and the ducts of all exocrine glands. Due to their large size and positioning, these surfaces are highly exposed to pathogen invasion, which has presumably led to the evolution of a multi-level defense ³. Nevertheless, many particles entering our body are harmless, such as food and commensal bacteria, and therefore must be tolerated. Thus, the mucosal immune system needs to uphold a delicate balance between inflammation and tolerance to fulfill its functions ⁴.

At the mucosal surfaces, highly specialized epithelial cells create a barrier that protects the body from the outside world. The epithelial cells form tight junctions that block invasion and actively get rid of invading microbes by secreting mucus and antimicrobial factors ⁵. The gel-forming mucins, which are highly glycosylated proteins with an ability to bind water, give the mucus its thick consistency and make it difficult for microbes to attach to the epithelium. Glandular columnar epithelial cells, called goblet cells, produce the mucins that are either kept anchored to the cell membrane or secreted ⁶. Secretory immunoglobulin (Ig) A, uniquely adapted for being transported through epithelial cells and to resist proteases, also represent a key first line of defense and is the most abundant antibody class at mucosal surfaces ⁵.

If pathogens succeed in crossing the epithelium, additional protection mechanisms are required to counter them. A substantial innate and acquired immune system is present in the subepithelial compartment, and the mucosal tissues are estimated to hold about 80% of all immune cells in a healthy individual. Most mucosal sites have organized lymphoid structures, where the induction of acquired immune responses is initiated, such as Peyer's patches in the intestine and the tonsils in the aerodigestive tract ^{3,5}.

Female reproductive tract

The female reproductive tract differs in many respects from other mucosal tissues as its role in reproduction has made certain adaptations necessary. It should protect against infectious agents while also allowing fertilization, implantation, pregnancy and parturition to take place ⁷.

Anatomy and histology

The female reproductive tract has two distinct compartments (Figure 1). The upper part consists of the endocervix, uterus, Fallopian tubes and ovaries. The lower part is comprised by the vagina and ectocervix. The upper compartment resembles other mucosal surfaces and is covered by a monolayer of mucus-secreting columnar epithelial cells with tight junctions, together with interspersed ciliated, non-secreting cells ⁸.

The lower compartment is instead lined with a stratified squamous epithelium and resembles the epidermis in skin, with layers of cornified cells in the outer part. In humans, these keratinocytes express several cytokeratins but they do not form the prominent keratin bundles seen in skin epidermis ^{8,9}. In contrast, a distinct keratinization is visible during certain periods of the hormonal cycle in mice ¹⁰. The superficial layers of the epithelium undergo a specialized apoptotic program, leading to loss of the nucleus and organelles, and the cells are weakly joined to each other ^{8,9}. Tight junctions are mainly present between the basal epithelial cells ⁷. The types of epithelia present in the upper and the lower part of the reproductive tract meet at the cervical transformation zone, a vulnerable site for dysplasia ¹¹.

The epithelial cells separate the underlying tissue from the lumen. The basement membrane attaches the epithelium to the tissue underneath, in which a dense population of stromal fibroblasts makes up the structural support. Leukocytes are found distributed throughout the stroma, with a higher proportion present in the upper reproductive tract ⁷.

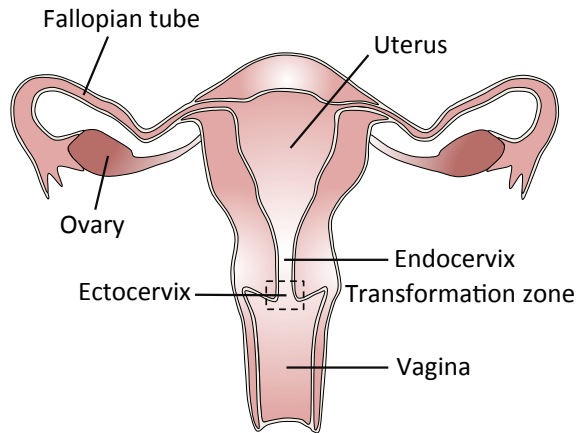


FIGURE 1. A schematic picture of the female reproductive tract (modified from ⁷).

Immunity in the vaginal mucosa

In vertebrates, the immune system is divided into an innate part and an acquired part. The innate responses are immediate and provide the first line of defense, while the acquired responses offer high specificity and memory. The two arms of immunity do not work independently of each other; rather, an efficient immune response requires an intricate cooperation of the two arms ². The following section describes immune responses within the vaginal mucosa in general, albeit with a primary focus on defense related to viral infections.

Innate immune responses

The epithelium together with the overlaying mucus mechanically prevents microbes from entering the body. Secreted components of the complement system and antimicrobial peptides can bind to microorganisms in the vaginal lumen, killing them before they reach the epithelium. It is mostly epithelial cells, glandular cells in the cervix and neutrophils that produce the antimicrobial peptides, such as calprotectin, lysozyme, lactoferrin, secretory leukoprotease inhibitor and defensin ¹².

The lower reproductive tract is colonized by bacteria, as opposed to the upper parts, which are more or less sterile. A normal vaginal flora helps to outcompete harmful microbes. The microbiota varies among women but the dominant commensal strains often belong to *Lactobacillus*. These bacteria produce lactic acid

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when metabolizing glycogen, released by epithelial cells, which keeps the pH acidic (3.5-5.0). The low pH is believed to inhibit several infections. Some species of lactobacilli also produce hydrogen peroxide, which can restrict the growth of certain unwanted bacteria^{10,13}.

The role of fibroblasts, located beneath the epithelium, is not clear but they may help to alert immune cells during an on-going infection. Uterine and cervical fibroblasts can produce cytokines and chemokines in response to pathogen-associated molecules¹⁴.

In contrast to most other mucosal tissues, the vaginal mucosa lacks organized lymphoid structures¹¹. Several types of immune cells can be found albeit at low numbers in the steady state. Dendritic cells (DCs) act as a critical link between innate and acquired immunity and multiple vaginal subsets exist, including intraepithelial Langerhans cells (LCs) and subepithelial DCs¹⁵. These, together with intraepithelial $\gamma\delta$ T cells and macrophages, patrol the vaginal mucosa.

Pathogen recognition

An important aspect of innate immunity is recognition of invading microbes. This pathogen-sensing function is highly dependent on pattern-recognition receptors (PRRs) that recognize structures typically displayed by pathogenic agents, often called pathogen-associated molecular patterns (PAMPs). These are evolutionary conserved molecules shared broadly by microbes, including bacteria, viruses, fungi and protozoa^{1,16}. Furthermore, these receptors can also detect host danger signals that are released due to stress, tissue damage and necrotic cell death¹⁷.

Even non-pathogenic commensals express PAMPs but somehow avoid triggering excess immune responses. The establishment of this host-microbe symbiosis has been suggested to occur through one or several mechanisms, including: anatomical location (such that beneficial microorganisms avoid contact with the immune system); structural differences (resulting in stronger stimuli to the PRRs offered by pathogenic PAMPs compared to beneficial microbes); active secretion of certain compounds by the commensals (such that the immune response is dampened); and/or additional danger signals provided by invasive microbes⁴.

The PRRs include Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), nucleotide-binding oligomerization domain (Nod)-like leucine-rich repeat-containing receptors (NLRs), C-type lectin receptors (CLRs) and absent in melanoma 2 (AIM-2)-like receptors. Targets identified are diverse and include polysaccharides, glycolipids, lipoproteins, nucleotides and nucleic acids. There are also some intracellular enzymes, such as oligoadenylate synthetase (OAS) proteins and cyclic guanosine

monophosphate-adenosine monophosphate (GMP-AMP) synthase (cGAS), which bind to nucleic acids².

Toll-like receptors

TLRs, or closely-related equivalent receptors, are present in both vertebrates and invertebrates¹. In fact, the name originates from homology with the Toll protein found in *Drosophila melanogaster*^{19,20}. The Toll protein was first recognized for its importance in embryonic development but was later found to be involved in antimicrobial defense in the fruit fly^{20,21}.

These transmembrane receptors usually take the form of dimers. Most TLRs are present as homodimers but some appear in heterodimer form. So far, ten human (TLR1-TLR10) and twelve murine (TLR1-TLR9 and TLR11-13) TLRs have been identified. In Table 1, the location and cognate ligands are shown. The subcellular location of TLRs correlates with the compartments in which their ligands are found. These receptors are found on various cells, although the pattern of expression differs among cell types²².

The TLRs consist of a leucine-rich ligand binding domain at the N-terminal and a signal transduction domain at the C-terminal²³. The TLRs are structurally similar to the interleukin 1 (IL-1) receptor and the C-terminal is called the Toll IL-1 receptor (TIR) domain due to this resemblance. The TIR domain connects TLRs to intracellular signaling. Activation of TLRs triggers TIR to associate with adaptor proteins such as myeloid differentiation factor-88 (MyD88), Toll receptor-associated activator of interferon (TRIF), TIR-associated protein (TIRAP) and Toll receptor-associated molecule (TRAM)²⁴.

All TLRs, except TLR3, act at least partly via MyD88. When MyD88 associates with a receptor it recruits kinases from the IL-1 receptor-associated kinase (IRAK) family, which are subsequently phosphorylated. The kinases dissociate from the receptor and interact with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6).

Downstream signaling leads to the activation of transcription factors nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1). This in turn results in expression of pro-inflammatory cytokines such as TNF- α and IL-1 α/β ^{16,22}.

Some TLRs signal in MyD88-independent pathways as well. These routes are instead dependent upon TRIF and may lead to activation of interferon (IFN) regulatory factors (IRFs), which promote transcription of IFN-inducible genes. Although, the MyD88-dependent pathway can also result in activation of IRFs, leading to production of type I IFNs. There are several type I IFNs but the best characterized are IFN- α/β ^{16,22}.

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Ultimately, TLR signaling leads to transcriptional activation or suppression of numerous genes, thereby coordinating the inflammatory response. Activation of TLRs plays a central role in the initiation and direction of acquired immunity. It leads to, for example, chemokine production to promote cell recruitment, as well as expression of major histocompatibility complex (MHC) class II and co-stimulatory molecules in antigen-presenting cells (APCs)².

Vaginal epithelial cells express several TLRs and may convey the message of a microbial invasion to the immune cells present in the subepithelial compartment¹⁸. Likewise, fibroblasts could potentially be involved in notifying the hematopoietic cell community¹⁴. Most immune cells also express a set of TLRs.

TABLE 1. Subcellular distribution of TLRs and examples of their ligands (modified from²²).

Receptor	Location	Natural ligands
TLR1/TLR2	Plasma membrane	Triacylated peptides
TLR2	Plasma membrane	Peptidoglycan, porins
TLR2/TLR6	Plasma membrane	Diacylated peptides, LTA, zymosan
TLR3	Endosome	dsRNA
TLR4	Plasma membrane	LPS
TLR4/TLR6	Plasma membrane	OxLDL
TLR5	Plasma membrane	Flagellin
TLR7	Endosome	ssRNA
TLR8	Endosome	ssNA
TLR9	Endosome	CpG-rich DNA
TLR10 (human)	Plasma membrane	Unknown, possibly anti-inflammatory ²⁵
TLR11 (mouse)	Plasma membrane	Profilin ^{26,27}
TLR12 (mouse)	Plasma membrane	Profilin ²⁸
TLR13 (mouse)	Endosome	23S rRNA

Abbreviations: LTA, lipoteichoic acid; dsRNA, double-stranded RNA; LPS, lipopolysaccharide; OxLDL, oxidized low-density lipoprotein; ssRNA, single-stranded RNA; CpG, cytidine phosphate guanosine; rRNA, ribosomal RNA

Acquired immune responses

The acquired arm of immunity responds to invading pathogens in a more precise way than innate immunity through antigen-specific receptors carried by its B and T lymphocytes. It can also offer memory and a more durable effect, in comparison to innate immunity, if long-lived memory cells develop. A subsequent infection usually results in a faster and more effective acquired immune response than that observed following the primary infection - the foundation that vaccination relies upon.

Antigen presentation

Antigen-specific presentation must occur in order for T cells to get involved in an immune response. The CD4⁺ T cells, central for acquired immune responses, need presentation via MHCII molecules that both professional APCs, such as macrophages, DCs and B cells, and non-professional APCs, e.g. epithelial cells and fibroblasts, express.

As vaginal mucosa lacks organized lymphoid structures, priming of naïve T cells is believed to take place in the draining lymph nodes^{29,30}. Antigen-bearing DCs migrate from the vagina to the draining lymph nodes and present antigen to the lymphocytes that travel to the site of infection through the bloodstream^{31,32}. The vaginal canal is drained by several lymph nodes, including the iliac, para-aortic and inguinal femoral lymph nodes^{33,34}.

Additionally, some reports indicate the ability of vaginal mucosa in carrying out antigen presentation. For instance, intravaginal (i.vag.) immunization was shown to induce protective immunity in lymph node-deficient mice³⁵. Furthermore, vaginal APCs could activate both naïve and memory T cells in a system using transgenic mice bearing T cell receptors specific for a MHC class II-restricted ovalbumin peptide³⁶. A recent study also indicated that priming of naïve CD8⁺ T lymphocytes occurred in the vagina without the involvement of other lymphoid tissues³⁷. Importantly, the presence of lymphoid aggregates has been described for both human and murine vaginal mucosa^{38,39}. Thus, it is likely that primary immune responses can occur locally in the vagina but further studies are required to pinpoint the precise underlying mechanism.

The vaginal mucosa appears to be rather tolerogenic⁴⁰⁻⁴². Still, some infections provoke vigorous T cell responses. Vaginal epithelial cells and fibroblasts may present antigens but there are no clear reports suggesting that they can activate naïve T cells. Likewise, macrophages and B cells do not seem to be responsible for vaginal primary immune responses. Instead, DCs are the key APC and most efficient activator of naïve T cells¹¹.

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It was previously thought that LCs could sample antigen from the vaginal lumen for presentation to T cells but there is no clear evidence supporting this³². It also appears that plasmacytoid DCs (pDCs) are dispensable for antigen-presentation⁴³. In fact, it has been shown that subepithelial migratory CD11b⁺ DCs most effectively prime CD4⁺ T cells in the draining lymph nodes³². Many viruses inhibit the host cell's ability to present antigen. DCs directly infected by a virus may also be incapable of presenting antigens as viruses can inhibit their activation and function. Instead, cross-presentation may be required for activation of CD8⁺ T cells. It permits presentation of exogenous antigen via MHCI without necessitating infection of the DC itself. The DC may acquire the antigen through ingestion of parts from lysed virus-infected cells.

T lymphocytes

A small population of CD4⁺ and CD8⁺ T cells is present in the vagina at all times^{30,38,44}. Nevertheless, as discussed in the previous section, priming of naïve T lymphocytes and clonal expansion appears to mainly take place in the draining lymph nodes. During a viral infection, an influx of T cells occurs, with the recruitment of IFN- γ -producing CD4⁺ T cells preceding that of cytotoxic CD8⁺ T cells^{31,45}. It has also been suggested that regulatory T cells (T_{Reg}) are involved in vaginal immune responses. Depletion of T_{Reg} cells lead to decreased homing of NK cells, pDCs, and CD11b⁺ DCs to the vagina during infection⁴⁶.

Previously, mainly effector memory T (T_{EM}) and central memory T (T_{CM}) cells were considered in the context of vaginal immunity. These two subsets are localized at different sites: T_{EM} cells circulate through non-lymphoid tissues and T_{CM} cells reside in secondary lymphoid organs⁴⁷. However, during recent years it has become apparent that a pool of tissue-resident memory T (T_{RM}) cells can remain within the vaginal mucosa after immunization or infection⁴⁸⁻⁵³. The generation of such pre-positioned memory cells may be of great importance for infections requiring more than potent antibody responses for protective immunity.

B lymphocytes and antibodies

B cells are present at low numbers within the vaginal mucosa under homeostatic conditions^{30,38}. However, the quantity of B cells and plasma cells can increase after infection and local vaccination^{51,54,55}. It also appears that B cells interact with T cells in an organized way under certain conditions, but the exact role of these cell clusters are not fully understood^{38,48}.

The most abundant antibody class in the vaginal lumen is IgG, while only low levels of IgA antibodies can be found, in contrast with other mucosal tissues. The dominant view is that vaginal IgG antibodies are mainly derived from the circulation. In both humans and mice, the neonatal Fc receptor present in the

female reproductive tract is considered to be responsible for the transcytosis of IgG from the blood to the vaginal lumen⁵⁶. The source of IgA is probably plasma cells present in the upper reproductive tract¹².

IgG antibodies can act to neutralize antigen through agglutination, by masking surface molecules used for invasion and/or by coating the antigen and thereby opsonizing it for phagocytosis. They can also activate the classical pathway of the complement system and/or induce antibody-dependent cell-mediated cytotoxicity, a mechanism whereby e.g. natural killer (NK) cells, macrophages and neutrophils actively lyse antibody-tagged cells.

B cells can bind to the antigen in native form but need to interact with T follicular helper (T_{FH}) cells in order to transform into efficient antibody-producing plasma cells via class switching and affinity maturation. The presence of T_{FH} cells has not been described for vaginal mucosa and it is likely that this type of interaction occurs in the draining lymph nodes.

Homing to vaginal mucosa

During homeostatic conditions, innate cells (monocytes, granulocytes and NK cells) as well as B and T lymphocytes circulate in the blood. Monocytes and granulocytes are not able to re-circulate between blood and tissue, whereas mature lymphocytes are thought to constantly travel back and forth between blood, peripheral tissues and secondary lymphoid organs until they encounter their specific antigen. A set of chemokines and adhesion molecules, involved in this type of normal immune surveillance, are constitutively expressed.

Upon infection or tissue damage, expression of chemokines and endothelial adhesion molecules will change and promote recruitment of innate and antigen-specific cells to the vagina. Many homing molecules associated with inflammation are shared among tissues, while others are tissue-specific. Several adhesion molecules involved in the extravasation process of rolling, activation, firm adhesion and endothelial transmigration have been documented for the vaginal mucosa. However, key questions related to T cell recruitment and residency in the vagina still wait to be answered. No specific homing pathway has been defined for the vaginal mucosa.

Adhesion molecules

Expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) has been documented on vaginal endothelial cells in both humans and mice^{38,57,58}. ICAM-1 is constitutively expressed and binds lymphocyte function-associated antigen 1 (LFA-1), present on various immune cells, while

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VCAM-1 is an inducible ligand for $\alpha 4$ integrins expressed on lymphocytes³⁸. In mice, IFN- γ has been shown to up-regulate the vaginal expression of both ICAM-1 and VCAM-1⁵⁷.

E-selectin, which can bind cells expressing $\alpha E\beta 7$ integrins, skin-associated cutaneous lymphocyte antigen (CLA) and P-selectin glycoprotein ligand-1 (PSGL-1), have also been detected in the vagina^{38,58,59}. It has been shown that integrin αE (CD103)-deficient mice not only lost their vaginal intraepithelial T cells, consistent with the known binding of $\alpha E\beta 7$ to epithelium E-cadherin, but also had a reduced number of subepithelial T cells⁶⁰.

Furthermore, there are also some contradictory reports regarding vaginal expression of mucosal addressin intercellular adhesion molecule 1 (MAdCAM-1)^{38,57,59}. This adhesion molecule is normally associated with binding of $\alpha 4\beta 7$ -bearing T cells in the gut mucosa. Expression of vascular adhesion protein-1 (VAP-1) and P-selectin has also been detected in human vaginal samples. VAP-1 is known to bind immune cells, possibly via sialic acid binding Ig-like lectin 9 (Siglec-9), while P-selectin binds P-selectin glycoprotein ligand-1 (PSGL-1)^{38,61}.

Chemokines

Chemokines are a family of small (8-10 kDa) cytokines that can induce chemotaxis when interacting with G-protein-coupled transmembrane receptors, found selectively on the surfaces of their target cells. They are grouped into four subclasses (C, CC, CXC and CX3C) according to the position of conserved cysteine residues at the N-terminal. Some chemokines only interact with one type of chemokine receptor, while others bind to several different receptors. Chemokines control the movement and localization of immune cells, such as entry into the circulation, emigration from the blood, positioning in the tissue and departure from the tissue.

The vaginal epithelium acts as a sentinel, with the ability to detect potential pathogens early on. In response to infection, epithelial secretion of CCL20 and CXCL8 appears to be an important signal for CCR6⁺ cells (mainly pDCs, DCs and lymphocytes) and CXCR1/CXCR2⁺ cells (mainly neutrophils), respectively, to move towards the epithelium.

Other chemokines associated with the attraction of innate cells include CCL2, which attracts CCR2⁺ monocytes, and CCL5, which binds CCR5⁺ NK cells. CCL5 is also known to be important for recruiting CD4⁺ T cells via CCR5. Beyond this, entry of CD4⁺ and CD8⁺ T cells into the vaginal epithelium seems to be highly dependent on the production of CXCL9 and CXCL10, binding to CXCR3⁶².

Hormonal control

The female reproductive tract stands under strict hormonal control, mainly by estradiol and progesterone. These sex hormones do not just direct reproduction but also have a great impact on local immune responses. As the levels fluctuate during the hormonal cycle, the ability of the vaginal mucosa to respond to infectious agents will vary. The hormonal cycle can be divided into two main phases: estrus and diestrus. Estrus is the period when estradiol dominates, whereas progesterone dominates during diestrus⁶³.

During diestrus, immune protection reduces in order to allow fertilization and pregnancy. Thus, women are more susceptible to reproductive tract infections throughout this period⁷. Progesterone-containing contraceptives may therefore increase the risk of sexually transmitted infection (STI) acquisition, as shown for vaginal transmission of human immunodeficiency virus (HIV)⁶⁴. In mice, it is widely recognized that progesterone increases susceptibility to STIs⁶⁵. Exogenous synthetic progesterone is therefore often used to establish such infections in animal models^{66,67}.

The number of immune cells in the lower reproductive tract appears to be constant during the hormone cycle⁷. However, one cell type affected by progesterone and estradiol is the epithelial cell. The two hormones regulate these cells' proliferation, apoptosis, secretions and capacity for responding to pathogens. In humans, the thickness of the squamous epithelium is relatively constant during the menstrual cycle⁷. In mice, estrus is characterized by a thick keratinized vaginal and ectocervical epithelium with low permeability, whereas diestrus is distinguished by a thin epithelium that is more permeable⁶⁶. Furthermore, epithelial expression of TLRs has been shown to fluctuate in mice due to varying levels of sex hormones¹⁸. This could potentially influence the ability to detect pathogens at an early stage.

The consistency and amount of mucus also varies during the hormonal cycle. During estrus the mucus is thin and watery with low viscosity that allows the sperm to move easily. During diestrus it is thick and viscous to stop any particles from moving upwards. The levels of certain molecules in the cervicovaginal fluid have also been found to change during the hormone cycle. A marked decrease of luminal antimicrobial peptides is for example seen during diestrus⁷.

Hormones must be considered when attempting to induce vaginal immunity by vaccination. In animal experiments, progesterone treatment is often used before i.vag. immunization³⁹. On the other hand, studies have demonstrated that administration of estradiol can enhance immune responses and induce better protection following immunization via nasal mucosa or systemic routes^{39,68,69}. This

would indicate that the sex hormones affect initiation of antigen-specific immune responses as well. Taken together, it seems that the period dominated by estradiol offers stronger immunity, while the influence of progesterone may be necessary for establishing vaginal immunity, at least via local vaccination.

Sexually transmitted infections

The World Health Organization (WHO) estimates that around one million individuals acquire an STI every day. There are over 30 different bacteria, viruses and parasites that cause these infections. Some are transferred by direct sexual contact, while others also spread via blood products or tissue transfer. Many of the infections can also be transmitted from mother to child during pregnancy and birth ⁷⁰.

The bacterial infections associated with the greatest incidence of illness are *Treponema pallidum* (causing syphilis), *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Trichomonas vaginalis* (causing vaginitis), which usually can be treated with antibiotics. The viral infections identified as the most serious conditions are hepatitis B virus, herpes simplex virus (HSV), HIV, and human papillomavirus (HPV), which are incurable even though symptoms often can be reduced by treatment ^{70,71}.

The vagina is a portal of entry for several pathogens but still relatively few establish infection in the vagina, which may be explained at least in part by its harsh milieu. Vaginal pathogens include HSV, *Trichomonas vaginalis* and the fungal infection *Candida albicans*. It is also believed that HIV can infect via the vaginal mucosa, possibly through DCs. The most common infections affecting the cervix are HPV, chlamydia and gonorrhea. The uterus, Fallopian tubes and ovaries are considered to be sterile sites, although microbes can sometimes reach this area, causing pelvic inflammatory disease ^{70,71}.

Genital herpes infection

Genital herpes is one of the most common STIs, with an estimate of over 500 million (11.3% global prevalence) infected individuals worldwide ⁷². There are however highly variable regional differences and in some high-risk populations the prevalence exceeds 80% ⁷³. The infection is nearly twice as common in women as in men, and prevalence increase by age ⁷². The view on genital herpes as an important threat to public health is highlighted by the fact that epidemiological studies have indicated that infected individuals are predisposed to HIV acquisition ⁷⁴⁻⁷⁶.

Pathogenesis

Genital herpes can affect both the inner and outer parts of the genitalia, with symptoms such as erythema, pain, and the appearance of vesicles and lesions. Occasionally, the infection leads to more serious complications, such as encephalitis, which can be particularly disastrous for immunocompromised individuals and neonates^{77,78}.

Genital herpes is a life-long infection, caused by HSV. After primary infection, the virus establishes itself latently within the nervous system and can cause recurrent disease episodes due to spontaneous reactivation. Generally, the most severe symptoms are associated with primary infection. However, it may pass unseen as some experience diffuse symptoms, meaning that many are unaware of the infection.

Herpes simplex virus type 2

There are eight viruses infecting humans within the *Herpesviridae* family: HSV-1, HSV-2, varicella zoster virus, Epstein-Barr virus, Kaposi's sarcoma-associated herpesvirus, cytomegalovirus and human herpes virus 6 and 7. The two types of HSV belong to the subfamily *α-herpesvirinae*⁷⁹.

The main targets of infection differ between HSV-1 and HSV-2, although they do overlap to some extent. HSV-1 typically enters the body through the oral mucosa or corneal epithelium. Oral herpes may express itself as blisters and ulcers with varying severity. Ocular herpes is a major cause of blindness and also the most important cause of sporadic encephalitis⁸⁰. HSV-2 is the leading cause of genital herpes and its main infection site is the vaginal and penile mucosa. However, in recent years studies have revealed that genital herpes associated with HSV-1 infection is prominent in some populations⁸¹⁻⁸³. If infected by one of the HSV subtypes, this is rarely followed by infection with the other one in the same anatomical region, although it is possible to have the two subtypes at different sites⁸⁴.

The inner part of the viral particle (Figure 2) contains a linear, double-stranded DNA genome surrounded by an icosahedral capsid. Outside the capsid is a matrix of proteins called the tegument. An outer lipid bilayer envelope surrounds the structure and has eleven different spike-like glycoproteins (gB-gM) incorporated within it, some of which are crucial for attachment and entry into target cells⁸⁵. The two subtypes of HSV are closely related and share several epitopes but there are some antigenic differences that allow serological discrimination, e.g. HSV-1 gG-1 and its HSV-2 counterpart gG-2⁸⁶.

The process of viral attachment and entry is believed to involve at least five of the virus envelope glycoproteins as well as engagement of several surface molecules on

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the host cell. The initial attachment is thought to be mediated by gB or gC, which can bind to heparan sulfate. Normally, this would be followed by interaction among gD and its receptors, such as herpesvirus entry mediator (HVEM), nectin-1 and nectin-2. The final entry step of membrane fusion is mediated by a machinery composed of gB and gH/gL^{85,87}.

Following viral entry, tegument proteins and the capsid reach the host cell cytoplasm. This ultimately leads to viral transmission of nucleic acids to the nucleus and replication. Three types of viral genes are transcribed: immediate-early (α), promoting the transcription process; early (β), involved in DNA replication; and late (γ), encoding structural components. Viral thymidine kinase (TK) helps enzymatically with DNA synthesis^{79,80}. If the virus is deficient in TK it fails to establish a productive infection within the nervous system. This type of virus strain (HSV-2 TK⁻) is commonly used for immunization studies in animal infection models^{88,89}. When new virions have assembled they are released via exocytosis⁸⁸.

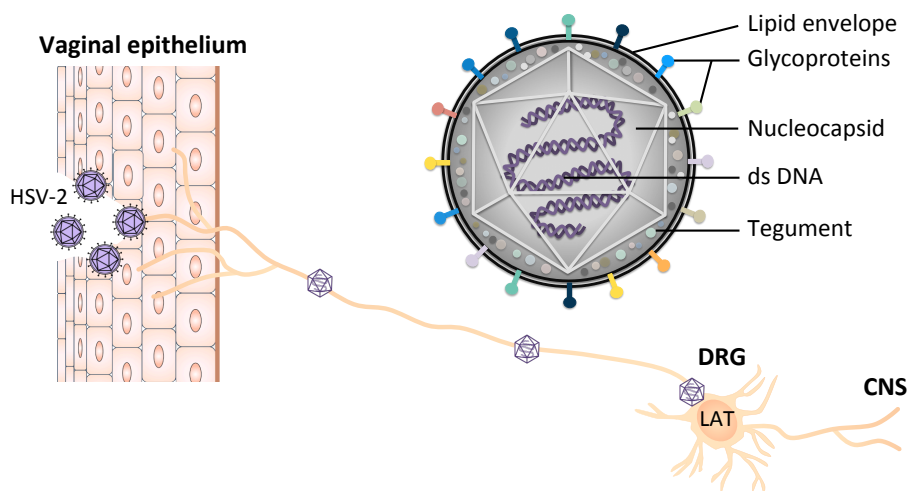


FIGURE 2. Schematic picture of HSV-2 (top right) and a sensory neuron innervating the vaginal epithelium (bottom). As the epithelial cells lyse during virus replication small abrasions opens up to the nerve endings. Retrograde transport moves the de-enveloped virus to the neuron cell body in the dorsal root ganglia (DRG), located in close proximity to the spinal cord, where it establishes latency. During latency the only transcribed HSV-2 mRNA is latency-associated transcripts (LAT). CNS= central nervous system.

Primary genital herpes infection

HSV-2 usually enters the body through the vaginal epithelial cells, where it also replicates. Replication culminates in host cell lysis, which may cause the symptoms associated with genital herpes infection. At this point, virions are also released and may reach the basement membrane, where they can be taken up into sensory nerve fibers. The virus travels retrograde along microtubules inside the axon as a de-enveloped capsid and releases its genetic material into the nucleus upon reaching the cell body of the neuron, where a period of latency follows (Figure 2). Once in the soma, the virus DNA circularizes and forms distinct objects called episomes. The virus normally does not lyse the neurons but rather exploits them as reservoirs^{77,80,90}.

Latent infection and reactivation

For reasons unknown, the virus occasionally reactivates. Production of lytic genes starts and the virus is transported anterogradely, generally back to the original site of infection, where it translocates from the nerve terminal into the epithelium. Upon release of infectious virions, the epithelial cells become reinfected and lytic replication leads to viral shedding⁷⁷. During this period, cell-free virus may spread to other sites in the genitalia, causing lesions, and can also be transmitted to another individual. The reactivation and shedding-process can result in symptoms but may as well be asymptomatic^{80,90}. Consequently, there is a risk of transmission from an infected to an uninfected individual even though apparent signs of viral reactivation are absent⁷⁷.

Previously, HSV-2 was thought to be largely silent during asymptomatic periods but mathematical modeling suggests that reactivation may occur in small numbers of neurons at any given time, leading to low but continuous release of HSV-2 into the vaginal lumen. Such release may not even be detectable. Even patients treated with high doses of antiviral drugs had frequent episodes of virus shedding despite the lack of symptoms⁷⁷.

Latency-associated transcript, or LAT, is the only HSV-associated transcript that is produced during the latent phase of infection (Figure 2). No protein is linked to this mRNA and the exact role of LAT is not completely resolved. Several studies have demonstrated that the capacity to produce these transcripts influences HSV reactivation rates and the recurrence phenotype⁹¹⁻⁹⁴. Nevertheless, it has been questioned whether quantification of LAT has any relevance for evaluating the outcome of latent infection⁹⁵.

The distinction seen for HSV-1 and HSV-2 regarding their main infection sites may be attributed to the preferential establishment of latency in different neurons. It has been shown that specific populations of neurons are more or less permissive for

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productive infection for the two types. Exchanging LAT between HSV-1 and HSV-2 also exchanges the neuronal preference. This may explain why the recurrence rate is lower for HSV-1 than for HSV-2 in the genital tract^{96,97}.

Autonomic neurons can also be infected by HSV-2 and are possibly the source of viral latency detected within the spinal cord, though it is not clear whether this part of the nervous system is relevant for initiation of recurrences^{92,98}.

Treatment

Drugs used to target HSV, such as acyclovir and penciclovir with their respective prodrugs valacyclovir and famciclovir, inhibit the replication of the virus by interfering with DNA polymerase in infected cells. These nucleoside analogues depend on the activity of the viral TK enzyme to start the process that converts the drug to its active form. Interaction with the virus-encoded DNA polymerase will subsequently lead to its inhibition and inactivation, thereby preventing further viral DNA synthesis. Some of the drugs can also be incorporated into the elongating viral DNA in their active form, which interferes with the process through an unknown mechanism^{84,99}.

Although effective, this approach requires a frequent intake to sustain inhibition of virus replication, as is not always feasible. Moreover, some HSV-2 strains have developed resistance to antiviral drugs and cannot be treated successfully^{84,99}.

Immunity to HSV-2

It is likely that both the non-hematopoietic and hematopoietic cells sense HSV-2 in the vaginal mucosa. The engagement of PRRs and downstream signaling set the tissue into an antiviral state and initiates an inflammatory response that ultimately leads to recruitment of a variety of immune cells¹⁰⁰.

Early immune responses

Recognition of HSV-2

Recognition of the virus is a crucial step for induction of immune responses that ultimately should lead to elimination of the virus from the site of infection. Both TLRs and other innate receptors have been suggested to be involved in HSV-2 detection¹⁰¹.

Certain TLR2 polymorphisms have been linked to increased viral shedding and lesion rates in patients with genital herpes, which might indicate that this cell surface receptor is critical for proper HSV-2 recognition¹⁰². The viral envelope glycoproteins gB and gH/gL have been shown to interact with the receptor¹⁰³. Epithelial cells,

pCs and NK cells have been suggested to respond to HSV-2, at least partly, in a TLR2-dependent manner. TLR2 activation leads to production of IL-6, TNF- α and IFN- α ¹⁰⁴⁻¹⁰⁶.

The HSV-2 DNA contains abundant unmethylated cytidine phosphate guanosine (CpG) motifs and can activate TLR9 inside cells, as seen for pDCs in particular¹⁰⁷. TLR9 is also likely to participate in recognition of HSV-2 by conventional DCs and macrophages^{104,108}, leading to production of type I IFNs (e.g. IFN- α) and type III IFNs (e.g. IFN- λ) through IRF and NF- κ B signaling pathways in the vagina¹⁰⁹. IL-12 is typically released after TLR9 activation as well¹¹⁰.

It has also been proposed that TLR3 could be involved in HSV-2 recognition but there is no clear evidence for this when it comes to vaginal cells¹⁰¹. However, the receptor may play a role in controlling HSV-2 infection within the nervous system^{111,112}.

MyD88-signalling downstream of TLRs appears to be critical for innate immune responses directed against HSV-2¹¹³. A functional pathway also appears to be required for induction of T helper 1 (T_H1) responses to genital herpes infection in mice, both in DCs and epithelial/stromal cells¹¹⁴. However, i.vag. immunization with a live attenuated HSV-2 strain generated comparable acquired immune responses as well as full protection in MyD88^{-/-} mice and immunocompetent mice, suggesting that additional signaling pathways are involved in HSV-2 recognition¹¹³.

Accumulation of double-stranded (ds) RNA has been found during HSV-1 infection, indicating that RLRs, such as RIG-I and melanoma differentiation-associated gene 5 (MDA-5), possibly play a role in HSV detection^{110,115}. These receptors appear to be activated by HSV-2 in fibroblasts and macrophages but it remains unknown if they participate in recognition of the virus within the vaginal mucosa¹¹⁶.

Cytosolic DNA sensors, DNA-dependent activator of IFN regulatory factor (DAI) and family member IFN-inducible 16 (IFI16), have shown to be activated by HSV-2 in primary vaginal epithelial cells¹⁰⁵. Virus-derived DNA and dsRNA have also been suggested to be recognized through DExD/H box helicase family DHX9, -36, -41 and -60 as well as RNA pol III and Ku70. Furthermore, C-type lectin mannose receptors have been proposed to mediate viral glycoprotein recognition¹¹⁰.

Innate cells

The epithelial cells are likely to respond first as they line the vaginal vault and are specifically targeted by the virus. During infection they have been shown to secrete pro-inflammatory cytokines, IFN- β and antimicrobial substances such as defensins. Tissue-resident DCs, macrophages and intraepithelial $\gamma\delta$ T cells also respond to the

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virus early on and produce type I IFNs and other cytokines. The intraepithelial $\gamma\delta$ T cells were deemed capable of bridging innate and acquired immunity to HSV-2 infection¹¹⁷. However, another study pointed out that they were dispensable for viral clearance¹¹⁸.

Neutrophils, NK cells, monocytes, DCs and pDCs are recruited to the site of infection. Out of these cells, pDCs are the most well equipped to respond to viruses through TLRs and also provide a burst of type I IFNs, which has been assumed to limit viral replication. Yet, the critical importance of pDCs for innate immunity and development of acquired immune responses to HSV-2 infection remains elusive^{43,107,119}. Neutrophils are recruited early on and appear to be involved in resolving genital herpes infection but only play a limited role in preventing HSV-2 spread to the sensory ganglia^{120,121}.

NK and/or NKT cells in addition to IL-15 have been shown to be important for immediate control of HSV-2 as well as initiation of acquired immune responses¹²²⁻¹²⁵. HSV-2 antigens have been reported to activate NK cells directly via TLR2¹⁰⁶. NK and/or NKT cells are responsible for the early source of IFN- γ but do not produce the amounts required to clear the virus infection¹²⁶.

Subepithelial DCs are critical for development of a T_H1 response directed towards HSV-2, while LCs are not. It appears that CD11b⁺ DCs migrate to the draining lymph nodes and present viral peptides to the CD4⁺ T cells³². Recruited inflammatory monocytes renew the vaginal DC population during infection and are known to drive vaginal T_H1 responses^{127,128}. A network of macrophages was also recently described to support clusters of vaginal-resident CD4⁺ T cells, probably through chemokine secretion⁵³.

Specific immune responses

B cell and antibody responses

The contribution of B cells and antibodies to immunity against genital herpes infection is evident but not fully understood. Challenge studies in animal models have demonstrated that passive transfer of immune serum can protect against vaginal delivery of HSV-2^{129,130}. Additionally, immunized μ MT mice, deficient in functional B cells, were shown to develop an ablated immune protection compared to their wild-type littermates after HSV-2 challenge. Furthermore, the presence of maternal antibodies was shown to reduce the transmission of HSV-2 to neonates^{131,132}. B cells also appear to play a critical role for induction of a vaginal T_H1 response⁴⁸. When B cells were depleted in mice after local immunization it mostly seemed to affect early control of the infection though¹³³.

IgA and IgG antibodies directed towards HSV-2 can be found in genital secretions after infection in both humans and mice¹³⁴. However, IgG is more abundantly found in the female reproductive tract and appears to be the main protective antibody class against HSV-2^{129,134,135}. As luminal antibodies encompass a constitutive barrier, with no requirement of preceding immune responses, they appear to be beneficial and act as the first line of HSV-specific defense to bind cell-free virus. In the latest human clinical vaccine trial a reduction of genital HSV-1 infection was seen, which correlated with neutralizing antibodies but not with T cell responses¹³⁶.

T cell responses

There is substantial evidence supporting T cells as critical for protection against genital herpes infection in humans. Individuals with impaired T cell responses, for example due to HIV infection, organ transplantation or chemotherapy, display increased recurrence frequency and viral shedding¹³⁷.

In mice, activated CD4⁺ T_H1 cells enter the vagina around day three post-infection, peaking a few days later. The signals responsible for vaginal recruitment of CD4⁺ T cells are unknown. Activated CD8⁺ T cells enter the vagina with slightly delayed kinetics and are reliant on local CD4⁺ T cells producing IFN- γ , which up-regulates the expression of CXCL9 and CXCL10^{31,138}. Both CD4⁺ and CD8⁺ T cells appear to be involved in resolution of primary vaginal HSV-2 infection. However, in most cases the virus has already been able to transfer itself to the nervous system and establish a latent infection.

In humans, both CD4⁺ and CD8⁺ T cells infiltrate the site of an active lesion during skin-related recurring episodes and can also persist long after viral clearance^{50,139-141}. In mice, CD8⁺ T cells have also been found surrounding dorsal root ganglia that are latently infected with HSV-2^{94,142}. In guinea pigs, persistent vaginal virus-specific T_{RM} cells have been found post-infection⁵¹. As discussed previously there are also some reports on T_{RM} cells in mice after immunization or HSV-2 infection⁴⁸⁻⁵³. It is thought that DCs and B cells offer MHCII-dependent re-stimulation of CD4⁺ memory T cells within the vaginal mucosa, without involvement of the draining lymph nodes⁴⁸.

The ability of cytotoxic CD8⁺ T cells to induce apoptosis of virus-infected cells by perforin- and/or Fas-mediated mechanisms would suggest that these cells are critical for protection against genital herpes infection¹³⁸. The depletion of CD8⁺ T cells in immune mice leads to a significant reduction of protection to a subsequent HSV challenge¹⁴³. In human skin, clearance of HSV-2 correlates with cytotoxic CD8⁺ T cells¹⁴⁰. It has also been shown that a CD8⁺ T cell response directed against a single epitope, expressed by a viral vector, conferred protection to a lethal viral

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challenge¹⁴⁴. Still, the importance of CD8⁺ T cells in HSV vaccination is up for discussion¹⁴⁵.

Instead, IFN- γ producing CD4⁺ T cells have emerged as the most critical cell type involved in vaccine-induced acquired immune responses¹⁴⁶⁻¹⁴⁸. Mice lacking CD4⁺ T cells rapidly succumbed to genital herpes infection, despite an i.vag. immunization regime that offers full protection in wild-type mice. These knockout mice could be rescued by administration of recombinant IFN- γ ¹³³. Likewise, mice depleted of CD4⁺ T cells after immunization were not protected¹⁴⁷. The use of neutralizing antibodies against IFN- γ has also been shown to result in weakened immunity^{126,138}. The dependency on T cells for virus resolution appears to be of a non-lytic nature in mice¹¹⁸. It seems that IFN- γ mostly acts on the vaginal stromal cells, rather than the hematopoietic cells, inducing an anti-viral state that reduces viral production and spread⁴⁸.

In summary, CD4⁺ T cells producing IFN- γ appear to be crucial for both natural and vaccine-induced immunity, while cytotoxic CD8⁺ T cells seem to be more involved in clearance of primary infection and recurrent HSV-2 disease episodes.

Viral immune evasion

Like most viruses, HSV has evolved several strategies to evade host defenses. It can interfere with PRR and type I IFN signaling at several levels by inhibiting or reducing activity of transcription factors, such as IRF3, IRF7, signal transducer and activator of transcription 1 (STAT1) and NF- κ B. The virus can also inhibit complement factors and secretion of antimicrobial peptides. Furthermore, it can block apoptosis of the host cell and hinder antigen presentation via the MHCI and MHCII pathways. Viral proteins involved in evasion mechanisms include infected cell protein 0 (ICP0), ICP27, ICP34.5 and virion shutoff protein (vhs). Some of the envelope glycoproteins, like gB, gC, gE/gI and gJ, have also been found to impede certain immune responses¹⁴⁹.

Most studies, related to HSV immune evasion, have been performed using HSV-1 and it is likely that HSV-2 acts in similar ways to render host immunity. However, there are some results specifically linked to HSV-2. For example, in acute genital herpetic lesions it was shown that type I IFNs were more or less absent, while high IFN- γ levels could be detected¹⁴⁵. In humans, viral ICP47 can bind to the transporter associated with antigen presentation (TAP), thereby preventing binding of antigenic peptides to MHCI¹⁵⁰. HSV-2 can also infect DCs, alter their expression of MHC molecules or induce apoptosis. Furthermore, HSV-2 has been reported to actively promote survival of epithelial cells^{151,152}.

Vaccine against genital herpes infection

The only vaccines licensed to prevent a STI are the HPV vaccines Gardasil and Cervarix. They are given by intramuscular (i.m.) injection and are believed to confer protection by inducing neutralizing antibodies transferred to the reproductive tract from the circulation¹⁵³. Despite numerous efforts, no cure or vaccine against genital herpes has been developed. Ideally, a vaccine will induce sterilizing immunity in the vaginal mucosa and prevent infection of dorsal root ganglia. A therapeutic vaccine that could dampen recurrences and lower viral shedding would also be a desirable option.

In animal experiments, mice or guinea pigs are most frequently used as HSV-2 challenge models^{154,155}. Models of rats have also been established but are less studied^{156,157}.

Antigen

Live vectors

A majority of all licensed human vaccines are based on live attenuated or inactivated pathogens, carrying the target pathogen's complete set of unique antigens and PAMPs. Vaccines based on live attenuated viruses are generally characterized by limited replication in the host and trigger activation of the innate immune system by danger signals, e.g. recognition of their nucleic acids by PRRs. Inactivated vaccines do not replicate within the host and are therefore often less effective. Even though vaccines currently in use generally demonstrate excellent safety, the development of formulations based on live or inactivated microorganisms still involves a degree of unpredictability regarding safety and stability. There will for example be a potential risk that an attenuated strain reverts to a pathogenic form and causes disease¹⁵⁸⁻¹⁶⁰.

Many forms of live attenuated and inactivated HSV vaccines have been tested in animal models (Table 2) and several offered significant protective immunity against genital herpes challenge¹⁶¹⁻¹⁷².

Subunits

Subunit vaccines are based on one or several parts of the pathogen. The antigen can be purified directly from the microbe or be produced using recombinant technology. Recombinant subunit vaccines can be constructed with proteins, peptides, DNA vectors, and other more complex structures, such as virus-like particles^{158,160}.

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A majority of all HSV-2 vaccines tested in pre-clinical settings and human clinical trials have been based on viral glycoproteins, which nowadays mostly are produced in recombinant form. These envelope proteins are highly exposed and make them suitable targets for antibody-mediated immune responses. Moreover, as the glycoproteins are involved in the initial steps of infection by facilitating attachment and entry into the host cells, a vaccine based on these antigens would potentially be able to stop the virus at an early stage. The most frequently used HSV-2 antigens are no doubt gB and gD. Some of the pre-clinical studies and early phase I/II human clinical trials studies in which HSV-2 glycoproteins have been included are listed in Table 2. Also, the subunit vaccines that have reached human phase III clinical trials are discussed below. However, there is a growing interest to exploit other HSV antigens as well, such as components of the tegument and capsid.

Adjuvants

Vaccines based on live attenuated/killed microorganisms or those that contain bacterial toxins typically act as potent stimulators of the immune system. The use of such vaccines has been a true success story, reducing the prevalence of some infections, such as polio, measles and diphtheria, by nearly 100%. Other types of infections, like HIV and malaria, have proven to be more challenging to control with traditional vaccines and require novel strategies.

The problem with new approaches focusing on highly purified antigens is that many of them are poor immunogens, particularly those based on recombinant peptides or proteins. Furthermore, when attempting to induce immunity within mucosal tissues it is necessary to overcome their tolerogenic nature. Initiation of efficient immune responses to poor immunogens may be enhanced by inclusion of a vaccine adjuvant. The name adjuvant comes from the Latin *adjuvare*, which means "to help". Adjuvants can have the capacity to boost, facilitate, accelerate, prolong or modulate an immune response to co-administered antigen.

Immunomodulation and focus on delivery are two main considerations for adjuvant formulations. Many adjuvants combine these two properties. There is a wide range of experimental adjuvants in use but only a few are currently included in licensed human vaccines: alum, aluminium salt; MF59, oil in water emulsion of squalene oil; AS03, squalene-based; AS04, monophosphoryl lipid A (MPLA) in combination with alum; and virus-like particles. At present, none of the vaccines applied via mucosal route contain any adjuvant.

Immunomodulators

An adjuvant can initiate or enhance an immune response but may also steer it in a certain direction. These immunomodulators function as signals of danger and are

often derived from pathogens or mimic pathogenic structures. By stimulating the innate immune system through PRRs, the desired end result is activation of APCs and subsequent initiation of acquired immune responses. By-passing PRR activation is also possible, by direct administration of recombinant cytokines/chemokines. What type of immune response the adjuvant should drive depends on the infection. Safe adjuvants that generate strong systemic antibody responses are available, like the most commonly used adjuvant alum, whereas there is a great need for adjuvants promoting cell-mediated immune responses. TLR agonists have been extensively used in pre-clinical vaccine research. A TLR4 agonist, MPLA, is included in AS04 that is now part of human vaccines against hepatitis B virus and HPV.

Delivery systems

Another focus lies on antigen deliverance. These types of adjuvant systems may for instance protect the antigen from degradation, transport the antigen over barriers, form a depot that prolongs antigen exposure and/or present the antigen to APCs in a way that better mimics natural infection. Adjuvants that exhibit such properties include alum, liposomes, virus-like particles, nanoparticles, immunostimulating complexes (ISCOMs) and emulsions.

Targeting TLR9 with adjuvants

In humans TLR9 is mainly expressed on B cells and pDCs, while multiple cells of the myeloid lineage, such as monocytes, macrophages and conventional DCs, express it in mice ¹⁷³. There are some reports of TLR9 expression by epithelial cells/keratinocytes and by fibroblasts derived from nasal and vaginal mucosa as well as from dermis, which could be of importance for vaccination when targeting the receptor via these routes ^{18,174-177}.

CpG ODN

In 1995 it was reported that certain CpG oligodeoxynucleotide (ODN) repeats stimulated B cells proliferation ¹⁷⁸. Later, TLR9 was identified as the primary receptor for CpG ODN ¹⁷⁹. Microbial genomes, both bacterial and viral, often have higher contents of unmethylated CpG islands than mammalian genomes, which more frequently are methylated. The discrimination between microbial and mammalian CpG motifs appears to relate to the ability of unmethylated/methylated CpG to co-localize with TLR9 in late endosomes, rather than the actual receptor binding ¹⁸⁰.

Upon TLR9 activation, production of pro-inflammatory cytokines, such as TNF- α , IL-6 and IL-8, and type I IFNs is initiated. Furthermore, MHCI, MHCII and co-stimulatory molecules can be up-regulated on the cell surface, facilitating antigen presentation. Synthetic forms of CpG have frequently been used as adjuvants. Different variants

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are used in humans and mice. For humans, four types of CpG have been described: D (also known as class A), K (also known as class B), C and P. These types differ with regards to both the backbone and sequence, and mediate slightly different responses. For example, type D induces strong pDC activation with IFN- α secretion, type K induces strong B cell activation with TNF- α and IL-6 secretion, type C activates both pDCs and B cells with IL-6 and IFN- α secretion, and type P activates both pDCs and B cells with IFN- α secretion¹⁷³

Several pre-clinical studies on HSV-2 vaccines have utilized CpG as adjuvant, some listed in Table 1. The documented ability of CpG to induce immune responses at mucosal surfaces makes it an attractive mucosal adjuvant candidate. CpG has recently been used in several human clinical trials, e.g. in vaccines and in immunotherapy for cancer treatment¹⁸¹.

IC31[®]

IC31[®] is a two-component adjuvant system that contains the synthetic peptide KLK, consisting of Lysine-Leucine-Lysine repeats, and the non-CpG motif-bearing ODN d(IC)₁₃ (ODN1a)¹⁸². The peptide was designed to mimic a naturally occurring cationic antimicrobial peptide. It was originally derived from sapecin B from *Sarcophaga peregrine* (flesh fly), but modified to increase its activity¹⁸³. The KLK peptide has been shown to activate human neutrophils and monocytes, possibly through binding to cell surface calreticulin¹⁸⁴. It induces T_H2-polarized immune responses to co-delivered antigens, has a depot effect at the injection site and enhances antigen uptake by APCs^{182,185,186}. The ODN1a has an unmodified phosphodiester backbone designed to avoid the systemic side effects of CpG motif-bearing ODNs^{182,186}. It promotes antigen-specific T_H1 responses.

The adjuvant effect from IC31[®] seems to be mediated by a KLK-mediated endocytotic uptake of ODN1a by DCs, and the subsequent ability of ODN1a to activate TLR9/MyD88 signaling pathway,^{182,186,187 186,188}. Importantly, the adjuvant has been tested in phase I clinical trials and was shown to induce T cell responses in humans¹⁸⁹.

Route of immunization

Most vaccines are given by i.m. or subcutaneous (s.c.) injection. Intradermal (i.d.) injection is also practiced to some extent, mainly for tuberculosis and rabies vaccines. There are also a few mucosal vaccines available today, protecting against cholera, polio, rotavirus infection and typhoid (oral) as well as influenza (nasal). All of these mucosal vaccines are based on live attenuated or killed virus/bacteria. In order to elicit mucosal T cell responses it seems that a mucosal route of

immunization would be favorable ¹⁹⁰. Various immunization routes have been explored pre-clinically for inducing genital herpes immunity, while i.m. injection has been the primary route tested in human clinical trials. Some of the other routes as listed in Table 2 and briefly discussed below have also practiced with some encouraging results.

Skin route

The close connection between the vaginal mucosa and skin makes cutaneous delivery of vaccines highly relevant. There are three main skin-related immunization approaches: transcutaneous, targeting both epidermis and dermis by forging a vaccine formulation to pass through the skin from the outside; i.d., injecting directly into the dermis; and s.c., injecting into the fat layer situated underneath the skin but above the muscle. The epidermis and dermis are highly populated by APCs, including LCs (mainly in the epidermis) and DCs (mainly in the dermis). The keratinocytes are also highly involved in the initiation of immune responses. Epidermal and dermal professional APCs appear to be more adapted to the generation of immune responses than those present in the s.c. or muscle tissues. Skin injections offer the opportunity to use vaccines with depot effect and several systems for i.d. administration have been developed, e.g. patches and microneedles. There are only a limited number of animal studies conducted on the potential of i.d. immunization for protection against HSV-2 ^{171,191}.

Nasal route

The subepithelial cell layer in the nose is highly vascularized and the venous blood reaches the systemic circulation directly, without passing the liver. A strong systemic immune response would therefore be anticipated after i.n. immunization ¹⁹². Furthermore, intranasal (i.n.) administration of vaccines can give rise to immunity in the female reproductive tract in both humans and mice. Most of these studies have focused on vaginal antibodies and it seems that the immunity provided through i.n. immunization is rather dependent on the humoral response ¹⁹³⁻¹⁹⁷. However, it was recently shown that i.n. immunization specifically yielded HSV-2-specific IFN- γ producing memory T cells in the vagina. The mechanism behind this induction of immunity at a distant site is unknown but suggests that the nasal and vaginal mucosa share homing receptors ⁵². Some studies have shown the i.n. immunization route to be superior over systemic and i.vag. vaccination ^{167,198,199}.

Vaginal route

Several studies have displayed the potential of i.vag. immunization for protection against genital herpes infection in mice and guinea pigs ^{169,200-202}. Some reports have shown that i.vag. delivery is more effective than i.n. and i.d. routes for protection against primary and recurrent genital herpes and/or eliciting specific vaginal immune responses. The induction of vaginal CD4⁺ T cell responses as well as tissue-

THEORETICAL BACKGROUND

resident memory cells also appears to be highly dependent on local vaccination^{54,171,197,203}, though the dependency on the hormone cycle must be considered^{66,196,204}.

Heterologous

Another option is to combine different routes for vaccination and some of these attempts are listed in Table 2. It might for instance be possible to generate potent antibody responses via one route and vaginal T cell responses by another. Another novel concept involves priming lymphocytes at a systemic site and pulling them to the vagina via I.vag. delivery of chemokines²⁰⁵. A combination of different vaccine modalities e.g., protein, DNA, live vector for prime-boost immunization, has also been considered for induction of immunity to genital herpes in mice^{206,207}.

Pre-clinical vaccine studies

In Table 2, some of the successful preclinical studies are listed alongside recent human trials.

Human phase III clinical trials

Two prophylactic HSV-2 subunit vaccines have completed phase III trials. The first one contained the antigens gB plus gD, with the adjuvant MF59. The vaccine elicited high antibody titers but did not provide protection to HSV-2 acquisition^{208,209}. The second vaccine contained the antigen gD in combination with the adjuvant AS04. In one study, HSV-1/HSV-2 seronegative women appeared to be protected by the vaccine but in a multicenter follow-up study no significant protection to HSV-2 was observed in the seronegative cohorts²¹⁰. However, significant protection to genital HSV-1 infection was observed, which correlated with neutralizing antibodies as already mentioned¹³⁶. There are a couple of ongoing therapeutic vaccine trials that have presented encouraging data, such as reductions in HSV-2 shedding^{211,212}.

TABLE 2. Pre-clinical and early phase I/II HSV-2 vaccine studies (modified from ^{206,207}).

Type	Antigen	Adjuvant/Virus	Route	Ref
<i>Preclinical</i>				
Protein	gB1	Non-ionic vesicles	i.n.	213
Protein	gB2	CpG	i.n./i.vag.	200,214
Protein	gD2-Fc fusion	CpG	i.n.	215
Protein	gB2 +/- or gD2	CpG	i.m.	216
Protein	gD2	CpG	i.vag.	202
Protein	gC2 + gD2	CpG + alum	i.m.	217,218
Protein	gD2	Liposomes + MPLA	s.c.	219
Protein	gD2	α -GalCer	i.n./i.vag.	220
Protein	gD2	Proteoliposomes	i.n.	221
Protein	gG2	CpG	s.c. + i.n.	222
Peptide	Epitope conjugate	Palmitic acid	i.vag.	223
Virus	Vaccinia virus expressing gD2	Live	i.d./i.vag.	171
Virus + protein	HSV-2 TK ⁻ (prime)	CXCL9 + CXCL10 (pull)	s.c. + i.vag.	205
DNA + virus	DNA (prime) + formalin-treated HSV-2 (boost)	MPLA + alum	i.m. + s.c.	224,225
DNA + protein	gD2 DNA (prime) + protein (boost)	Liposomes	i.m. + i.n.	226
<i>Phase I/II prophylactic</i>				
Virus	HSV-2 mutated for UL5, UL29	Live non- replicating	s.c./i.m.	201,227,228
DNA	Modified gD2 DNA	Ubiquitin-fused plasmid	i.d.	229
Virus	HSV-2 mutated for ICP10	Live replicating	s.c.	230,231
<i>Phase I/II therapeutic</i>				
Protein	gD2 + ICP4	Iscomatrix MM2	s.c.	232
Peptide	32 HSV-2 peptides	HSP70 + QS-21 saponin	i.d./s.c.	191,233
DNA	gD2, UL46, UL47	Cationic lipid	i.m.	234

α -GalCer: alpha-galactosylceramide

Systems vaccinology

Systems information regarding innate and acquired immune responses elicited after vaccination can help understanding the mode of actions of successful vaccines, which in turn may provide insights into the design of new vaccines. The emergence of high-throughput “omics” technologies, such as transcriptomics, proteomics and metabolomics, combined with advances in systems biology analysis has provided an opportunity to analyze immune responses at a system level. Network analysis and predictions, plus the exploration of signaling pathways, will help in understanding the molecular signatures indicative of vaccine-induced protective immunity^{160,235}. Molecular signatures and correlates of vaccine-induced immune responses have recently been investigated for several licensed human vaccines such as the yellow fever vaccine and an influenza vaccine^{236,237}. Systems biology has also recently been employed to study the mode of actions of vaccine adjuvants (reviewed in Olafsdottir T et al., *Vaccine*, 2015). A whole genome transcriptomics analysis of CpG and α -GalCer in the murine female genital tract unraveled common and unique molecular signatures of these two immunomodulators, previously shown to serve as equally potent vaginal adjuvants for protection against genital herpes^{202,220,238,239}. It is envisaged that systems vaccinology could provide invaluable information to inform rational development of vaccines against genital herpes and other STIs.

AIMS

The main objective of this thesis was to investigate immunization strategies for immunity to primary and recurrent genital herpes infection and to dissect molecular patterns linked to protective immunity against HSV-2.

Specifically:

- To compare nasal and skin routes of immunization with a novel adjuvant for generation of protective immunity to primary genital herpes infection in mice.
- To assess the potential of nasal immunization for induction of high avidity neutralizing antibody response and protection to primary and recurrent genital herpes infection in guinea pigs.
- To define the early molecular and immune cell profiles of the vaginal mucosa upon vaginal administration of an experimental gold standard vaccine in mice.

KEY METHODOLOGIES

Animals (Paper I-III)

There are essentially two different challenge models used in HSV-2 research, mouse and guinea pig, both of which were used in the work included in this thesis. In Paper I-III, an in-bred mouse strain, C57BL/6, was used. In addition, a mouse strain deficient in functional B cells, μ MT on C57BL/6 background²⁴⁰, as well as out-bred Dunkin-Hartley guinea pigs were used in Paper II. Figure 3 displays an overview of the experimental outline. Animals were used in accordance with ethical permits obtained from the Ethical Committee for Animal Experimentation in Gothenburg, Sweden.

Immunization studies (Paper I-III)

Antigen

Paper I-II: A recombinant form of HSV-2 gD protein was used for immunizations. The recombinant protein was truncated, including the ectodomain and part of the transmembrane domain. The production was performed by the Mammalian Protein Expression core facility, University of Gothenburg. Details regarding the production can be found elsewhere²²¹. Briefly, the gene, encoding amino acids 1–342, was amplified by PCR using DNA from HSV-2 strain 333. The product had an added C-terminal His₆-tag. The expression vector was transfected into Chinese hamster ovary K1 cells. Nickel-based affinity purification was performed to retrieve the polyhistidine-tagged protein from cell supernatants.

Cell line

African green monkey kidney cells (GMK-AH1), an adherent epithelial cell line, were grown to confluent monolayers and used for virus production, titration and neutralization assays.

Virus

Paper I-II: Live wild-type virus, HSV-2 strain 333, was used for challenge studies. Paper III: Live attenuated virus, HSV-2 TK⁻ strain Lyon, was used for immunizations. Both strains were grown in cell culture flasks. Viral stocks were prepared by a freeze-thaw cycle, followed by removal of cellular debris by centrifugation, and stored at -80°C. In order to verify that the HSV-2 TK⁻ preparation lacked the TK gene the virus was grown in the presence of varying concentrations of acyclovir.

Immunization schedule

Paper I

The stock solution of the adjuvant IC31[®] was provided by Intercell AG, Austria. Mice were immunized via three different routes: i.n., s.c. and i.d. Two different doses (low and high) of IC31[®] were used for the s.c. and i.d. routes, while the volume limited the i.n. route to the low dose only. Skin injections were made at the base of the tail, using one and two sites for the s.c. and i.d. routes, respectively. Three immunizations were performed, ten days apart.

Paper II

Two variants of the adjuvant CpG ODN, with different sequences, were used: CpG 7909 for guinea pigs and CpG 1826 for mice. Immunization was performed via i.n. route. Three immunizations were performed, seven days apart.

Paper III

Mice were pre-treated with progesterone. Live attenuated HSV-2 TK⁻ was delivered i.vag. once. The dose of HSV-2 TK⁻ has previously been tested in challenge studies with a fully virulent HSV-2 strain 333 and is known to induce complete protection.

Analysis of antigen-specific immune responses (Paper I-II)

Samples were collected three to four weeks after the final immunization and used for evaluation of antigen-specific antibody and T cell responses.

Sample collection for antibody evaluation

Paper I: Blood samples were retrieved from mice by tail vein rupture. Whole vaginal tissues (cervix excluded) were collected from sacrificed mice and used for saponin-extraction. Paper II: Blood was collected from guinea pigs by puncturing the hind leg *v. saphena*. Swab samples were collected from the vaginal vaults of guinea pigs.

Isolation of lymphocytes

Paper I: Spleens were collected from sacrificed mice. Paper II: Blood was withdrawn directly from the heart in guinea pigs under deep sedation, just prior to euthanasia. The blood was used for isolation of peripheral blood mononuclear cells (PBMCs). Additionally, spleen and genital draining lymph nodes were excised from sacrificed guinea pigs. Cells were isolated from the lymphoid organs.

Proliferation assay

Paper I-II: Cells retrieved from lymphoid organs and blood were seeded in 96-well plates and re-stimulated with gD antigen. The level of cell proliferation was estimated by measuring [6-³H] thymidine incorporation at 72 (guinea pig) or 96 h (mice). Paper I: Cell supernatants were collected at 96 h and used for cytokine

protein analysis. Paper II: Cells were collected from the cultures at 24 and 48 h for analysis of cytokine mRNA expression.

Antibody levels and avidity by ELISA

Paper I-II: The content of gD-specific IgG antibodies were determined in sera and vaginal swabs by an indirect ELISA. Paper II: ELISA, with the addition of a potassium thiocyanate (KSCN) elution step, was also used to measure relative avidity of the gD-specific IgG antibodies in sera²⁴¹. Avidity index was defined as the concentration (M) KSCN needed to dissociate 50% of bound antibodies.

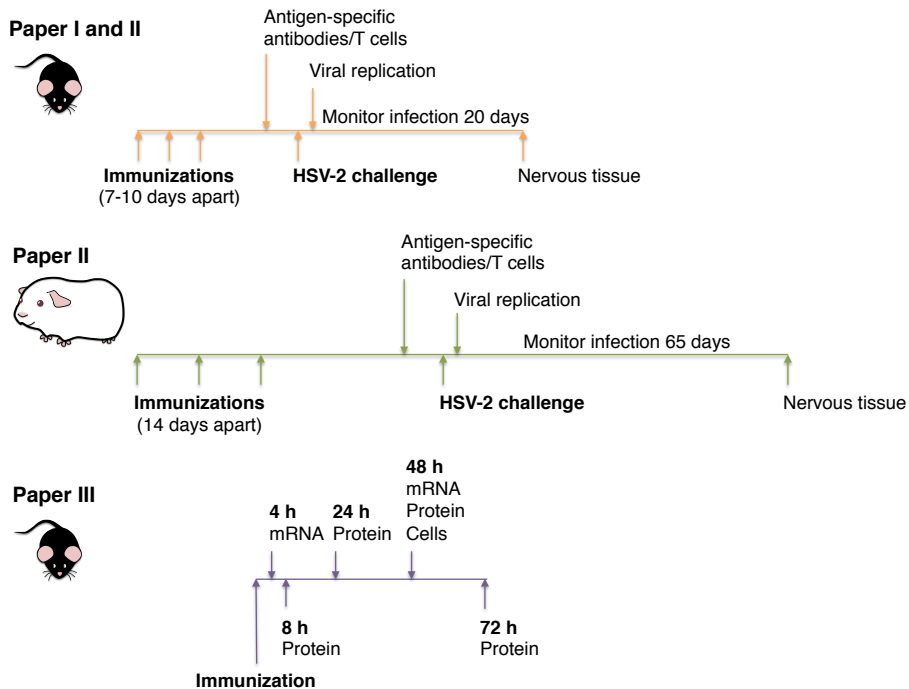


FIGURE 3. Experimental design for immunization studies in Paper I-III.

Antibody avidity by surface plasmon resonance (SPR)

Paper II: SPR was used to detect binding between immobilized gD antigen, captured via direct or indirect (via His-tag) on a sensor chip, and purified guinea pig IgG antibodies flowing over it. The technology is based on an optical phenomenon that occurs when light at a certain angle is reflected off a thin metal film (a gold spot on the sensor chip), leading to total internal reflection. The light excites surface

plasmons (electron oscillations that exist between any two materials). The internal reflection generates a wave in the thin metal film that extends into the liquid flowing above. The excited surface plasmons are sensitive to the refractive index change at the surface of the thin metal film. Thus, the angle of the light required for SPR will be impacted by the refractive index change when antibodies bind to the antigen that is in contact with the chip surface. The changes will be tracked in real-time by a detector. The change of the angle required for SPR is defined as response unit (RU), which reflects the surface density of bound antibodies. Relative avidity was determined by taking the average maximal binding during the association phase divided by the dissociation rate constant (kD) to yield the avidity score ($[RU \times s]$).

Mapping of epitope targeting by competitive SPR

Paper II: Purified guinea pig IgG antibodies were injected into both channels of the SPR sensor. This was followed by injection of previously characterized gD-specific mouse monoclonal antibodies. The right channel was used as a reference channel to record the background binding. The blocking activity of each guinea pig IgG sample was calculated for each monoclonal antibody as a percentage using the formula: $[1 - (RU \text{ MAb binding to guinea pig IgG-coated chip} / RU \text{ MAb binding to control chip})] \times 100$.

Neutralizing antibodies

Serum samples were serially diluted and pre-incubated with HSV-2 strain 333. The virus was then applied to GMK-AH1 cells and after 72 h cells were stained with crystal violet, and the cytopathic effect examined. Neutralization was considered as the highest dilution of sera at which a 50% reduction of plaques, relative to virus control, was seen.

Cytokine ELISA

Paper I: Supernatants from stimulated splenocytes, collected after gD re-stimulation, were analyzed for IFN- γ and IL-5 levels by a sandwich ELISA.

Cytokine mRNA analysis

Paper II: Total RNA, isolated from guinea pig cells re-stimulated with antigen post-immunization, was converted into cDNA and analysed with primers specific for IFN- γ and IL-5.

HSV-2 challenge (Paper I-II)

Mouse infection model

Paper I-II: Mice are generally used for the study of basic immune responses as well as protection level against genital herpes infection post-immunization. The access to reagents and genetically modified mice makes this model attractive. However, the mouse model can only be used to study primary HSV-2 infection, as mice do not develop recurrent disease. Furthermore, the mice require pre-treatment of progesterone to make them susceptible to the infection. Symptoms of disease normally emerge after a few days, peaking around 7 days post-infection. The duration of the challenge experiments were 14-20 days, during which mice were monitored daily for symptoms of disease.

Guinea pig infection model

Paper II: Guinea pig HSV-2 infection resembles that of humans, with a primary and latent phase of the disease. In contrast with mice, guinea pigs are not dependent on hormone pre-treatment in order to be susceptible to infection. If using a high dose of a neurovirulent virus strain, as in our studies, a fraction of the naive control group may display severe primary infection requiring euthanasia. However, some of the control guinea pigs normally survive and establish a productive latent infection within the nervous system. The first 14 days are considered as the primary infection period, which will be followed by spontaneous reactivation episodes causing recurrent disease and viral shedding. Guinea pigs are often followed for several months post-infection (three months in our studies). Thus, the model is highly relevant to study prophylactic or therapeutic vaccination. Still, there are some limiting factors. Firstly, read-outs are more difficult due to lack of reagents. Secondly, immunological differences between humans and guinea pigs are not mapped very well.

Viral replication and shedding

Paper I-II: To determine replication of the virus in the vagina during the acute phase, vaginal samples collected 3 days post-infection and assessed in a viral plaque assay. Paper II: In one of the guinea pig experiments, samples were also retrieved twice a week during the latent phase of infection (13 days in total) in order to study viral shedding by real-time PCR.

Nervous tissues

Paper II: Guinea pigs surviving the HSV-2 infection were sacrificed approximately 65 days post-infection and the lumbosacral dorsal root ganglia were collected. Lumbosacral dorsal root ganglia and the spinal cord corresponding to the same region of the spine were also collected from challenged mice. The tissues were used to establish the presence of virus within the nervous system by real-time PCR.

Real-time PCR analysis viral presence

Paper I-II: Purification of total RNA or co-purification of RNA/DNA was performed for collected nervous tissues. Isolated RNA samples from guinea pig dorsal root ganglia was converted into cDNA and analyzed with primers specific for HSV-2 LAT and host IFN- γ expression. DNA samples from vaginal swabs (guinea pig), ganglia (guinea pig and mouse) and spinal cord (mouse) were analyzed for the presence of HSV-2 gB DNA.

Study on early immune responses (Paper III)

Samples

Whole vaginas (cervix excluded) were excised from sacrificed mice at 4, 8, 24, 48 or 72 h after immunization. Samples were used to study immune responses at gene (4 and 48 h), protein (8, 24, 48 and 72 h) and cellular level (48 h).

Genome wide expression microarray

Total RNA was extracted from the vaginal tissues and the quality assessed before submitted for genome-wide gene expression analysis by hybridizing to Affymetrix Mouse Gene 1.0 ST arrays. The analysis was performed at the SCIBLU Genomics core facility (Swegene Centre for Integrative Biology at Lund University, Sweden). All raw intensity files (CEL) were pre-processed and statistically analyzed before used for further analysis. The calculated p-values were transformed to Q-values by correcting for multiple testing. Q-values were overlaid on the annotated network of Gene Ontology (GO) followed by applying a reporter algorithm to evaluate statistical significant of each GO category.

Ingenuity pathway analysis (IPA)

The entire microarray data set for 4 and 48 h with associated Q-values ($Q < 0.001$) and Log_2 -fold changes were submitted for IPA. Patterns in gene expression can be identified by relating the significantly altered genes to the information found in the Ingenuity knowledge database that consists of manually reviewed publications.

Protein analysis by antibody array

Proteins were extracted from vaginal tissue in the presence of protease inhibitors and samples were individually analyzed for 111 proteins using an antibody array. Each sample was added onto a blocked membrane covered with arrayed antibody supports, after which a cocktail of biotinylated antibodies were allowed to attach. Following addition of streptavidin conjugated to a peroxidase enzyme, the substrate luminol was added and the resulting chemiluminescent signal detected by an image system.

In silico prediction of immune cell populations in complex tissue

The collection of transcriptional profile of various immunological cell lineages and activation states (GSE15907) were retrieved from the ImmGen project²⁴², pre-processed and normalized. With this dataset, transcriptional signatures of each specific cell phenotype were identified. The genes that had a p -value < 0.01 and a fold change >2 were considered as transcriptional signatures of the cell population.

The identified signature genes for each phenotype used to do an *in silico* prediction of potential immune cells present in the murine vagina after HSV-2 TK⁻ inoculation. The cumulative transcriptional changes for each signature gene and the rest based on differential gene expression of virus treatment and control were compared and evaluated statistically. The cell populations with an adjusted p -value $< 1^{-10}$ were considered significant.

Immune cell analysis by flow cytometry

Cells from vaginal tissues were isolated by mechanical and enzymatic dissociation. Cells were blocked with anti-CD16/32 followed by staining with conjugated antibodies directed towards: CD3, CD4, CD11b, CD11c, CD45, CD103, CD115, F4/80, Ly6C, Ly6G, MHCII, NK1.1, TCR β and TCR δ . The live/dead stain 7AAD was also included. The analysis was performed on a BDTM LSR-II cytometer.

Statistical analysis

In experiments with two groups data were analyzed with a two-tailed unpaired t test (95% CI). A one-way ANOVA followed by the Tukey multiple comparison test (CI 95%) was used for experiments with more than two groups. Survival data (Paper I) were analyzed with the Kaplan-Meier method, with subsequent pairwise comparisons by the Gehan-Breslow-Wilcoxon test and correction for multiple comparisons by setting a Bonferroni corrected threshold. Survival data (Paper II) were analyzed with the Kaplan-Meier method and the Log-rank test. Correlations were computed by nonparametric Spearman test. Differences were considered statistically significant at p values of < 0.05 (*), < 0.01 (**) and < 0.001 (***)

RESULTS AND DISCUSSION

The below text summarizes the major findings of the three papers included in this thesis, and as such should not be deemed exhaustive.

Paper I

Nasal and skin immunization with gD adjuvanted with IC31[®] for vaccine-induced protection to primary HSV-2 infection

There is currently a very limited choice of adjuvants with high potency and acceptable safety tested in humans. Further, most available adjuvants merely elicit antibody responses. This poses challenges for development of vaccines against infections that require IFN- γ responses and possibly the formation of antigen-specific cytolytic CD8⁺ T cells, such as genital herpes. IC31[®] is a novel adjuvant that combines a synthetic anti-microbial peptide, called KLK, and a TLR9-targeting non-CpG ODN. In combination, the two components result in an adjuvant that forms a depot at the injection site and promotes a mixed T_H1/T_H2 immune response, dependent on TLR9/MyD88-signaling^{182,186,243}. The IC31[®] adjuvant has previously been included in a subunit tuberculosis vaccine and found to elicit protection in both mouse and guinea pig models of tuberculosis infection²⁴⁴. Importantly, it has also been shown to safely induce long-lasting T cell responses in humans.

So far, pre-clinical and clinical studies including IC31[®] have been restricted to the most common parenteral routes of immunization. The current study is the first side-to-side comparison between the nasal-mucosal route and skin delivery using IC31[®]. It is also the first report providing evidence that IC31[®] holds potential as an adjuvant for induction of protective immunity in a mouse model of genital herpes infection.

Female C57BL/6 mice were immunized three times with recombinant HSV-2 gD antigen alone or adjuvanted with IC31[®] via either i.n., s.c., or i.d. route. Two different doses of IC31[®] were used for the skin routes, while only the low dose could be used for nasal delivery due to volume restriction. Antigen-specific antibody and cellular responses were evaluated three weeks after the third vaccination.

All three immunization routes were able to induce a vaginal and systemic gD-specific IgG antibody response (Figure 4). The skin routes (s.c. and i.d.) induced a greater amount of antibodies than nasal delivery, with the high dose of IC31[®] injected i.d. giving rise to the highest IgG antibody levels. Interestingly, the route played a major role in IgG isotype class switching. While both s.c. and i.d. injection of adjuvanted gD induced a mixed IgG1/IgG2c response, i.n. immunization only

RESULTS AND DISCUSSION

elicited IgG1 antibodies at levels similar to antigen alone (Figure 4). In C57BL/6 mice the IgG2c isotype is an indicator of a T_H1 response, which would suggest that skin immunization more strongly steered the immune response in this direction ²⁴⁵. Furthermore, IgG2c antibodies have been shown to have greater HSV-1 neutralizing capacity than IgG1 antibodies ²⁴⁶. The s.c. and i.d. immunization with gD combined with the high dose of IC31[®] also gave rise to systemic antibodies that significantly neutralized HSV-2 *in vitro*. This indicates that the higher adjuvant dose induced a broader or more potent antibody response.

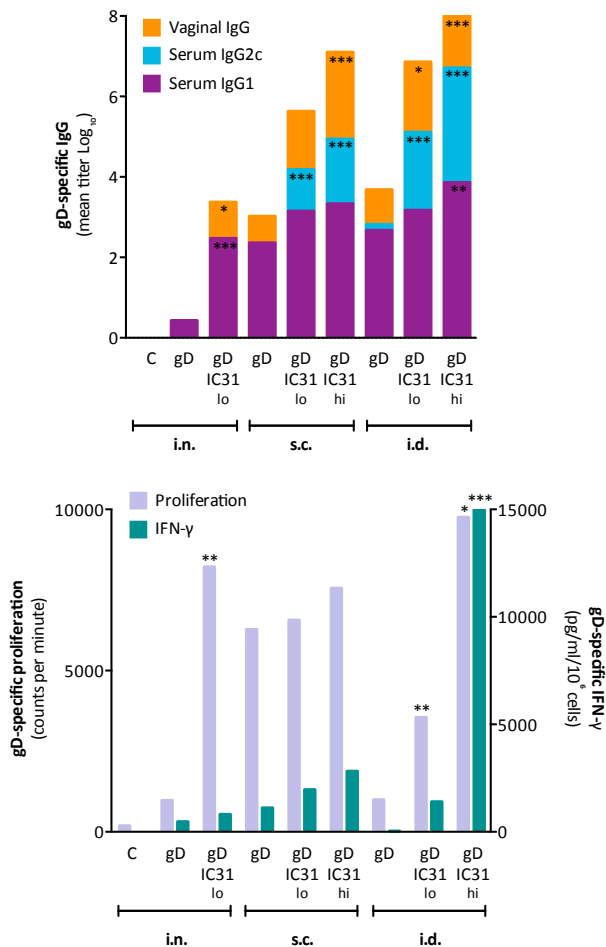


FIGURE 4. Antigen-specific immune responses post-immunization. IgG antibody levels specific for gD antigen, measured by ELISA (top). Splenocyte proliferative and IFN- γ responses after re-stimulation with gD antigen *in vitro* (bottom).

The notion that the skin immunization routes directed the immune response more towards T_H1 was supported by the analysis made of gD-specific proliferative responses and IFN- γ production. Both i.n. and i.d. immunization induced a significant proliferative response after antigen re-stimulation but a significant IFN- γ level was only observed following i.d. immunization (Figure 4). However, the IFN- γ production was clearly dependent on administration of the high IC31[®] dose, which could not be given via the nasal cavity.

Despite having seen that skin vaccination appeared to drive the immune response more strongly towards T_H1 , all three immunization routes appeared to generate protection against genital herpes infection when IC31[®] was included (Figure 5). Both i.n. delivery as well as s.c. (low and high dose) and i.d. (low dose) injection resulted in low disease scores and 84-92% survival. However, only i.d. immunization with gD adjuvanted by the high dose of IC31[®] offered 100% survival of challenged mice. This group was also the only one displaying significant reduction of vaginal HSV-2 replication post-infection (Figure 5). In contrast, control mice and the groups given antigen alone displayed severe signs of infection and required euthanasia in a majority of the animals.

Previous studies have shown that nasal mucosa can be utilized as an inductive site for vaginal immunity^{167,193-199,220}. Here, we showed for the first time that IC31[®] functions as a nasal adjuvant and when combined with gD antigen, was able to partially prevent primary HSV-2 infection. It is unlikely that IC31[®] forms a depot in the nasal cavity; rather the adjuvant effect observed probably relates to its ability to stimulate innate immune responses via TLR9. Due to volume restriction we were only able to use the low IC31[®] dose, which was 1/10 of the high dose, for i.n. immunization. Therefore, it is difficult to conclude how i.n. delivery compares with skin injection as we could observe a dose-dependency effect for the s.c. and i.d. routes. Still, the results concerning IgG isotypes indicated that skin targeting directed the immune response more strongly towards T_H1 , as IgG2c was found explicitly in mice immunized by the s.c. and i.d. routes, with both the low and the high IC31[®] dose.

Natural immunity may not be able to prevent genital herpes infection but plays an important role for controlling it. Symptoms associated with the acute phase of infection are generally the worst and acquired immune responses are thereafter often able to dampen recurrent disease episodes. This encourages the belief that vaccine-induced immunity would be able to lower HSV-2 acquisition rates. It is well known that virus-specific memory $CD4^+$ and $CD8^+$ T cells express the skin-homing molecule CLA in HSV-2 infected humans^{247,248}. This implies that it might be favorable to induce such cells before exposure to the virus, which a vaccine targeting skin could offer. Previous reports have also documented that i.d.

RESULTS AND DISCUSSION

immunization specifically can induce a T_H1 -dominated immune response²⁴⁹⁻²⁵². Nevertheless, as there are no reports of CLA^+ T cells in the vaginal mucosa it is uncertain whether skin immunization alone would be able to generate sufficient protection in humans.

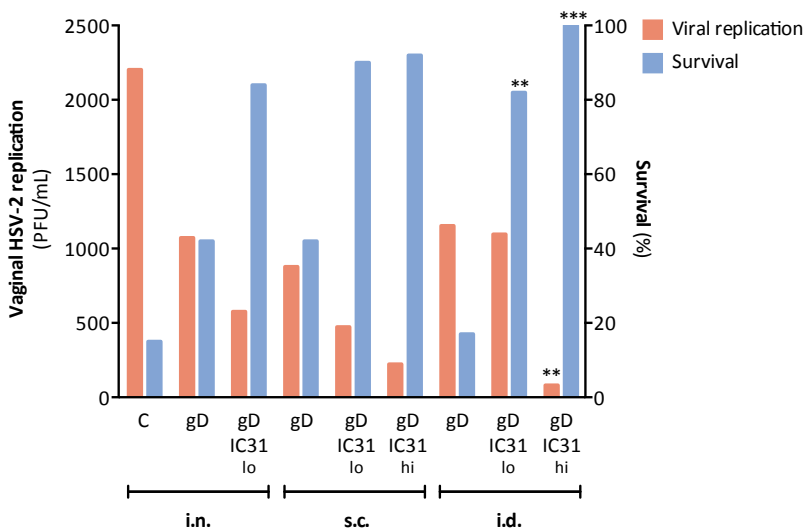


FIGURE 5. Disease progression after i.vag. HSV-2 challenge. Vaginal secretion samples were retrieved three days post-infection and tested for viral replication by plaque assay. Disease symptoms were recorded daily for 20 days and mice were sacrificed as soon as displaying severe signs of infection.

We found that the survival rates following HSV-2 challenge were high after both s.c. and i.d. immunization, although it was only the i.d. route (both $IC31^{\circ}$ doses) that induced significant protection compared to antigen alone. Interestingly, the mice i.d.-immunized with the high $IC31^{\circ}$ dose displayed full survival and were the only group that displayed a significant reduction of vaginal HSV-2 replication as well as induction of a gD-specific $IFN-\gamma$ response post-immunization. These results support previous studies showing that skin delivery is a highly efficient route of immunization when including $IC31^{\circ}$ as adjuvant^{186,243,244}.

We conclude that $IC31^{\circ}$ can serve as a highly efficient adjuvant in combination with HSV-2 gD protein for induction of protective immunity against genital herpes infection in mice. The study provided evidence that the adjuvant holds potential for delivery via both nasal mucosa and skin. These results lay the foundation to further test $IC31^{\circ}$ as an adjuvant for vaccines against genital herpes. The next step would be to test the potential of these immunization approaches in the HSV-2 guinea pig model, to see whether latent infection could also be prevented. Additional studies will also be needed to elucidate the mechanisms behind the observed protection.

Paper II

Nasal immunization for induction of high avidity neutralizing antibody response and protection to primary and latent HSV-2 infection

In this project we expanded on i.n. immunization for protection to genital herpes infection. To assess whether latent HSV-2 infection could be prevented, we set up a guinea pig challenge model in the lab. The HSV-2 disease outcome in guinea pigs closely resembles that of human with latency induction and spontaneous recurrences, and as such the model is highly relevant to study the impact of vaccination. In addition, complementary experiments were performed in the well-established mouse model.

Our group has previously reported that i.vag. immunization with recombinant HSV-2 gD antigen, combined with TLR9 agonist CpG ODN, generates close to full protection against primary genital herpes infection in mice²⁰². Different forms of CpG have been widely used as experimental adjuvants and are known to provoke strong T_H1 responses in most settings. We have also demonstrated that the i.n. route was highly efficient for induction of protective immunity when using gD together with the invariant NKT cell agonist α -GalCer²²⁰.

In this study, groups of female guinea pigs were i.n. immunized with HSV-2 gD protein alone or in combination with CpG ODN. Antigen-specific cellular and antibody responses were evaluated post-immunization. Cells, isolated from blood and lymphoid organs, displayed proliferative response and IFN- γ expression after antigen re-stimulation *in vitro* in the CpG-adjuvanted guinea pigs. In contrast, no responses could be detected in cells derived from the controls or from the group given antigen alone (Figure 6).

Vaginal gD-specific IgG antibodies were exclusively found in the adjuvanted group. Interestingly, the systemic IgG from the CpG-adjuvanted group exhibited functional antibodies displaying high avidity and potent HSV-2 neutralizing activity (Figure 6). Furthermore, by using competitive SPR with known mouse neutralizing monoclonal antibodies we could map antibody-targeted discontinuous gD epitopes that have been previously demonstrated to have HSV-2 neutralizing property (Figure 7)^{253,254}. The guinea pig IgG antibodies almost completely blocked the binding of two of the monoclonal antibodies (DL11 and MC5), both targeting epitopes that have been correlated to potent neutralizing IgG antibodies in a recent human vaccine trial²⁵⁵. It is of great importance to know that findings generated by the guinea pig model may be able to predict what can be expected from a vaccination strategy in humans.

RESULTS AND DISCUSSION

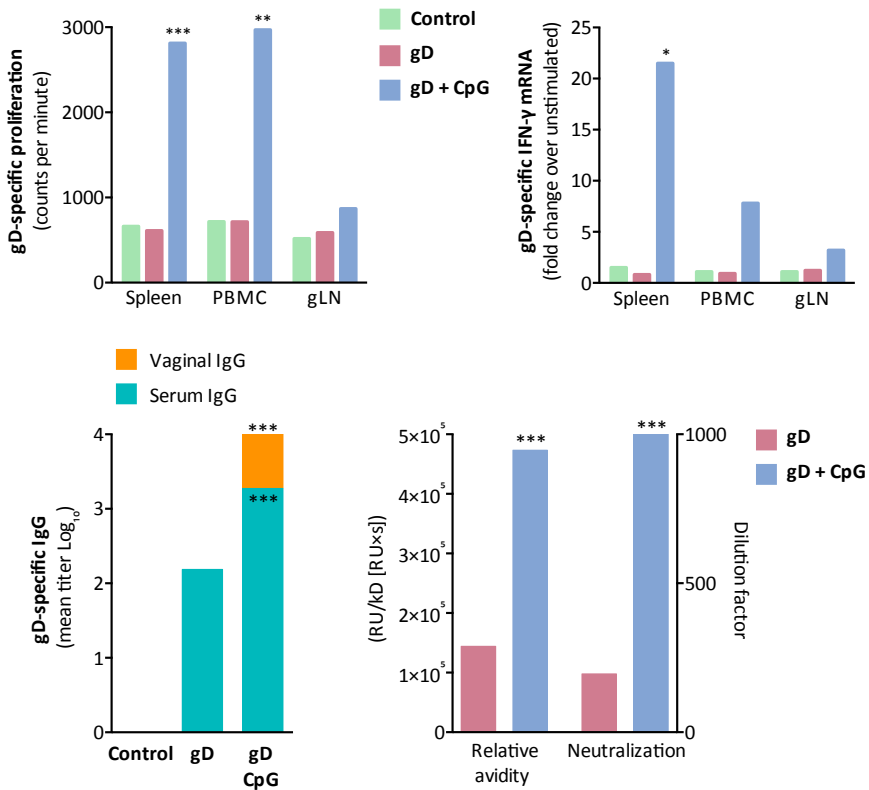


FIGURE 6. Antigen-specific immune responses in guinea pigs post-immunization. Cells were re-stimulated with gD *in vitro*, after which proliferative response (top, left) and IFN- γ mRNA expression (top, right) were assessed. Levels of gD-specific IgG, in the vagina and blood, were measured by ELISA (bottom, left). The relative avidity of purified IgG was analyzed by SPR and the capacity of serum to inhibit HSV-2 replication *in vitro* was determined by a HSV-2 neutralization assay (bottom, right). gLN=lymph nodes draining the genital tract.

The importance of B cells and/or antibodies for protection following nasal immunization was assessed in mice. Following a vaginal challenge, immunized μ MT mice developed severe HSV-2 infection similar to the control group, contrary to immunized wild-type mice that demonstrated close to full protection. It has been suggested that antibodies play a central role in inducing protection after i.n. immunization but it could also relate to a reduced capacity to induce vaginal T_H1

responses in the absence of functional B cells^{48,195}. Following HSV-2 challenge, the guinea pigs i.n. immunized with gD plus CpG demonstrated strong protection to primary infection, with no or mild symptoms of disease, virtually undetectable viral replication in their vaginal secretions and 100 % survival (Figure 8). In comparison, the controls and the group given gD alone demonstrated severe symptoms, a majority of which required euthanization. A majority of the survivors in these two groups turned out to be latently infected by the virus. Several guinea pigs in the adjuvanted group also demonstrated signs of latent infection, although recurrent outbreaks were either absent or at low frequency, and no or only low levels of latency markers (HSV-2 LAT and host IFN- γ mRNA) were detected in their dorsal root ganglia (Figure 8).

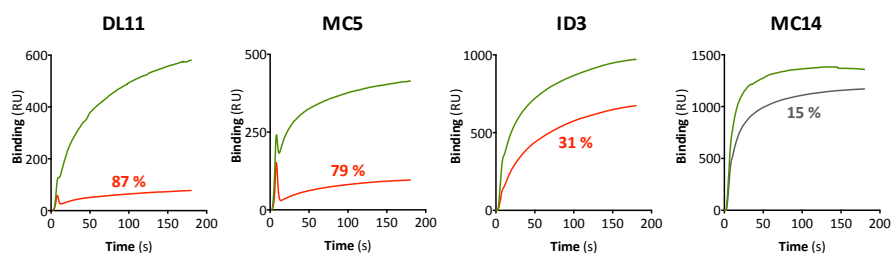


FIGURE 7. Mapping of epitopes targeted by gD-specific guinea pig IgG in competitive SPR analysis. Purified serum IgG antibodies from the adjuvanted group were allowed to associate with immobilized gD, followed by previously characterized neutralizing monoclonal antibodies. The monoclonal antibodies were considered as blocked when binding to gD was reduced >20 % (DL11 and MC5). Partial blocking was displayed in one instance, for which only two guinea pig samples could reduce binding (ID3). One monoclonal antibody retained its capacity to bind to gD (MC14).

We were able to show that i.n. immunization with recombinant gD antigen together with TLR9 agonist CpG ODN elicited strong protection to primary HSV-2 infection in guinea pigs. The immunization approach was also successful in reducing recurrent disease and establishment of latency in the DRG. Importantly, one third of the immunized guinea pigs did not demonstrate signs of latent infection.

RESULTS AND DISCUSSION

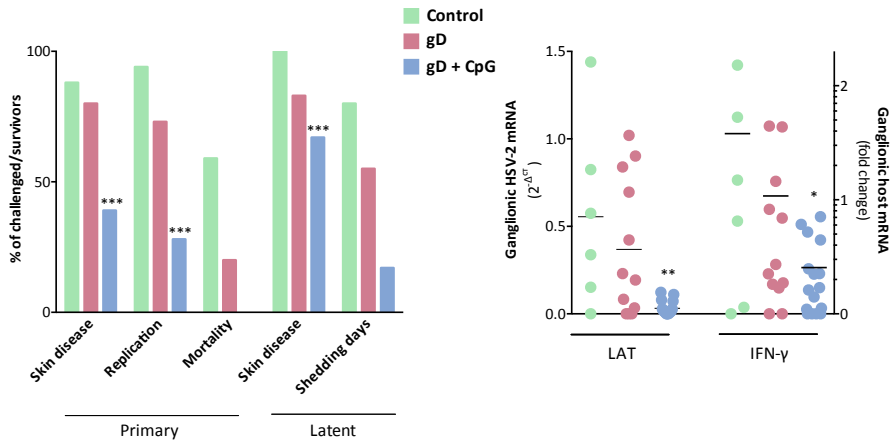


FIGURE 8. Disease progression (left) and latency within the nervous system (right) in guinea pigs following a high dose HSV-2 challenge.

Various vaccination strategies have been successful in eliciting protective immune responses to primary HSV infection in mice, while only a limited number of studies provided evidence on reduction of latent infection in the guinea pig model. Human clinical trials conducted so far have focused entirely on parenteral routes (mostly i.m.) of immunization. In mice, mucosal immunization has proven to be highly efficient. Interestingly, delivery of a live attenuated HSV-2 strain via nasal mucosa was shown to specifically elicit vaginal T_{RM} cells, only seen previously following i.vag. immunization⁵². In guinea pigs, only few mucosal immunization studies were reported and most of them have been based on administration of live attenuated virus^{170,171,256}. It is of great interest to evaluate whether mucosal subunit vaccines have the capacity to elicit protective immune responses to genital herpes, not just to primary infection but also to latent infection. For this purpose, immunization approaches displaying potential in mice must be transferred to the guinea pig model.

The HSV-2 gD antigen has offered only limited protection in human clinical trials, and the inclusion of other proteins should therefore be considered in a subunit vaccine in order to increase the antigen breadth. However, the choice of adjuvant and route of immunization play major roles for vaccine efficacy and must also be evaluated thoroughly. This study indicates that nasal immunization holds potential for induction of protection against primary genital herpes, establishment of latency, and recurrent genital herpes. The importance of using a potent mucosal adjuvant, such as TLR9 agonist CpG ODN, is also evident.

Paper III

Vaginal molecular imprints and immune cell trafficking early after HSV-2 TK⁻ immunization

Gene expression profiling and systems biology have been used to uncover the mode of action of successful human vaccines^{236,237}. Previously, our group mapped the molecular signatures of the experimental vaginal adjuvants CpG ODN and α -GalCer²³⁹. These adjuvants have formerly been described to efficiently induce immunity in the genital herpes mouse model, when co-delivered with HSV-2 gD antigen i.vag.^{202,220}. In the current study, we strived to delineate early innate molecular and cellular signatures in the murine vagina after delivery of a live attenuated HSV-2 strain (HSV-2 TK⁻), known to induce gold standard immunity in mice⁸⁹. There are still many outstanding questions regarding initiation of vaginal immunity and cell recruitment induced by this gold standard vaccine. For this reason, complex vaginal tissue was used in this study for analysis of transcriptional changes, cytokine-associated proteins and cell populations post-immunization.

Already 4 h post-immunization we could identify a set of around 100 genes that differed from the control group, with a stringent statistical threshold ($Q < 0.001$). After 48 h the number of changed genes had increased to over 1500. A majority of the significantly altered genes at 4 h were also found at 48 h (Figure 9).

When exploring the transcriptional data by GO enrichment analysis, an up-regulation of terms related to innate immune and inflammatory responses was evident after HSV-2 TK⁻ immunization. Many of these genes could be linked to cytokine/chemokine signaling, antigen presentation, DC maturation, tissue homing, TLR-signaling and cellular motility. By IPA we found that genes related to immune cell chemotaxis and migration were prominent among the significantly up-regulated ones (Figure 9).

When analyzing vaginal extracts by an antibody array, a burst of cytokine-associated proteins was seen 8 h post-immunization. This response appeared to involve a wide range of chemokines, interleukins and growth factors. Significantly up-regulated proteins included cytokines IL-2, IL-4 and leukemia inhibitory factor (LIF), and chemotaxis-related CCL6 and endoglin. The number of proteins found at elevated levels at 24 and 48 h were similar, and those significantly up-regulated included chemotaxis-related CCL5, CCL19, CXCL1, CXCL2, CXCL16, endoglin, ICAM-1 and VCAM-1. CXCL1 and CXCL2 are well-known neutrophil recruiters, while CCL5 mainly is related to T cell recruitment and activation of NK cells.

RESULTS AND DISCUSSION

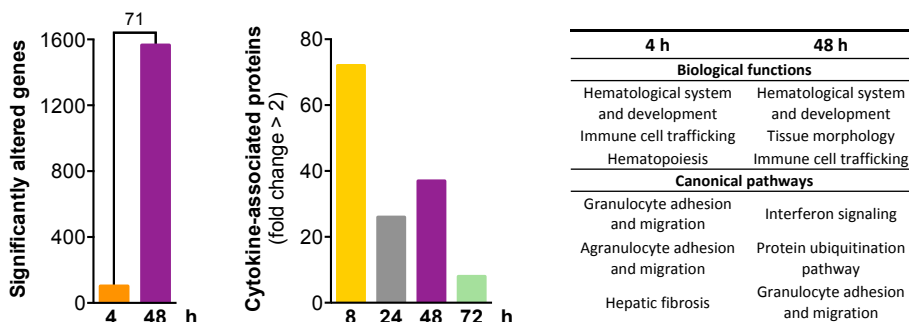


FIGURE 9. Vaginal transcriptional changes and up-regulation of cytokine-associated proteins post-immunization with HSV-2 TK. The left panel displays the number of significantly changed genes ($Q < 0.001$) at 4 and 48 h, with the number of common up-regulated genes indicated, and the number of up-regulated proteins at 8, 24, 48 and 72 h. The right panel displays the pattern of up-regulated genes for both time points, according to IPA. The table includes the top biological functions and canonical pathways, in which up-regulated genes were found enriched.

Interestingly, CCL19 (binds CCR7) and CXCL16 (binds CCR6) are mostly associated with DC and T cell recruitment to secondary lymphoid organs but are also linked to the development of organized lymphoid structures in peripheral tissues²⁵⁷⁻²⁵⁹. Thus, it is likely that HSV-2 TK induced a specific inflammatory milieu within the vaginal mucosa, facilitating antigen-presentation and cell interactions required to initiate an immune response. The up-regulation of the gene for *Glycam1*, an adhesion molecule on high endothelial venules, also supports this notion. Importantly, there are reports describing lymphoid aggregates within vaginal tissue.

Based on immunohistochemical staining, showing an increase in cells following immunization, we decided to analyze vaginal cells by flow cytometry at 48 h (Figure 10). The frequency of CD45⁺ cells tripled after immunization and a marked up-regulation of MHCII/expansion of MHCII⁺ cells was observed. This MHCII⁺ population appeared to consist of various APCs (DCs, monocytes, macrophages and possibly LCs). A high expression of Ly6C was apparent within the MHCII⁺ population, which suggested recruited inflammatory monocytes (CD11c⁻, F4/80⁻) as well as monocyte-derived macrophages (CD11b⁺, CD11c⁻, F4/80⁺, CD11b⁺) and DCs (CD11c⁺, F4/80⁻). We also observed higher frequencies of neutrophils, NK cells, NKT cells and T cells. Furthermore, we could confirm the presence of CD103⁺ DCs, described as migratory DCs in the gut mucosa, that were predicted by an *in silico* analysis of transcriptional changes. However, we did not find that this cell population expanded in the draining lymph nodes (at 48 h) and their role for vaginal immunity remains unknown.

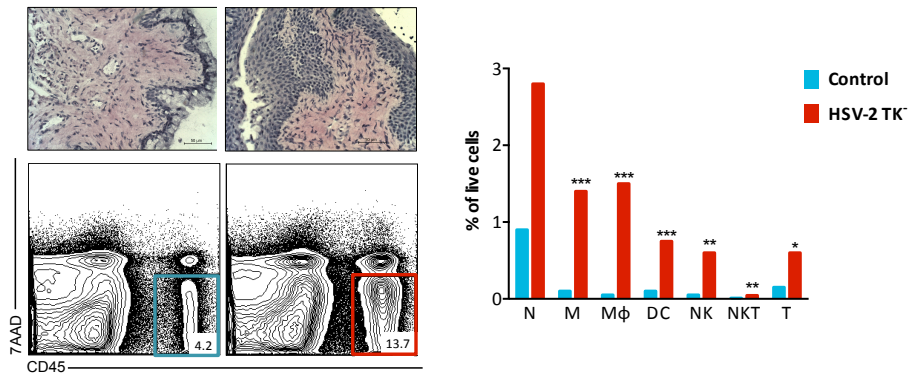


FIGURE 10. Immunohistochemical staining of vaginal tissue (top, left panel) and the expansion of live CD45⁺ cells by flow cytometry analysis (bottom, left panel) at 48 h post-immunization. The CD45⁺ cells were further characterized by flow cytometry (right panel). Results are presented as the frequency of live vaginal cells (7AAD). N=neutrophils (CD3⁻, CD11b⁺, CD11c⁻, TCRb⁻, Ly6G⁺, MHCII⁻), M=monocytes (CD3⁻, CD11c⁻, F4/80⁺, Ly6C^{hi}, MHCII⁺), MΦ=macrophages (CD3⁻, MHCII⁺, F4/80⁺, Ly6C^{hi}, CD11b⁺), DC=dendritic cells (CD3⁻, CD11c⁺, F4/80⁻, MHCII⁺), NK=natural killer cells (CD11b⁻, CD11c⁻, NK1.1⁺, TCRb⁻), NKT=natural killer T cells (CD11b⁻, CD11c⁻, NK1.1⁺, TCRb⁺), T=T cells (CD11b⁻, CD11c⁻, NK1.1⁻, TCRb⁺).

This study proposes imprints of i.vag. HSV-2 TK⁻ immunization, which may provide information regarding vaginal homing and clues as to how to efficiently initiate protective immune responses to genital herpes infection. Figure 11 is an attempt to summarize the findings derived from this study, and place them in the context of what is known from the literature about vaginal immunity to HSV-2. Understanding the mechanism of action of experimental gold standard immunization regimens, known to evoke protective immunity in the female reproductive tract, may inform the rational design of vaccines.

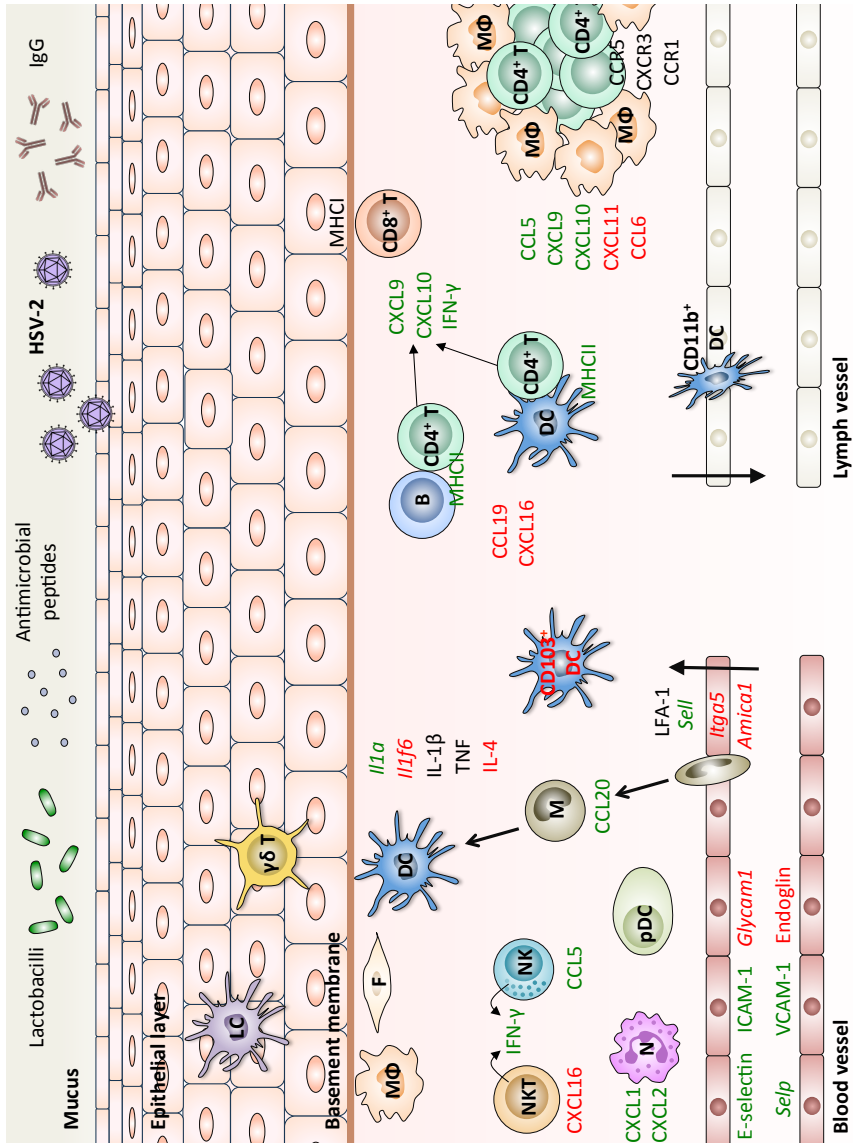


FIGURE 11. A schematic representation of innate (left) and acquired (right) immune parameters induced in the vaginal mucosa upon local immunization with HSV-2 TK. The results from Paper III are placed in the context of previous studies regarding immunity to HSV-2. Cytokines, chemokines and adhesion molecules that were detected on gene or protein level are marked in green (supported by previous findings) or red (potential novel markers for initiation of protective immunity). The molecules marked in black text indicate previous findings that were not specifically confirmed by our study. Tissue-resident DCs, MΦ, LCs and $\gamma\delta$ T cells patrol the tissue in steady state. Upon recognition of HSV-2, these cells and perhaps also epithelial cells and stromal fibroblasts produce pro-inflammatory cytokines and type I IFNs. The inflammatory response leads to expression of chemokines and endothelial adhesion factors. Neutrophils, monocytes, pDCs, NK cells and NKT cells are recruited to/expand at the site of infection. These cells help control the infection and produce cytokines/chemokines, which promotes involvement of cells from the acquired arm of immunity. Inflammatory monocytes are known to renew the DC population during infection¹²⁷. It is generally believed that CD11b⁺ DCs pick up antigen and migrate to the draining lymph nodes, where activation of B and T cells occurs. Vaginal lumen IgG antibodies are probably mainly translocated from the circulation. Activated antigen-specific lymphocytes travel to the site of infection via blood, where IFN- γ producing CD4⁺ T cells play a crucial role for controlling HSV-2 infection. CD8⁺ T cells are dependent on CD4⁺ T cells for recruitment and activation, and are probably involved in clearance of the infection. Vaginal DCs and B cells are known to maximize T_H1 responses⁴⁸. There are reports of lymphoid aggregates within the vaginal mucosa, which indicate T_{RM} cells and possibly local antigen presentation^{38,39}. Such clusters of CD4⁺ T cells (expressing CCR1, CCR5 and CXCR3) have been seen to be surrounded by MΦ, possibly secreting chemokines⁵³. It is believed that recall immune responses can be triggered locally but it is unclear whether naïve T cells can be primed, even though recent reports support it³⁵⁻³⁷. Some of our results support the formation of organized lymphoid structures (CCL6, CCL19, CXCL16 and *Glycam1*). Moreover, the role of other adhesion molecules in vaginal homing (endoglin, *Itga5* and *Amica1*) warrants further studies. DC=dendritic cell, F=fibroblast, LC=langerhans cell, M=monocyte, MΦ=macrophage, N=neutrophil, NK=natural killer cell, NKT, natural killer T cell, pDC=plasmacytoid DC, and T=T cell.

CONCLUDING REMARKS

It is estimated that over 500 million people are affected by genital herpes worldwide. This chronic infection can create great physical and psychological distress but may also lead to serious conditions in infants and immunosuppressed persons. Despite numerous efforts, there is currently no vaccine against genital herpes available for human use. The need for an effective HSV-2 intervention strategy is highlighted by the fact that genital herpes appears to facilitate HIV transmission. The prevalence of HSV-2 is nearly twice as high in women as in men. Thus, the understanding on how to elicit vaginal immunity to this infection is critically important. This thesis attempted to address alternative immunization approaches for generation of protective immunity to genital herpes infection in the female reproductive tract, and to pinpoint the early molecular and cellular signatures of early immune responses in the vagina after local vaccination.

Our protection studies in animal models show that both nasal and skin vaccine delivery holds potential for induction of protective immunity to genital herpes infection. Importantly, immunization via nasal mucosa could limit recurrent disease and establishment of viral latency in the guinea pig model. Given the fact that all human vaccine trials so far centered on intramuscular route of immunization, alternative routes of immunization such as mucosal or intradermal merit further investigation. It should be also noted that although a vaccine with the ability to elicit sterilizing immunity is highly desirable, a vaccination approach that may only yield partial protection would likely have a significant impact on control of genital herpes.

We could further show the usefulness of two TLR9-dependent adjuvants, IC31[®] and CpG ODN, to potentiate immune responses towards the HSV-2 gD antigen. Both of these adjuvants also elicited protective immunity to genital herpes infection via nasal-mucosal route. Furthermore, IC31[®] appeared to be even more well suited for dermal administration, presumably due to its depot forming property at the injection site in addition to its immunostimulatory effect. As both of these adjuvants have been tested in human clinical trials in other contexts, our results encourage further investigation for future development of a vaccine against genital herpes infection. So far, MF59 (an oil-in-water emulsion) and AS04 (TLR4 agonist MPLA in alum) were used for intramuscular immunization in humans with limited success. Hence, development of novel vaccine adjuvants, especially those with the ability to elicit mucosal immunity represents an important unmet need.

While immune responses directed to HSV-2 gD appear to be important for protection, it is of significant importance to expand the choice of HSV-2 antigens (such as immediate early proteins and other envelope glycoproteins) included in

CONCLUDING REMARKS

vaccines beyond gD to achieve a broad complete protection. Another area requiring further research is characterization of antibody and T cell responses in patients displaying asymptomatic and symptomatic genital herpes as well as in volunteers who received the exploratory herpes vaccines in order to pinpoint what constitutes a protective immunity to genital herpes. Further, more efforts need to be devoted to development of immunization strategies to induce protective immune responses in the female reproductive tract as the portal of entry of the virus. Our results from microarray analysis and large-scale protein analysis on vaginal tissue following an experimental protective immunization unraveled early signatures of vaginal immunity.

It is my hope that the information resulted from this thesis work can shed light on development of new vaccine strategies to prevent HSV-2 infection in humans. As outlined above, there however remain a number of unmet needs for vaccine research on genital herpes that require to be addressed.

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