Mucosal Vaccine Approaches for Prevention of HIV and SIV Transmission

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Abstract: Optimal protective immunity to HIV will likely require that plasma cells, memory B cells and memory T cells be stationed in mucosal tissues at portals of viral entry. Mucosal vaccine administration is more effective than parenteral vaccine delivery for this purpose. The challenge has been to achieve efficient vaccine uptake at mucosal surfaces, and to identify safe and effective adjuvants, especially for mucosally administered HIV envelope protein immunogens. Here, we discuss strategies used to deliver potential HIV vaccine candidates in the intestine, respiratory tract, and male and female genital tract of humans and nonhuman primates. We also review mucosal adjuvants, including Toll-like receptor agonists, which may adjuvant both mucosal humoral and cellular immune responses to HIV protein immunogens.

Keywords: HIV, SIV, nonhuman primates, mucosal adjuvants, rectal, vaginal, oral, nasal immunization TLR agonists, vaccine vectors, delivery vehicles.

1. INTRODUCTION

The vast majority of human immunodeficiency type 1 (HIV) transmissions occur across mucosal barriers, whether we consider mother-to-child transmission or adult sexual transmission, and significant HIV replication occurs in the gastrointestinal mucosa [1-5]. Blood transmission is now mostly restricted to intravenous drug use, as transmission through blood products has been virtually eliminated thanks to the careful selection of donors and screening of blood products before their use. In the setting of mucosal transmission, different surfaces can be breached by the virus and these surfaces are very different in histological structure and immunologically competent cells that are available for their protection. This translates in different efficiencies of infection at different sites for the same virus amount, with rectal infection being significantly more efficient than oral [6, 7]. Because HIV can spread in a few days to other compartments, immunological containment cannot rely on anamnestic systemic responses, as these responses may become effective too late [8, 9]. An effective vaccine needs to be able to establish resident mucosal memory cells that can relatively quickly block viral replication and peripheral spread after infection has occurred. Once persistent systemic infection is established, it seems unlikely that immunological control can eradicate the infection, even in the setting of an attenuated virus that can control an incoming wild type virus [10]. Mucosal routes of vaccination, which provide more significant antigen-specific mucosal responses than systemic vaccination, may be better suited to achieve these goals [11]. However, for protection against HIV, induction of antiviral responses in both mucosal and systemic compartments may be necessary to achieve virus control, and it is unlikely that only humoral or only cell-mediated immunity will be sufficient for this task. This review covers different HIV vaccine candidates administered via mucosal routes that have been or are currently being explored in humans and Nonhuman Primates (NHP) in the attempt to control HIV or Simian Immunodeficiency Virus (SIV) infection and disease progression.

2. RECOMBINANT VECTORS FOR HIV ANTIGEN DELIVERY

Thanks to the advent of recombinant DNA technology, in addition to the traditional approach of using an inactivated pathogen mixed with an adjuvant, there are now numerous ways to expose the immune system to a specific antigen or antigens. Immunization can deliver antigens as expressed from naked DNA molecules containing the sequences for the antigens, as purified proteins (not discussed here), or as part of a recombinant microbe that expresses the antigen we intend to target as part of its own makeup [12-15]. This last possibility permits the use of live vectors as vehicles for selected proteins of a specific pathogen, an approach that results in a more prolonged stimulation of the immune system than non-replicating antigens can accomplish. This approach also provides the option of choosing recombinant
bacteria or viruses known to enter the body via a specific route and known to stimulate preferentially certain immune responses, therefore tailoring the stimulated quality and quantity of immunity at a particular site. Understanding how microbial components, also referred to as microbe-associated molecular patterns (MAMPs), can interact with specific pattern recognition receptors (PRR) in the host and stimulate distinct innate immunological pathways with subsequent diverse adaptive immune responses permits the selection of vectors suited to stimulate the response with protective characteristics, once the correlates of protection are defined [16-18]. The affected PRR, specific for the vector, are strictly linked to what can be described as the adjuvant signature of a certain vector, which influences both innate and adaptive responses more than the recombinant antigen inserted in the vector. Toll-like receptors (TLR), which can be differentially engaged by lipids, carbohydrates, nucleic acids or other molecules unique to microbes, are a subcategory of PRR. For instance, Gram-negative bacteria are likely to activate the TLR4 pathway due to their lipopolysaccharide (LPS) content [16, 19]. Flagellin in some bacteria permits stimulation of TLR5, while single-stranded RNA viruses can activate TLR7 and TLR8 receptors, which in turn activate the Type I interferon (IFN) response [20, 21]. Unmethylated CpG motifs present in viral DNA are recognized by TLR9 [21]. In the paragraphs below we will review the different delivery systems that have been investigated as candidate HIV vaccines.

2.1. Recombinant Bacterial and Viral Vectors

Use of bacteria with attenuated pathogenic potential as vectors to deliver pathogen-associated antigens is a promising approach to vaccination [13, 14]. A variety of well-studied bacteria are available, especially for the enteric route [22]. The biology of expression systems in different bacteria is also well characterized. Furthermore, continual expression of recombinant proteins appears to be a more efficient way to expose the immune system to the selected antigen resulting in more potent immune responses [23, 24]. Bacteria with tropism for a specific body compartment can be selected, depending on where the immunity is desired. Among the bacteria that are considered for engineering HIV vaccines to administer orally are attenuated strains of Salmonella typhimurium and Shigella flexneri [25-30]. The vaginal use of Gram-positive Lactobacillus jensenii, has been investigated for the prevention of HIV infection, not as a vaccine vector but as a delivery vehicle for the expression of the entry inhibitor cyanovirin-N [31, 32]. This approach reduced by ~ 60% the transmission rate of CCR5-tropic SHIVSF162P3, suggesting that engineered bacterial commensals might be used similarly to achieve protection against pathogens. Lastly, Bacillus Calmette and Guerin (BCG), which is an attenuated version of Mycobacterium bovis, has been explored as a vector for oral or intradermal antigen delivery, as it is one of the few vaccines that are given at birth and, if successful as a vector, could accomplish dual immunizations early in life [33-36]. However, it should be noted that BCG as a vaccine vector is contra-indicated in healthy breast-feeding infants with HIV-infected mothers because BCG immunization by either systemic or oral routes induces persistent immune activation, including enhanced frequencies of activated CCR5+ CD4+ T cells (preferred HIV target cells) in the circulation, and oral immunization of neonatal macaques with a BCG-SIV vector increased susceptibility to oral SIV infection [37, 38].

When considering viruses as vectors, a few options are available, some being DNA viruses, others being RNA viruses [15, 39]. A few poxviruses have been explored as HIV candidate vaccines, including canarypox, fowlpox and Modified Vaccinia Ankara (MVA) virus [40-44]. These viruses work well when administered via systemic routes, especially in prime-boost heterologous immunizations [42, 44-48]. Their use via mucosal routes is less established but fowlpox has been given to NHP via the nasal route and MVA has been utilized nasally, orally, rectally, and vaginally with some level of protection obtained [44, 46, 47, 49-52]. Adenoviruses have also been extensively explored as vectors, and given their tropism for the respiratory tract and intestine, they seem particularly suited for mucosal immunization [53-62]. Among viruses of the Herpesviridae family, the rhesus Cytomegalovirus (CMV) is the most advanced in preclinical trials with evidence of stimulation of immunity that can clear infection in at least 50% of NHP that received parenteral vaccination and were subsequently infected rectally with SIV [63-65]. However, due to its biology, it does not lend itself well to mucosal immunization. Within Herpesviridae, Varicella Zoster virus (VZV) has also been considered as a possible vector and administered subcutaneously and intra-tracheally with significant results [66]. The human live attenuated oral Poliovirus, a virus in the family of Picornaviridae, is one of the most successful mucosal vaccines, and both Poliovirus and Vesicular Stomatitis virus (VSV), a member of the Rhabdoviridae family, have been explored as mucosal viral vectors in macaques with partial efficacy [67-70].

A confounding factor that may reduce immune responses to HIV transgene products delivered via bacterial or viral vectors is pre-existing anti-vector immunity, either as a result of natural exposure or vaccination [71-73]. Pre-existing immunity is believed to be responsible for the poor results obtained in humans orally immunized with live-attenuated Salmonella enterica vaccines [22, 74, 75]. To overcome immunity to vaccine vectors, the most common strategy has been to administer higher doses, although this may compromise safety and preclude commercialization. High doses may also induce greater levels of anti-vector antibodies that obviate vector boosting by the same route. Prime-boosting with heterologous vectors may therefore be more effective [76-78]. Another approach found effective for priming HIV-specific B and T cells without inducing anti-vector immunity in vaccination regimens has been to use DNA as the priming vehicle [79, 80].

2.2. Recombinant DNA

DNA vaccines have a number of demonstrated and potential advantages over more conventional vaccine formulations. DNA is a relatively stable molecule, facilitating storage and handling, and the ease of safe manufacture, scale-up and distribution of DNA vaccines makes this technology attractive. Stimulation of both humoral and cellular responses is usually observed, as antigens are expressed in their native form and correctly presented to the
immune system [12, 81]. DNA vaccines expressing HIV genes have been investigated in humans for their safety and ability to prime or boost virus-specific immune responses [82-84]. Several Simian Immunodeficiency Virus (SIV) or Simian-Human Immunodeficiency Virus (SHIV) DNA vaccine constructs have been evaluated for their ability to induce protection against challenge with SIV or SHIV [85-88]. Macaques have been inoculated with various plasmids expressing a partial viral genome and various SIV or HIV Envelope (Env) genes. In some cases, DNA vaccination was followed by administration of recombinant bacterial or viral vectors or Env protein in regimes defined as heterologous prime-boost [45, 89-102].

Although the results obtained thus far with DNA vaccination in animal models are promising, there is clearly a need to increase the potency of this technology, especially for induction of antibodies. A number of studies indicate that the magnitude of the antigen-specific response can be modulated by using codon-optimized sequences for the antigen production, by the addition of genes for certain cytokines and by administering the DNA via electroporation, an approach that has significantly reduced the amount of DNA required for systemic immunization while increasing efficacy in expression of the antigen encoded by the DNA [89, 92, 94, 103, 104]. Interestingly, it has proved possible to use electroporation for buccal DNA delivery in the oral cavity of guinea pigs and mice, which generated more antigen-specific IgA in vaginal secretions, IgG in serum and CD8+ T cells in blood than did topical DNA application [105]. However, there are no devices that could be used similarly to deliver DNA vaccine directly into the mucosa of the small intestine, rectum or vagina. For mucosal administration at these sites, DNA must be shielded from nucleases in the lumen, for instance through encapsulation in a protective delivery vehicle. For this purpose, cationic liposomes (discussed below) have most often been utilized to administer DNA vaccines via mucosal routes in NHP.

3. MUCOSAL ADJUVANTS

To avoid induction of T regulatory cells and tolerance [106], mucosal vaccines, like parenteral vaccines, must contain immune stimulatory motifs that bind to host PRR to elicit pro-inflammatory mediators and up-regulation of co-stimulatory molecules required for activation of naive lymphocytes by antigen-presenting cells (APC) in regional lymph nodes [107]. The cytokines and chemokines produced by local cells play a critical role in modulating the adaptive response by influencing the development of specific T-helper cell subsets which differentially promote cellular and humoral responses [108, 109]. For example, Th17 cells promote IgA responses [110-113] whereas Th1 cells preferentially induce IgG. In the presence of TGFβ, which is enriched in mucosal tissues [114], pro-inflammatory cytokines typically induce Th17-type responses [108, 115]. Thus, vaccines or infectious agents that generate strong inflammatory responses and Th1-biased IgG responses in the systemic compartment often elicit Th17 cells and IgA responses when administered at mucosal surfaces [116-118].

Administration of the same antigen and adjuvant by mucosal or parenteral routes may also produce qualitatively different immune responses because the composition of innate cells, which express unique combinations of PRR, varies in mucosal and systemic tissues [119-122]. The responsiveness of innate cells to PRR stimulation also differs between mucosal and systemic compartments. For instance, intestinal macrophages, but not peripheral macrophages, are refractory to TLR stimulation [114, 123]. The majority of cells first contacted by vaccines applied to mucosal surfaces are epithelial cells, and apical expression of PRR and responsiveness to MAMPs varies considerably among these cells in mucosal compartments [121, 124, 125]. For these reasons, results obtained after administration of vaccine and adjuvant in one mucosal tissue cannot necessarily be extrapolated to another mucosal tissue.

3.1. TLR Agonists

Protein and peptide immunogens typically lack sufficient innate stimulatory properties and must be co-administered with adjuvants to trigger an adequate inflammatory response by innate cells. Molecules that bind to PRR are a rational choice for adjuvants, and a variety of MAMPs, especially TLR ligands [109], have been explored as adjuvants for parenterally administered HIV or SIV subunit immunogens in humans and NHP [126-131]. However, only a few have been evaluated in vaccine efficacy trials [127] or tested for the ability to adjuvant immune responses to mucosally administered antigens in humans or NHP.

It should be noted that B and T lymphocytes express TLR [132, 133], and TLR agonists can directly modulate functions of these cells [134]. Humans and macaques have similar cellular TLR distributions and responsiveness to TLR ligands [135-138]. However, there are some striking differences between mice and primates [137-139], especially for TLR4, TLR7 and TLR8. Results of some mouse vaccination studies must, therefore, be interpreted cautiously.

TLR2 can bind more MAMPs than other TLRs due to its propensity to form heterodimers with TLR1 or TLR6 [140]. Among its ligands are bacterial lipoproteins/peptides (TLR2/1) and fungal zymosan and β-glucan (TLR2/6). In NHP, TLR2/1 agonists have not shown much promise as mucosal adjuvants for enhancing antibody responses to soluble proteins. Rectal or sublingual co-administration of the TLR2/1-binding lipoprotein, PAM3CSK4, with β-galactosidase or ovalbumin (OVA), respectively, in macaques failed to generate antigen-specific antibodies in serum or secretions [141]. PAM3CSK4 also did not induce antibody responses to nasally co-administered keyhole limpet hemocyanin (KLH) in macaques, although a TLR7/8 agonist did [141]. However, TLR2/6 agonists might be more effective as mucosal adjuvants. Vaginal immunization of mice with the TLR2/6 agonist, FSL-1, improved vaginal IgA responses to co-delivered tetanus toxoid, whereas the TLR2/1 agonist, peptidoglycan, did not [142]. Others have reported that FSL-1 was more effective than PAM3CSK4 for generating Th17 and Th1 cells in the intestine of mice [143], and TLR6 stimulation promotes Th17 responses in the lung [144]. Mucosal administration of TLR2 agonists conjugated to antigens could also be more effective than admixed agonist mixtures for generating immune responses. Vaginal immunization of mice with a TLR2 ligand conjugated to a Herpes Simplex Virus 2 (HSV-2) peptide was shown to generate local HSV-2-specific memory CD8 T cells [145].
Intragastric immunization with antigen incorporated in β-glucan particles [146] also improved specific antibody responses, possibly by enhancing M cell uptake and induction of local immune responses [147]. Stimulation of M cells with a variety of TLR2 agonists has been shown to enhance apical-to-basolateral transcytosis of microparticles and recruitment of dendritic cells (DC) in follicles below [148, 149]. There is also an intriguing possibility that parenteral immunization with TLR2 agonists might generate antigen-specific lymphocytes that home to the intestine. Human peripheral blood B cells cultured with TLR2 ligands have been shown to produce J chain and IgA, up-regulate expression of CCR9 and CCR10 chemokine receptors and migrate toward CCL25 and CCL28 [150], chemoattractants respectively produced by epithelial cells in the small and large intestine [151-153]. Splenic DC of mice have also been reported to acquire the ability to imprint CD8 T cells with the αβ TCR and CCR9 small intestinal-homing phenotype after culture with PAM3CSK4 [154]. On the other hand, TLR2 agonists may not be desirable components of HIV vaccines. Human T cells express TLR2 [133], and direct stimulation of activated naive and memory CD4 T cells from blood with TLR2 ligands has been found to result in proliferation, up-regulation of CCR5 expression, and enhanced HIV replication in vitro [155-159]. Mucosal or parenteral immunization with TLR2 agonists could therefore transiently increase both inflammation and numbers of HIV target cells in vaccine recipients, rendering them more susceptible to HIV infection during this time.

TLR3 binds double-stranded RNA [160, 161], and TLR3 ligands based on polyinosinic-polyctydidylic acid (pIC) have demonstrated great potential for use as adjuvants in mucosal or systemic HIV vaccines. Type I IFNs produced in response to TLR3, TLR7 or TLR9 ligation promote cross-presentation of antigens to CD8 T lymphocytes by DC [162], but pIC has been found optimal to TLR7 and TLR9 agonists for generating gag-specific CD8 T cells in blood and lung of NHP immunized with gag protein by the subcutaneous (s.c.) route [131]. In NHP, TLR3 agonists have also augmented systemic IgG responses to parenteral VLP vaccines [163]. In mice, pIC has acted as a nasal, sublingual and vaginal adjuvant to enhance mucosal and systemic antibody responses to HIV gp140 or other protein immunogens [142]. TLR3 agonists have not yet been tested as mucosal adjuvants for preventative HIV vaccines in NHP or humans. However, studies of inactivated nasal influenza A vaccines formulated with the TLR3 agonist, rintatolimod (aka Ampligen®), indicate that TLR3 agonists can function as nasal adjuvants in primates [164, 165]. In humans, nasal administration of rintatolimod three days after nasal immunization with the FluMist influenza vaccine augmented nasal IgA responses [166]. Importantly, vaccination with TLR3 agonists at sites of mucosal HIV transmission may also be safe. In non-vaccinated Rhesus macaques, rectal administration of the pIC analogue, PICLCL, just before or at the time of rectal HIV challenge tended to decrease susceptibility of macaques to infection, despite the presence of more activated CD4+ T cells and αβ TCR memory CD4+ T cells in the rectal mucosa and blood [167]. In SIV-infected macaques on antiretroviral therapy (ART), therapeutic immunization of the palate and lingual tonsils with PICLCL and inactivated SIV particles resulted in a 2-log reduction in rebound viremia after cessation of ART [168], although a TLR9 agonist with SIV particles failed to do so. Primary human endocervical and ectocervical epithelial cells cultured with pIC have also been reported to up-regulate IFNβ [169], a cytokine recently shown to protect macaques against vaginal SHIV transmission [170]. Taken together, these studies suggest that innate responses generated by TLR3 agonists administered with vaccines in the nasal, oral, rectal, and vaginal mucosae may augment humoral and cellular immune responses without increasing susceptibility to HIV infection. Additional studies evaluating the ability of TLR3 agonists to enhance immunogenicity and efficacy of mucosal SIV or SHIV vaccines in NHP are warranted by these encouraging results.

TLR4 agonists [171] include LPS, detoxified monophosphoryl lipid A (MPL), and the synthetic analogue, glucopyranosyl lipid A (GLA). Both MPL and GLA have been found to enhance humoral and CD4+ T cell responses to parenterally administered HIV Env or SIV gag proteins in humans and macaques [126, 128, 131], albeit to a lesser degree than TLR3, TLR7/8 or TLR9 agonists. Whether TLR4 ligands can act as mucosal adjuvants in humans or NHP is unknown. In mice, there is conflicting data regarding the ability of TLR4 agonists to function as nasal adjuvants [142, 172, 173]. In minipigs [174], which have a respiratory tract more similar to humans [175], GLA did not enhance antibody responses to nasal protein immunogens. However, it is unclear how translatable these results are to humans. Determining whether TLR4 agonists can function as mucosal adjuvants for HIV vaccines will require more definitive studies in humans or NHP.

The TLR5 agonist, bacterial flagellin [176], can directly modulate B cell responses [20] and has enhanced humoral responses to i.m. influenza vaccines in humans [177]. There is some evidence that flagellin expressed as a fusion protein with antigen might function as an adjuvant in the nasal cavity of primates [178]. In studies with guinea pigs, nasal immunization with HIV virus-like particles (VLPs) constructed to express a membrane-anchored bacterial flagellin generated greater levels of Env-specific serum IgG and vaginal IgA than VLPs lacking or admixed with flagellin [179]. Flagellin has also augmented local T-cell responses to peptides administered in the mouse vagina, but efficient delivery at this site required epithelial thinning and disruption by medroxyprogesterone and Nonoxynol 9 [180]. In the intestine, flagellin may enhance transport of particles by M cells [181] but it has not yet been tested for ability - as a surface coating - to enhance uptake of delivery vehicles containing vaccine.

The TLR7/8 agonist, resiquimod (R848), has enhanced systemic immune responses to HIV proteins co-administered parenterally in macaques [128-131]. In mucosal immunization studies with macaques, R848 given with β-galactosidase by the rectal route or with ovalbumin by the sublingual route did not elicit antibody responses [141]. However, R848 did function as a nasal adjuvant for KLH in these animals. Importantly, nasal immunization with R848 enhanced KLH-specific serum IgG and vaginal IgA responses without generating adverse inflammatory responses in the nasal cavity [141]. R848 may therefore be safe as a nasal adjuvant in humans. R848 is also one of the few TLR...
agonists identified as a vaginal adjuvant [183]. Vaginal ring delivery of HIV gp140 protein with R848 in sheep significantly increased levels of anti-gp140 IgG in serum and IgA in vaginal secretions [183]. More studies of R848 as a mucosal adjuvant for HIV vaccines are warranted by these encouraging results.

TLR9 agonists [184], unmethylated synthetic CpG oligodeoxynucleotides (CpG ODNs), have also augmented humoral and cellular immune responses to parenterally administered HIV and SIV proteins or virus particles in macaques [128, 131, 185]. In mice, CpG ODNs have also been reported to function as nasal, oral, intestinal and vaginal adjuvants [142, 172, 186], but these results may be misleading as different innate cells in mice and primates express TLR9 [138, 187]. The type of CpG can also influence results. There are 4 classes of CpG ODNs which differ in their ability to activate B cells and to induce production of TNFα or IFNs by plasmacytoid DC (pDC) [187]. Type B CpGs induce TNFα production by primate pDC whereas Type C induces more IFNα [188-190]. CpG-C may not be ideal for mucosal HIV vaccines. Vaginal administration of CpG-C in nonvaccinated macaques enhanced the risk of vaginal SIV transmission [191], albeit in larger doses than those typically used for immunization. Supplementation of inactivated SIV particles with CpG-C did not enhance SIV-specific humoral or cellular responses or improve the ability to control rectal SIV infection in macaques given tonsillar vaccinations [192]. Therapeutic parenteral or tonsillar vaccination of SIV-infected macaques on ART with CpG-C and inactivated SIV particles also failed to generate immune responses that reduced rebound viremia after cessation of ART, although a TLR3 agonist did so [168, 185]. Whether mucosal immunization with CpG-B ODNs may generate more beneficial anti-HIV innate and adaptive responses in humans or NHP is unknown. It is noteworthy that CpG-B has been administered in the human nasal cavity without producing adverse side effects [193].

### 3.2. Cytokines

Cytokines have been tested in many vaccine studies for their ability to enhance immune responses and protective efficacy of SIV and SHIV DNA vaccines. The first cytokine shown to improve the efficacy of a DNA vaccine in NHP was IL-2, expressed as a fusion protein with the Fc of human IgG1 to increase its half-life [194]. Both soluble IL-2/Ig and DNA encoding IL-2/Ig were able to adjuvant humoral and cellular responses induced by an i.m. SHIV DNA vaccine, and each reduced viral replication and disease progression after intravenous SHIV89.6P infection [194, 195]. We found that supplementing a nasal SHIV DNA vaccine with IL-2/Ig DNA induced greater frequencies and more durable virus-specific IFNγ- and TNFα-producing CD8+ T cells in the rectum and blood of NHP after nasal boosting with SHIV MVA [49]. Better protection against disease development was observed in these animals after rectal SHIV89.6P infection, and control was associated with frequencies of vaccine-induced gag-specific CD8+ T cells.

Toward the goal of generating even greater HIV-specific CD8+ T-cell responses with DNA vaccines, the Th1-promoting IL-12 cytokine has also been evaluated as an adjuvant in NHP, but mostly by the i.m. route, and with conflicting results regarding its ability to improve CD8+ T-cell responses and vaccine efficacy [196-199]. We tested DNA encoding IL-12 as a nasal adjuvant for the DNA prime in a DNA/MVA vaccination strategy in NHP. When compared to animals nasally-primed with SHIV DNA and nasally-boosted with MVA, co-priming with IL-12 and SHIV DNA did not induce detectable differences in virus-specific cellular or humoral responses before or after rectal infection with SHIV89.6P, nor did it alter viremia or the course of disease progression [49]. The lack of IL-12 effects in this study could not be attributed to degradation of DNA in the lumen of the nasal cavity because the DNA was protected by formulation in cationic liposomes, and identical co-administration of IL-2/Ig DNA and SHIV DNA in another group of animals did enhance T-cell responses and control of infection [49].

IL-15 has been explored as an adjuvant for augmenting CD8+ T-cell responses to DNA vaccines in NHP. However, in macaques primed by the i.m. route with IL-15-adjuvanted SHIV or SIV DNA, little difference has been noted in the virus-specific CD8+ T-cell responses [196, 200, 201], and vaccine efficacy for preventing vaginal or rectal SIV infection was not significantly improved [196, 201]. We found that nasal immunization of macaques with IL-15-adjuvanted DNA followed by nasal MVA boosting generated greater control of rectal SIVmac251 infection than the same regimen given by the i.m. route [47]. While this could be attributed to the use of a mucosal route for vaccine delivery, it is also possible that IL-15 may enhance responses to DNA vaccines given at mucosal surfaces. On the other hand, nasal priming with IL-2- and IL-15-adjuvanted SIV DNA did not elicit different cellular (or humoral) responses compared to macaques primed with GM-CSF, IL-12 and TNFα. After a nasal boost with MVA-SIV and vaginal challenge with SIVmac251, both regimens were found equally effective for control of infection [51]. The inclusion of soluble IL-15 in both a colorectal SIV peptide prime and colorectal SIV-MVA boost also failed to induce higher quality CD8+ T-cell responses or to enhance control of rectal SIV infection when compared to animals immunized with non-adjuvanted peptide and MVA [202]. However, the IL-15 rectally applied in this study was not protected by formulation in a protective delivery vehicle. Therefore, the bioactivity of IL-15 may have been reduced by proteolysis in the rectal lumen. Alternatively, the 300μg dose of IL-15 repeatedly administered may have been too large. IL-15 has been shown to be immunosuppressive when given in high doses [203].

To augment humoral responses to HIV DNA or subunit vaccines, several cytokines which directly stimulate B cells have been tested as mucosal adjuvants in animals. Of these, B-cell Activating Factor (BAFF) and A Proliferation-Inducing Ligand (APRIL) have not proven effective as nasal adjuvants in mice for enhancing HIV-specific antibodies in serum or secretions when given in soluble form with HIV Env protein [204]. Thymic Stromal Lymphopoietin (TSLP) co-administered by the nasal route with HIV gp140 in mice did induce impressive increases in Env-specific systemic IgG and vaginal IgA antibodies [204]. However, in NHP, nasal immunization with TSLP and KLH infrequently generated KLH-specific IgG or IgA antibodies in serum or secretions [141]. Nasal immunization of macaques with human IL-1α, GM-CSF and an HIV peptide also did not increase IgA
antibodies in the rectum or vagina [205], despite promising results in mice [206]. The use of human cytokines in macaques or administration of potentially non-optimal cytokine doses may be partly responsible for this disappointing outcome. Like IL-15, high doses of GM-CSF can also suppress immune responses, including IgA responses [207, 208].

A noteworthy molecular adjuvant used to enhance Env-specific systemic IgG and vaginal IgA antibodies generated by systemically administered DNA vaccines or HIV VLPs in mice is the CCL28 chemokine [209, 210], which attracts CCR10-expressing lymphocytes to the large intestine, and to the mouse uterus [211]. Recently, in macaques, co-delivery of CCL28 DNA with SIV DNA by electroporation was shown to enhance SIV-specific serum IgG and vaginal IgA responses, and after vaginal challenge with SIV Δenv, these animals had lower peak viremia and rapidly controlled viremia to undetectable levels [212]. Hopefully, this chemokine will be evaluated in future studies to determine if it can augment immune responses and improve efficacy of mucosal vaccines.

3.3. Attenuated Bacterial Enterotoxins

Promising adjuvants for mucosal HIV vaccines include attenuated bacterial enterotoxins such as the Escherichia coli heat-labile double mutant toxin, dmLT [213] and cholera toxin (CT) derivatives [214, 215], which function as adjuvants in the murine nasal cavity, sublingual mucosa, intestine and female genital tract [215-217], and typically generate mixed Th1/Th2/Th17 responses. The precise mechanisms through which these enterotoxin adjuvants exert their immune stimulatory activity is still under investigation [213, 214, 218]. Most cannot be administered in the human nasal cavity as local inflammatory responses can result in Bell's palsy [219, 220]. However, like dmLT, they should be safe in the intestine, oral cavity and probably the vagina [221]. There is evidence that the innate and adaptive responses generated by LT-based adjuvants might be favorable for control of HIV infection. Rectal immunization of macaques with SHIV peptides and the first iteration of dmLT, the single mutant mLT (aka LTR192G), was shown to generate local CTL which controlled rectal SHIVΔenv infection [222]. Therapeutic transcutaneous vaccination of ART-treated SIV-infected macaques with DNA-encoding SIV proteins and the LT holotoxin has also been found to dramatically reduce viremia following cessation of ART [223]. It is not widely appreciated that these enterotoxins can be used as parenteral adjuvants [213]. Indeed, there is a possibility that dmLT-adjuvanted parenteral HIV vaccines might generate HIV-specific intestinal immune responses. Intradermal immunization of mice with peptide and dmLT (but not CpG) was recently shown to generate αβ7-expressing peptide-specific CD4+ T cells in the circulation, followed by increases of these cells in the small and large intestine [224]. Intradermal immunization of mice with dmLT-adjuvanted inactivated poliovaccine also generated polio-specific intestinal IgA responses [225]. However, it remains unclear whether immunity induced by parenteral vaccines supplemented with dmLT would be as protective as mucosal vaccines containing this adjuvant.

3.4. Chitosan, Endocrine, and ISCOMs

Some bioadhesives that reduce mucociliary clearance and improve the retention time of vaccines at mucosal surfaces have also been reported to function as adjuvants in mice [226]. The mucoadhesive, chitosan [227], is a chitin-derived cationic polymer that can enhance both vaccine retention and uptake at columnar epithelial cell surfaces by transiently opening tight junctions [228]. In humans, nasal insufflation of a lyophylized diptheria toxoid formulated with chitosan modestly increased titers of neutralizing antibody in serum and IL-5 producing T cells in blood of humans [229]. However, chitosan is weakly inflammatory in the human nasal cavity [230], and nasal administration of chitosan with an HIV gp140 protein in humans did not elicit anti-Env antibodies [231]. Generating strong humoral responses to mucosally administered HIV proteins formulated in chitosan will likely require supplementation with more potent adjuvants.

Endocrine (aka Eurocrine L3) is an emulsion of the anionic lipids, mono-olein and oleic acid, that forms liposomes less than 100nm in diameter. In addition to enhancing uptake of antigens at mucosal surface, Endocrine has some immune stimulatory activity, which has been linked to activation of danger-associated molecular pattern molecules by RNA released from damaged cells at the site of administration [232]. Therapeutic nasal vaccination of HIV-infected humans with HIV gag peptides in Endocrine has been reported to increase lymphoproliferative responses in blood, and gag-specific serum IgG, rectal IgA and nasal IgA antibodies, although very modestly [233]. In mice, Endocrine has impressively adjuvanted antibody responses to HIV VLPs given in the nasal cavity [234]. However, in macaques, four nasal immunizations with Endocrine and the same VLPs did not elicit systemic or vaginal antibodies [235]. It is therefore unclear whether Endocrine has sufficient stimulatory activity to elicit strong distal mucosal antibodies to nasally administered HIV proteins in humans.

Saponin derivatives in the ISCOM delivery vehicle (see below) have proinflammatory activity, and have functioned as adjuvant for ISCOM-formulated antigens given to mice by systemic or mucosal routes [236, 237]. However, ISCOMs also have not yet been tested for adjuvant activity at mucosal surfaces of primates. In addition, better results have been obtained in mice when mucosally-administered ISCOM vaccines were supplemented with an additional adjuvant [217, 238].

4. FORMULATIONS FOR MUCOSAL DELIVERY OF VACCINES

Although mucosal vaccination should provide more optimal mucosal immunity than systemic immunization, the same innate physical and chemical barriers that inhibit pathogen entry at mucosal surfaces pose obstacles in the efficient uptake of mucosal vaccines (reviewed in [239]). Physical impediments include commensals, mucus, mucociliary clearance and epithelial cell barriers which may consist of a single layer of columnar epithelium with impermeable tight junctions, as in the intestine, or multiple layers of permeable squamous epithelium that lack tight junctions, which can be up to 50 cells deep in the vagina.
[240]. Chemical constraints include degradative enzymes (proteases, glycolases and nucleases) produced by both the local microbiota and epithelial cells, the host defense antimicrobial peptides and proteins produced by epithelial cells [241-243], and, for peroral (ingested) vaccines, the low pH and bile acids in the stomach [239]. A variety of protective vehicles have been utilized with success to deliver intact HIV or SIV antigens across these barriers, as discussed below.

4.1. Enteric Capsules

One solution to protect peroral vaccines against hydrolysis in the stomach is to administer them in a neutralizing bicarbonate solution, which has worked well for bacterial vaccines, such as the Dukoral® whole-inactivated cholera vaccine and the live-attenuated Ty21a Salmonella typhi vaccine [244, 245]. Peroral administration of freeze-dried vaccine in enteric-coated capsules that are resistant to hydrolysis in low pH solutions has also been used to preserve vaccine integrity during transit through the stomach, and is another method commonly used to orally deliver the live-attenuated Ty21a vaccine [245]. Enteric capsules typically consist of gelatin or Hydroxy-Propyl-Methylcellulose (HPMC) capsules containing lyophilized antigen and stabilizing agents. Surfaces of the capsules are coated with various mixtures of Eudragit® methacrylic acid esters that are insoluble at low pH [246]. Capsules coated with Eudragit® formulations that dissolve at a specific pH and after a certain period of time can be used to deliver vaccines to specific segments of the small intestine and even the distal colon [247, 248]. In macaques, intragastric administration of freeze-dried adenovirus type 5 (Ad5) expressing HIV Gag or Env in capsules coated with Eudragit® solutions designed to dissolve in the jejunum has elicited antigen-specific lymphoproliferative responses in blood and specific IgA in saliva, and vaginal secretions despite significant loss of vector activity, possibly due to the absence of stabilizing excipients [247]. Recombinant adenovirus-associated viruses have also been delivered successfully in the intestine of macaques using enteric capsules [249]. An obvious disadvantage of this approach, though, is that the contents of capsules are released in the lumen where they may be degraded before being internalized.

4.2. Poly(lactide-co-Glycolic Acid) Particles

A delivery vehicle that does not release its contents until after internalization at mucosal surfaces would be advantageous for induction of immune responses. Apical surfaces of intestinal M cells are largely devoid of mucus, and these cells readily transcytose bacteria- and virus-sized particles into underlying lymphoid follicles for phagocytosis by DC [11, 250]. This includes antigens encapsulated in poly(lactide-co-glycolic acid) (PLG) biodegradable particles [251], which slowly release their contents following internalization by Antigen-Presenting Cells (APC). M cells have also been identified in the nasopharyngeal tonsil (adenoids) of humans [252] and may similarly mediate uptake of PLG particles applied in the nasal cavity. For vaginal immunization, available data suggest that hydrophobic surfaces of particles should be modified with polyethylene glycol or other substances for optimal diffusion through mucus [253, 254], and smaller (20-40nm) particles may better penetrate vaginal epithelium and reach draining lymph nodes [255]. However, the utility of PLG particles as protective delivery vehicles for vaccines is limited because organic solvents are required for antigen incorporation, and the lactic and glycolic acids released during particle dissolution can further degrade antigen [251]. Hence, while the immunogenicity of some HIV peptides and the adjuvant activity of some TLR agonists is retained [248, 256], encapsulated HIV Env proteins or DNA vaccines are typically denatured [239, 257]. This may also explain why oral immunization of humans with PLG particles containing an HIV V3 peptide dose of 1mg failed to elicit detectable immune responses, although the lack of an adjuvant was more likely responsible [258]. In mice, a converse strategy of adsorbing DNA onto the PLG particle surface has been reported to be superior to naked DNA for nasal immunization [259]. It is unlikely, though, that proteins or DNA vaccines could be effectively administered in this way in the harsher luminal environments of the oral cavity, intestine and vagina.

4.3. Cationic Liposomes

Phospholipid bilayer-based liposomes with an aqueous core (reviewed in [260]), and often supplemented with cholesterol to increase stability, have been used with success in NHP to deliver SIV peptides by the rectal route [217] and DNA vaccines by numerous mucosal routes [50, 88, 202]. In humans, freeze-dried liposomes containing protein immunogen have also been administered perorally in enteric capsules for induction of salivary IgA antibodies [261]. However, mucosal delivery of vaccines incorporated within liposomes may primarily induce T cell responses as liposomes fuse with cell membranes resulting in cytosolic delivery of antigen [260]. Positively charged (cationic) liposomes, rather than negatively charged (anionic) liposomes, are most often used for vaccine delivery because DNA and most protein or peptide antigens are negatively charged and readily associate with cationic liposomes through electrostatic interactions. Cationic liposomes have also been reported to be more efficiently internalized by DC due to their ability to bind to heparan sulfate proteoglycans on the surface of these cells [251]. Epithelial cells also express heparan sulfate proteoglycans. Mucosal immunization with liposomal DNA may therefore result in transfection of epithelial cells, which can function as APC [262-264], but may be sloughed in a matter of days. Nonetheless, we and others have found that SIV DNA encapsulated in cationic liposomes can successfully generate SIV-specific T cells when delivered in the rectum, small intestine, oral cavity, vagina or nasal cavity of macaques [50, 88]. However, we should note that administration of naked DNA in the primate nasal cavity is also effective for induction of antigen-specific T cells [47, 49, 51, 52].

4.4. Immune Stimulatory Complexes (ISCOMs)

ISCOMs [265] are 40-50nm nanoparticles consisting of cholesterol, phosphatidylcholine and Quillaja saponin derivatives [238], which form cage-like structures through
4.5. Influenza Virosomes

Influenza virosomes are 150-200nm phosphatidyl-choline-enriched liposomes supplemented with the influenza A Neuraminidase (NA) and Hemagglutinin (HA) viral proteins [270, 271]. The HA assists in targeting of virosomes to APC due to its recognition of sialic acid residues abundantly expressed on DC and macrophages, and it likely enhances mucosal uptake of virosomes through binding to sialic acid-containing molecules ubiquitously expressed on apical surfaces of epithelial cells [272]. In mice, virosomes containing incorporated DNA, proteins or peptides have induced humoral and cellular immune responses after delivery by the nasal, pulmonary, sublingual or vaginal routes [273-276]. To generate stronger antibody responses, antigens can also be expressed on the surface of the virome through integration into the lipid bilayer [270], although they would be unprotected. In one study, HIV peptides were covalently attached to the influenza proteins on the outer surface of gp120 and saponin containing virosomes. However, nasal immunization of macaques with these surface modified virosomes did not produce good antibody responses [277]. In contrast, i.m. priming and nasal boosting of macaques with gp41 membrane proximal external region (MPER) peptide and an immunodominant domain-deleted gp41 integrated into the virome lipid bilayer was successful for generating protective neutralizing and transcytosis-inhibiting antibodies [278] in rectal and vaginal secretions [279]. In humans, a similar i.m./nasal vaccination regimen with virosomes containing only the MPER peptide elicited more modest mucosal antibody responses [280]. Whether this may have been due to the presence of pre-existing anti-influenza antibodies in serum or the upper respiratory tract is unclear. In humans, pre-existing serum antibodies have been correlated with reduced systemic IgG responses to influenza following i.m. immunization with the Inflexal V® influenza virome vaccine [281]. However, in other clinical studies with adults who presumably had pre-existing anti-influenza HA antibodies, excellent serum antibody responses have been generated to non-influenza antigens after i.m. administration of virosome-based hepatitis A virus and malaria vaccines [282, 283]. Additional studies will be required to determine the extent to which pre-existing mucosal and serum IgG antibodies to influenza proteins may limit the utility of the virome as a mucosal delivery vehicle in humans.

Disadvantages of delivering mucosal vaccines in the above liposome-based vehicles are the increased costs associated with vaccine manufacture and short shelf-life [284]. While new lipid-based nanovesicles are in development to circumvent these issues [284], the problem still remains that encapsulation of proteins inside liposomes and PLG particles that are primarily internalized by DC not only shields them from degradative enzymes but also from B cells. New methods for delivery of HIV Env proteins to mucosal B cells are urgently needed to more effectively generate anti-Env antibodies in the intestine and genital tract.

5. ORAL AND RECTAL ROUTES OF SIV VACCINATION

Immunization in the oral cavity and gastrointestinal mucosa using peroral administration has significant appeal because of the simplicity of administration and therefore its feasibility even in settings with limited health care resources [285]. Rectal immunization, important because the rectum is a site of HIV transmission, primarily stimulates responses in the intestine, and it is not excessively complicated in terms of administration, as the vaccine could be formulated in suppositories, a common delivery tool for therapy [286]. However, it would not be as well received as oral immunization. Immunization in the large intestine through peroral administration is feasible, though, as the vaccine can be formulated in enteric-coated capsules that specifically dissolve in the colon or rectum [248], as discussed above.

Gastrointestinal immunization in the context of the NHP model for AIDS has been explored with a variety of platforms, including DNA, peptide, proteins, viruses, and bacteria. These candidate vaccines were delivered exclusively to the oral cavity, tonsils, small intestine, rectum or the entire gastrointestinal (GI) tract. It is unclear to what extent oral vaccination, whether targeted to the oral cavity or the GI tract can protect in the context of HIV/SIV infection. The highest antibody titers are usually achieved at the mucosal site of antigen exposure and decrease at distant sites [287-292]. Although HIV replicates at significant levels in the intestinal mucosa, it enters the body predominantly via the genital tract or the rectum, requiring a more diversified immunity than pathogens that infect exclusively by the oral route [5]. The rationale for considering an oral vaccine capable of stimulating both mucosal and systemic immunity comes from humans and NHP vaccine studies [293-295] and from data involving highly exposed but persistently seronegative (HEPS) uninfected individuals [296-298], which suggest that it is unlikely that vaccine approaches that stimulate a single arm of the immune system will provide effective prevention of chronic systemic infection [293-295, 299-308]. Achieving humoral and cellular mucosal responses at different mucosal sites in addition to systemic responses, which is possible with oral immunization, may result in protection from chronic infection, as this combination has been suggested key to HIV resistance in highly exposed sex workers and discordant heterosexual couples [296, 297]. In the next paragraphs, we will review what we have learned so far when oral and intestinal immunization approaches were investigated in preclinical trials.
We used SIV and SHIV recombinant DNA+MVA via the oral cavity, the small intestine and rectal routes, using a SHIV challenge rectally and an SIV challenge either rectally or vaginally [46, 50, 88, 309]. We determined that mucosal and systemic responses to multiple viral antigens produced by SHIV or SIV DNA vaccine boosted by a matched recombinant MVA can be induced to different degrees after rectal or mixed systemic/mucosal vaccination in male and female animals. However, mucosal antibody responses were sporadic and short-lived. After challenge, we observed control of viremia and delay of CD4+ T-cell loss and AIDS. When the vaccinated animals were compared to controls, on average a significant reduction in viremia was observed. Post-challenge immunological correlates of protection were systemic anti-SIV Gag + Env CD4+IL-2+, CD4+/IFNγ, and CD8+/TNFα T cells and vaginal anti-SIV Gag + Env CD8+ T-cell total responses. When a similar SIV vaccination, further boosted by mLt-adjuvanted inactivated SIV particles, and given via the oral route was directly compared to the small intestinal route in female animals challenged vaginally, we found that immunizations generated mucosal SIV-specific IgA at different sites and limited levels of SIV-specific IgG antibodies in plasma and mainly against Gag and Pol proteins after particle immunization [50]. No SIV-specific IgG antibodies were detected in secretions. Oral immunization was effective in inducing SIV-specific IgA in vaginal secretions and generated greater IgA responses in rectal secretions and saliva when compared to the small intestinal immunization route or vaginal and nasal secretions evaluated in the same study. These immunizations stimulated systemic T-cell responses against Gag and Env, albeit to a different extent, with oral immunization inducing a higher magnitude response. SIV-specific T cells producing IFNγ dominated these responses. Vaccination also induced CD4+ and CD8+ T-cell responses in the rectal and vaginal mucosa with greater functional heterogeneity than in blood samples. Rectal T-cell responses were significantly greater in the orally vaccinated animals than in the other animals. The most balanced, higher-magnitude vaginal T-cell responses were observed after intestinal vaccination. Of the routes tested, oral vaccination provided the most overall diverse and significant response to the vaccine. After vaginal challenge with SIVmac251, 50% of the orally vaccinated animals suppressed viremia to undetectable levels and viremia did not rebound after CD8+ T-cell depletion, while suppression of viremia occurred to a significantly lower degree in intestinally vaccinated animals and in controls [46]. Regardless of the route of vaccination, mucosal vaccinations prevented loss of CD4+ T central memory cells and CD4+αβ γ T cells and reduced immune activation. None of the orally vaccinated animals had developed AIDS after 72-84 weeks of infection, when the trial was closed.

When considering viral vectors given via the oral route, an interesting approach was provided by recombinant live attenuated oral polioviruses, each expressing a small SIV peptide and covering, when combined in a single vaccination, the entire Gag, Pol and Env protein sequences [67]. Oral immunization of 7 Cynomolgus macaques with this vaccine stimulated both humoral and cell-mediated systemic and mucosal responses. These responses provided protection from vaginal challenge in some animals, which required a significantly higher number of challenges to become infected, and 2 were completely protected from infection, while 2 other animals showed significant reduction of viremia after infection. All remained disease free by week 48, when 50% of the 12 controls had developed AIDS [68].

Vaccination of the palate and lingual tonsills with aldrithiol 2 (AT-2)-inactivated SIVmac particles mixed with CpG adjuvants was tested in Chinese Rhesus macaques [192]. Anti-SIV rectal IgA were of limited magnitude and the titer did not increase after challenge. Fifty percent of the animals were fully protected from homologous rectal challenge. In the infected animals, viremia control was limited and transient [192]. A SIV-based, Single Cycle Immunodeficiency Virus (SCIV) was also used as an oral spray administered at the tonsils and followed by oral challenge [310]. Although reduction of peak viremia was observed, this immunization provided very limited benefits compared to others. This route of vaccination provided slightly better results when a recombinant MVA vaccination was used in the SHIV model, where better viremia control and protection from CD4+ T-cell loss was observed. In both cases, systemic and mucosal responses were stimulated. The easier ability to protect against CXCR4-tropic SHIVs compared to SIV needs to be kept in mind when these different outcomes are considered [311].

Rectal immunization of macaques using SIV and HIV peptides mixed with mLt induced responses at multiple mucosal sites and although it did not prevent infection, viremia after challenge was significantly reduced to undetectable levels in the intestinal mucosa and in blood [222]. These investigators observed equally significant results when they tested a combination of multiple SIV Gag and HIV Env and Tat peptides and the attenuated vaccinia virus strain NYVAC expressing SIV gag/pol and HIV env genes. Excellent T-cell responses in blood, colon and mesenteric lymph nodes were observed and delayed dissemination of SHIV occurred after rectal challenge [312]. When focusing on rectal vaccination with viral vectors, significant results were also observed in macaques rectally immunized with SIV gag, env and rev-expressing replication-competent adenovirus type 5 (Ad5), which stimulated secretory IgA at multiple mucosal sites and memory cell-mediated responses both systemically and mucosally [61]. Importantly, when combined with parenteral SIV gp120 immunization, this study showed that although the antibody responses were non-neutralizing, vaccination induced antibodies that mediated Antibody-Dependent Cellular Cytotoxicity (ADCC), Antibody-Dependent Cellular Viral Inhibition (ADCVI) and transcytosis inhibition [61].

Among the bacterial vectors investigated for oral immunization, recombinant live attenuated Salmonella typhimurium strains expressing SIV Gag and Env fragments have been evaluated for their capability to stimulate anti-SIV immunity when given alone or combined with boosting by recombinant MVA [25-29, 313]. Cell-mediated responses were induced in blood and colonic mucosa but these responses did not provide control of viremia after rectal or intravenous challenge. Shigella flexneri strains have been investigated to deliver DNA vaccine plasmids in a mouse model and succeeded in stimulating both cell-mediated and humoral responses [30]. However, data in NHP are not yet available.
An interesting approach to HIV vaccination is the use of recombinant mycobacterial vectors, which could simultaneously achieve vaccination for TB and HIV. A single oral dose of an attenuated Mtb-SIV vaccine given to infant macaques during the first week of life stimulated immune responses to TB and SIV antigens, and anti-SIV responses increased after MVA-SIV boosting. T-cell responses reached the highest magnitude in the intestine and in oral lymph nodes. Mucosal SIV-specific IgA could be detected in saliva and intestinal secretion, while IgG was present in blood. Significantly lower peak viremia after challenge correlated with pre-challenge SIV Env-specific salivary and intestinal IgA responses and higher-avidity SIV Env-specific IgG in plasma, and in these animals CD4+ T-cell populations were better preserved over time compared to non-controlling animals [35, 36].

6. VAGINAL SIV OR SHIV VACCINATION

In studies with NHP, vaccine-mediated protection against vaginal SIV or SHIV infection has been associated with the induction of CD8 T-resident memory (Trm) cells and local anti-Env IgA- or IgG- antibody responses, especially to gp140, in the female genital tract [10, 279, 314-316]. Transudated serum IgG antibodies may also contribute to protection in genital tract tissues if they can neutralize virus, mediate phagocytosis or kill infected cells through ADCC [127, 317, 318]. These immune effectors could be generated by systemically administered replication-competent viral vectors that have some propensity to replicate in the female genital tract. Even replication-incompetent viral vectors, such as the SIV-expressing NYVAC poxvirus vector, can induce specific CD8 T cells in the genital tract of primates following parenteral immunization [319]. However, a replication-competent Ad5-SIV generated more SIV-specific CD8 T cells in vaginal tissues of macaques when immunization was performed by the vaginal route [61], consistent with reports that more virus-specific CD8 T cells are generated at sites of immunization [248, 320, 321]. Parenteral immunization with non-replicating vectors and protein immunogens is also ineffective for generating IgA or IgG plasma cells in the female genital tract [127, 287]. The vaginal route has been found optimal for induction of local IgA and IgG antibody responses in the female genital tract of NHP and women [287, 289-291]. In several animal studies, vaginal immunization with DNA or other non-replicating vaccines has also proved more effective than parenteral immunization for preventing infections by vaginally transmitted pathogens [322-324].

Vaginal immunization can, however, be challenging. The phase of the menstrual cycle may need to be taken into account for some vaccine antigens. Vaginal immunization of women with a whole inactivated cholera vaccine containing CTB has been found equally effective for generating local CTB-specific antibodies when immunization was performed during either the mid-follicular or mid-luteal phase of the menstrual cycle. However, no antibodies were generated to cholera LPS in women immunized during the mid-luteal phase [290]. The reason for this is unclear. It may be related to reduced uptake of free LPS or contact of cholera vibrios with the epithelium during the luteal phase, a time when mucus is more viscous [325]. Mucus interferes with the ability of many antigens, including HIV particles [326], to penetrate the epithelium and contact DC. In studies tracking the fate of tracer proteins or nanoparticles applied in the vagina of mice or macaques, very little tracer has been detected in the stroma of the vagina or ectocervix. Most of it remains sequestered in mucus at apical epithelial surfaces or within the superficial layers of sloughing epithelium [255, 326-328]. For this reason, induction of antibody responses in the female genital tract will require large doses of antigen, in addition to adjuvant. Even large doses of HIV Env protein administered by the vaginal route in the absence of adjuvant have proved relatively ineffective for generating local IgA- or IgG-antibody responses in humans and NHP [231, 329-331].

Generating strong local antibody responses with the vaginal immunization route has also proved difficult using viral-vectorized vaccines, including HIV-expressing canarypox in humans [332] and replication-competent SIV Ad5 [61]. Vaginal administration of SIV DNA in cationic liposomes, MVA and SIV particles formulated with mLTA adjuvant in macaques did elicit SIV Env-specific vaginal IgA responses in some animals, but they were of low magnitude and did not persist [50]. Vaginal immunization of macaques with HPV pseudovirions expressing SIV gp120 env, gag and pol generated negligible anti-Env IgA in vaginal secretions [333]. Recently, however, sheep immunized with vaginal rings that release gp140 and R848 adjuvant within 24 hours were shown to develop impressive levels of HIV gp140-specific IgA and IgG in vaginal secretions [183]. In serum, high concentrations of anti-gp140 IgG but not IgA were also found in these animals. It would be interesting to know if this immunization strategy could confer protection against SHIV infection in macaques.

Since low doses of antigen can stimulate T-cell responses, vaginally administered SIV vaccines have, not surprisingly, proved more effective for generating antigen-specific CD4+ and CD8+ T cells in the genital tract of NHP [46, 61, 333]. Evaluating cells in genital tract tissues of humans is difficult in clinical studies. However, vaginal immunization of women with HIV gp140 conjugated to the Mycobacterium tuberculosis heat shock protein 70 has been shown to generated gp140-specific CD4+ and CD8+ T cells in blood [334]. Interestingly, the innate antiviral restrictive enzyme, APOBEC3G, and chemokines (CCL3 and CCL5) which down-regulate CCR5 expression by CD4+ T cells were up-regulated in these women. In addition, CD4+ T cells from the blood of these women were found to be more resistant to HIV infection in vitro when compared to their pre-immunization T cells [334].

Vaginal immunization using viral vectored vaccines can also generate T cells in the large intestine [50, 61, 335]. In a small study with macaques, vaginal administration of replication-incompetent helper-dependent adenoviral vectors expressing HIV gp140 induced Env-specific CD4+ T cells in the rectum, and this vaginal immunization regimen was as effective as i.m. vaccination for reducing acute viremia after rectal challenge with SHIV162P3 [335].

Despite the induction of local antiviral CD8 T cells, non-replicating vaginal SIV vaccines have not prevented vaginal SIV transmission or significantly reduced viremia in NHP. Only vaginal immunization with the nonpathogenic SHIV89.6
has proved effective for this [336]. Interestingly, SHIV$_{89.6}$ does not generate Th1-biased responses, in contrast to most SIV vaccines. CD4$^+$ T cells recruited to the genital tract of SHIV$_{89.6}$-immunized macaques include T regulatory cells, and following SIV$_{mac251}$ infection these cells suppress local CD4$^+$ T-cell proliferation and inflammation, allowing local CD8$^+$ T cells to eliminate infected cells in the genital tract without increasing the pool of available activated CCR5$^+$ CD4$^+$ target cells [337]. Replication-incompetent vaccines designed to mimic the cellular responses generated by SHIV89.6 and induce Env-specific antibodies to CCR5-tropic viruses may be ideal for vaginal immunization, and achieving protection against HIV in the female genital tract.

### 7. PENILE VACCINATION

In the last few years, there has been considerable progress identifying modes of HIV transmission in the male genital tract [338-341] and the local immune effectors that could potentially contribute to protection at this site [340, 342-344]. In the urethral mucosa, IgA plasma cells dominate over IgG plasma cells [345], but male genital tract secretions, like those in the female genital tract, typically contain more IgG due to transudation (or possibly FcRn-mediated transport) of large amounts of serum IgG [346]. Thus, i.m. vaccination can generate IgG antibodies in these fluids [347]. However, HIV resistance in men has been linked to HIV-neutralizing IgA in foreskin [298, 348], and induction of local HIV-specific IgA responses will likely require that vaccine be administered locally or by another mucosal route. Unfortunately, there is very little information on this topic. The male genital tract is the most understudied site for vaccine administration. In the single penile immunization study that we are aware of, a pediatric nasogastric feeding tube was used to topically apply chimeric SIV p27-Ty yeast transposon virus-like particles conjugated to cholera toxin B subunit (CTB) [349] in the urethra of male macaques [350]. Two urethral immunizations induced p27-specific IgA and IgG antibodies in urethral fluids and serum. Interestingly, p27-specific lymphoproliferative responses were also detected in blood, and these were comparable to those in animals vaccinated by the i.m. route with p27-Ty particles and alum [350]. This study clearly demonstrates the feasibility of generating virus-specific humoral and cellular responses by immunization in the male genital tract. However, more practical methods are needed for vaccine delivery at this site.

Additional studies evaluating the immune responses generated in the male genital tract after delivery of vaccines by more practical immunization routes would also inform HIV vaccine development. It is known that i.m. immunization with non-replicating Ad26- and Ad35-SIV vectors can generate gag-specific CD4$^+$ and CD8$^+$ T cells in foreskin of macaques [351], and i.m. vaccination would provide serum IgG antibodies at this site. To generate both antigen-specific T cells and IgA plasma cells in the male genital tract, nasal immunization may be optimal to oral immunization. In men orally immunized with the live attenuated Salmonella typhi vaccine, only low levels of Salmonella-specific IgA antibodies were generated in semen [347]. In studies using the highly immunogenic CTB as an antigen, nasal immunization of men has been found more effective than oral immunization for generating IgA antibodies in urogenital fluid [292]. More studies will be needed to determine which vaccination route or combination of routes would be most effective for generating protective immunity to HIV in the male genital tract.

### 8. RESPIRATORY TRACT VACCINATION

Antibody responses generated by mucosal immunization of humans or NHP with protein immunogens can be remarkably confined to the site of immunization [288, 352, 353]. For instance, immunization of one palatine tonsil in humans generates IgG and IgA antibody-secreting cells in the immunized tonsil but not in the non-immunized tonsil [352]. Administration of vaccine in a single nostril of humans similarly induces greater levels of nasal IgA antibodies in the immunized than in the non-immunized nostril [354]. Considering this, and that unique combination of receptors are utilized for homing of lymphocytes to different mucosal compartments [355, 356], it should not be surprising that there is even less cross-talk between the intestine and the genital tract. Indeed, in women and female macaques, oral or rectal immunization with live-attenuated Salmonella Typhi [357-359], non-adjuvanted inactivated influenza vaccine [360], CTB [289-291] or SIV p55 gag protein with CT adjuvant [361] has not consistently elicited specific antibodies in cervical and vaginal secretions. Local vaginal immunization has been found optimal to oral and rectal routes for induction of genital tract antibody responses, but vaginal immunization has not generated antibodies in the rectum [289-291]. This is problematic as a vaccine intended to prevent sexual transmission of HIV should ideally induce antibodies in both the rectum and genital tract of men and women. The nasal route began to attract attention as a possible means to accomplish this when it was reported that nasal vaccination of mice could generate IgA plasma cells in the intestine [362], IgA antibodies in vaginal secretions [362], CTL in mesenteric and iliac lymph nodes [363] and protection against vaginal HSV-2 infection [364]. It was subsequently confirmed that nasal immunization in humans and NHP could also induce specific antibodies in the rectum and both the male and female genital tract [290, 292, 365, 366].

Nasal vaccination would also be more practical and acceptable worldwide than rectal or vaginal vaccination. Unfortunately, after Bell's palsy was reported in study subjects receiving nasal vaccines with the LTK63 enterotoxin adjuvant [220], some have viewed nasal HIV vaccine development as being futile. However, numerous nasal vaccines are in clinical development, and they have been confirmed safe in the human nasal cavity. These include recombinant chimpanzee Ad and MVA vectors expressing respiratory syncytial virus (RSV) antigens [367], recombinant Sendai virus expressing human parainfluenza virus type 1 antigens [368], inactivated influenza vaccines adjuvanted with Neisseria meningitidis outer membrane vesicles [369] or squalene [370], a Shigella LPS-containing vaccine [371], and live attenuated RSV, Bordetella pertussis and influenza vaccines [372-374]. Developing an HIV nasal vaccine should therefore be quite feasible.

As one would expect, nasally-administered replication-competent vaccines, such as attenuated SHIVs, have proved most effective in NHP for preventing or controlling mucosal infections by SHIV$_{89.6}$ or SIV$_{mac239}$ [336, 375]. Cynomolgus
macaques nasally immunized with replicating poliovirus vectors expressing SIV genes also developed both T-cell responses and mucosal antibodies, that together were likely responsible for the sterile protection or significant control of infection observed following vaginal SIVmac251 challenge [68]. Non-replicating vaccines can also provide protection against rectal or vaginal immunodeficiency virus infections when applied simply as liquid drops in the nasal cavity, and perhaps as an aerosol in the lungs [376]. We have found that nasal priming of Rhesus macaques with DNA expressing SHIV or SIV VLP followed by nasal boosting with MVA-SHIV or -SIV resulted in significant control of rectal SHIV89.6 or vaginal SIVmac251 infection [49, 51, 52]. Nasal administration of these vaccines seeded both the rectal and genital tract mucosa with virus-specific T cells, and these were likely responsible for control because only very low levels of systemic and mucosal antibodies were generated by these DNA/single MVA boost vaccines. Importantly, nasal DNA/MVA immunization generated more T cells in the colorectal mucosa and significantly better control of infection when compared to i.m. immunization with the same vaccine components [47].

Interestingly, some non-replicating vaccines that failed to protect macaques when given solely by the i.m. or nasal route have demonstrated greater efficacy when given by both routes. For example, i.m. priming with SIV gag and IL-12 encoding DNA followed by nasal boosting with a vesicular stomatitis virus (VSV) vector expressing SHIV genes generated greater protection against SHIV89.6 than did nasal immunization with the VSV construct alone [377, 378]. A SHIV DNA/fowlpox vaccine that did not result in control of SHIV162P3 vaginal infection when the components were given only by the i.m. or nasal routes did significantly reduce viremia in pig-tailed macaques immunized with the DNA by the i.m. route and the fowlpox by the nasal route [44]. Excellent antibody-mediated protection against vaginal SHIV162P3 transmission was obtained in Chinese Rhesus macaques vaccinated by the i.m. route with influenza virosomes containing a gp41 MPER peptide and recombinant gp41 lacking the immunodominant domain, but better protection was generated by i.m. priming followed by nasal boosting [279]. In studies focused on induction of antibodies for preventing mother-to-child breast milk transmission, i.m. DNA/MVA vaccination of lactating female macaques followed by nasal boosting with gp120 and R848 adjuvant has been found to generate more gp120-specific IgA antibodies in breast milk when compared to i.m. boosting [379]. However, serum IgG antibodies generated by nasal immunization were less effective at mediating ADCC. Simultaneous parenteral and nasal vaccination may be one approach for generating immune effectors in both the systemic and mucosal compartments. We have found that simultaneous i.m. and nasal DNA/MVA administration resulted in more dramatic control of viremia when compared to immunization by the nasal route alone [52].

CONCLUSION AND FUTURE PERSPECTIVES

The studies reviewed above clearly support the fact that mucosal administration of replication-competent T-cell-inducing SIV/SHIV vaccines can be just as efficacious, if not better [46, 222], than parenteral vaccination for control of mucosal immunodeficiency virus infections. Given the efficacy of SIV DNA/MVA delivered by the intragastric or buccal routes for controlling vaginal SIV infection [46], peroral HIV vaccine development may deserve more attention because an ingested vaccine would contact tissues in both oral cavity and intestine. In the absence of safe, replication-competent vaccines that would induce strong Env-specific mucosal antibody responses in addition to T-cell responses, there is a need to identify Env immunogens and adjuvants that can stimulate production of these antibodies in the intestine and genital tract, which should improve efficacy of HIV vaccines [279, 380]. Vaccines delivered by the nasal route can induce T cells that control rectal or vaginal SIV or SHIV infections [46, 47, 49, 52], and because the nasal route can also generate antibodies in the rectum and genital tract, the identification of safe adjuvants for nasally-administered Env proteins could have a large impact on vaccine development. However, without a clearly suitable small animal model, NHP will likely be required for adjuvant studies. Vaccines that additionally generate multifunctional serum IgG antibodies that transudate into mucosal tissues would further augment protective immunity to HIV [381]. Whether simultaneous mucosal and parenteral vaccination strategies will be required for this is unclear, but should be explored. The ability to shorten vaccination regimens by performing simultaneous immunizations rather than prime-boost immunizations with antibody-inducing Env immunogens and T cell-inducing DNA plasmids or viral vectors should also be investigated because vaccine regimens that require numerous boosts are less likely to result in full compliance. There has been considerable progress in the design of recombinant Env immunogens that can induce HIV-neutralizing antibodies [382], and with improved mucosal delivery systems for these, the goal of an HIV-free generation might be achieved.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Macrosal Vaccine Approaches for Prevention of HIV and SIV Transmission

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