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Engineering immunity in the mucosal niche against sexually transmitted infections

Renuka Ramanathan and Kim Woodrow*

Department of Bioengineering, University of Washington, Seattle, WA, USA

Abstract

The mucosal surfaces of the genital tract are the site of entry to over 30 different bacterial, parasitic, and viral pathogens that are the cause of sexually transmitted infections (STIs) including HIV. Women and adolescent girls are more severely impacted by STIs than men due in part to a greater biological susceptibility for acquiring infections and differences in disease sequelae. While it is widely accepted that preventative vaccines against the most commonly transmitted STIs would have a major impact on decreasing the global health burden of STIs for women worldwide, several challenges preclude their development. The female genital tract is a complex niche of microflora, hormonal influences, and immune tissues and cells that result in a mucosal immune system that is distinct from other mucosal sites and from our systemic immune system. An appreciation of these differences and their effect on shaping mucosal immunity to sexually transmitted pathogens is an important determinant for the design of effective STI vaccines. Here we describe the anatomy and mucosal immune system of the female reproductive tract, and discuss bioengineering strategies to design mucosal vaccines that overcome delivery challenges and coordinate the presentation kinetics and compartmentalization of antigens and adjuvants to relevant mucosal immune cell subsets. In particular, we describe recent progress in understanding the role of specific mucosal dendritic cell subsets in facilitating immune responses to pathogenic microbes in the genital mucosa. We also discuss the development of pathogen-mimicking materials that may be useful for engineering protective immunity in this mucosal niche.

INTRODUCTION

Sexually transmitted infections (STIs) are a significant cause of morbidity and mortality globally, where over a million new infections are acquired daily and lead to other adverse health consequences including increased risk for HIV acquisition.^{1–6} In the United States, there is a high incidence of STIs among youth ages 15–24 and the economic burden to the healthcare system is estimated to be \$16 billion annually.^{7,8} Safe and effective vaccines have the greatest potential to reduce the worldwide health burden of STIs including HIV. In particular, women and adolescent girls, who are disproportionately impacted by STIs, would benefit considerably from the availability of vaccines.⁹ Topical delivery of drugs and vaccines may expand the toolbox of prevention strategies to protect the site of infection. However, vaccines are available for only two of the over 30 different pathogens that are

*Correspondence to: woodrow@uw.edu.

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transmitted sexually. A critical barrier to the development of effective STI vaccines is the lack of immunological correlates associated with protection against the many diverse mucosally transmitted pathogens. In addition, the unique immunological environment of the female genital tract mucosa imposes challenging constraints for vaccine design. Here we discuss barriers and opportunities for developing vaccines to protect against mucosal pathogens that are transmitted sexually. In this review, we focus specifically on the challenges in eliciting protective immune responses from topical administration of vaccines to the vaginal mucosa and discuss bioengineering strategies to elicit protective immunity to STIs.

THE ROLE OF REPRODUCTIVE TRACT ANATOMY IN THE DEFENSE AGAINST PATHOGENS

The vast majority of infectious pathogens invade through mucosal surfaces lining the nasal and oral cavities, the gut, and the genitourinary tract. In immunocompetent individuals, a range of innate and adaptive immune defenses recognizes and responds to these foreign pathogens.¹⁰ In the female reproductive system, these defenses are uniquely organized to protect the host against infectious bacteria, fungi, and viruses, while maintaining tolerance toward spermatozoa and a semiallogeneic fetus. The organization of the reproductive tract, including its anatomy, hormonal control over the tissue environment, presence of antimicrobials and mucus, lymphatics, dominant antibody types, and cellular composition collectively defend the reproductive tract against sexually transmitted pathogens. Compartmentalization within the mucosal immune system also suggests that immune responses are likely to be strongest at and proximal to the site of mucosal immunization. For example, a human clinical trial identified that vaginal immunization leads to greater and more robust local mucosal IgG and IgA antigen-specific antibody responses than parental immunization or immunization at a distal mucosal site such as intranasally.^{1,3,5} This result provides strong rationale that eliciting local protective mucosal cellular and antibody responses may require immunization in the target mucosa. An understanding of the key features of the female reproductive system will inform development of biomaterials for intravaginal drug and vaccine delivery, which may have the potential to modulate mucosal immunity.

The female reproductive anatomy can be subdivided into two distinct compartments, which differ in function (Figure 1). An anatomical appreciation of both the upper and lower female reproductive tracts is critical in understanding mechanisms of pathogen infection, and informing the design of novel biomaterials that can access the necessary immunological tissues and cells. The upper genital tract is classified as a type I mucosal surface and shares features common to the gut, small intestine, colon, and lungs.^{11–14} The upper female reproductive organs include the endocervix, uterus, oviducts, and ovaries. As a type I mucosal surface, the upper genital tract is comprised of one layer of columnar epithelial cells, which are joined by tight junctions.^{11,13,15} The lower genital tract is classified as a type II mucosal surface and shares features common to the cornea, oral cavity, and esophagus.^{13,14} The lower female reproductive organs include the ectocervix and the vagina, which are comprised of multiple layers of non-keratinized and squamous epithelial cells,

that cover a basement membrane. As squamous epithelial cells are turned over, they are renewed from basal epithelial cells in the basement membrane.^{13,16} One of the many functions of this multi-layered squamous epithelium is to protect the underlying tissue from disruption during intercourse.^{10,11} An anatomical feature of murine reproductive tracts that differs from humans is that the murine upper female reproductive organs include the endocervix and uterine horns.^{11–13,17}

A critical region of the genital tract is the transitional zone between the upper and lower genital tract, which is marked by the change from columnar to squamous epithelial cells near the cervix.^{11–14} It is thought that the transitional zone is the most immunologically active region of the entire female reproductive system. Unlike the close association of the upper tract columnar epithelial cells, tight junctions do not join the squamous epithelial cells. This subtle variation in epithelial tissue allows the escape of small molecules from the lumen into the tissue. While recent research has shown that HIV virions are able to transcytose through the vaginal epithelium to access target tissues, some studies postulate that the lack of tight junctions between the squamous epithelial cells enable HIV virions to pass between cells and access Langerhans cells (LCs) and CD4+ T cells in the lamina propria.^{11,12,18,19} Alternatively, secretion of inflammatory cytokines during infection, such as TNF- α , has been implicated in loosening epithelial cell junctions and facilitating easier transepithelial pathogen transport.^{11,15}

Dendritic Cell Subsets of the Reproductive Tract

While epithelial cells themselves play an important role in mucosal immunity in the reproductive tract,^{13,16,20–22} the major mediators of inflammation and immunity are the professional antigen-presenting cells, most notably dendritic cells (DCs).²³ DCs are thought to be the most efficient of the professional antigen-presenting cells. Unlike macrophages, only DCs possess the unique capacity to migrate to draining lymph nodes after antigen uptake to prime B and T cells. A summary of DC subsets in different regions of the body is presented in Figure 2. At steady state, DC precursors exit the bone marrow and enter circulation, eventually entering lymph nodes and differentiating into various DC subsets. DCs can be subdivided into two categories—tissue DCs, which reside in the mucosa, skin, and internal organs; and blood DCs, which are found in blood and lymphoid tissues. Mucosal DCs have been studied and phenotypically described well in murine models, which will be the focus of this review. It is important to note, however, that there are key differences in receptors and cell surface markers between human and mouse mucosal DCs.

In the murine reproductive mucosa, tissue DCs have been subdivided into vaginal epithelial dendritic cells (VEDCs) and submucosal dendritic cells (SMDCs). VEDCs sit in the vaginal epithelium and play a key role in antigen sampling from the lumen (Figure 1). VEDCs share similarities to Langerhans cells found in the skin, but are distinct in a few key features.²⁴ First, VEDCs express much lower levels of Langerin than Langerhans cells found in skin epithelium.^{24,25} Second, while both cell types show morphology consistent with round, cytoplasmic granules, Langerhans cells contain distinct Birbeck granules that are rod-shaped and contain striations.²⁵ Third, while most DC precursors are monocytes, in non-inflammatory conditions VEDCs are renewed from non-monocyte bone-marrow derived

precursors. Under inflammatory conditions such as during HSV-2 infection, VEDCs, like skin Langerhans cells, differentiate from monocytes. Finally, VEDCs express low levels of CD207 and have a generally more activated phenotype than skin Langerhans.^{23–25}

MHC class II⁺ CD11b⁺ DCs found within the submucosal lamina propria of the mouse vaginal mucosa are referred to as SMDCs.²³ SMDCs share similarities to dermal DCs found in skin and are characterized by surface expression of MHC class II, CD11c, and DC-SIGN in humans, CD123 in rhesus macaques, and CD11b in murine models.^{20,23} In an elegant experiment by Zhao et al. (2003), it was demonstrated that after genital HSV-2 infection, MHC class II⁺/CD11c⁺/CD11b⁺ submucosal DCs migrated to draining lymph nodes where they primed CD4⁺ T cells and initiated a Th1 response.²⁶ While a thorough rationale for why DC precursors migrate to tissues and then differentiate into DCs is not completely elucidated, it is hypothesized that in the absence of inflammatory stimuli, constitutive expression of chemokines regulate the steady-state recruitment of DCs into tissues.²³ Cytokine-induced recruitment of monocyte-derived DCs during inflammation increase the local population up to 100-fold. One important distinction between local SMDCs and recruited monocyte-derived DCs is that the latter is recruited to sites of inflammation in a CCR2-dependent manner and in response to type I interferon.²⁷ Furthermore, rather than stimulation of naive T lymphocytes, these recruited DCs are responsible for restimulation of Th1 effector and memory T cells.

In general, cell recruitment to infected tissues is a multi-step process. During tissue inflammation, the chemokine CCL20 is produced and induces the recruitment of monocyte-derived DCs.²⁸ Monocytes or monocyte-derived DCs travel through blood vessels toward CCL20 via the CCR6 receptor. Monocytes bind selectin on endothelial cells via adhesion molecules and extravasate across the endothelium.¹⁰ After recognizing and phagocytosing antigens, DCs downregulate CCR6 and upregulate expression of CCR7, which allows them to home to lymph nodes. These mature DCs further present antigen on MHC class II molecules and co-stimulatory molecules on their surface to engage TCRs and provide priming and co-stimulation of T cells.

Differences in the Lymphatic Structures that Guard Mucosal Surfaces

A major component of immune responses to vaccination at mucosal surfaces is the architecture of lymphatic vessels that drain the tissue. One of the most marked differences between the vaginal mucosal environment and other mucosal compartments is the composition of organized lymphoid structures, including secondary lymphoid organs. The nasopharynx and bronchial passage and the gastrointestinal tract contain organized lymphatic structures, which are termed the bronchial-associated lymphoid tissues (BALT) and the gut-associated lymphoid tissues (GALT), respectively.²⁹ In addition to an extensive network of lymphatic associated with the BALT and GALT, these mucosal sites have large lymphoid aggregate structures similar in tissue organization and function to lymph nodes. For example, Peyer's patches in the gut are lymphoid aggregate structures that contain specialized cells that are highly efficient in antigen uptake.³⁰ These highly specialized cells, known as M cells, are able to phagocytose antigen from the lumen of the bronchial and gastrointestinal tract and deliver antigen to DCs in secondary lymphoid structures. Peyer's

patches in the gut and lymphoid aggregates in the subepithelial space of the nasal and bronchial tract are able to efficiently scrub lymph drained from these tissue for antigens, and subsequently promote drainage to systemic lymph nodes.

In contrast to the BALT and the GALT, the reproductive tract does not have associated lymphoid tissues or specialized M cells. Two major sets of lymph nodes drain the reproductive tract tissues. The iliac lymph nodes, comprised of the caudal, sacral, and lumbar lymph nodes, drain the upper two-thirds of the reproductive tract. The inguinal lymph nodes are responsible for draining the lower third of the vagina.²³ It has been reported that the uterus contains a lymphoid structure containing B and T cells, but the exact role and function is unclear. For example, during post primary infection with virus, foci of T and B lymphocytes and DCs have been observed to organize near the epithelium, providing rapid response to secondary infection.¹³ The lack of organized lymphoid structure in the reproductive tract limits the migration of leukocytes and lymphocytes in and out of the tissue. Therefore, the role of professional antigen-presenting cells in aiding with efficient immune responses to pathogens and vaccines in this compartment is undeniable: DCs are responsible for vaccine uptake, processing, and presentation. However, in the absence of inflammation, the insufficient numbers of steady-state professional antigen-presenting cells in reproductive tissues may be a major challenge facing immunization in the vaginal tract.

Role of Mucus and Maintenance of Vaginal pH

In addition to reduced lymphatic drainage of intravaginally delivered vaccine antigens, poor mucus penetration traps vaccines, which can be subsequently degraded by enzymes. Reproductive tissues, like other mucosal surfaces, are constantly producing mucus to promote ciliary clearance as well as to perform other immune defense mechanisms. In contrast to type I mucosal surfaces where mucus is produced by Goblet cells, in the reproductive system mucus is secreted from epithelial cells in the vagina and from cervical crypts, which extend down into the lower genital tract during the ovulatory phase of the menstrual cycle.^{11,13} Mucus secretions are composed of a number of different mucin proteins, which are encoded for by the MUC1 gene.³¹

A straightforward mechanism of defense in the genital tract is the restricted diffusivity of opsonized pathogens in cervicovaginal mucus.³² Mucin proteins are heavily glycosylated large, and complex proteins that entangle and entrap pathogens in the lumen until they are eventually secreted.¹³ Similar to antimicrobial and chemokine production, mucus production is also influenced by different stages of the menstrual cycle. For example, mucus viscosity is low prior to and during ovulation,¹¹ which is important for sperm motility and moving upward in the lower vaginal tract. The cervical mucus plug plays an important role acting as a physical barrier to lower the incidence of pathogen transmission to the upper female reproductive tract where pregnancy can be compromised.³³

The presence of mucus also has a significant impact on the local pH of the vaginal environment, which is generally maintained by commensal host bacteria.¹¹ The predominant bacterium in the reproductive tract is *Lactobacillus*, which is responsible for lactic acid production that maintains the low acidic pH in the vagina. Normal pH in the female reproductive tract is maintained in the range of 4–5.³⁴ In murine models, the vaginal pH is

slightly higher, between 6.2 and 6.5. Bacterial infection has been shown to alter the local pH. An important aspect of the local acidic pH is charge shielding of HIV virions, which possess an overall negative surface charge.³⁵ Charge shielding by mucin proteins slows HIV diffusion through mucus. Interestingly, the presence of semen buffers the vaginal pH to alkaline conditions (pH 8–9). As such, HIV virions that are present in semen are likely in an environment more conducive to transmission.

Variability in mucus and vaginal pH can arise from a number of factors, including hormonal regulation during the lifecycle.³⁶ While the normal pH of the vaginal environment is acidic, it has been noted that the local pH transiently becomes more alkaline during ovulation, in the presence of semen post-coition, and during certain infections such as bacterial vaginosis.^{37,38} The impact of vaginal pH and mucus on virus-like particle diffusion has been well characterized in *ex vivo* models. Shukair et al. reported that while HIV-1 particles exhibited reduced diffusivity in cervicovaginal mucus (CVM) as compared to that in a saline solution; this phenomenon was not dependent on the presence of HIV envelope glycoproteins.³⁹ Moreover, they found that virion diffusion directly correlated to pH. Separate studies confirmed that HIV-1 and HSV-1 virions were immobilized in low pH CVM, characterized by a lactic acid-rich environment.³⁵ However, virions freely diffused in mucus near neutral pH. Further studies revealed that IgG freely diffused through vaginal mucus only transiently and weakly interacted with mucin proteins. However, the presence of IgG (even non-neutralizing IgG) within mucus was essential in trapping and immobilizing virions.⁴⁰ Leukocyte motility in cervical mucus and their ability to penetrate the mucus layer have also been documented. For example, the measured motility coefficient of neutrophils in human midcervical mucus is $\sim 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ and increases up to fourfold in the presence of a formylated tripeptide chemokine.⁴¹ Leukocytes were also observed to completely penetrate across a mucus layer of 500 μm thickness but not larger. These data support the effective migration of leukocytes in mucus, which may play an important role in immunity and infection.

Antibody Production

Antibody production at mucosal surfaces varies in different mucosal tissues. In many type I mucosal surfaces, such as the gut mucosa, IgA is the predominant antibody subtype. IgA produced in the subepithelial space is transcytosed as dimeric IgA by the poly Ig receptor (pIgR) across the gut epithelium.⁴² In contrast, the predominant antibody type produced in reproductive tract tissues is IgG. Control of IgG presence in the vaginal lumen is not fully understood. However, it has been shown that IgG can be paracellularly transported from the subepithelial space to the surface of the mucosa. It has also been shown that IgG can be transcytosed bidirectionally across the vaginal epithelium by receptors expressed on vaginal epithelial cells⁴³ (Figure 1). It is possible that both mechanisms of IgG transport act together to provide protective concentrations of neutralizing antibodies in the lumen and to transport opsonized pathogens back into the lamina propria where they may be taken up by professional antigen-presenting cells via the Fc γ receptor.⁴⁴

BIOENGINEERING INTRAVAGINAL VACCINES FOR SEXUALLY TRANSMITTED INFECTIONS

Several strategies have been employed to improve or promote the specific effector mechanisms required for protective immunity in the genital mucosa. One strategy is focused on local enrichment of effector immune cells. A recent study by Shin et al. demonstrated a new vaccine strategy for sexually transmitted diseases termed 'prime and pull'.⁴⁵ In the 'prime and pull' paradigm, vaccines are subcutaneously injected to evoke a systemic immune response ('prime'). This is followed by topical application of chemokines CXLC9/10 to recruit ('pull') and establish a local T-cell population, which can provide long-term memory and curb the spread of HSV-2 to neurons and prevent clinical manifestations of disease. This strategy allows for systemic 'priming' and active 'pulling' of effector T lymphocytes to the genital mucosa in order to respond efficiently during viral infection. However, this strategy requires pairing mucosal delivery of agents with a subcutaneous immunization, rather than local delivery of the immunization itself within the reproductive tissues.

A separate strategy focuses on the administration of protein or peptide biologics to recruit leukocytes (specifically antigen-presenting cells) into tissues to promote vaccine uptake, processing, and presentation. The observation that strong adjuvants are required to potentiate mucosal immune responses to non-replicating subunit vaccines administered to the vaginal mucosa suggests a critical role for mucosal DCs in shaping the immune response in the genital tract. In the context of vaccines, research has shown that antigen delivery at the site of infection may be important in imprinting T cells during priming with the ability to home back to the site of pathogen encounter.^{46,47} The specific recruitment of immature or not fully differentiated antigen-presenting cells minimizes nonspecific inflammation, which may be a result of upregulated inflammatory cytokine and chemokine secretion. One approach has been to administer chemokines, which promote chemotaxis of APCs from distal sites to sites of immunization. For example, CCL20 is commonly used as a chemoattractant for CCR-6 bearing DCs and macrophages.^{48–51} CCL20 is a chemokine ligand endogenously produced at basal levels in the body by a variety of cell types. It has been demonstrated that CCL20 production can be upregulated in the presence of other pro-inflammatory signals such as TNF- α .^{52,53} *In vitro*, CCR6 agonists such as macrophage inflammatory protein (MIP)-3 α have been shown to promote chemotaxis of CCR6-bearing cells.^{28,50,51,54,55} Research groups have shown that particles, which slowly release CCL20 over time are able to recruit monocytes and immature DCs, as well as bone marrow-derived DCs.⁵⁶ Furthermore, studies have shown the ability to recruit DCs and macrophages to sites of immunization *in vivo* with CCL20.^{28,57} Aside from CCL20, studies have also evaluated the potential for antimicrobial β -defensins to act as DC chemoattractants.^{52,58,59} Although β -defensins are constitutively expressed as antimicrobial agents by mucosal epithelial cells⁶⁰ and are also CCR6 agonists,⁶¹ their affinity for CCR6 is much lower than CCL20.⁶²

The use of molecular adjuvants to boost the immune response by stimulating upregulation of co-stimulatory molecules is common and well studied.^{63–66} CpG, a TLR9 agonist, is a commonly used vaccine adjuvant via systemic and mucosal routes of administration. Many

lines of evidence suggests that mucosal vaccines delivered in the presence of strong adjuvants such as CpG, which stimulates epithelial cells via the TLR9 receptor, induce cytokine secretion and increased infiltration of activated CD8⁺ T-cells.^{67,68} Furthermore, intravaginally delivered CpG has been shown to recruit DCs.⁶⁹ However, molecular adjuvants nonspecifically stimulate the immune system by a number of mechanisms of action by recruiting and activating a broad range of cell types. This nonspecific mechanism of cell activation, and its application for vaccine delivery strategies, is controversial due to potential tissue toxicity.^{70,71} Other general mucosal adjuvants apart from CpG have also been used to enhance co-stimulation of lymphocytes including cholera toxin and monophosphoryl lipid A (MPLA), a TLR4 agonist.^{2,72} More recently, polycationic polymers such polyethylenimine have been employed as potent mucosal adjuvants.⁷³ A major component of selecting adjuvants for intravaginal vaccination is their mechanism of action, and whether the molecular targets engaged by general mucosal adjuvants are distributed on vaginal epithelial cells.

Recently, research has focused on leveraging molecular targets presented on vaginal epithelial cells as potential targets for adjuvanting function. Lipopeptides are self-adjuvanting vaccines synthesized by conjugating synthetic peptide immunogens with lipid agonists for toll-like receptor 2 (TLR-2), a pattern recognition receptor highly expressed by vaginal epithelial cells.^{70,71,74} Di- and tri-acylated peptides have shown enhanced affinity for TLR-2, and the hydrophobic lipid tails allow for enhanced cell penetration and uptake. Furthermore, intravaginally delivered HSV-2 lipopeptides show enhanced immune responses, including improved priming of functional T-lymphocytes.

NANOPARTICLES FOR MUCOSAL VACCINE DELIVERY

Intravaginal drug and vaccine delivery has been a major focus of addressing sexually transmitted pathogens.^{75–78} In the context of microbicides, topical administration of antiretrovirals may allow for delivery of agents directly at the site of infection. While several intravaginally delivered vaccines have shown the ability to elicit immune responses in murine models, these systems still face many challenges including poor vaginal retention, limited access to critical immune cells, and degradation by proteases. A major focus of improving delivery, both systemically and mucosally, has been on the development of nanoparticle systems formulated to deliver drugs or antigens. Here we review the key properties of nanoparticles that may be advantageous in vaccine delivery by intravaginal administration, and examine the development of vaccine-loaded nanoparticles designed to overcome challenges related to mucosal delivery. Nanocarriers loaded with drug, protein, and adjuvants have been synthesized in variety of sizes and materials including liposomes, nanolipogels, polymeric nanoparticles, and micelles (Figure 3). These nanocarrier systems can be largely categorized as polymeric and liposome carriers, which have been used in a range of mucosal delivery applications.

Nanoparticle Platforms for Delivery of Mucosal Antigens and Adjuvants

Liposomes have prolific use as vaccines, and a specific category of liposomes known as virosomes, which are vesicle composed of natural phospholipids and fused with specific

viral coat proteins, have been employed to create a clinically approved influenza vaccine and a Hepatitis A vaccine.⁷⁹ In these applications, virosome technology imitates natural virus membrane structure without delivering genetic material that would cause infection.⁸⁰ The ability to incorporate hydrophilic agents into the aqueous core and hydrophobic agents into the lipid bilayer, makes liposomes attractive for delivering a range of physicochemically diverse compounds relevant for vaccines.^{81,82} In addition, the diversity of modified lipids that can be incorporated into liposomes allows them to be easily surface conjugated to targeting moieties or antigens, as well as exhibit fusogenic or pH-dependent functionality.^{83,84} Furthermore, liposomes are prepared under aqueous and mild conditions that help to preserve the integrity of labile vaccine antigens that may be sensitive to denaturation or degradation. While liposomes possess many advantages they show limited serum stability *in vivo*, resulting in their inability to provide long-term sustained release.⁸⁵ Recently, interbilayer crosslinked multilamellar vehicles (ICMV) were developed to address these challenges observed with simple liposomes.⁸⁶ ICMVs consist of multiple lipid bilayers that are chemically crosslinked to create rigid membranes encapsulating an aqueous core. Compared to simple liposomes, ICMVs demonstrate greater loading capacity of protein antigens such as ovalbumin (OVA). ICMVs also demonstrate greater serum stability and sustained OVA release *in vitro* out to 30 days, which may predict their potential for long-term release of protein cargo *in vivo*. A robust immune response was mounted in mice administered ICMVs loaded with OVA and containing the potent adjuvant MPLA anchored into the lipid bilayer. In a separate study, mucosal administration of ICMVs carrying OVA in combination with two TLR-based adjuvants, improved antigen delivery from the lung to draining lymph nodes and stimulated > 10-fold greater CD8+ T-cell responses in draining lymph nodes than soluble protein.⁸⁷ Using an adoptive transfer model of OT-1-luc cells, researchers found that this approach to vaccination also elicited heightened expression of $\alpha 4\beta 7$ mucosal homing integrin and immune responses in distal mucosal sites, including the vagina. These results suggest that mucosal delivery of antigen in highly stable liposomes can drive cross-presentation. Furthermore, it provides evidence supporting the existence of a common mucosal immune system, which allows for vaccination at one mucosal site to impart specific immunity at other mucosal sites.

As an alternative to liposomal delivery systems, polymer-based nanoparticles also have a number of attributes that are relevant for mucosal vaccine delivery. In particular, several nanoparticle systems have been used for delivering drugs and biologics topically to the vaginal mucosa with excellent efficacy and low cytotoxicity. In the acidic conditions of the vagina, one strategy that may be useful for antigen delivery is encapsulation within a pH-triggered material that releases vaccine cargo. For example, Eudragit polymers, composed of methacrylic acid and methyl methacrylate, showed the ability to load model hydrophilic and hydrophobic dyes in a pH-dependent manner.⁸⁸ Both dyes showed enhanced release in the acidic conditions of the vaginal pH. The range of polymers available for incorporation in nanoparticles also allows for the selection of materials with surface biofunctionality that is amenable to antigen release within intracellular compartments. For example, polypropylene sulfide (PPS) nanoparticles conjugated to OVA induced cytotoxic T-lymphocyte responses in the lung and spleen after intranasal delivery.⁸⁹ Moreover, conjugation of a TLR5 ligand as an adjuvant showed enhanced immune responses in distant mucosal compartments including

the vagina and rectum. In this formulation, OVA antigen was conjugated to the surface of PPS nanoparticles via a disulfide bond, which is readily reduced in the intracellular environment. Finally, calcium phosphate nanoparticles that are self-adjuvanting and loaded with a HSV-2 glycoprotein-derived antigen elicited a protective systemic and mucosal immune response following viral challenge *in vivo*.⁹⁰ A major consideration in the selection of polymer materials for vaginal delivery is whether the systems promote maximal distribution along the length of the reproductive organs and sustained release, increasing the opportunity for encapsulated cargo to be delivered to target cells. In a gene therapy application, PLGA nanoparticles loaded with high levels of siRNA exhibited highly efficient gene silencing after intravaginal delivery in a murine model. PLGA-siRNA vehicles knocked down the expression of MAPK1 along the entire length of the vaginal lumen and up into the uterine horns. Moreover, it was reported that PLGA nanoparticles were able to successfully penetrate into the epithelial tissue.⁹¹ In another example, Ham et al. showed that PLGA nanoparticles loaded with RANTES sustained release and exhibited anti-HIV activity. *Ex vivo* tissue models showed that RANTES-loaded PLGA nanoparticles exhibited a significant improvement in tissue uptake and permeation, as well as efficient migration to the basement epithelium, when compared to soluble RANTES.⁹²

Leveraging Surface Chemistries to Overcome the Mucus Barrier

Mucus production and turnover at mucosal sites is imperative to rapid clearance of foreign bacterial and viral pathogens. As previously discussed, charge shielding by mucin proteins, viscosity, and low-affinity interactions with IgG play a role in trapping or slowing diffusion of virions such as HIV, which are on the same order of magnitude in size as many vaginal nanoparticle platforms. As such, identifying the key characteristics that allow nanoparticles to overcome the mucus barrier is imperative for vaginal immunization. To date, the field remains polarized as to which physical and chemical material properties are most important. Mucoadhesion was initially considered to be an important characteristic enabling prolonged retention of nanocarriers.^{93–96} However, since mucus turnover in the vaginal tract is rapid, mucoadhesive particles may actually promote entrapment by mucin proteins and eventual expulsion of particles from the reproductive tract, rather than prolonged retention or penetration of the vaginal epithelium. Recently, mucus-penetrating particles (MPPs) have been engineered to overcome delivery in the vaginal tract by employing strategies to surface functionalize nanoparticle with moieties that promote high diffusivity in mucus and improved association with the vaginal epithelium.^{97,98} MPPs are composed of polymeric nanoparticles that are surface functionalized with a low molecular weight PEG, a hydrophilic polymer. Low molecular weight PEG has been shown to promote mucus penetration whereas high molecular weight PEG may lead to chain entanglement with mucin proteins and result in mucoadhesion.^{99,100} Surface modification of nanoparticles with PEG can employ covalent conjugation strategies or methods that promote phase-separation and partitioning of the hydrophilic PEG polymer to the surface of hydrophobic polymer nanoparticles. Cu et al. demonstrated in an *in vivo* side-by-side comparison of intravaginal nanoparticle delivery that PEG-modified PLGA nanoparticles showed increased association with the vagina while avidin-modified PLGA nanoparticles and unmodified PLGA nanoparticles were recovered predominantly in cervicovaginal lavages.⁹⁷ Intravaginal delivery of fluorescent MPPs demonstrated enhanced ability to penetrate mucus and access

the deep folds in the rugae of the vagina compared to conventional polymeric nanoparticles.⁹⁸ In the same study, acyclovir-loaded MPPs resulted in a threefold increase in protection against HSV-2 viral challenge in the murine genital tract than delivery of soluble drug.

While these studies suggest the use of a strategy to overcome the mucus barrier, the application of MPPs for vaginal delivery of vaccines remains to be realized. Furthermore, while MPPs have been shown to improve association with the vaginal epithelium, they are still faced with a secondary barrier of a complex, stratified, squamous epithelium across which to transport antigen. PEGylation of nanoparticles is commonly used to prolong circulation in blood by reducing opsonization, and reducing monocyte and macrophage uptake.^{101,102} MPPs, with PEGylated surfaces, may in fact reduce cellular uptake—ultimately reducing the delivery of antigen to target cells. Future studies of vaginal MPP delivery should focus on comparing MPP uptake by local cells to conventional NPs, cell-free and cell-mediated trafficking of particulates to lymph nodes, and the impact of antigen delivery to vaginal APCs after formulation in MPPs.

Exploiting Receptor-mediated Delivery across a Complex Mucosal Epithelium

While the type I and type II epithelia of the female reproductive tract are imperative in defending against foreign pathogens, they also serve as a barrier to vaginally delivered vaccines. A major focus in mucosal drug delivery design is to engineer systems that transport cargo across the epithelium without relying on the use of mucosal adjuvants, which may enhance delivery by compromising the integrity of the epithelium. Recently, several studies have evaluated mechanisms of transcytosis that utilize recycling receptors expressed by cells at mucosal sites. These recycling receptors are targeted to promote transepithelial transport from the apical to basal membrane, where they may access organized lymphoid structures or antigen-presenting cells in the lamina propria.

A recent study evaluated the delivery of gp140 across the nasal epithelium by conjugating the protein to transferrin.¹⁰³ Transferrin conjugated gp140 engaged the transferrin receptor (CD71), expressed on the nasal mucosal epithelium, promoting active delivery of cargo across the epithelium and ultimately improving systemic and vaginal immune responses to intranasal vaccination. Several other groups have exploited this same receptor by functionalizing nanoparticles with transferrin for the application of drug delivery to the brain.^{104,105} Active transcytosis of nanoparticles after oral delivery has also been shown to improve delivery across the type I intestinal epithelium.¹⁰⁶ In this study, PLA-PEG-based nanoparticles were modified with Fc protein, which binds the neonatal Fc receptor (FcRn). The authors found that Fc-modified nanoparticles successfully crossed the intestinal epithelium and entered the lamina propria after oral delivery, and were able to reach systemic circulation. FcRn is expressed by several organs,¹⁰⁷ including the reproductive tract where bidirectional transcytosis of IgG has been shown in a murine model.³⁴ Importantly, the Fc–FcRn interaction is strongest at an acidic pH and it is hypothesized that the encounter occurs in acidified endosomes, rescuing IgG from lysosomal degradation. However, given the acidic pH of the human vagina, it is likely that Fc–FcRn interactions may also occur at the surface of the vaginal epithelium. Surface protein modification of nanoparticles to engage receptors to promote active delivery across a complex epithelium

has been shown to be successful at other mucosal surfaces, and may be a strategy to enhance delivery to the lamina propria for vaginally delivered vaccines. Future studies may also aim to elucidate the specific synergy between active transcytosis and nanoparticle formulation of antigens. For example, a head-to-head comparison of intravaginal delivery of soluble antigen and nanoparticle-formulated antigen, both modified to target a recycling receptor, would inform the relative contribution and importance of nanocarriers versus transepithelial transport.

DC-targeted Nanoparticles for Mucosal Vaccination

In order to mount an effective immune response to vaginal vaccination, antigens must be taken up by relevant APCs, specifically DCs located in the lamina propria. The advantages of formulating antigen in nanoparticles include optimizing antigen in carriers that are preferentially taken up by target DCs due to size, surface charge, or morphology.^{108,109} A number of studies have evaluated the impact of size on cellular uptake, and it is generally observed that nanoparticles <500 nm are preferentially taken up by DCs.¹¹⁰ Specifically, after subcutaneous administration, it was observed that DEC205+ DCs preferentially internalize 20 and 40 nm diameter nanoparticles in draining lymph nodes. In contrast, 1 μ m particles showed heightened uptake in F4/80+ macrophages. A follow-up study by the same group evaluated uptake of 50 and 500 nm nanoparticles after intratracheal instillation in pulmonary APC subsets.¹¹¹ The results showed that 50 nm diameter nanoparticles were more significantly taken up by lung APCs, and specifically by DCs in draining lymph nodes. Both studies evaluated the use of these nanoparticles in immunotherapeutic strategies, and demonstrated that smaller-sized nanoparticles resulted in more favorable immune responses. Consideration of nanoparticle size can have a major impact on intravaginal mucosal vaccines. Apart from potentially improving passive targeting of vaginal DC subsets, engineering smaller-sized nanoparticles also increases the surface area to volume ratio—allowing immobilization of a higher density of antigens or targeting ligands. Further, *in vitro* studies have also suggested that engineering positively charged surfaces might be a strategy for improving nanoparticle uptake for larger-sized particulates,^{112,113} which may have the capacity to more strongly induce DC maturation. However, exclusively engineering nanoparticles with a positive surface charge may lead to improved uptake in all cells due to their interaction with the negatively charged cell membrane, and may not skew toward DC targeting.

While consideration of nanoparticle size and surface charge allows for passive targeting of APCs, it has also been demonstrated that nanoparticle systems can be surface functionalized with moieties that target their cargo to receptors uniquely expressed by APCs. For example, a recent study evaluated the delivery of plasmid DNA-loaded chitosan nanoparticles after intranasal administration against nucleocapsid protein and found that by immobilizing a fusion protein specific for the DEC-205 receptor allowed for enhancing targeting specificity to DCs after nasal immunization.¹¹⁴ Several other studies show the ability to employ nanoparticle surface functionalization to target to various DC receptors relevant in humans, *in vitro* and *in vivo*, including DC-SIGN and Fc γ .^{115,116} While active targeting of DCs has been shown to be successful in these studies, it remains to be realized if similar approaches can be used in vaginal nanoparticle delivery of antigens. This is largely due to the fact that

while various vaginal DC subsets have been explored, there still remains a great deal of uncertainty as to which subsets promote tolerance versus immunity. Furthermore, the specific and unique expression of potential target receptors on vaginal DCs still remains to be thoroughly characterized.

SUMMARY AND FUTURE OUTLOOKS

Formulation of antigens in nanocarriers for immune stimulation has been widely explored. However, the adaptation of these strategies in the context of the specific delivery challenges in the vaginal mucosal environment may require finding synergies between multiple approaches to address efficient mucus penetration, epithelium transcytosis, and dendritic cell uptake. Nanocarrier systems provide an innovative approach for developing pathogen-mimicking materials for engineering mucosal immunity. The diversity of materials used to synthesize carrier system in addition to formulation of biologics and signaling molecules enable the ability to target mucosal DCs and potentially modulate their function toward a specific immune response. Many co-stimulatory molecules being evaluated as mucosal adjuvant candidates present significant delivery challenges that include stability and solubility, local toxicity, and requirement for targeting of specific tissues and cells while limiting systemic absorption. In addition, there is strong rationale to deliver a combination of maturation stimuli to mimic natural pathogens and realize potential adjuvant synergy. Nanocarrier delivery systems have the capacity to address challenges of co-delivering antigens with adjuvant combinations, and modulating their presentation kinetics and compartmentalization in specific mucosal DC subsets. Elucidating the mechanisms in which the interpretation of antigen and adjuvant agents in the mucosa causes immunity or tolerance will broaden our understanding of mucosal biology and the engineering of effective mucosal vaccines.

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FIGURE 11.

Unique features of the reproductive tissues include type I and II mucosal epithelium in the upper and lower genital tract, respectively. Type I mucosal tissues include a single layer of columnar epithelial cells while type II mucosal tissues consist of multiple layers of stratified squamous epithelial cells. The luminal side of the genital tract is coated in a layer of mucus, which is constitutively produced in the reproductive tissues. Various immune cells populate the vaginal epithelium and the submucosal tissues including vaginal epithelial dendritic cells (VEDCs), macrophages, and submucosal dendritic cells (SMDCs). The reproductive tissues have mechanisms for bidirectional transcytosis of IgG across the vaginal epithelium by the neonatal Fc receptor (FcRn).

FIGURE 2 I.

Dendritic cells subsets include blood DCs, tissue DCs, and specialized mucosal DCs in the vaginal tract. VEDCs in the reproductive mucosa are identified by cell surface markers MHC II, CD11c, and CD207. Submucosal DCs (MHC II⁺ CD11c⁺ CD11b⁺) play a key role defending against primary infections by sampling antigen and trafficking to draining lymph nodes. During inflammation, circulating undifferentiated monocytes are rapidly recruited into the reproductive tissues to provide response to secondary infection and restimulation of effector T lymphocytes.

FIGURE 3 I.

Nanocarrier platforms have been synthesized for efficient encapsulation of various cargo including protein and peptide antigens, vectors and inactivated microbes, as well as a variety of adjuvants for co-stimulation of T lymphocytes. These systems can be synthesized from lipids or polymers and range in size to include micelles, nanoparticles, and microparticles. Finally, many of these systems can be surface functionalized with a range of ligands to provide mucus penetration, cell targeting, or endosomal escape.