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Review

Mucosal HIV vaccines: A holy grail or a dud?

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ABSTRACT

The mucosal immune system appears to be a major target of the HIV infection. Therefore, a strong pre-existing anti-HIV immune response in mucosal compartments might be able to prevent HIV infection. Conflicting views regarding the mechanisms of protection at mucosal sites, inferred by the contradictory results of mucosal vaccines in human clinical trials, attests to our lack of knowledge in understanding the human mucosal immune system. In this article, we briefly review the function of innate and adaptive immune responses and discuss current strategies and potential adjuvants in designing and delivering HIV vaccines through mucosal routes.

Contents

1. Introduction

Despite the extensive efforts put forward thus far, major challenges still exist concerning HIV vaccine design. Meanwhile, the relevance between innate and adaptive immune responses and their corresponding correlates of protection continue to be debated. The high HIV-1 mutation rate and resulting antigenic heterogeneity among viruses circulating throughout the world poses a significant challenge to vaccine development. The majority of HIV infections occur via vaginal or rectal transmission [1,2], and therefore, many researchers believe that a strong pre-existing anti-HIV immune response in mucosa-associated lymphoid tissue (MALT) may be able to prevent HIV infection [3]. Although the architecture of nasal, bronchial, and gut-associated lymphoid tissues is varied, homing and chemokine receptors such as $\alpha4\beta7$ (LAMP-1), $\alpha4\beta1$, and CCR1-CCR10 make a functional connection between these mucosal compartments [4,5]. Therefore, it is thought that immunization at one mucosal site might lead to the induction of immune responses

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in other mucosal sites due to disseminate of precursors of antibody-producing cells from the inductive to effector mucosal sites.

Mucosal membranes in the reproductive tract contain antibodies, T cells, beta-defensins, lactoferrin, lysozyme, and various enzymes which play crucial roles as the first line of defense against HIV-1 [6]. The breakdown of this barrier permits HIV virus entry into the apical luminal side. Microfold (M) cells (groups of cells present at basal membrane sites) play a crucial role in transcytosing antigens, including HIV-1 particles, to the lamina propria [7]. The neonatal Fc receptor, intestinal enterocytes, and the intraepithelial dendritic cells (DCs) may also transcytose HIV-1 particles into the lamina propria, a major source of lymphocytes and dendritic cells, which may thus act as a potential reservoir for HIV-1 infected cells [8–10]. Over 70% of CD4T cells, predominantly memory CD4T cells, become depleted in mucosal tissues a few days after HIV infection, causing permanent damage to the immune system [11–15]. These findings suggest that mucosal immunity is critical for protection against HIV infection [16–18]. In this article, we briefly review mucosal innate and adaptive responses to HIV-1 and address current mucosal vaccine strategies and their challenges. We also address adjuvants used in mucosal HIV vaccine studies over the past few years, and comment on the challenges associated with developing effective mucosal adjuvants.

2. Innate immunity and control of HIV infection

Upon the initiation of viral infection, including HIV-1, the body requires a few days to develop and expand effector T and B cells. During this critical timeframe, innate immune responses play an important role in controlling the infection [19]. Dendritic cells and natural killer (NK) cells are the two main elements of the innate immune system which act as the first line of defense against HIV infection [20].

Gamma delta T cells (γδ T cells) are another component of the innate immune response which play important roles against viruses and other microorganisms [21,22]. These cells are primarily found in the gastrointestinal mucosa and shown to comprise up to 50% of intraepithelial lymphocytes and about 10% of the lamina propria lymphocytes [23]. However, 1–10% of CD3+ cells in peripheral blood of healthy individuals are also γδ T cells [24]. The γδ T cells recognize antigens via their TCR but they are not MHC restricted, and it appears that they detect whole proteins and not peptides displayed by MHC molecules. Various γδ T cells phenotypes have been described, and several types are found throughout the human body, although the role of each phenotype has not yet been completely elucidated [21]. Mucosal γδ T cell responses contribute predominantly in the earliest stages of infection, and act as a link between innate and adaptive immune responses. However, if the infection becomes established, the majority of γδ T cells are deleted in mucosal sites, and the rest of γδ T cells do not respond to infection and stay anergic [21,25,26].

It have been shown that stimulation of γδ T cells with phosphocarbohydrates releases cytokines such as INF-γ and TNF-α which regulates HIV replication [25]. In addition, γδ T cells are able to induce maturation of dendritic cells and activate specific αβ T cell responses [25]. In a study by Li et al. [27] the frequency of γδ T cells and their function (isopentenyl pyrophosphate-responsive) were compared in both HIV-infected individuals and healthy individuals. The results showed a positive correlation between the number of CD4+ T cells with γδ T cell frequency and function. Furthermore, a reverse correlation was detected between viral loads and γδ T cell counts and function, indicative of the anti-HIV efficacy of γδ T cells.

Macrophages are also potent cells of the innate immune system that initiate and regulate immune responses [28,29]. Macrophages are not infectable in intestinal tract and play a role as primary effector cells in innate immunity [30]. The large number of DCs in the vagina, and macrophages in the vaginal subepithelium, secrete cytokines such as TNF-α and INF-γ that may not only block HIV transmission and replication but may also attract T and B cells to mucosal sites [31–34]. Macrophages, dendritic cells, natural killer cells also secrete chemokines such as RANTES, MIP-1α, and MIP-1β (CCL 5, 3, and 4) which can bind to M-tropic-CCR5 or T-tropic-HIV-1-CXCR4 co-receptors and accordingly mediate HIV infection *in vitro* [35]. Some studies also showed that high amounts of these chemokines may down-regulate the cell-surface expression of the CCR5 receptor [36]. Furthermore, NK cells are able to destroy HIV-infected cells directly or through antibody dependent cellular toxicity (ADCC) [37]. These findings support the view that innate immunity may control HIV-1 replication.

The presence of DCs within the mucosa or in draining lymph nodes is responsible for the initiation and induction of mucosal immune responses against foreign antigens including pathogens and vaccines [38]. Immature DCs congregate and migrate into the epithelial layers and capture antigens by either phagocytosis, macropinocytosis, or endocytosis. Capture of antigen by DCs upregulates the expression of maturation markers such as CD80, CD86, and MHC-II, and consequently activates DCs which interact with local lymphocytes or travel to secondary lymphoid organs expressing CCR7 ligands. Thereafter, activated DCs present antigens to, and activate T cells [39].

NK cells are found in a variety of mucosal tissues including the lungs, the intestinal tract, nasal mucosa, and the uterus [40,41]. NK cells in the respiratory and lower reproductive tract express CD56dim, CD16+, while NK cells in the gastrointestinal tract, lymphoid tissues and upper female reproductive tract express CD56bright, CD16+/− markers. NK cells expressing CD56dim (classical NK cells) mediate cellular cytotoxicity, cytokine production, and ADCC function while NK cells expressing CD56bright (helper NK cells) generate a variety of cytokines and play an important role in directing immune responses [42,43]. Although NK cells in peripheral blood have been comprehensively studied, the function of NK cells within mucosal sites is not yet clear. Some studies have shown a direct correlation between decreases in the numbers of NK cells and disease progression in HIV patients [44]. Notably, preserved or even increased numbers of NK cells were detected in HIV-1 resistant individuals and long-term nonprogressor (LTNP) patients [45]. Recent studies have shown that NK cells play important roles in the differentiation and maturation of various subpopulations of DCs. The outcome of the cross-talk between NK and DCs results in the coordination and activation of both innate and adaptive immune responses, but the mechanisms by which NK–DC interactions may control HIV infection is not yet clear [20,46,47]. A recent study by Cella et al. [48], demonstrated that some human NK cells located in MALT are able to proliferate and express a variety of cytokines (IL-22 and IL-26) and mitogenic and anti-apoptotic molecules. In addition, the interaction between NK cells and epithelial cells results in the mucosal production of IL-10. Consequently, this results in constrained inflammation and potential protection of mucosal sites from infection. Therefore, it appears that an ideal HIV vaccine should not only induce strong adaptive immune responses, but also restore and enhance the components of innate immune system in mucosal sites. Despite extensive studies about innate immunity in peripheral blood, questions on the mechanisms of antiviral effects by the innate arm of the immune system, and its associated cell subsets in mucosal sites, remains unanswered.

3. Mucosal vaccines and tolerance

Administration of large doses of antigens by the oral or intranasal route induces in experimental animals a state of mucosal

(oral) tolerance, defined as systemic unresponsiveness to antigens first encountered by the mucosal route. This phenomenon prompts a frequently asked question: Can mucosally administered vaccines induce a state of mucosal tolerance manifested by decreased systemic immune responses? Several important facts must be considered in providing an objective response.

First, the induction of mucosal tolerance displays marked and important species-dependent differences. Mice, rats and guinea pigs can be easily tolerized, while chickens, cattle, and rabbits are refractory to the tolerance induction [49]. For several reasons, only a handful of studies have been performed in humans [50]. Antigens used in animal experiments such as ovalbumin or bovine gammaglobulin cannot be used in humans due to pre-existing immunity. Consequently, as a true neo-antigen, keyhole limpet hemocyanine (KLH) has been used in five published studies [50]. Remarkably concordant results indicated that the ingestion or intranasal application of large doses of KLH in humans induces a "split" tolerance manifested by a systemic T cell unresponsiveness but priming for both mucosal as well as systemic antibody responses. Furthermore, mucosal tolerance cannot be induced in human or animals previously immunized by the systemic route [50]. This finding is of utmost importance to the mucosal vaccinology: the temporal sequence of the antigen exposure determines the quality of the ensuing response. In other words, it is unlikely that mucosal immunization would suppress antibody-and/or T cell-mediated responses in individuals with pre-existing immunity. For example, this would be the case with an intranasally administered influenza vaccine. In addition it appears that most, if not all currently available vaccines used in humans exhibit their protective effect through the production of specific antibodies [51]. On the other hand, initial mucosal immunization of immunologically naïve individuals not previously exposed to HIV with a potential HIV vaccine might have an undesirable effect on the induction of cell-mediated, CTL-dependent immunity which appears to be of importance in the HIV infection. To prevent such an outcome, mucosal immunization should be preceded by the initial systemic priming. Finally, the induction of mucosal tolerance in humans has been explored with a single neo-antigen, KLH, administered either orally or intranasally. Rectal, genital or sublingual immunization routes with vaccine-relevant antigens, and the use of mucosal adjuvants or immunoregulatory molecules (e.g., cytokines) has not been addressed in humans.

4. Humoral and cell-mediated immune responses in mucosal sites

Over the past 25 years, many candidate HIV-1 vaccines have been evaluated in human clinical trials, but strong correlates of protection have not yet been demonstrated. These approaches relied mostly on the parenteral delivery of immunogens, however, thus far, only a few vaccine trials have been designed to characterize immune responses at mucosal sites. Mucosal vaccine delivery against HIV infection is hampered by the difficulty in analyzing these types of immune responses in humans [52,53]. Whereas systemic immunization induces mostly immune responses in peripheral and systemic sites, mucosal delivery of immunogens triggers primarily mucosal immune responses [54].

It has been shown that IgA is the predominant antibody in the majority of mucosal secretions [55,56]. Mucosal IgA antibody is generated primarily in the mucosal epithelial compartment and transported across the epithelial cell boundary into external secretions by interacting with the polymeric immune globulin receptor (pIgR) [57]. Some studies revealed a correlation between the high level of secretory IgA (S-IgA) and protection in high-risk individuals who remain seronegative [58–60]. These studies concluded that this S-IgA may interact with and potentially neutralize HIV-1 on mucosal surfaces and within epithelial cells capable of internalizing IgA-bound HIV-1. Conversely, other studies dispute the role of specific IgA antibodies in HIV-1-exposed seronegative individuals, and suggest there is no evidence for *in vivo* functionality of IgA-mediated intraepithelial HIV-1 neutralization [61–64]. Although the above-mentioned studies add to the uncertainty regarding the role of mucosal IgA antibodies in HIV infection, it has been shown that S-IgA antibodies are able to protect animals from intestinal rotavirus infection by blocking virus replication inside the cell during IgA transcytosis, unlike the neutralizing mechanisms associated with IgG antibody, which block cellular attachment of the virus [65]. It has been hypothesized that besides neutralizing viruses, IgA may also block infection by conveying viral particles into the lamina propria and then eliminating them into exocrine secretions, to be excreted from the body as immune complexes [66,67]. This suggests that the mechanism of IgA-mediated protection may have a broader scope than that provided by IgG-mediated neutralization. Therefore, IgG-oriented neutralizing antibody assays might not be appropriate for evaluating IgA-mediated activity in mucosal sites.

Specific anti-HIV IgG antibodies in mucosal sites may control HIV-1 infection through neutralization or/and as mediators of ADCC. Previous studies showed that HIV-1 infected women with high titers of specific IgG antibodies in both sera and cervical fluids exhibited lower genital viral loads [68,69]. It is interesting to note that although IgA is the dominant antibody in external secretions (3–5 g IgA is secreted per day in humans), the frequency of specific anti-HIV IgA is significantly lower than specific IgG antibodies, in not only sera but also most external secretions [70]. This antibody pattern is preserved in non-human primates, as some studies have shown that IgG, and not IgA, is the predominant specific antibody in the genital secretions of HIV-infected chimpanzees or in the intestine of SIV-infected macaques [71,72]. It showed that systemic administration of neutralizing monoclonal antibodies of IgG is able to protect macaques after systemically or intravaginally SHIV challenge [73–75]. Whether HIV-specific IgA is able to influence mucosal protection or not, it would be appropriate to conclude that HIV-specific IgG isotypes is able to control HIV infection in the genital but perhaps not intestinal tract.

Cell-mediated immune responses in mucosal sites are also known to play important roles in the control of viral infection, replication and persistence. Several studies have shown that mucosal CD8+ and CD4+ T cells inhibit HIV-1 or SIV entry at mucosal sites, and prevent infection as a result. A recent study has shown that induced T cell responses against Gag and Vif proteins are directly correlated with lower viral loads and higher CD4+ T cell counts [76]. In another study, macaques immunized with a live-attenuated SIV vaccine expressing nef (SIVmac239Δnef) were able to effectively control viral replication after challenge with a highly pathogenic heterologous isolate. All immunized animals showed a broad, but low frequency of CD8+ T cells against viral proteins by using IFN-γ ELISPOT and MHC-I tetramer staining [77]. These results confirm that a broad T cell epitope repertoire vaccine might be effective in controlling HIV infection.

Cytotoxic T cells (CTLs) recognize and destroy infected cells by various mechanisms, including perforin-mediated killing as well as through the secretion of antiviral cytokines [78,79]. CD4+ T helper cells (Th) secrete cytokines which provide support for the generation and preservation of CD8+ T cells and B cells. The antigens contained in traditional vaccines are frequently processed by endosomal proteases and not cytosolic proteosomes. Therefore, such antigens are presented via MHC class II and not MHC class I, resulting in a lack of CTL responses [80]. The expression of MHC class II molecules is limited to APCs while MHC class I molecules are present on the surface of all nucleated cells. As a result, CTLs are able to eradicate a variety of infected cells [81].

In a recent study by Li et al. [82], female macaques were intravaginally inoculated by SIV. The virus was found to replicate in cervical tissues and established a persistent infection in the lymphatic tissues in the absence or delay of CTLs in the early stages of infection. This work suggested that a local effector immune response at the site of infection depends on the timing, ratio, and spatial colocalization of specific CTLs.

It appears that the route of vaccination is also important in the induction of T cells in systemic or mucosal sites. A number of studies suggest that mucosal vaccination induces CD8+ T cell migration into mucosal sites, while systemic vaccination generates specific T cells mainly in secondary lymphoid organs and peripheral tissues [83,84].

However, in a study Sun et al. [85], the kinetics of the specific mucosal T cell immune responses was evaluated after intramuscular immunization of macaques with a variety of HIV immunogens. They showed that systemic immunization of macaques with recombinant adenovirus serotype 5 (rAd5) is able to induce a high frequency of CD4+ and CD8+ T cell immune responses in the colonic mucosa. In another study by Kaufman et al. [86], strong specific CD8+ T cell responses were detected within mucosal surfaces following intramuscular immunization. In this study, mice and rhesus macaques were intramuscularly immunized with a recombinant adenovirus (rAd) vector expressing SIV Gag. Immunized animals were able to induce strong functional CD8+ T cell responses at multiple mucosal effector sites. In addition, mucosal homing receptors were up-regulated and these cells were shown to migrate from systemic to mucosal sites. Therefore, systemic immunization strategies may also be able to induce mucosal immunity even though it is not clear if the induction of mucosal immunity by systemic immunization is as protective as mucosal immunization.

Memory T lymphocytes migrating into mucosal compartments showed up-regulated β7 integrin and CD69 activation marker expression while memory T lymphocytes migrating to systemic compartments displayed a different immunophenotype [87]. The interaction of α4β7 homing receptors with mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) on Peyer's patches and the post-capillary venules of the intestinal tract modulates accumulation and migration of CD4+ and CD8+ T cells into mucosal compartments [88]. However, the functional consequences of these homing receptors are not well understood as memory T cells expressing these homing receptors are often retained in peripheral and not in mucosal sites [89].It should be noted that mucosal immune responses may be induced concomitantly with diminished systemic T cell immune responses, thereby permitting IgA-mediated containment without stimulation of systemic immunity [50,90,91]. There is substantial immunophenotypic diversity among mucosal T cells in Peyer's patches, lamina propria and intraepithelial compartments. It is thought that different subsets of CD4+ and CD8+ T cells may change their function and reflect the specialized needs of the adaptive immune responses. However, the roles of these subsets are relatively unknown [92–94]. Although the nature of the protection required against HIV-1 is not yet concrete, it appears that strong innate as well as cellular (effector and memory) and humoral immune responses in both mucosal and peripheral compartments are needed to subsequently down-regulate HIV replication.

5. Genital delivery of HIV immunogens

MALT represents a unique compartment of the immune system, and is composed of anatomically distinct lymphoid organs which serve as crucial inductive sites of mucosal immune responses [95,96]. Although the genital and intestinal tracts are of a common embryologic origin, both male and female genital tracts lack inductive mucosal sites analogous to intestinal Peyer's patches [97].

While the majority of HIV infections occur through the genital and rectal tracts, most exposures to HIV do not result in infection. This may be due to protection afforded by an intact mucosal epithelium, or/and innate and adaptive mucosal immune responses present in these sites [98]. Therefore, it appears that a strong immune response in mucosal sites may be important for the protection against the sexual transmission of HIV-1.

As discussed earlier, several discrepancies exist regarding the presence levels and functional significance of IgA antibodies to HIV-1 in the genital tract secretions. However, it is believed by many scientists that both S-IgA and IgG are important components for protection against invading pathogens [65].

The local administration of vaccine antigens into the vagina of animals or human volunteers has been shown to predominantly result in the development of specific antibodies in local secretions, but in most cases the immune response is not disseminated to remote mucosal sites or to the systemic compartment. Furthermore, local immunization via the male genital tract is unlikely to be practical [99–101]. However, there are some reports indicating strong immune responses after vaginal administration of HIV vaccine candidates in animals. In a study directed by Kato et al. [102], mice were immunized via the genital mucosa by an HIV peptide vaccine and cholera toxin (CT) as an adjuvant. HIV-1-specific IgA antibody was detected in fecal samples and vaginal washes. In addition, substantial levels of HIV-1-CTL responses were detected in immunized mice. In a recent study, HIV gp140 protein was entrapped into a polymeric gel (rheologically structured vehicles) and administered vaginally to rabbits. This vaginal delivery system was able to induce specific systemic and mucosal IgG as well as IgA antibody responses in genital secretions [103].

Analysis of the molecular forms of IgA antibodies in murine mucosal tissues and even sera indicated that unlike in humans, these were principally polymeric though a minor amount of monomeric IgA was reported [104]. The existence of polymeric IgA-secreting plasma cells in the subepithelial tissues of the female genital tract, chiefly in the endocervix, and to a lesser extent in the fallopian tubes and uterus, has been well documented, and pIgR, the membrane precursor form of the secretory component (SC), has been demonstrated in the overlying epithelium [105]. Thus in these location, and in the penile urethral glands of the male genital tract, S-IgA is assembled and secreted into the lumen [106]. Although S-IgA is the principal class of immunoglobulin in most external secretions, IgA and IgG levels in the female genital tract vary during the estrous cycle in animals and humans [107,108]. As shown in Table 1, the immunological properties of the human intestinal tract, and male and female reproductive tract, alter dramatically in response to hormonal fluctuations during the menstrual cycle, method of sample collection, and method of sample processing [107]. As a consequence, the levels of IgG and IgA antibodies in intestinal or genitourinary secretions vary significantly and therefore should be considered in the effective protection of respective mucosal sites.

Cell-mediated immune responses to HIV in the female genital tract have been comprehensively examined while the male reproductive tract, and its purported role in HIV infection and transmission, has been examined in far less detail [109].

The uniqueness of humoral immune responses in male and female genitourinary tracts may thus affect the potency of vaccines if administered by this route. In a phase I randomized trial, 34 females were vaccinated nasally or vaginally by recombinant protein HIV-1 gp160MN/LAI with or without DC-Chol, a cationic lipid 3beta-[N-(N′,N′-dimethylaminoethane) carbamoyl] cholesterol adjuvant. Although the vaccine did not show any adverse

Table 1
Comparative immunological features of human intestinal vs. human male and female genital tracts.

	Intestinal tract	Genital tract
Ig isotopes	IgA > > > IgG	IgG > IgA. In male and female secretions
Amounts[a]		
IgG (μg/ml)	1–4	10–467 (F)[b], 16–33 (M)[b]
IgA (μg/ml)	143–827	21–118 (F), 11–23 (M)
Molecular forms of IgA	S-IgA > > > pIgA > mIgA	S-IgA = pIgA = mIgA
	IgA1 > IgA2 (small intestine) IgA2 > IgA1 (large intestine)	IgA1 > IgA2 (IgA2 dominant in the female secretions)
Ig origin		
	Local > > > systemic	Local = systemic (F), systemic = local (M)
Hormonal regulation		
	–	+++ (F), + (M)
Antibody-secreting cells		
Main localization		
Dominant isotope	Lamina propria	Endocervix (F), urethral glands of litre (M)
	IgA > > > IgG	IgG ≥ IgA (F), IgA (M)
Epithelium		
	Single layers of polarized enterocytes	Stratified multilayered cells in vagina and ectocervix
Lymphoepithelial inductive sites	+++	–
DC/Langerhans cells		
DC-SIGN	+++	–
CCR 5	+++	+
Macrophages		
CD 14	–	+
CD 89 (FcαR)	–	+
CD 16 (FcγR)	–	+
CD 4	–	++
HIV infectable	–	+
Plasma cells	IgA > > > IgG	
	IgA1 > IgA2 (small intestine)	IgG > IgA endocervix
	IgA2 > IgA1 (large intestine)	IgA2 > IgA1

[a] The amounts of IgA and IgG are highly variable as they depend on the method of sample collection, processing, hormonal status, and method of measurement [70].
[b] M: Male, F: Female.

events, no anti-envelope IgA antibody was detected in sera, saliva, or cervico-vaginal and nasal secretions [50,110].

6. Rectal delivery of HIV immunogens

To date, most rectal HIV vaccine studies have not shown broad antibody titers, and only modest levels of local IgG and IgA titers have been reported [111,112]. Of note, a study by Hamajima et al. [113] showed a detectable level of both cellular responses and antibody titers in systemic and mucosal sites after rectal vaccination with a DNA vaccine encoding HIV antigens. In a study by Wang et al. [114], a few macaques were rectally vaccinated with either SHIV DNA alone or SHIV-DNA followed by modified vaccinia virus Ankara (MVA) expressing SHIV. However, only one macaque showed modest IgA and IgG antibody titers. In a phase I study, volunteers were primed intramuscularly with a VLP (viral-like particle) vaccine expressing HIV p17/p24 at months 0, 2, and 6, followed by two rectal boosts at months 10 and 11 [115]. None of the individuals showed broad humoral or cellular immune responses. It appears that so far, rectal vaccinations only induce modest immune responses in large animals and humans. In addition, there is a paucity of human mucosal adjuvants and delivery systems. This, and the difficulty in quantifying effector cells in rectal tissues, combined with intricacies in the route of inoculation, are some of the major challenges associated with rectal vaccination.

7. Nasal delivery of HIV-1 immunogens

Various clinical and preclinical studies have shown that intranasal immunization (instilled by drops or sprays) induces immunity not only locally in the nasal-associated lymphoid tissue and in the lung, but also in the female genital tract [116–119]. In addition, smaller doses of antigen have been shown to elicit antibody titers equivalent to those elicited by other mucosal routes of immunization [120]. Another major advantage of intranasal immunization is that this route of immunization is easier than rectal or vaginal routes and eliminates the use of needles [121].

Various approaches including peptide antigens, DNA vaccines, live bacterial and viral vectors have been evaluated by intranasal immunization [122–124]. Polymeric nanospheres have also been applied as a nasal vaccine delivery system. It has been reported that intranasal immunization with inactivated HIV-1-capturing nanospheres (concanavalin A-immobilized polystyrene nanospheres) induced specific IgA antibody in vaginal washes of immunized mice [125]. A neutralizing antibody response was also detected in vaginal washes of intranasally immunized mice against HIV-1 isolate IIIB [125]; however, it is commonly thought that mouse models are not appropriate systems in which to evaluate HIV-1 neutralizing antibodies. Similarly, macaques were intranasally vaccinated with SHIV-capturing nanospheres and then challenged with a pathogenic virus (SHIV KU-2). Elevated levels of IgA and IgG antibodies were detected in the sera of immunized macaques. Additionally, these vaccinated macaques showed a higher frequency of CD4+ T cells and lower viral loads compared to control macaques [126]. In another intranasal immunization study, mice vaccinated with gp120 protein carried by nanoparticles (gamma-glutamic acid) showed strong CD8+ T cell immune responses in systemic sites. This vaccine was able to induce memory T cells which remained in circulation over 7 months after vaccination [127].

Recent reports have suggested that combinations of mucosal and systemic immunizations may enhance both mucosal and sys-

temic immune responses [128–130]. In a recent study, macaques were immunized with the combined intramuscular-nasal DNA-MVA protocol followed by a rectal challenge with SHIV 89.6P. All vaccinated animals were able to induce memory CD4+ T cells and significantly control the viral load [131]. In another study, intranasal/oral administrations of macaques with Ad5 vector-expressing SIV genes (*env/rev*, *gag*, and *nef*) elicited higher levels of cell-mediated immune responses at systemic and mucosal sites compared to macaques immunized only orally. In addition, animals primed by intranasal/oral administrations exhibited lower viremia compared to other groups [132].

The site at which APCs take up antigen likely influences the quality of immune responses. The appearance of antigen-specific S-IgA at distant mucosal sites following intranasal immunization is thought to be due to the homing of antigen-specific B cells from the nasal-associated lymphoid tissue. In a mouse study, intranasal immunization with HIV envelope peptide antigens along with IL-1α, IL-12, and IL-18 generated specific IgA antibodies in saliva, fecal extract and vaginal lavage samples [133]. A human clinical trial has also reported that intranasal vaccination with CTB induced specific IgA antibodies in the female genital tract and rectal mucosa, and that intranasal immunization produced higher levels of specific IgG in serum, compared to other mucosal immunization routes [119].

However, a major obstacle in the development of mucosal vaccines is that antigens applied to mucosal membranes generally induces relatively weak immune responses. To generate potent immune responses through nasal immunization, improved mucosal adjuvants and/or delivery systems are required (see the adjuvant section). In addition, intranasal vaccination has the potential to cause side effects such as Bell's palsy, and damage to olfactory nerves and the nasal epithelium has been described elsewhere [134,135]. Recently, two human clinical trials based on nasal vaccination of HIV-1 antigens were terminated dues to safety concerns (http://clinicaltrials.gov/ct2/results?term=HIV+vaccine+nasal). Such side effects should not discourage the development of vaccines and adjuvants for nasal administration, since this route of immunization has shown promising results in animals.

8. Oral delivery of HIV-1 immunogens

Oral vaccination has the ability to induce both mucosal and systemic immune responses, in addition to being safer, easy to administer and not requiring sterile needles. Furthermore, oral vaccines could more easily meet the immunization needs of affected people in developing countries, where access to proper medical care and vaccine storage is frequently limited [136]. Although oral vaccines have several attractive features, studies on their use have been limited due to several challenges such as the induction of tolerance, lack of safe and effective mucosal adjuvants, a requirement for large doses of antigens, and the stability of antigens against the harsh conditions of the gastrointestinal tract [137,138]. It is for these reasons that only a limited number of oral vaccines are currently licensed, compared to many parenteral vaccines [138]. It is not yet clear why some antigens administered orally induce immune responses in the GI tract while others induce tolerance. It is thought that multiple mechanisms such as presentation of antigens by non-professional APCs, lack of costimulatory activity by mucosal antigens, or antigen-microbial interactions that are occurring continuously at the large intestinal may induce tolerance. As well, alterations in delayed-type hypersensitivity (DTH) and the induction of suppressive cytokines such as IL-10 may diminish the frequency of systemic T cells, which are the dominant target of mucosal tolerance [139–141]. The type of T-helper (Th) cells in lymph nodes or Peyer's patches play an important role in the expression of IgA or IgG antibodies [142,143]. Th1 cells secrete IL-2, IL-12 and INF-γ which consequently increase the level of IgG and activate CTLs, while Th2 and T-regulatory (T-reg) cells secrete IL-4, 6, 10, and TGFβ, resulting in B cell isotype switching and upregulation of IgA antibody production [144]. Therefore, when designing a vaccine, it should be decided which arm of the immune response would be of greatest benefit in controlling the infection.

Over the past few years, a number of vaccine delivery vehicles such as lipid vesicles or polymeric nanoparticles have been identified as being effective at eliciting mucosal immune responses following oral administration [138,145,146]. These vehicles act as immunostimulants while preventing the degradation of immunogens by enzymes in the gastrointestinal tract. They are thought to interact with M cells in delivering their immunogens to antigen-presenting cells. With this mucosal delivery approach, immunogens are released slowly and antigen encapsulation may promote increased phagocytosis [147]. Recombinant or attenuated strains of various bacteria such as *Salmonella*, *E.coli*, and *Lactobacilli* have also been used to deliver HIV-1 antigens into the intestine over the past decade. While some interesting results have been reported for these oral delivery systems, immune responses against the delivery bacteria eventually predominated over time [148]. Oral delivery of recombinant viruses such as adenoviruses (Ad), poxviruses, and polioviruses encoding specific HIV antigens has also been tested in several oral vaccine studies [91,149,150]. While these viral vectors showed promising results, pre-exposure to these viruses may result in an undesirable outcome marked by a gradually elevated response against such carrier viruses. Oral delivery of DNA vaccines encoding HIV antigens has been also evaluated in animals in various studies [151–153]. Nevertheless, due to relatively low uptake of DNA from the intestinal tract, limited amounts of B and T cell immune responses have been detected [154].

So far, only a limited number of orally administered vaccines against HIV-1 have been tested in human trials. In a phase I study, 33 HIV-seronegative volunteers were primed orally three times with a polymerized V3 peptide derived from HIV-1 isolate MN in biodegradable microspheres, followed by a systemic boosting. However, none of individuals showed broad humoral or cellular immune responses in mucosal sites [155]. In a phase I study by Wright et al. [150], 84 individuals were vaccinated with live canarypox vectors expressing HIV-1 p55, p15, gp41, and gp120, systemically and/or mucosally via the nose, mouth, vagina, or rectum. No strong mucosal IgG or IgA antibodies were detected, and only sera IgG was detected against the canarypox vector in some individuals. In another study, 18 healthy individuals were immunized orally with a single dose (5×10^6 to 1×10^{10} CFU) of *Salmonella typhimurium* vector expressing HIV gag protein. Although a moderate response was seen in a few individuals (2/18 volunteers responded to Gag peptides by IL-2 ELISPOT), none of the volunteers showed strong immune responses as measured by ELISA, or B and T cell ELISPOT against a pool of Gag peptides [156].

Our group recently reformulated a lipid-bile vesicle system (bilosomes) to deliver antigens orally. Bilosomes are liposome-like vesicles but the chemical stability of their structure provides them with a significant advantage over conventional liposomes [157]. Their structures are similar, with the difference that the presence of bile salts (sodium deoxycholate) in the structure of bilosomes protects peptide immunogens from the detrimental effects of stomach pH and GI digestive enzymes [157,158]. Because of this chemical difference, bilosomes appear to be resistant to the harsh conditions of the GI tract and promote antigen uptake by M cells within the small intestine.

We have previously developed a multivalent HIV vaccine based on env and gag hypervariable regions [159]. Despite a broad cellular and humoral immune response in mice and macaques, IgA and IgG antibody titers were suboptimal in mucosal sites. In an attempt to increase the antibody titers in mucosal sites, this vaccine candidate

was entrapped into an orally delivered lipid-based system. Our preliminary results indicated that the group of mice that were primed and boosted orally with liposome-entrapped multivalent HIV-1 peptides and the group that were primed orally with liposome-entrapped multivalent HIV-1 peptides and boosted systemically with multivalent HIV-1 peptides plus adjuvant R848 displayed elevated levels of IgA titers in lung lavage and fecal samples. Interestingly, the intramuscular boost induced specific anti-viral CD8+ T cell responses in systemic sites (manuscript in preparation). The magnitude of the induced immune response in the intestinal compartment is dependent upon the nature of the entrapped antigen, the bilosome formulation employed, and the presence of adjuvants in the final formulation. While this approach still requires improvement, it may open the door to a new generation of efficacious orally administered vaccines.

9. Mucosal adjuvants

Adjuvants are becoming more important in modern vaccine formulations as more subunit and recombinant vaccines are being developed. The weak immunogenicity of some antigens requires an enhancement of the immune response, making adjuvants an integral part of any newly developed vaccine. Adjuvants modulate the immune response by promoting the prolonged release of antigens, targeting APCs, and directing the immune response towards a Th1 or Th2 response. Therefore the incorporation of an appropriate adjuvant in an HIV vaccine will allow for the induction of protective cell-mediated and antibody-mediated responses. The classical function of an adjuvant is to slowly release an antigen by either forming an environment that prevents degradation, or by forming a depot that allows the antigens to be released over time. Aluminum based salts (alum) represent this group of adjuvants. The majority of adjuvants licensed by the Food and Drug Administration (FDA) are alum-based, and they generally have an acceptable safety record [160]. The tendency of alum for depot formation at the injection site precludes its use as a mucosal adjuvant, making it of limited value for oral HIV vaccines, where mucosal immunity is of greater importance. In fact, the addition of alum to a short amino acid sequence (ELDKWA) of HIV gp41 has significantly reduced IgA secretion in the intestinal tract following intramuscular administration in BALB/c mice [161], which indicates that alum may interfere with mucosal immune response of some antigens. The failure of the classical adjuvants in the induction of the desired type of mucosal and systemic immune responses against HIV drives the development of new carriers that will safely deliver the antigens of choice, and/or induce a specific immune response. In this section, the latest findings on cholera toxin (CT), heat-labile toxin (LT), TLR agonists, cytokines, and other promising mucosal adjuvants in the quest to develop a HIV vaccine are discussed.

To date, the most potent mucosal adjuvants are LT and CT. The incorporation of *Escherichia coli* LT into a vaccine targets the M cells responsible for antigen sampling and uptake in intestinal sites [162]. CT has also been used as an adjuvant and it functions using a similar mechanism to *E. coli*'s LT [111]. One of the few oral vaccines licensed for human use worldwide is Dukoral®, which contains a whole-cell killed *Vibrio cholerae* and cholera toxin B (CTB) subunit. The wide application of the vaccine proved not only safe but also efficacious as the rate of protection was 100% for children under the age of 5, 85% for adults up to 6 months following vaccination, and 60% for adults up to 2 years following vaccination [163]. The protection is predominantly mediated by IgA antibodies that neutralize the bacteria and its toxin [164], thereby preventing colonization of the intestinal tract and binding of CT to intestinal cells. Induction of specific neutralizing IgA antibodies has been the aim of many researchers developing vaccines against pathogens that use mucosal surfaces as a site of entry. The success of this vaccine, particularly the extended duration of protection at a mucosal surface, has inspired many researchers to seek an effective mucosal vaccine for other diseases. Indeed, CT or its derivatives has greatly enhanced the immunogenicity of mucosal HIV [165], influenza [166], *Campylobacter* [167], and *Proteus* [168] vaccine candidates. A synergistic effect was observed when antigens were either linked to the toxin [169], or in preparations mixed with the toxin, as long as both the antigen and the toxin were present at the same time and on the same mucosal surface [169]. Both CT and LT enhance antigen transfer by increasing the permeability of the intestinal epithelium [170], which increases the antigen transfer rate across the epithelium, and by a marked increase in antigen presentation by professional APCs and non-professional enterocytes [171]. Once presented, immunomodulation by CT affects both B and T cells. B cells switch their antibody isotype to IgA and increase its production, while T cell effects are more complex and include both activation and inhibition of various cytokines [172]. The presence of CT also increases DC antigen presentation and induces the secretion of IL-1β [173]. The adjuvant and stimulatory properties of IL-1β further modulate the immune response induced by CT [173]. Despite the apparent similar mechanism of the action of CT and LT, it has been found that CT induces a Th2 and T-reg immune response with increased IL-4, -5,-6 and -10, while LT induces a mixed Th1 and Th2 response [174].

CT and LT toxicity remains a concern for their use in human vaccines, although they are well tolerated in animals. As little as 5 μg of CT will cause diarrhea in human subjects, while 1 mg of CT will cause temporary diarrhea in 4-weeks old piglets [175]. The development of the less-toxic derivative of CT, non-toxic B subunit of cholera toxin (CTB), has increased its appeal as a mucosal adjuvant in HIV vaccine candidates. The conjugation of CTB to gp41 increased the magnitude of the mucosal IgA and systemic IgG immune responses against HIV gp41 following intra-nasal vaccination when compared to gp41 alone or gp41 mixed with CTB preparations [165]. The superior immune response of conjugated CTB may be due to the enhanced uptake of the larger antigen [169]. Another approach based on CT is the fusion of the gene of the active (A1) subunit of the CT to the gene of a synthetic analogue *Staphylococcus aureus* A protein [176]. The full enzymatic activity of the CTA1 component and selective B-cell targeting by DD-dimer act together to increase the cell-mediated and antibody immune responses against administered antigens [177]. CTA1-DD, mixed with HIV-1 envelope glycoproteins (Env), administered nasally in mice and cynomolgus macaques, exhibits increased production of IgA in vaginal and bronchial alveolar lavage [178]. In the same experiment, the parenteral administration of Env protein in RIBI adjuvant (oil-in-water) increased serum IgG but had no effect on mucosal IgA [178], demonstrating the importance of choosing a suitable adjuvant to target the desired immune response.

TLR molecules, with their ability, despite their limited number, to sense the presence of a pathogen and direct the immune response have been the target of many newly developed vaccine candidates' formulations. Targeting one or more TLR's would not only increase the magnitude but also the quality of an immune response, leading to up-regulation of chemokine and cytokine production required for DC maturation. Overall, this process results in enhanced antigen presentation and an increase in cellular, mucosal, and humoral immune responses.

CpG is a DNA motif that differentiates bacterial DNA from eukaryotic DNA. The increased frequency and methylation of this motif is recognized by TLR9, which in turn activates a variety of cells including DC, macrophages, monocytes, and spleenocytes [179]. The TLR9 signaling pathway leads to IL-1β and INF-γ secretion, polarizing the immune response to a Th1 type [179]. *In vitro*, CpG activates B cells and significantly increases MHC-II expression and

antibody production [180]. The use of CpG as a mucosal adjuvant against HIV infection has shown promising results in mice [16,181–183]. Dumais et al. [16], immunized mice with gp120-depleted HIV mixed with CpG intranasally. The immunized group had a significantly higher IgG in the serum and IgA in both the sera and vaginal washes compared to the controls, an increased IFN-γ, MIP-1α and β production in lymphocytes isolated from the genital tract [16], and cleared an intra-vaginal challenge with a surrogate vaccinia virus (VV) expressing HIV-1 gag [16]. These promising results indicate, in principle, that a strong mucosal immune response is important for clearing of HIV infection. Intranasal immunization of BALB/c mice by Horner et al. [183] with HIV gp120 mixed with or conjugated to CpG, showed increased secretory IgA in vaginal washes and fecal samples, and increased IgG titers in the sera of vaccinated mice. The intranasal vaccination of mice with inactivated HIV-1 virus mixed with CpG induced a potent CTL immune response in the cervical tissue and iliac lymph nodes [182]. The immune response induced was strong enough to clear homologous and heterologous intra-vaginal recombinant VV challenge [182].

TLRs can be stimulated by a plethora of pathogen-associated molecular patterns (PAMPs), but the use of synthetic molecules is highly desirable due to safety, consistency, and the flexibility in modifying these synthetic molecules. *Mycoplasma*-derived macrophage activating lipopeptide of 2 kDa (MALP-2) is a synthetic TLR 2/6 agonist that has potent mucosal adjuvant properties capable of stimulating CTL responses better than CTB after intranasal administration, as it stimulates the secretion of pro-inflammatory cytokines that recruits neutrophils, followed by T and B cells [184]. The use of MALP-2 as an intranasal adjuvant augmented IgG serum titers and S-IgA titers in vaginal and lung lavages of BALB/c mice against HIV-1 p17 [185]. The antibodies were able to block biotinylated-p17 from binding to its receptor, which suggests some neutralizing activity [185]. These results make MALP-2 attractive as a mucosal adjuvant.

The glycolipid α-galactosylceramide (α-GalCer) is a synthetic ligand that induces NK cells and induces DC maturation *in vivo* [186]. The success of α-GalCer in potentiating humoral and cell-mediated immunity against influenza and adenoviruses [187], in addition to a complete prevention of EG7 tumor after intra-nasal administration [187], has encouraged its use as an adjuvant in HIV vaccines [188,189]. Intra-nasal or oral immunization with gp120 peptide mixed with α-GalCer induced a strong systemic antibody response after only one immunization, and improved mucosal and systemic antibody levels after three repeated immunizations. Although no clinical studies using α-GalCer with HIV vaccine candidates have been conducted yet, the α-GalCer adjuvant had no adverse effects in an anti-cancer Phase I clinical trial when administered orally [190]. The mechanism of adjuvant activity of α-GalCer administered orally is still unknown, but its intramuscular adjuvant activity was abolished in both INF-γ receptor knockout and CD1d knockout mice [189], reflecting the role these two molecules play in α-GalCer's adjuvant effects.

Cytokines have been also used mucosally to steer the immune system towards an increase in local CTL activity and/or increased IgG and IgA titers. The cytokines IL-1, IL-2, INF-γ, and granulocyte macrophage-colony stimulating factor (GM-CSF) have been used as HIV vaccine adjuvants with reported success [133]. BALB/c mice immunized intranasally with a synthetic HIV *env* peptide in a mixture of IL-1α, IL-12, and IL-18 had a significant increase in serum IgG and fecal IgA and IgG1 titers compared to controls [133]. The addition of IL-12 to a recombinant vector expressing HIV gp160 has increased the local CTL responses in mice vaccinated intra-rectally but not in mice vaccinated intra-muscularly [191]. Despite these promising results, the main obstacle in delivering cytokines is that they are proteins that will be affected by gastrointestinal conditions easily, thus requiring a delivery vehicle. Another concern is the safety of cytokines as they show toxic effects with increased doses, which resulted in deaths in a clinical trial when injected into subjects [192]. Combined with the relatively higher cost of cytokines, the above issues make their use as an adjuvant difficult.

Over the past few years, a number of delivery vehicles such as lipid vesicles, multiple emulsions, polymeric nanoparticles, micelles, and dendrimers have been identified as being effective at eliciting mucosal immunity following oral administration [138,145,146,157,193]. Some vehicles act as immunostimulants, while preventing the degradation of immunogens by enzymes in the GI tract. They are thought to interact with M cells to deliver their associated immunogens to antigen-presenting cells (APCs). With this mucosal delivery approach, immunogens are released slowly, and antigen encapsulation may also promote increased phagocytosis. The slow release of antigen by these vesicles may avert the need for a vaccination boost [147,194].

For a mucosal vaccine, liposomes represent many of the desirable features needed in an adjuvant. Liposomes are spherical vesicles composed of lipid bilayers enclosing an aqueous compartment. This unique structure allows the incorporation of a wide variety of antigens and compounds, making them an ideal vehicle for drug delivery and as an adjuvant. The entrapment of an antigen within a lipid bilayer protects the antigen, ensuring its safe delivery and enhanced antigen uptake by the immune system, especially by DC and macrophages [195]. The use of liposome-encapsulated HIV components has induced encouraging immunological responses [196]. The entrapment of Gag p24 in a cationic liposome compound increased CD4+ T cell populations in spleen, lymph nodes, and peripheral blood, and increased central memory CD8+ in peripheral blood [196]. In another study, HIV gp160 envelope protein administered with a cationic liposome adjuvant intranasally or intra-vaginally induced a significant increase in IgA levels, in addition to neutralizing antibodies and CTL responses [50,110]. The liposome encapsulating HIV gp160 prepared by Sakaue et al. [197] also induced an increase in S-IgA and systemic IgG accompanied by an increase in CTL response. Overall, the use of liposomes offers a versatile and controllable mucosal adjuvant.

A successful HIV vaccine will likely require the activation of multiple arms of the immune system. This ideal vaccine will require an adjuvant that has the potency to generate the required immune responses and maintain the required safety profile. Thus far, systemic immunization, regardless of the adjuvant used, has not produced sufficient immune responses to protect against HIV infection, making a mucosal adjuvant a necessary component of a successful vaccine.

10. Concluding remarks

Three decades after the discovery of HIV-1 neither the effective HIV vaccine nor the correlates of protection against the infection have been conclusively established. Acceptance of the fact that HIV-1-infection is acquired dominantly through the mucosal sites of the genital and intestinal tracts should focus future studies to the induction of protective humoral and cellular immune responses at the sites of viral entry. In general, systemic immunization generates low humoral responses at mucosal sites with the exception of secretions of the genitourinary tract: IgG antibodies from the circulation represent the dominant isotype in genital secretions and may exhibit their protective function as evidenced in animal models. Conversely, it has been demonstrated that the mucosal administration of antigens induces preferentially IgA responses at the site of antigen encounter as well as in secretions of anatomically remote mucosal sites. Although the protective effect of IgA antibodies specific for HIV-1-dervied antigens has been demonstrated *in vitro*,

the presence and protective role of IgA antibodies in the genital tract secretions of highly exposed but persistently seronegative individuals remains controversial.

The marked depletion of CD4+ regulatory T cells may further accentuate compromised humoral as well as cell-mediated protective responses by suppressing CTL activities in mucosal tissues. Furthermore, initial mucosal HIV-1 immunization of immunologically naïve individuals may induce a state of mucosal tolerance dominated by T cell hyporesponsiveness. However, systemic immunization preceding mucosal antigen encounter is likely to prevent this undesirable outcome. Furthermore, this sequence of immunization – systemic priming followed by mucosal boosting, is likely to stimulate protective humoral responses in both systemic and mucosal compartments. Regrettably, mucosal delivery of HIV vaccines has not been greatly explored in comparison to the systemic route. Such mucosal delivery has been attempted in only a limited number of studies mainly due to the relatively low uptake of antigens from mucosal surfaces and the unavailability of effective mucosal adjuvants approved for use in humans. Notwithstanding the difficulties associated with the collection, processing and precise quantitative measurement of humoral immune responses (especially of virus-neutralizing antibodies), the evaluation of immune responses in external secretions and mucosal tissues should be a compulsory component of immunization protocols, irrespective of the route of HIV-1-vaccine administration. Addressing these critical outstanding issues will require additional well-designed studies on mucosal vaccines.

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