Induction of Intestinal Immunity by Mucosal Vaccines As a Means of Controlling HIV Infection

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Abstract

CD4⁺ T cells in the mucosa of the gastrointestinal (GI) tract are preferentially targeted and depleted by HIV. As such, the induction of an effective anti-HIV immune response in the mucosa of the GI tract—through vaccination—could protect this vulnerable population of cells. Mucosal vaccination provides a promising means of inducing robust humoral and cellular responses in the GI tract. Here we review data from the literature about the effectiveness of various mucosal vaccination routes—oral (intraintestinal/tonsillar/sublingual), intranasal, and intrarectal—with regard to the induction of immune responses mediated by cytotoxic T cells and antibodies in the GI mucosa, as well as protective efficacy in challenge models. We present data from the literature indicating that mucosal routes have the potential to effectively elicit GI mucosal immunity and protect against challenge. Given their capacity for the induction of anti-HIV immune responses in the GI mucosa, we propose that mucosal routes, including the nonconventional sublingual, tonsillar, and intrarectal routes, be considered for the delivery of the next generation HIV vaccines. However, further studies are necessary to determine the ideal vectors and vaccination regimens for these routes of immunization and to validate their efficacy in controlling HIV infection.

HIV Epidemics and Treatment

HIV INFECTION HAS RESULTED in the death of millions since the early 1980s, and has been responsible for significant morbidity and decline in quality of life for millions more. Each year, an estimated 50,000 people are newly infected with the virus in the United States alone, adding to the 34 million currently living with the virus worldwide.^{1,2} Even with the increasing numbers and classes of highly effective antiretroviral therapies (ART), the virus is still responsible for 1.8 million deaths annually. The development of effective, affordable, and accessible preventive and therapeutic modalities is greatly needed.

The most effective therapeutic approach against HIV continues to be administration of ART. Introduced in 1996, combination antiretroviral drugs have significantly decreased the incidence of opportunistic infections and the mortality associated with HIV/AIDS.³ Although ART represents a major victory in the fight against HIV infection, it does not offer a cure as it fails to clear the virus from the infected hosts.⁴ It has also contributed to a more than 3-fold increase in Medicaid/Medicare expenditure on HIV care from \$2.5 billion in 1995 to \$8 billion in 2004,⁵ and represents 73% of

the \$619,000 average lifetime cost of care.⁶ This high cost of care is prohibitive in areas of low to middle income, limiting access to ART among a substantial, and critical, proportion of the HIV-infected population.⁷ Vaccination represents an attractive, cost-effective alternative for high-risk individuals and those unable to regularly access these expensive medications.

Need for Vaccines to Control HIV Epidemics

Rather than endeavoring to treat HIV infection with ART after infection has already been established, prophylactic strategies would offer significant benefits to communities in areas with a high HIV burden. Recent studies on ART as prophylactic drugs have shown its potential for reducing HIV infection rates,^{8–11} but this strategy requires long-term, consistent adherence and would be prohibitively costly for the large numbers of high-risk individuals. Additionally, studies of ART prophylaxis have demonstrated that targeted populations experience undesirable side-effects (nausea, vomiting, decreased bone density),¹² exacerbating poor adherence to the drug regimen. As a result, HIV infection that occurs among cohorts receiving ART prophylaxis is often

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found in patients with low or undetectable levels of the ART drugs in their plasma.^{11–14}

Immunization, on the other hand, requires a limited number of treatments, curtailing adherence concerns and obviating long-term medication costs. Two major categories for immunization against HIV infection are prophylactic and therapeutic vaccines. While not mutually exclusive, prophylactic vaccines focus on preventing viral entry and the initial stages of HIV infection, while therapeutic vaccines seek to limit replication of the virus once the virus has penetrated the initial port of entry, established infection, and/or spread to lymphoid and other distant tissues.

Although over the past two decades the focus of HIV vaccine research efforts has shifted from prophylactic to therapeutic and recently back to prophylactic vaccines, an effective HIV vaccine would need to both block the initial infection and prevent viral replication and spread. This can be achieved only by a vaccine that elicits both humoral and cellular immune responses. Antibodies are capable of preventing viral entry, but alone may not block all virus particles and provide sterilizing immunity. Those viruses that succeed in penetrating the antibody shield can rapidly establish infection in long-lived cellular reservoirs. Cellular effectors, such as CD8⁺ cytotoxic T lymphocytes (CTLs), antibody-dependent cellular cytotoxicity (ADCC), and other functions targeting virus-infected cells, must be established to clear infected cells and block viral replication, dissemination, and disease progression. Indeed, abrogating viral replication is critical for any effective vaccine strategy against HIV, as high levels of HIV replication, even early in the course of infection, serve as an indicator of poor prognosis.¹⁵ This is especially pertinent in light of recent findings that demonstrate the seeding of long-lived viral reservoirs within the first 3 days of simian immunodeficiency virus (SIV) infection in macaques.¹⁶ In fact, even when ART was initiated at 3 days postinfection in these animals, cessation of ART 24 weeks later resulted in a resurgence of viremia, indicating that very early ART treatment is ineffective in preventing the establishment of these long-lived viral reservoirs.

Much research has been done to elucidate the optimal means of inducing high-avidity humoral and CTL responses, as well as recruiting the helper CD4 T cells that are essential for the development and maturation of the B cell and CTL populations. Numerous immunogens have been developed and many vaccine regimens have been tested in animal models and in human clinical trials. Yet, only a few have been evaluated in large phase IIB or phase III trials, namely VAX003, VAX004, RV144, and STEP/Phambili.^{17–20} The former three trials were developed to confer protection against HIV acquisition by induction of antibodies with (RV144) or without (VAX003/4) cell-mediated immune responses. The STEP and Phambili trials, on the other hand, were designed to induce CD8 T cell responses to control virus replication and lower virus burden, rather than prevent infection. It is important to note that these four trials aimed to prevent HIV acquisition or disease in uninfected high-risk individuals. There are also therapeutic vaccines and other immunotherapeutic strategies intended to suppress disease progression or eradicate virus reservoirs in patients with established chronic HIV infection; the development of such therapeutics faces its own specific hurdles and is reviewed elsewhere.²¹

While no vaccine efficacy was observed in VAX003 and VAX004,^{17,18} an increased rate of HIV acquisition was observed in the STEP vaccine recipients as compared to placebo controls, resulting in the early termination of the STEP and Phambili trials.¹⁹ By contrast, the RV144 vaccine offers a glimpse of hope, with a 31.2% reduction in risk of HIV acquisition, mainly from heterosexual transmission, among the vaccine recipients.²⁰ The efficacy is of short duration and the vaccine did little to reduce the degree of viremia in those who contracted the infection. The RV144 vaccine utilized a prime-boost strategy to deliver a replication-deficient canarypox virus vector expressing HIV Gag, Env, and protease, followed by bivalent recombinant gp120 proteins of subtypes B and E via the intramuscular route.²² A case-control study of the RV144 trial revealed that the only immune parameter associated with reduced risk of HIV infection was a high level of plasma IgG against the V1/V2 region of the virus Env protein gp120.²⁰ In contrast, a high level of Env-specific plasma IgA was inversely correlated with protection. Notably, it was recognized that serum Env-specific antibody titers were greater in those patients with detectable populations of interleukin (IL)-2 secreting, Env-specific, effector memory T cells in the peripheral blood.²³ However, assessment of antibodies and cellular immune responses in the mucosa was not performed.²⁴ It is unknown if a more durable and higher degree of protection against HIV acquisition may be achieved with a mucosal route of immunization. Considering that the intestinal lymphoid tissues are one of the key sites supporting HIV replication, induction of antiviral antibodies, IL-2 secreting helper T cells, and CTLs at this site may improve vaccine efficacy by reducing not only the initial virus acquisition but also blunting the spread and replication of breakthrough virus that reaches the gastrointestinal (GI) tract.

In this review we discuss vaccination routes and strategies intended to induce mucosal immunity in the intestinal tract with the purpose of guiding future HIV vaccine development efforts. Evidence from the literature is presented that indicates the effectiveness of mucosal routes of vaccination (intra-intestinal, oral, intranasal, and intrarectal) in inducing high avidity antibody and CTL responses against a broad array of HIV antigens at the intestinal mucosa. In addition, a number of studies examining these mucosal routes of vaccination have demonstrated vaccine-mediated resistance against recombinant vaccinia expressing simian immunodeficiency virus (SIV)/HIV antigens or simian immunodeficiency virus/simian-human immunodeficiency virus (SIV/SHIV) challenges in murine and simian models, respectively.

The Importance of Gastrointestinal Mucosa in HIV Infection

HIV produces its destructive effects through infection of immune cells that express the CD4 receptor, the coreceptors CCR5 or CXCR4, and in some instances the gut homing integrin $\alpha_4\beta_7$.^{25–27} The major population targeted by the virus is CD4⁺ T lymphocytes. Given the vital role of CD4⁺ T cells in the host defense against pathogens and cancer, HIV-induced progressive and chronic depletion of such T cells results in the collapse of the immune system, leading to opportunistic infections, malignancies, and ultimately death.²⁸ An SIV model of infection suggests that immediately after

exposure, the virus is shuttled from the genital mucosa to the lymph nodes and then to the intestinal mucosa where it rapidly replicates in the effector memory CD4⁺ T cells and forms permanent reservoirs.^{29–32} Of note, both rectal and intravenous infection with the virus also results in the preferential targeting and depletion of gut-associated lymphoid tissue (GALT) CD4 T cells.³²

After 2 to 4 weeks of HIV infection the viral load peaks and then declines to reach a viral set point within 6 months. At the height of viremia, the peripheral CD4 T cell count drops but soon recovers to a normal level. In contrast, a more dramatic depletion of memory CD4 T cells occurs in the GALT at this early stage of infection.³³ Henceforth, there is a constant battle between the immune system and the virus, leading to a chronic immune hyperactivation. In the absence of ART, within 2 to 10 years the vast majority of infected individuals will invariably succumb to AIDS with its hallmark of rampant increase in the plasma viral load and massive loss of CD4 T cells readily measurable in the blood. Given the significant role that the GALT plays in the pathogenesis and progression of HIV infection, it represents an ideal target for intervention by vaccination (Fig. 1).

Selective Depletion of CD4 T Cell Subsets

In the past few years, several studies have focused in more depth on the activity of HIV at mucosal surfaces. There are ample data in the literature demonstrating that among the different CD4 T cells subsets, IL-17-producing Th (Th17) cells, which predominate in the intestinal mucosa, are one of the key determinants contributing to virus replication in experimental SIV infection as well as during natural HIV infection. When Brenchley *et al.* compared Th17 cells in SIV-infected and -uninfected rhesus macaques (RMs), significantly lower percentages of Th17 cells were observed in the intestinal mucosa of the infected animals.³⁴ Such depletion was not detected in sooty mangabeys that experience a

non-pathogenic SIV infection. In RMs, the loss of Th17 cells occurred early within the first week after rectal infection with pathogenic SIV_{mac251}, and did not recover over time, even after ART. The low Th17 cell frequency correlated with high viral loads and AIDS progression in the animals.^{34,35} In addition, a high percentage of Th17 cells express the integrin $\alpha_4\beta_7$, and during SIV infection this subset is depleted and harbors more viral DNA, indicating preferential infection of the $\alpha_4\beta_7^{\text{hi}}$ Th17 subset.^{36,37}

Lower numbers of Th17 cells were also found in HIV⁺ human subjects as compared to healthy controls, similar to the phenomenon seen in SIV-infected RMs. Moreover, comparable to RMs, Th17 cells are lost in the intestinal mucosa in humans during HIV infection,³⁴ but the percentages of Th17 cells in the periphery are not significantly affected, and in some patients long-term ART partially restores this cell population.^{38–40} A recent study in our laboratory demonstrates that the CCR6⁺ Th17 cells that express high levels of HIV receptors (CD4, CCR5, and the integrin $\alpha_4\beta_7$) but lack anti-HIV CC-chemokines are more susceptible to HIV infection than Th1 cells.⁴¹ Furthermore, once the Th17 cells are lost, HIV control with an antiretroviral drug alone is not sufficient to restore this cell population; rather, a combination of anti-retroviral drugs and IL-1 β , IL-6, and IL-23 is needed for full expansion of Th17 cells to the levels seen in uninfected controls.⁴² HIV infection also damages the intestinal architecture by inducing apoptosis of enterocytes and eliminating mucosal repair mechanisms mediated by Th17 cells and their cytokines IL-17 and IL-22.^{43–45} Because the integrity of the mucosal barrier is compromised, bacterial products such as lipopolysaccharide (LPS) can translocate into the periphery and fuel the chronic immune activation that contributes to AIDS progression.^{46,47}

An effective vaccination strategy against HIV should provide protection to the intestinal Th17 population as these cells predominate in the intestinal mucosa, play a critical role

FIG. 1. Summary of the four mucosal routes of vaccination discussed herein to induce immune responses against HIV or simian immunodeficiency virus/simian-human immunodeficiency virus (SIV/SHIV) models in the intestines, the primary site of HIV replication, and one of the key targets for vaccine-induced immune responses. Although some degree of compartmentalization exists, the mucosal immune system by and large is interconnected, such that vaccination at multiple mucosal sites can induce immune responses in the intestines. Modified from www.studyblue.com/notes/note/n/innate-immunity-and-inflammation/deck/2498874.

Sublingual/Tonsillar Vaccination:
- Delivery: MVA, Ad5, AT-2-inactivated SIV, single cycle SIV, DNA, CT adjuvant, TLR agonist adjuvant (CpG, polyI:CLC)

- Pros: Simple administration
- Cons: Unproven, not widely studied, complicated by swallowing of vaccine, potential induction of tolerance

The intestines are the primary site of HIV replication and thus should be one of the key targets for vaccine-induced immune responses against the virus

Intrarectal Vaccination:

- Delivery: Peptides, LT or CT adjuvants, MVA, Ad5
- Pros: Extensively studied, highly immunogenic, potential use in infants
- Cons: More invasive than other mucosal routes, need development of vaccine formulations and delivery systems for widespread use

Intranasal Vaccination:

- Delivery: DNA, DNA+LAMP, DNA with IL-2Ig or IL-12, MVA, adenovirus, fowl-pox+vaccinia, CT and LT adjuvants
- Pros: Non-invasive, extensively studied, certified for use against influenza virus
- Cons: May require nebulizer, risk of CNS inflammation

Intraintestinal/Swallowed Vaccination:

- Delivery: Salmonella, PLG microparticle, enteric-coated adenovirus, MVA, hepatitis E, attenuated *M. tuberculosis*
- Pros: Easily administered, extensively studied, used in clinically approved vaccines against mucosal pathogens
- Cons: Need to resist digestion, potential induction of tolerance

in mucosal integrity, and are preferentially targeted by HIV in the early stages of infection. We propose that an approach that not only blocks interactions with the conventional receptors CD4 and CCR5/CXCR4 but also disrupts HIV envelope binding with $\alpha_4\beta_7$ on Th17 cells should be considered. In addition, inducing CD8 and CD4 T cell populations that produce anti-HIV chemokines (MIP-1 α , MIP-1 β , RANTES) might provide protection to Th17 cells, given that most Th17 cells do not produce anti-HIV chemokines.^{39,41} Nonetheless, a minor subset of IL-17⁺ cells is capable of producing anti-HIV chemokines⁴¹ and possibly other antiviral factors that confer some level of resistance against HIV. Elicitation and expansion of this population by vaccination may be advantageous; however, the types of immunogens, adjuvants, and delivery systems capable of predominantly eliciting this Th17 subset or other desirable Th subsets are not at all known. Vaccination capable of inducing production of other anti-HIV soluble factors, such as β -defensins, also should offer some degrees of protection. β -defensin 2, in particular, is of interest, since this molecule has been shown to affect HIV infectivity at different stages of the virus life cycle and its receptor is CCR6, which is expressed by most Th17 cells.^{48,49} Because this important research area is still at its infancy and to date has received little attention, the rest of this review will discuss available data on mucosal vaccination,

with emphasis on mucosal routes for induction of anti-HIV antibody and T cell responses in the GI mucosa.

Mucosal Vaccination Routes to Elicit GI Mucosal Immune Responses

To protect the GALT from depletion of vulnerable lymphocyte populations (such as the Th17 subset) during the early phase of HIV infection, vaccination should seek to induce effective antiviral responses localized to the intestines. We propose that this can be most effectively accomplished through mucosal vaccination. Indeed, a number of successful and efficacious mucosal vaccines have been developed for clinical use to combat pathogens in the intestines and other mucosal sites (Table 1).

In a 1978 paper, Bienenstock *et al.* initially described the theory of the joint mucosal immune system, which postulated that there was a linkage and crosstalk between the various mucosal surfaces of the body, namely, the GI, respiratory, and urogenital tracts.⁵⁰ Furthermore, the theory suggests that introduction of antigens at one mucosal site may result in the induction of immune responses at both local and distant mucosal sites. Therefore, it should be possible to induce an effective GI immune response by targeting vaccines to the intestinal mucosa as well as more distant mucosal sites. Yet,

TABLE 1. MUCOSALLY DELIVERED VACCINES CURRENTLY AVAILABLE FOR CLINICAL USE AND THEIR STRATEGY/DOSING INFORMATION AS DESCRIBED IN FOOD AND DRUG ADMINISTRATION AND WORLD HEALTH ORGANIZATION DOCUMENTS^a

<i>Pathogen</i>	<i>Vaccine strategy and dosing</i>	<i>Year</i>	<i>References</i>
Poliovirus	Sabin vaccine/OPV: Live attenuated mono/bi/trivalent viral vaccine 3 doses orally at 1 month intervals, and boosts Associated with rare cases of iatrogenic paralysis	1961	117–118
Rotavirus	RV1/Rotarix and RV5/RotaTeg: Live attenuated mono/pentavalent virus vaccine 2–3 doses at 4 week intervals, no boost	2006	119–121
<i>Vibrio cholera</i>	Dukoral: Killed monovalent (O1) whole cell suspension Adjuvanted with subunit B of cholera toxin 2–3 doses orally at 1–6 week intervals, and boost Administered in bicarbonate buffered water	1991	122–123
<i>Vibrio cholera</i>	Shanchol: Killed bivalent (O1/O139) whole cell suspension Adjuvanted with subunit B of cholera toxin 2 doses orally at 2 week intervals, no boost No buffer used	2009	124–125
<i>Salmonella typhi</i>	Ty21/Vivotif: Oral live attenuated bacterial formulation 3–4 doses of an enterically coated capsule Taken at 2 day intervals, boost after 5 years	1983	126–128
Influenza virus	LAIV/Flumist: Live attenuated quadrivalent viral vaccine 1–2 intranasal doses at > 1 month interval Annual revaccination is required for protection	2003	129

^awww.who.int/biologicals/areas/vaccines/poliomyelitis/en/
www.fda.gov/downloads/biologicsbloodvaccines/vaccines/approvedproducts/ucm133539.pdf
www.fda.gov/downloads/biologicsbloodvaccines/vaccines/approvedproducts/ucm142290.pdf
www.who.int/cholera/vaccines/Briefing_OCV_stockpile.pdf
www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM142807.pdf
www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm123743.pdf

there is also evidence that some degree of compartmentalization is exhibited by the mucosal immune system with important implications on the choice of routes for mucosal vaccines.^{51,52} For example, oral immunization efficiently generates antibody responses in the small intestine and not in the respiratory tracks, whereas nasal immunization is considered effective for inducing antibodies in the respiratory and reproductive tracts but not in the GI tract. Nonetheless, certain vectors have been tested for the intranasal route to produce intestinal immune responses. Recombinant *Streptococcus gordonii* expressing the p27 SIV protein delivered via the intranasal route has been reported to induce both CD4 and CD8 responses in the mesenteric lymph nodes of mice.⁵³ Mice vaccinated intranasally with adenovirus serotype 5 (Ad5) expressing the rotavirus R4 gene demonstrated more robust rotavirus-specific IgG and IgA responses, as well as greater protection against rotavirus-induced disease, than those receiving the vaccine via intramuscular or oral applications.⁵⁴

Further examination of the mechanisms that underlie the mucosal crosstalk and compartmentalization has elucidated a pathway by which mucosal vaccines induce immunity in the intestines. The secretion of retinoic acid, a vitamin A derivative, by mucosal dendritic cells (DCs) up-regulates the expression of gut-homing receptors, the integrin $\alpha_4\beta_7$ and CCR9 (in the small bowel only), on the surface of antigen-specific B and T cells.^{55,56} These gut-homing receptors bind to MAdCAM-1 and CCL25, respectively, both of which are expressed on intestinal high endothelial venules, permitting extravasation of responding cells into the mucosa. Recruitment of lymphocytes to GI surfaces is primarily a function of GALT DCs, as DCs isolated from peripheral lymph nodes were incapable of secreting retinoic acid and inducing gut-homing receptors on activated lymphocytes. Hence, the selective recruitment and localization of antigen-specific B and T cells to mucosal sites are determined by tissue-specific homing receptors and chemokine receptors along with their ligands, of which expression is regulated differentially at the distinct sites with participation of tissue-specific DCs, endothelial cells, and other cell types.

Notably, there are also data to suggest that mucosal delivery of vaccines increases the avidity of the mucosal CTL response. Ranasinghe *et al.* demonstrated that vaccination through the systemic route results in heightened IL-4 and IL-13 secretion, which corresponded to lower CTL avidity, whereas mucosal vaccination induces greater IL-2 production, which increased CTL avidity.⁵⁷ High-avidity CTL responses, detected in the mesenteric lymph nodes, have been shown to correlate with an ~20-day delay in the acute peak viremia following an SHIV-ku2 rectal challenge in macaques, though no effect on viral load set-points was apparent.⁵⁸

An insightful mucosal vaccination study in macaques utilized an *env/rev/gag* recombinant Ad5 prime, administered sublingually, intranasally/intratracheally, intravaginally, or intrarectally followed by an intramuscular monophosphoryl lipid A (MPL)-adjuvanted gp120 protein boost.⁵⁹ In all groups, Env/Gag-specific CD4 and CD8 central memory T cell populations were induced in the blood, and were significantly larger than those in nonvaccinated animals. In the mucosa, there was similar evidence of Env-specific memory T cell populations induced following the two priming vaccinations

and the first boost (28 weeks) among intranasal/intratracheal, sublingual, and intrarectal groups. Intravaginally induced mucosal responses appeared to be partially compartmentalized to the genital mucosa. All routes induced Env-specific humoral responses in the blood with measurable neutralizing antibodies, antibody-dependent cellular cytotoxicity (ADCC), and antibody-dependent cell-mediated viral inhibition (ADCVI) activity as well as Env-specific IgA in the mucosal secretions. When repeatedly challenged with SIV, all of the vaccinated animals but one became infected: this uninfected animal possessed exceptionally high levels of high avidity IgA across all mucosal sites surveyed. Acute viremia was lower among the mucosally vaccinated animals, as compared to the unvaccinated subjects, and was inversely correlated with antibody avidity, while greater antibody titers correlated with delay of infection. However, chronic viremia was not affected. Given the capacity of mucosal routes to induce high avidity antibody and memory T cell responses in both the mucosa and the periphery, mucosal immunization offers a promising means for HIV vaccination.

Mucosal Vaccination Routes

Oral vaccination

Oral vaccination refers to the administration of a vaccine via one of the following routes: (1) intrainestinal administration, in which a vaccine is swallowed to deliver it to the surfaces of the intestine, (2) tonsillar, and (3) sublingual vaccination, wherein the vaccine is applied directly to these mucosal surfaces. Administration of vaccine via these routes is noninvasive and well tolerated.

Intrainestinal vaccination. In an early study in a murine model, recombinant *Salmonella* expressing HIV Gag elicited HIV Gag-specific Th1 and Th2 type CD4 responses, as measured by production of interferon (IFN)- γ /tumor necrosis factor (TNF)- α and IL-4/IL-5, respectively.⁶⁰ Env delivered by a *Salmonella* vector was also found to induce a predominantly CD4 T cell response, which can enhance B cell activation as well as produce anti-HIV chemokines⁶¹; however, this response alone was likely an ineffective means of controlling infection as limited antiviral effector populations (CTL or antibody secreting cells) were induced. Vaccination of rhesus macaques with *Salmonella* expressing Env and Gag failed to confer protection, although the challenge was introduced intravenously and the mucosal response was not studied.⁶²

Two other studies compared intrainestinal to intramuscular vaccination using *Salmonella* or PLG microparticles. Mice were immunized with a construct of HIV *env* DNA encoded by *Salmonella* or encapsulated in PLG microparticles. Oral delivery of these vaccines resulted in significantly greater IgA and Env-specific IFN- γ ⁺ CD8 T cell responses in the intestines.^{63,64} Notably, intramuscular vaccination with the PLG-*env* vaccine failed to prevent viral entry and proliferation in mice challenged with recombinant vaccinia expressing HIV Env, whereas the animals that received this vaccine via the oral route had viral titers that were 100-fold less than those animals in the intramuscular or vehicle-control groups.⁶⁴

Several groups have investigated the efficacy of replicating viral vectors expressing HIV proteins in the setting of intrainestinal vaccination.^{65–67} For instance, a replicating

host range-mutant Ad5 vector expressing SIV Gag/Env/Nef—encapsulated in an enterically coated tablet for oral delivery—was given in a two dose prime via oral/oral or intranasal/oral followed by an intramuscular SIV gp120 protein boost.^{66–67} This vaccine regimen resulted in induction of gut homing ($\alpha_4\beta_7^+$) CD8 T cells in the periphery, and the percentage of these gut homing cells in the blood was diminished significantly by week 38 of the vaccine course. The decline is possibly due to migration of these populations to the gut mucosa, although this was not examined in the study. Anti-SIV gp120 IgA antibodies were found in rectal secretions of these vaccinated animals, with the intranasal/oral group exhibiting earlier detection and a greater fold increase in antibody-specific activity. Even though the antibodies were nonneutralizing, they demonstrated ADCC and viral inhibition (ADCVI) activity, as well as the capacity to inhibit viral transcytosis, and all of these were remarkably enhanced in the intranasal/oral vaccinated group.⁶⁷

Even though multiple parameters of mucosal immune responses were observed in the vaccinated animals, upon rectal challenge with SIV_{mac251} at 6 weeks after the last vaccine dosage, all animals became persistently infected.⁶⁶ Acute peak and chronic viremia levels were lower in vaccinated animals as compared to controls, and animals vaccinated intranasally/orally exhibited lower acute viral loads than the oral/oral group. Nonetheless, the difference was only about 1 to 1.5 log. Although replication-competent vectors are generally pursued for the ability to provide more sustained and higher production of antigens at the desired target site, this particular Ad5 mutant vector replicated poorly in the gut, as indicated by the limited duration of shedding in stool samples (only 1 to 3 days). Whether a fully replicating vector may offer greater protection efficacy is unknown, but the use of replicating viral vectors comes with important safety concerns. More importantly, the utility of Ad5 vectors in general has been disfavored since a replication-defective MRK Ad5-gag/pol/nef vaccine evaluated in the STEP trial resulted in enhancement of HIV acquisition. In particular, since a higher rate of HIV infection was observed among Ad5-seropositive vaccines, preexisting immunity to the adenovirus vector has been implicated in causing increased CD4 T cell activation and providing more susceptible target cells for the virus at the mucosal site of entry. With contrasting data from different studies, this issue remains controversial.^{68–71} Neutralizing antibodies to Ad5 and other serotypes are found in significant portions of the population in both developed and developing nations,⁷² raising serious safety concerns for any vaccines with this vector type.

A number of nonreplicating viral vectors have also been investigated. Replication-incompetent hepatitis E virus represents a potential oral vaccine vector candidate, as it is naturally transmitted by the fecal to oral route, and normally infects the epithelial cells of the small intestine. Indeed, when recombinant hepatitis E expressing HIV Env was used to vaccinate mice via the oral route, it resulted in robust IgA responses in the intestine/fecal extracts. Moreover, a high percentage of CTLs was recruited to the Peyer's patches and mesenteric lymph nodes.⁷³ Similarly, CD4/CD8 T cell and IgA/IgG antibody responses have been observed in the intestines of infant macaques that swallowed a highly attenuated *M. tuberculosis* expressing SIV Gag followed by a nonreplicating modified vaccinia Ankara (MVA) boost en-

coding SIV gag/pol/env.⁷⁴ On the other hand, contradictory findings were seen in a study conducted by Lin *et al.* in which mice vaccinated with replication-deficient chimpanzee adenovirus via intramuscular or oral routes showed greater induction of both GALT and systemic Gag-specific IFN- γ^+ CD8 responses in the intramuscular animals.⁷⁵

Although swallowing of a vaccine represents a highly accessible means of administration, certain considerations must be taken into account when designing such a vaccine. One such concern is the delivery of the immunogens through the stomach, which can serve as a natural barrier to proper vaccination due to its acidic and proteolytic environment, as well as the small intestines due to the presence of pancreatic proteases and mucins.⁷⁶ To counteract vaccine degradation, sodium bicarbonate solution intake can be used prior to vaccination in order to neutralize gastric acid.⁷⁷ Alternatively, enteric-coated capsules have been developed to utilize the digestive tract's pH gradient, releasing the viral vector only after it has passed through the highly acidic and proteolytic environment of the stomach and has reached an environment where it can induce both humoral and cellular intestinal immunity.⁷⁸

Another potential issue for swallowed vaccines is the development of tolerance, rather than a protective immune response, thus compromising the efficacy of vaccination.⁷⁹ Nonetheless, while cellular responses may be absent in such cases, induction of humoral responses have been reported regardless of the immune status.⁸⁰ Given the effectiveness of poliovirus and rotavirus vaccines, it is clear that swallowing of vaccine can function as a means of protection against viruses with significant GI pathogenesis. However, evidence supporting the effectiveness of the intrainstestinal route for vaccines against HIV or SIV is still insufficient.

Sublingual and tonsillar vaccination. Though not widely studied in the context of HIV vaccination, vaccines delivered to the oral mucosa, such as sublingual and tonsillar vaccines, have been shown in a select few studies to induce immunity against mucosal pathogens of the respiratory and genital tracts.^{81–83} Again, these vaccine routes are easily accessible and noninvasive, making them candidates for widespread vaccination strategies.

One strategy used an SIV/IL-2/IL-15 DNA prime followed by SIV Gag/Pol/Env-expressing MVA and AT-2-inactivated SIV.^{84,85} This vaccine, when delivered to the buccal mucosa of RMs, induced greater and more functionally diverse rectal IgA responses as compared to intranasally, intrainstestinally, and intravaginally delivered vaccine, although the differences were not statistically significant.⁸⁴ This oral vaccine also generated equivalent vaginal IgA responses when compared to intravaginal administration, and overall induced IgA in multiple mucosal sites. SIV Gag/Env-specific CD4 and CD8 responses in the rectal mucosa were demonstrated in all vaccinees, although these responses were greatest in orally vaccinated animals. When challenged intravaginally with repeated low dose SIV_{mac251}, intestinally vaccinated animals were reported to withstand a greater number of challenges before infection occurred.⁸⁵ On the other hand, after the animals became infected, the intestinal group was least protected from disease, and a greater degree of disease control was observed in orally and nasally vaccinated animals with 50% reaching undetectable levels of viremia. Moreover,

100% of the orally vaccinated animals did not progress to AIDS and survived until the end of the study, more than 75 weeks after infection.

The sublingual epithelium is another potential vaccination site, as it contains a large population of mucosal DCs, which are expanded by administration of adjuvants such as cholera toxin (CT).⁸² Sublingual vaccination of mice using OVA with or without CT demonstrated CT-dependent induction of OVA-specific antibody-secreting cells in the proximal, but not distal, mesenteric lymph nodes (MLNs). B cell activation in the distal MLN was observed only following bicarbonate-buffered intrainestinal vaccination with OVA/CT, not when the same vaccine was delivered without a buffer. An equivalent response was observed in mice vaccinated intranasally with OVA/CT. In BALB/c mice, sublingual delivery of formalin-inactivated influenza virus and the adjuvant mCTA/LTB (cholera toxin subunit A/labile toxin subunit B) activates intestinal responses.⁸⁶ Influenza-specific IgA antibodies were found in a number of mucosal secretions, including the feces, and populations of IgA-producing B cells were recruited to both the small and large intestines.

Though the Ad5 vector vaccination strategies have raised significant concerns, Gag-specific CTLs were observed in the mucosal (submandibular LN) and systemic compartments of B6 mice sublingually immunized with Ad5 expressing HIV-Gag, regardless of prior exposure to the viral vector.⁸⁷ It was noted that coadministration with a recombinant toll-like receptor (TLR) agonist *Eimeria tenella* antigen (rEA) enhanced the immune responses of both pre-immune and naive mice, but intestinal responses were not evaluated. Nonetheless, the efficacy of sublingual vaccination may be reduced by unintentional swallowing of the vaccine during administration.

Another approach to oral delivery consists of direct application of vaccine to the palatine and lingual tonsils in order to induce mucosal immunity, which can circumvent the accidental swallowing of vaccine that occurs during sublingual administration. In a 2009 study, Chinese RMs were vaccinated via the tonsils with AT-2-inactivated SIVmac239 (wild type or deltaV1V2) with CpG vs. control adjuvant at six sequential time points.⁸⁸ Upon rectal challenge with the homologous SIVmac239, approximately 50% of all vaccinated animals were protected from infection, with no additional benefits observed from the V1/V2 removal or the CpG adjuvant. Most vaccinated animals exhibited a low concentration of SIV-specific IgA in the rectal fluid, but no boosting effect was observed following virus challenge. The vaccinated animals also exhibited some degree of viral control, with a modest and very transient reduction in viremia as compared to controls during the first 2 weeks. However, all animals exhibited a similar decrease in blood CD4 T cell counts.

A similar finding has been reported in macaques vaccinated with two primes of single-cycle immunodeficiency virus (SCIV), a modified SIV virus that can undergo only one round of replication followed by two recombinant adenoviral vector (expressing SIV *gag-pol/env*) boosts, all delivered in a tonsillar spray.⁸⁹ Following SIVmac239 challenge, this time via the tonsillar route, vaccinated animals had reduced viral peaks, but viral loads at early and late set-points were not significantly different from those of the unvaccinated controls. Importantly, the tonsillar route did not offer an obvious advantage over the systemic administration of one SCIV

prime (i.v.) and one Ad-SIV boost (i.m.), which yielded similar degrees of viral load reduction.

In the SHIV/89.6P model, protection against infection has also been observed in Mamu-A*01⁺ macaques following two palatine tonsil vaccinations with MVA expressing SHIV-derived *gag/env/pol/tat/nef* genes.⁹⁰ Vaccinated animals were rectally challenged 9 months after the last vaccination, and those vaccinated via the tonsils exhibited reduced peak and chronic viral loads as well as significantly better control of CD4 depletion than the unvaccinated subjects. However, the SHIV/89.6P challenge has been found to be relatively easy to protect against with different vaccines, including the MRK Ad5 vaccine that was developed into the STEP trial.⁹¹ Assessment of vaccine efficacy with multiple virus challenge models, including heterologous and more stringent viruses, is necessary to gain a better understanding of the full potency of any vaccines tested in nonhuman primates.

Overall, both the sublingual and tonsillar routes of vaccination have the capacity to induce SIV/SHIV-specific mucosal and systemic immune responses, as well as to prevent infection and/or control viral loads to varying degrees. Vaccination through these routes may also induce immune tolerance, necessitating extensive screening and optimization to identify immunogens, vectors/delivery systems, and vaccination protocols, such as heterologous prime/boost, that will best induce HIV immunity.⁹² Further investigation into the safety of these routes is also required, given that there are no vaccines utilizing these routes currently available in the clinical setting. In addition, more comparative studies are required to evaluate the efficacy of these routes as opposed to the other mucosal and systemic routes. Nonetheless, further examination is warranted principally for vaccine approaches with encouraging evidence of reducing virus acquisition and/or delivering complete virus suppression. Vaccines that simply reduce viral loads, as exemplified by many vaccines discussed above, may not be viable candidates for future clinical trials due to many difficult-to-resolve strategic reasons, including the requirement to delay or withhold ART from infected trial participants and the costly and arduous necessity to assess long-term clinical benefits from vaccine-induced viral load reduction.⁹³

Intranasal vaccination

As with oral routes of vaccination, the intranasal route is highly accessible, and a diverse body of work exists to support its efficacy as a means of mucosal vaccination.^{53,54} Although there have been reports of mucosal compartmentalization restricting induction of intestinal immune responses by intranasal immunization, a recent study by Ruane *et al.* showed that intranasal immunization stimulated $\alpha_4\beta_7$ expression on T cells in the lung and the mediastinal lymph nodes and induced cell migration to the GI tissues.⁹⁴ This activity was mediated by lung DCs, in a process similar to that exhibited by mesenteric lymph node DCs. Consistent with this concept, DNA encoding HIV-1 *gag* fused to lysosome-associated membrane protein (LAMP/*gag*) delivered intranasally to neonatal mice was shown to elicit IgA, IgG, and IFN- γ ⁺ T cell responses in the intestines.⁹⁵ The conjugation of *gag* to LAMP modulates the uptake and presentation of *gag* antigens, enhancing the production of both IgA and IgG in comparison to a pure *gag* DNA formulation.

Systemically delivered vaccines have also been shown to induce mucosal responses in the GI and reproductive tracks. Immunization with recombinant adenovirus vectors expressing SIV Gag via the intramuscular route was shown to result in antigen-specific functional cellular responses, consisting mainly of CD8 effector and memory T cells, at the intestinal and vaginal tracts in mice and rhesus monkeys.⁹⁶ Upon virus challenge, these cellular responses corresponded to a reduction of peak and set-point viral loads and decreased progression to AIDS.⁹⁷

Comparison of a combination of systemic and intranasal routes (intramuscular/intramuscular, IM/IM; intramuscular/intravenous, IM/IV; intradermal/intradermal, ID/ID; intranasal/intranasal, IN/IN; intranasal/intramuscular, IN/IM) for prime-boost vaccination with recombinant fowlpox encoding HIV *gag/env/pol/tat/nef* followed by a recombinant vaccinia virus encoding *gag/pol* in a murine model suggests the primacy of heterologous IN/IM prime-boost vaccination strategies.⁹⁸ The IN/IM strategy induced greater numbers of long-lasting antigen-specific IFN- γ -secreting T cell populations in the spleen and genitoretal nodes than the other four vaccination groups. A subsequent study reported that mucosally targeted immunization (IN/IM and IN/IN) induced CTLs of higher avidity, as determined by a tetramer dissociation assay, than a peripheral vaccination strategy such as IM/IM.⁹⁹

In an SIV challenge experiment in rhesus macaques, intranasal vaccination has shown the capacity to reduce virus replication, but not virus acquisition.¹⁰⁰ Hence, the number of low-dose repeated SIV challenges needed for infection in animals receiving DNA (*SIV + IL-2 + IL-15*) prime followed by MVA (*SIV gag/pol/nef*) boost intranasally was similar to those in the intramuscular and the unvaccinated control groups. However, the intranasal vaccination prevented early depletion of both colorectal and circulating memory CD4 T cells, reduced plasma viral RNAs, and delayed the progression of disease. When compared to intramuscular administration, intranasal vaccination also resulted in a slightly better control of viral replication among Mamu-A*01-negative animals. Consistent with the well-documented contribution of the Mamu-A*01 allele in controlling SIV,^{101,102} vaccinated Mamu-A*01-positive animals exhibited lower virus replication, but the levels were similar between the intranasal and intramuscular groups.

Likewise, Bertley *et al.* reported that a SHIV DNA prime followed by an MVA (*SIV Gag/Pol* and *HIV Env*) boost via the intranasal route induced Env-specific cellular responses in the rectal secretions of vaccinated male macaques.¹⁰³ Systemic cellular responses were not observed consistently among all vaccinated animals, but were enhanced by coadministration of IL-2/Ig DNA during priming. Though SIV-specific IgA was induced in the rectal secretions of the majority of vaccinated subjects, systemic IgG responses were less robust, even in those groups vaccinated with IL-2/Ig or IL-12 DNA adjuvants. Following a high-dose SHIV/89.6P rectal challenge, IL-2/Ig-supplemented animals exhibited the greatest control of viral replication and CD4 depletion, suggesting the utility of IL-2/Ig as a mucosal vaccine adjuvant, but again no protection was observed against virus infection. Altogether, these results indicate that intranasal vaccination can both recruit cellular and humoral responses to the intestines and other mucosal surfaces, while conferring partial

control of viral replication. The capacity of this route to prevent virus acquisition is yet to be proven.

As with other immunization routes, there are safety concerns about vector-induced pathogenesis and preexisting immunity to vectors, especially the Ad5-based vector.^{68–70,104} A study of intranasal vaccination of a group of Ad5-seropositive mice with an Ad5 vector expressing Ebola antigens suggested that induction of a mucosal immune response was feasible.¹⁰⁵ Indeed, in the presence of preexisting anti-Ad5 vector immunity, intranasal vaccination provided protection against lethal Ebola challenge, while oral or intramuscular routes did not. Nonetheless, given the results of the STEP trial, where seropositivity to the Ad5 vector was correlated with a higher rate of HIV infection in the vaccinated group,¹⁹ vector-specific responses will continue to raise serious concerns in the HIV vaccine arena. As such, further investigation into the safety and efficacy of the intranasal route with and without preexisting vector immunity is necessary.

There are also additional considerations to be made for vaccination via the intranasal route. Specialized equipment, such as nebulizers, may be required for the administration of these vaccines. More importantly, intranasal vaccination and use of intranasal adjuvants have the potential to cause adverse side-effects. Intranasally administered toxin adjuvants such as CT and *Escherichia coli* heat-labile toxin (LT) showed greater induction of inflammatory responses than “parenteral adjuvants such as CpG oligodeoxynucleotides, polymerized liposomes, microparticles and interleukins.”¹⁰⁶ However, these toxin adjuvants can also induce inflammatory responses in the facial nerves, as demonstrated in human intranasal HIV and TB vaccine trials in which subjects experienced transient Bell’s Palsy after the use of detoxified LT.^{107,108} In addition, CT was detected in brains of mice following intranasal CT administration and was thought to have migrated via retrograde neuronal transport through the olfactory neurons.^{109–111} Newer adjuvants such as the carbohydrate formulations Advax-M¹⁰⁶ and chitosan derivatives^{112,113} may provide alternative candidates for safe and effective intranasal adjuvants. Intranasal administration of viral vectors such as vaccinia, fowlpox, and adenovirus can result in viral infection and inflammation of the CNS through the same mechanism.¹⁰⁴ Therefore, any potential intranasal vaccine candidates must be stringently evaluated for safety.

Intrarectal vaccination

As with the aforementioned mucosal routes, administration of vaccines via the intrarectal route has also been examined in the context of mucosal HIV vaccination. Though more invasive, intrarectal vaccination has the potential to induce robust anti-HIV immune responses.

Intrarectal vaccination of macaques with HIV/SIV Env, Gag, and Pol peptides with modified LT adjuvants resulted in the recruitment of peptide-specific CTLs to the GALT.^{114,115} Furthermore, the immune responses induced by intrarectal versus parenteral vaccination demonstrate significant compartmentalization. Intrarectal vaccination of mice with MVA induced more high-avidity antigen-specific CTLs in the GALT than in mice vaccinated via the subcutaneous route; however, the opposite was observed in the spleen.¹¹⁴ A similar finding is observed in mice vaccinated intrarectally

with multiepitope HIV peptides with or without CT adjuvant as compared to those vaccinated subcutaneously with incomplete Freund's adjuvant.¹¹⁶ However, intrarectal vaccination with this formulation resulted in equivalent splenic responses, suggesting its capacity to induce peripheral immunity. In addition, CTL responses in lamina propria and Peyer's patches were persistent for at least 6 months post-vaccination and conferred resistance to a recombinant HIV gp160-expressing vaccinia challenge model. The recruitment of high avidity CTLs to the intestinal mucosa following intrarectal vaccination with the above peptide/LT formulation conferred resistance to SHIV challenge in macaques, lowering viral RNA in the colon, jejunum, and blood to below detectable levels and preventing CD4 depletion, as observed 6 months postchallenge.^{114,115}

Despite the body of evidence suggesting the efficacy of vaccine delivery at anatomically distant sites (intranasal or oral) in the induction of anti-HIV immunity, it has been reported that it is difficult to attain intestinal immunity as robust as the response achieved through intrarectal vaccination, a result of immune system compartmentalization. Macaques primed with an SIV *env/rev/gag* recombinant Ad5 delivered intrarectally followed by an intramuscular gp120 boost exhibited the highest Env-specific T cell responses, both central and effector memory subtypes, in the rectum and jejunum as well as the greatest levels of IgA in their rectal, nasal, vaginal, and salivary secretions when compared to subjects primed via the sublingual, intranasal/intratracheal, or intravaginal routes.⁵⁹ Among vaccinated macaques, all but one individual became infected with SIV after nine cycles of a repeated low-dose intrarectal SIV_{mac251} challenge. This animal was a member of the intrarectal vaccine group and was notable for its comparatively high avidity responses, levels of Env-specific IgA at all of the mucosal sites surveyed, and Env-specific serum antibody titers. Even though protection was not observed in all animals, mucosal vaccination via any route resulted in decreased peak viremia.

While seemingly a physiologically ideal site of vaccination, rectal immunization may face social barriers to successful worldwide implementation. The administration of rectal vaccines has the potential to be perceived as uncomfortable or undesirable in a number of sociocultural environments. Nonetheless, if solid protection against HIV infection and disease can be achieved and easy-to-use rectal suppositories for vaccine delivery are developed, this route of immunization would likely gain ready acceptance. Moreover, if an efficacious HIV vaccine is proven to require multiple boosts over many years, administration of the initial priming as rectal vaccines early in life to infants or children is a sensible strategy to pursue. Like other mucosal vaccines, rectal vaccines also present additional benefits of needle-free vaccination that does not necessitate extensive training for the healthcare providers, improves safety by eliminating needle-stick injuries and cross-contamination, and reduces the overall vaccination costs.

Summary

Given the staggering morbidity and mortality associated with the HIV epidemic over the past 30 years, it is clear that the development of novel prophylactics and treatment options is imperative. The current generation of ART represents

an effective means of suppressing viral proliferation in infected individuals; however, it is not an ideal prophylactic strategy given that the cost of treatment limits its accessibility, and that lapses in compliance, sometimes associated with the onset of unpleasant side-effects, can compromise preventative efficacy. Instead, a new generation of efficacious HIV vaccines would be a more sustainable, cost-effective, and tolerable means of tackling the global HIV epidemic.

A large body of research has concluded that HIV is predominantly a mucosal disease, with much of the immune system destruction characteristic of the pathogen localized to the GI tract.^{29–32,33} As such, induction of protective cell-mediated and humoral immunity at this site should be one of the primary goals in HIV vaccine development, as it represents a viable means of protection against viral spread, uncontrolled viral replication, and CD4⁺ T cell depletion. Already, a number of vaccines leveraging mucosal delivery have been developed to combat mucosal pathogens, such as poliovirus, typhoid, cholera, rotavirus, and influenza virus.^{117–129} From the literature presented herein, we believe that these mucosal routes represent a promising approach for the development of the next generations of HIV vaccines. Each of the routes discussed herein (intraintestinal/swallowed, oral, intranasal, intrarectal) has shown the potential to induce humoral and cellular responses in the mucosa of the GI tract, but also at other mucosal surfaces and in the systemic compartment.

There remain immense scientific challenges for the development and optimization of mucosal vaccination protocols, beginning with the identification of the ideal routes of vaccine delivery. While the literature suggests that intrarectal vaccination is the most robust inducer of local or intestinal mucosal immunity, a practical formulation and delivery system is needed for routine use in the clinics. The intranasal and intraintestinal or swallowed routes are both relatively noninvasive and have demonstrated the capacity to limit viral proliferation in murine and nonhuman primate models of HIV infection. Though some efficacy has been reported in studies of sublingual and tonsillar vaccination, this literature is not extensive and further study is warranted. The ideal classes of immunogens, vectors, and delivery vehicles also have yet to be determined for either of these routes, though it is evident that protein-only strategies will not suffice. Intestinal and nasal delivery of DNA and protein formulations may be more effective, but the use of a bacterial vector, bicarbonate buffer, or enterically coated tablet would be necessary for intestinal delivery. Viral vectors also show promise for intestinal and nasal administration, as a result of their ability to migrate and replicate in the mucosa; however, extensive safety testing must be conducted, especially in the case of intranasal vaccination where certain vectors carry the risk of causing neuronal damage.

In summary, the need for effective HIV vaccines remains unfulfilled. Mucosal routes provide a promising avenue to pursue for improving the vaccine efficacy against HIV, a virus that establishes infection, replicates, and rapidly forms long-lived reservoirs in the GALT. However, there are an array of scientific obstacles to overcome, requiring solid commitments and investments on basic and vaccine mucosal immunology to drive the field forward. Such research efforts would advance further development of not only HIV vaccines but also other vaccines against mucosal pathogens.

Acknowledgments

We thank Dr. Mirosław Gorny (NYU) for critical reading of the manuscript. This work was supported in part by the U.S. Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research and Development, the Department of Veterans Affairs Merit Review Award (C.E.H.), NIH Grants F31 AI085958 (Y.A.) and R01 AI-084807 (C.E.H.), and the AGA-Eli and Edythe Broad Student Research Fellowship (J.P.). Dr. Catarina E. Hioe is a recipient of a VA Research Career Scientist Award.

Author Disclosure Statement

No competing financial interests exist.

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