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Current Efforts on Generation of Optimal Immune Responses against HIV through Mucosal Immunisations

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Abstract

Currently, over 40 million HIV-infected individuals are found around the globe, with an additional 15 000 daily infections. There is a general consensus that the most effective way to prevent new infections is to introduce a prophylactic vaccine. It is also generally agreed that both cytotoxic T lymphocytes (CTLs) and neutralising antibodies are important to mediate protection. The neutralising antibodies must be broadly reactive to neutralise multiple primary isolates. There is also increasing agreement that CTLs and neutralising antibodies should be present at mucosal sites of HIV entry, the draining lymph nodes and systemically. The route of immunisation is important when determining the site where protection is desired, i.e. the female genitourinary tract versus the male or female rectum versus systemic tissues, as are the type of HIV-related antigens, immunopotentiating adjuvants and delivery systems. Finally, multiple vaccine delivery systems may be required to be administered through both mucosal and parenteral routes to

induce optimal immune responses and protection against HIV infection through rectal, vaginal or systemic routes of transmission. This review discusses current efforts on the generation of optimal immune responses against HIV in the genitourinary and intestinal tracts using mucosal immunisations alone or combinations of mucosal and parenteral immunisations.

1. Introduction

Currently, over 40 million HIV-infected individuals are found around the globe, with an additional 15 000 daily infections. Presently, there is no effective vaccine to prevent HIV infection and acquired immunodeficiency syndrome (AIDS). The goal of vaccination is to generate adaptive immune responses that lead to immunological memory. However, the mechanisms of generation and maintenance of innate, adaptive and memory responses in the female genital tract and rectum, the most prevalent routes of HIV transmission, are not well understood. Although more than 35 HIV vaccine clinical trials have been performed using protein- and DNA-based vaccines, as well as live recombinant vectors, few, if any, have included a mucosal route of immunisation.[1]

Because HIV/SIV (simian immunodeficiency virus) has been shown to infect cells within mucosal membranes of the vagina and rectum as well as systemic lymphoid tissues,[2-4] it is expected that both local and generalised systemic immunity would be required to prevent infection and/or disease. Local production of IgA could result in transport of secretory (S)IgA and protection of the vaginal and rectal mucosa. In support of this concept, the presence of anti-HIV SIgA has been correlated with resistance to HIV infection in partners of HIV-infected individuals.[5] Moreover, in rhesus macaques, protection against SIV challenge correlated with increased numbers of SIV-specific IgA-secreting cells in iliac lymph nodes.[6] In addition, intracellular neutralisation of HIV transcytosis across epithelial cells was demonstrated to be mediated by dimeric IgA and IgM against the HIV envelope protein (env).[7]

By far the majority of current vaccinations are performed through parenteral routes. However, mucosal vaccination offers several benefits over parenteral routes of vaccination, including ease of administration, the possibility of self-administration, eliminating the chance of injection with infected needles, and induction of mucosal as well as systemic immunity. For instance, the nostrils, as opposed to the rectal or vaginal routes, are readily exposed and available for administration of vaccines by health professionals or even by self-administration. Thus, in terms of ease of administration, the intranasal route resembles the oral route. However, although the oral route has been used for drug delivery for centuries and is much preferred, intranasal immunisation generally requires much lower doses of antigen, with important implications for many, often costly, recombinant antigens. Lower doses are possible by the intranasal route mainly because intranasal immunisation, as opposed to oral, does not expose antigens to low pH and proteases.

Another important benefit of intranasal immunisation, in addition to induction of systemic immune responses, is the induction of potent responses both in the upper and lower respiratory tracts and the genital tract, through, as yet, undefined mechanisms.[8] Indeed, it appears that in contrast to local immunisation of the genital tract, intranasal immunisation induces vaginal and systemic responses that in some cases are more potent than those induced by vaginal immunisations.[9-14] Thus, compared with rectal or vaginal immunisations, the

intranasal route is more readily accessible, culturally more acceptable, and induces better mucosal and systemic immune responses. However, this route may not be ideal for induction of rectal immunity.

There are several important safety and immunological issues that need to be addressed when oral or intranasal routes of immunisation are considered. There is general concern that intranasal administration may cross the blood-brain barrier through the olfactory bulb and cause serious inflammatory responses at this site.[15-17] In addition, immunisations through the intranasal or oral routes with inert protein antigens in the absence of appropriate immunopotentiating adjuvants may cause tolerance, rather than immunity.[18,19] However, using carefully selected adjuvants or delivery systems may alleviate these concerns.

Mucosal vaccines have to overcome several formidable barriers in the form of significant dilution and dispersion, competition with a myriad of various live replicating bacteria, viruses, inert food and dust particles, enzymatic degradation and low pH before reaching the target immune cells. Therefore, it has long been established that vaccinations through mucosal membranes require potent adjuvants to enhance immunogenicity, as well as delivery systems to decrease the rate of dilution and degradation and to target the vaccine to the site of immune function.

In this review, in the first place, mucosal immune inductive and effector sites of the female genital tract and rectum, as relevant to HIV transmission, will be defined. To understand the mechanism of penetration of HIV and related viruses through these mucosal barriers, the multiple barrier model against HIV penetration and spread will be discussed. The roles of IgA versus IgG in mucosal protection against HIV and the importance of mucosal versus parenteral routes of immunisation for induction of optimal mucosal immunity will be examined. As CD4+ T cells are specifically targeted and eradicated by HIV, the importance of the interaction of mucosal B cells with CD4+ T cells in HIV infection and immunity will also be considered. As one of the hallmarks of mucosal vaccination is trafficking of lymphocytes to distant mucosal and systemic lymphoid tissues, B-cell and T-cell trafficking following HIV or SIV infection or vaccinations will be reviewed. The link between innate and adaptive responses has been the focus of recent intense research and thus it will be discussed in the context of mucosal HIV vaccines. Finally, current efforts on mucosal vaccinations alone or in combination with parenteral immunisation with HIV-related protein-, DNA- and RNA-based vaccines will be reviewed.

2. Mucosal Immune Inductive and Effector Sites Relevant to HIV Transmission

The uptake of a vaccine at the mucosal surface by or through the epithelial layer, followed by uptake and presentation by antigen-presenting cells, are the first steps in the initiation of an immune response after vaccination or infection (figure 1). The anatomy of the respiratory, gastrointestinal and genitourinary tracts differ with regard to their epithelial cells as well as the composition of the various antigen-presenting cells and lymphocytes in the various mucosal tracts.

The nasal mucosa are drained by lymphoid tissue in the pharynx, which forms an incomplete circular structure called the Waldeyer's ring. This lymphoid tissue is aggregated to form masses of lymph node called tonsils. Unlike peripheral lymph nodes, which are not directly associated with the mucosal lumen, the surface epithelium of the tonsils, similar to the mucosal-associated lymphoid tissue (MALT) of the gastrointestinal tract (e.g. Peyer's patches), is in direct contact with the lumen. The palatine tonsils and adenoids are covered with lymphoepithelium consisting of ciliary and non-ciliary epithelial cells, goblet cells and microfold (M) cells, the latter show-

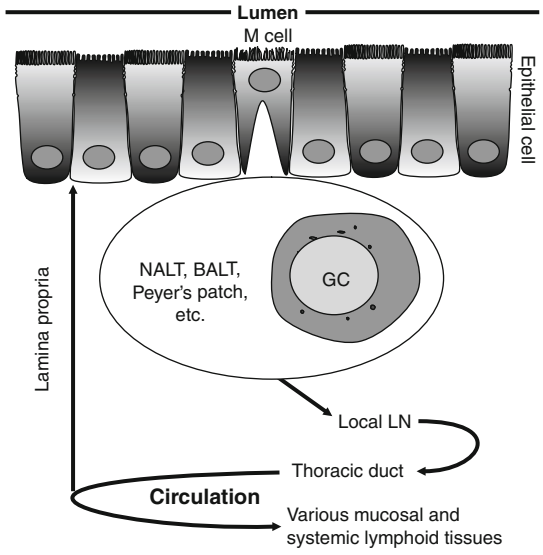

Fig. 1. General structure and process of mucosal antigen sampling. Columnar epithelial cells overlying the intestinal Peyer's patches, nasal-associated lymphoid tissue (NALT) or bronchus-associated lymphoid tissue (BALT) are interspersed with microfold (M) cells that take up particulate antigens and deliver them to the underlying lymphoid cells. In the case of B cells, this results in the generation of a germinal centre (GC) reaction and the trafficking of the activated B cells through local draining lymph nodes, the thoracic duct and the general circulation to reach various mucosal and systemic lymphoid and non-lymphoid tissues, including the lamina propria in the vicinity of the original antigen-sampling site. **LN** = lymph node.

ing many invaginating lymphoid cells.[20] Dendritic cells are numerous within and underneath the epithelial layer of the tonsils and are in close contact with the neighbouring B and T cells.[21] It is currently not known how activated cells traffic from the upper or lower respiratory tracts to the female genital tract following intranasal immunisation.

In the oral cavity, the lymphatic vessels of the parotid and submandibular glands drain to superficial and deep cervical lymph nodes.[22] In addition to the lymphoid aggregates in the epithelium, local draining lymph nodes also represent important inductive sites for local and systemic immunity following application of antigens to the oral cavity. Lymph from the end of the tongue drains to the superior deep cervical lymph node, whereas lymph from the tip of the tongue drains to the submental lymph node. Lymph from the sides and the middle of the tongue drains to the inferior deep cervical lymph node and to the submandibular lymph node, respectively.[22]

Similar to the small and large intestines, the rectal mucosa is covered with a single layer of epithelial cells. Interspersed within the epithelial layer are intraepithelial T cells, as well as dendritic cells or their dendrites reaching through to the lumen at the apical side. In the space on the basolateral side, underneath the epithelia, in lamina propria, are found B cells, plasma cells, T cells, macrophages and dendritic cells. This is the immune effector site of the rectal mucosa. The rectal mucosa of several mammalian species, including humans, contains macroscopically invisible solitary lymphoid nodules that resemble Peyer's patches of the small intestine in their cellular structure and phenotype and thus may serve as the immune inductive sites of rectum. These structures are overlaid with M cells that are specialised in antigen uptake.[23] Of note, both the rectal and vaginal mucosa are drained by the iliac lymph nodes, and there is indirect evidence that SIgA-secreting cells in the vaginal mucosa originate from the solitary lymphoid nodules of the rectum.[24-26]

In non-human primates,[27] as well as in humans,[28] the rectal and small intestinal lamina propria contain high numbers of CD69+ macrophages that are concentrated under the single layer of epithelial cells (enterocytes), whereas cells with dendrites, which are far fewer in number (most likely dendritic cells), form a reticular framework throughout the lamina propria. The rectal mucosa may serve as a vaccine delivery route, and because the vaccine does not have to go through the entire digestive tract and the intestine, lower amounts of antigen are required for intrarectal compared with oral immunisations. However, it may not be an

attractive route of immunisation for socioethical reasons (figure 2).

The vaginal mucosa is covered with multilayered squamous epithelia. However, the uterus, cervix and fallopian tubes are covered with pseudosquamous and simple columnar epithelia. Underneath the epithelial layers of the vagina, uterus and fallopian tubes is the lamina propria compartment, i.e. the effector site, comprising a large array of B cells, CD4+ and CD8+ T cells and antigen-presenting cells (APC).[29] The presence of lymphoid aggregates in the female genital tract has also been reported, although whether these aggregates have follicle-associated epithelium, as is the case with nasal-associated lymphoid tissue (NALT) and Peyer's patches, remains to be elucidated.[29,30] Dendritic cells and CD8+ cells with cytotoxic activity are found interspersed within the squamous epithelium of the vagina.[31-33] Thus, the vaginal mucosa contains dendritic cells as well as cytotoxic T lymphocytes (CTL) and can mount antiviral cytotoxic T-cell responses that can be protective.

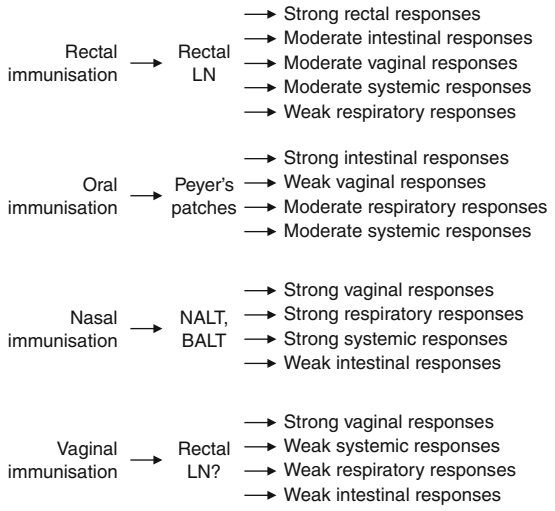

Fig. 2. Various routes of immunisation have specific inductive sites and generate differential local or distant mucosal and systemic immune responses. **BALT** = bronchus-associated lymphoid tissue; **LN** = lymph node; **NALT** = nasal-associated lymphoid tissue.

The vagina is considered to be a component of the common mucosal immune system, and oral immunisation in mice with microparticles has been shown to induce a vaginal antibody response.[34] In addition, intranasal immunisation with microparticles also induced antibodies in the lower genital tract of mice.[35] Although there is no evidence to indicate the presence of lymphoid follicles or M cells in the vaginal mucosa,[36] intravaginal immunisation in humans induced local antibody responses.[12] However, intravaginal immunisation protocols in small animal models have not normally met with great success, despite the use of novel delivery systems and adjuvants,[37-39] although a more recent report showed that vaginal or rectal, but not intranasal or intramuscular, immunisations with alphavirus-based replicon particles encoding HIV-1 gag protected against intravaginal challenge with vaccinia virus encoding HIV-1 gag.[40]

Moreover, the local immune response in the vagina is subject to significant hormonal regulation, with major changes in local antibodies at different stages of the menstrual cycle.[41] A study in mice showed that the intranasal route of immunisation was more effective than the intravaginal route for the induction of immune responses in the vagina.[13] In female humans, the intranasal route of immunisation may be exploited for the induction of genital tract antibody response.[14] Thus, although the vaginal mucosa contains the necessary immunological machinery to mount a local immune response, intranasal immunisation appears to be a more suitable route.

3. The Multiple Barrier Model against HIV Penetration and Spread

Based on the current body of knowledge regarding the primary infection with HIV or SIV, it is thought that immunological responses may be required at multiple barriers. SIgA will be required to neutralise HIV in the rectal and genital lumen or block adherence to the epithelial layers. If the HIV

virions cross the epithelial barrier, B and cytotoxic T cells are required in the lamina propria effector site to combat the infection. If the HIV virions are not eradicated at these sites, they spread to local draining lymph nodes and from there spread systemically. Thus, effector B and T cells are required at the inductive sites and systemic compartments as well.

To neutralise or eradicate HIV, B cells must produce mucosal IgA and IgG as well as systemic IgG antibodies to prevent HIV adherence to target cells. However, CTL will be mostly required for the eradication of virus-infected cells once the target cells (i.e. CD4+CCR5+ or CD4+CXCR4+ cells) are infected in either mucosal effector sites, the draining lymph nodes or the systemic compartment. Thus, to induce optimal protection against HIV infection, an effective vaccination strategy should generate both effector B cells and CTL at mucosal sites of HIV entry, the draining lymph nodes and the systemic compartments. As discussed below, a combination of mucosal and parenteral immunisation may be required to induce such responses.

4. The Role of IgA versus IgG in Mucosal Protection against HIV

It is well established that IgA is the predominant immunoglobulin isotype in the gastrointestinal tract. However, the effector arms of the humoral immune response in the female genital tract are believed to include both IgA and IgG, which may result in either direct virus neutralisation or virus elimination through antibody-dependent cellular cytotoxicity (ADCC). Although rapid local effector B-cell responses are required for protection, it is not clear whether vaginal IgG or IgA responses detected in most vaccination studies are induced locally or systemically. Also the germinal centre requirement as well as the inductive sites where female genital tract-targeted isotype switch from IgM to IgA or IgG occurs, is unknown. In this regard, a role of HIV-specific IgA in protection against HIV infection in Kenyan sex workers has been suggested.[42] It is important to note that cross-clade HIV-specific neutralising IgA has been detected in mucosal and systemic compartments of HIV-exposed, persistently seronegative subjects.[43] Once maturation and differentiation of antigen-specific B cells occur in the inductive sites, these cells should migrate to the mucosal effector sites to exert their effector function. This migration occurs through the expression homing and chemokine receptors on B cells leading them to the effector sites.

The requirement for isotype-switched B cells at rectal or vaginal mucosa may be difficult to meet, however, given the following findings. Decreased numbers of IgA+ cells or IgA-secreting cells have been observed in the rectal or intestinal mucosa of SIV-infected macaques. This decrease in the number of IgA+ cells is associated with an increase in the number of IgM+ cells.[44] A similar characterisation of the B cells in the vaginal/uterine mucosa in HIV or SIV infection has not been reported. Moreover, the process of isotype switch from IgM to IgG or IgA in the female genital tract is unknown. However, because of a significant decrease of CD4+ T cells also in the female genital tract following infection with SIV, it is likely that, similar to the intestinal mucosa, the process of isotype switching is also impaired at this site.

Although not demonstrated with antibodies in mucosal secretions, both IgG and IgA antibodies can exert cytotoxic effector functions through the ADCC mechanism. FcR-bearing cells armed with antibodies can kill virus-infected cells by ADCC, an immune mechanism known to occur *in vitro* and *in vivo*.[45-51] Overall, these findings show that IgA and IgG antibodies directed against HIV surface envelope glycoproteins are required to neutralise HIV virions directly or lyse HIV-infected cells through ADCC.

5. Mucosal versus Parenteral Routes of Immunisation for Induction of Optimal Mucosal Immunity against HIV

Several lines of evidence argue for the mucosal routes of vaccination being superior to parenteral routes of vaccination for the generation of mucosally produced antibodies. It has been shown that local administration of vaccines to mucosal tissues may induce the strongest local immunity at the site of immunisation.[52-54] Intravaginal immunisation in humans can induce local antibody responses.[12] Based on the concept of a common mucosal immune system,[55] intranasal immunisation can be a potential route since it induces local immunity not only in the nasal-associated lymphoid tissue and the lung, but also in the female genital tract in rodents,[35,55-58] non-human primates[59] and humans.[14,60] In addition, the intranasal route of immunisation may be more effective than the intravaginal route for the induction of immune responses in the vagina.[13] Importantly, intranasal immunisations have shown protection against SIV in non-human primates.[61]

It has been suggested that systemic priming followed by mucosal boosting significantly enhances mucosal as well as systemic responses induced by either mode of immunisation alone.[62] However, it has also been shown that parenteral immunisation causes antigen-specific cell-mediated suppression of an intestinal IgA response.[63] Nonetheless, it has been shown that mucosal priming followed by systemic boosting can enhance mucosal and systemic responses induced by either routes alone, or by systemic priming followed by mucosal boosting.[64,65] Mucosal followed by systemic immunisations significantly enhanced mucosal and systemic immune responses in a rhesus macaque[66] and in a murine model.[67] How this enhancement is achieved is currently unknown. It may be hypothesised that the reason why parenteral immunisations are effective for protection against mucosal pathogens is due to prior mucosal exposure to the mucosal pathogen, potentiating the responses after parenteral immunisations. In support of this hypothesis, induction of mucosal immunity by inactivated poliovirus vaccine through parenteral immunisation is dependent on previous mucosal contact with live virus.[68]

6. Trafficking of B and T Cells Relevant to HIV Infection and Immunity

Both B and T cells express homing and chemokine receptors that direct their trafficking to specific mucosal and/or systemic compartments. The role of expression of homing receptors, such as $\alpha 4\beta 7$ on B cells, on the induction of B-cell immunity at the appropriate sites for exerting B-cell effector functions is well established.[69,70] However, how B-cell trafficking and homing plays a role in memory B-cell responses in the genital tract inductive and effector sites or rectum is not well established. Few IgA+ or IgG+ cells expressed CCR2, CCR3 or CCR9, although CCR4, CCR5 and CXCR3 were more significantly expressed on IgG+CD19+ B cells than IgA+CD19+ cells.[71] CCR10 has also been implicated in directing IgA-secreting plasma cells to mucosal sites.[72] The above evidence demonstrates that activated effector B cells express both homing and chemokine receptors that play an important role to direct them to effector sites.

T cells are also known to express homing and chemokine receptors. Vaginal herpes simplex type 2 infection of mice induced migration of both B cells and memory type CD4+CD44+ T cells to the genital tract.[73] The chemokine receptor CCR4 was shown to be important for trafficking of systemic, but not mucosal, memory CD4+ T cells.[74] Expression of the mucosal homing receptor $\alpha 4\beta 7$ by murine CD4+ T cells has been shown to delineate a memory phenotype,[75] and $\alpha 4\beta 7$+CD45RA-CD45RO+CCR7-CD27+ memory T helper-1 (T_h1) cell responses were correlated with better antibody responses after parenteral immunisation with inactivated poliovirus in subjects orally pre-immunised

with live poliovirus.[76] Overall, these data suggest that distinct expression of homing and chemokine receptors directs B and T cells to mucosal effector sites.

7. Mucosal Protein-Based Vaccines against HIV

In this review a distinction is made between immunopotentiating adjuvants and delivery systems. Immunopotentiating adjuvants include mutant toxins, CpG (C poly G) nucleotide motifs, small chemical molecules and various saponins. Delivery systems include polylactide co-glycolide (PLG) microparticles, oil in water emulsions, immunostimulatory complexes (ISCOMS) and proteosomes. A number of immunopotentiating adjuvants have been used to enhance the immunogenicity of protein-based vaccines against HIV. These include, but are not limited to, mutants of enteric bacterial-derived enterotoxins, CpG, plant lectins and QS21.

While conventional approaches to vaccine development have been based on biochemical, immunological and microbiological methods, they have proven laborious and time-consuming, and allow only for the identification of few abundant antigens. Recent progress in DNA sequencing and subsequently in bioinformatics have resulted in advances in vaccine development. The availability of the whole sequence of a bacterial genome led to using the genomic information to discover novel antigens that had been missed by conventional methods of vaccine development. This novel approach, now termed reverse vaccinology, is involved the *in silico* analysis of the microbial genome sequence.[77-79] This approach has already resulted in the identification of immunogenic antigens as potential candidates for a vaccine against *Neisseria meningitidis*.[80] Thus, this approach holds great promise for future vaccine development in general, including HIV vaccine development.

Mutants of *Escherichia coli*-derived heat labile enterotoxin (LT) have been shown to be potent adjuvants for inducing mucosal and systemic immune responses. In order to retain the adjuvanticity of these molecules but reduce their toxicity, several mutants have been generated by site-directed mutagenesis. LTK63 is the result of a substitution of serine 63 with a lysine in the A subunit, which renders it enzymatically inactive and non-toxic.[81-85] LTR72 is derived from a substitution of alanine 72 with an arginine in the A subunit and contains about 0.6% of the enzymatic activity of wild-type LT. LTR72 is shown to be 100 000-fold less toxic than wild-type LT in Y1 cells *in vitro* and 25–100 times less toxic than wild-type LT in the rabbit ileal loop assay.[55] The ability of LT mutants to induce CTL responses against HIV-1 gag (p55), following intranasal, oral or intramuscular immunisations has been reported.[86]

Interestingly, LTK63 and LTR72 had diverse effects when used as mucosal adjuvants for oral versus intranasal immunisations; LTK63 induced stronger CTL responses following intranasal immunisation with p55 compared with LTR72. Conversely, LTR72 induced stronger CTL responses against p55 when given orally, and it also induced local CTL responses. Thus, it appears that some ADP-ribosyl-transferase activity of the LT mutant may be required for oral, but not for intranasal, immunisations if induction of CTL responses is the objective. These studies showed that intranasal immunisation with protein vaccines and LT mutant adjuvants can be an effective means for the induction of cell-mediated immunity against HIV.

It was also shown that LTK63 used as a mucosal adjuvant for intranasal immunisations with HIV envelope glycoprotein gp140 induced significant B- and T-cell responses.[66] In another study, pre-existing immunity to LTK63 did not affect its potency as an adjuvant, when used for intranasal immunisation with a second vaccine, soon after.[87] Staats et

al.[10,88] demonstrated that intranasal administration of an HIV-1 peptide vaccine (T1Sp10 MN) with cholera toxin in mice induced significant levels of antipeptide serum IgG titres and HIV-1MN neutralising responses. Vaginal antipeptide IgG and SIgA titres were also induced. In another study, the intranasal route was superior to the vaginal, gastric or rectal route of immunisation for induction of systemic and mucosal anti-HIV-1 peptide responses.[89] Thus mutants of enterotoxins hold promise as mucosal adjuvants in vaccine development against HIV infection.

Unmethylated CpG in the context of selective flanking sequences are thought to be recognised by cells of the innate immune system to allow discrimination of pathogen-derived DNA from self DNA.[90] It has been shown that cellular responses to CpG DNA are dependent on the presence of toll-like receptor (TLR)9.[91] The T_h1 cell adjuvant effect of CpG appears to be maximised by conjugation to protein antigens.[92] Importantly, CpG also appears to have potential for the modulation of pre-existing immune responses, which may be useful in various clinical settings, including allergies.[93] A recent review discusses in detail the interaction of CpG with TLR9 on dendritic cells.[94]

CD8+ T-cell-mediated cross-clade protection was observed following intranasal immunisations with HIV env antigen plus CpG and intravaginal challenge with Vaccinia virus expressing HIV env.[95] Moreover, intranasal immunisations with CpG plus HIV antigen protected against intravaginal challenge with VV-gag.[96] Interestingly, inactivated influenza virus was as effective as CpG for enhancing anti-HIV B- and T-cell responses following co-administration with simian/human immunodeficiency virus (SHIV)-like particles intranasally.[97] The CD4+ and CD8+ T-cell responses following immunisation with the adjuvant CpG clearly indicate a T_h1 cell response.[96-98] CpG used for intranasal immunisation with envelope glycoprotein gp120, either in combination or as a conjugate, induced serum antibody responses and SIgA responses in the vaginal and faecal samples.[98] These data suggest that CpG used as an adjuvant may be used alone or in conjunction with other immunopotentiating adjuvants or delivery systems in an HIV vaccine in future human clinical trials.

Plant lectins have been considered as both targeted delivery systems and immunopotentiating adjuvants in HIV vaccine research. The plant lectin, *Ulex europaeus 1* (UEA1) with specificity for the α-L-fucose sugars, acts as an adhesin and selectively binds to intestinal M cells, which have the specific capacity to take up particulate antigens from the intestinal lumen and overlie the Peyer's patches.[99] Enhanced mucosal and systemic B- and T-cell responses were observed following intranasal immunisation of mice with HIV peptides together with UEA1 entrapped in polylactide co-glycolide (PLG) microparticles.[100] Moreover, concanavalin A-immobilised nanospheres were also shown to enhance responses against co-captured HIV antigens following immunisations of mice or rhesus macaques.[101] Thus, the use of plant lectins in primate models has been limited, and whether it can be safely used in an HIV vaccine remains to be investigated.

The biodegradable and biocompatible polyesters, the PLGs, may be prime candidates for the development of microparticles as delivery systems, since they have been used in humans for many years as resorbable suture material and as controlled-release drug delivery systems.[102-107] In contrast to alum, PLG microparticles were effective for the induction of CTL responses in rodents.[108-110] It has also been reported that macrophages that carry microparticles to lymph nodes can mature into dendritic cells.[111] In addition, uptake of PLG microparticles into dendritic cells *in vitro*[112] and *in vivo* has been demonstrated.[113] It is assumed that the uptake of microparticles into APCs underpins the ability of the

particles to perform as vaccine delivery systems/adjuvants.

This approach allowed the induction of significantly enhanced antibody titres in mice with adsorbed p55 gag from HIV-1.[114] Whether this approach is effective in humans is currently not known.

Liposomes are phospholipid vesicles that have been evaluated both as adjuvants and as delivery systems for antigens and adjuvants.[115,116] Liposomes have been commonly used in complex formulations, often including monophosphoryl lipid A (MPL), which makes it difficult to determine the contribution of the liposome to the overall adjuvant effect in inducing anti-HIV responses following intranasal immunisations.[117] Intranasal immunisations with HIV envelope glycoprotein gp160 formulated in proteosomes and/or emulsomes containing MPL induced IgG and IgA antibodies in serum, vaginal, lung and intestinal washes and faecal pellets.[117] Nevertheless, several liposomal vaccines based on viral membrane proteins (virosomes) without additional immunostimulators have been extensively evaluated in the clinic and are approved as products in Europe for hepatitis A and influenza.[118] Intranasal immunisations with gp160-encapsulated liposomes induced mucosal and systemic B- and T-cell responses in a murine model.[119] Intranasal immunisation of mice with HIV env formulated in cholera toxin B subunit (CTB)-associated GM1 lipid vesicles enhanced mucosal IgA responses in nasal and intestinal tissues.[120] The ISCOMs are derived from *Quillaja saponaria* (Quil A) and have been shown to induce anti HIV env IgA responses in the genital tract of mice following intranasal immunisations.[121,122] A liposomal formulation with encapsulated gp160 in haemagglutination virus induced CTL and neutralising antibody responses following intranasal immunisation of mice.[119] However, a potential problem with ISCOMs is that inclusion of antigens into the adjuvant is often difficult and may require extensive antigen modification.[123]

The use of cytokines as adjuvants for experimental HIV vaccines has also been investigated, where various cytokines, e.g. interleukin (IL)-1, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor, used together with HIV-1 antigens, were shown to be effective adjuvants.[124-126]

Inactivated HIV- or SHIV-capturing nanoparticles have been produced and used to induce vaginal antibody responses in mice and rhesus macaques following intranasal immunisations.[101,127] Moreover, inactivated HIV-1 plus CpG adjuvant induced genital CTL and antibody responses in mice that were subsequently protected against vaginal challenge with recombinant vaccinia virus.[95,96] There have also been examples of intranasal immunisations with recombinant BCG bacteria encoding an HIV-1 antigen[128] or heat-inactivated bacteria conjugated to HIV env.[129] Reports on these approaches in non-human primates are scarce and thus their viability as an effective anti HIV-1 vaccine for human use remains to be explored. As stated above, live attenuated or inactivated HIV-1 viruses will most likely not serve as a vaccine candidate for human use because of serious safety concerns.

Virus-like particles are another candidate for an anti-HIV vaccine. Virus-like particles are produced by transfecting eukaryotic cells, yeast, insect or mammalian cells with DNA encoding a gene of interest, usually delivered by baculovirus or vaccinia virus. The virus-like particles are then formed, without a genome, in the cell and are either secreted or remain in the cell to be purified. Virus-like particles made of HIV gag alone or with HIV env expressed on the surface of gag-virus-like particles have been developed and used for intranasal immunisations in animal models.[130,131] As a different approach, intranasal immunisations of mice with chimeric influenza haemagglutinin (HA)/SHIV vi-

rus-like particles induced mucosal and systemic humoral and cellular responses.[132] An inherent problem with most studies using baculovirus-based virus-like particles is that no or insufficient efforts were made to purify virus-like particles in the absence of baculovirus. Baculoviruses induce innate antiviral effects and can clearly enter mammalian cells, and although they do not replicate, the DNA enters the nucleus, thus raising concerns about host cell chromosomal integration.[133]

8. Mucosal DNA-Based Vaccines against HIV

Immunisation with DNA has several advantages over immunisation with proteins, including the induction of potent CTL responses in small animal models.[134,135] DNA offers the potential for improved vaccine stability and reduced costs for vaccine production. Moreover, compared with attenuated viruses as delivery vehicles for HIV genes, plasmid DNA offers a safe alternative. Clinical trials involving intramuscular immunisation with DNA vaccines have already been performed in humans and these appear to be safe and well tolerated at the doses tested.[136,137] However, although DNA vaccines have proven potent in small animal models, the potency in larger primates, including humans, has been relatively ineffective.

There are several approaches to increase the potency of DNA vaccines. Modification of the plasmid DNA vector to increase expression levels has resulted in increased immunogenicity *in vivo*. Changing the nucleotide sequence of certain genes to better reflect preferential codon usage in mammalian cells can result in markedly higher levels of expression in eukaryotic cells *in vitro*[138] and, when incorporated into a DNA vaccine vector, can increase immunogenicity substantially.[139-141] Modification of the HIV *gag* gene produced a potent DNA vaccine that expressed 100- to 1000-fold higher levels of protein compared with the wild-type gene *in vitro* and induced significant immune responses *in vivo* in non-human primates.[139]

To enhance the immunogenicity of DNA-based vaccines, adjuvants and delivery systems can also be used. These can include various proteins including cytokines, small molecule compounds, lectins or DNA plasmids encoding immunologically active proteins such as cytokines, chemokines, costimulatory molecules and PLG microparticles.[142] Vaccination with DNA encoding HIV env complexed with UEA 1-poly lysine induced B- and T-cell responses following intranasal immunisations.[143] Intranasal immunisation with DNA plasmid encoding gp160 together with IL-12 and GM-CSF induced IgA responses in faecal and vaginal washes and a T_h2 cell type response.[144] Moreover, oral priming immunisation with DNA followed by boosting with recombinant vaccinia virus (rVV) induced mucosal and systemic antibody responses.[145] Plasmid DNA encoding macrophage inflammatory protein-1α given intranasally together with plasmids encoding HIV antigens enhanced SIgA responses.[146] Plasmid DNA encoding HIV-1 envelope antigen formulated with Pol-L-Lysine given three times intranasally was more effective than soluble DNA in inducing env-specific CTL responses in lungs, lower respiratory lymph nodes, cervical lymph nodes, submaxillary gland lymph nodes and spleens.[147] The use of chitin microparticles along with HIV DNA given intranasally has also been reported.[148]

Local (cervical lymph nodes) and systemic (spleen) B- and T-cell responses were observed when plasmid DNA encoding HIV-1 gag adsorbed to PLG microparticles was administered intranasally.[149] To investigate a possible mechanism for the enhanced immune responses induced following intranasal immunisations with PLG DNA, the cells that expressed gag protein in local and systemic lymphoid tissues were phenotypically identified (our unpublished observation). In the immunostaining studies of cervical lymph nodes and spleen, the

majority of gag-expressing cells were CD11b+, suggesting that this population is responsible for uptake and expression of DNA following intranasal immunisation with PLG-DNA. Although CD11b is expressed by many cell populations, it is primarily considered a marker for tissue macrophages and dendritic cells, which are both professional antigen-presenting cells.[109,150,151] However, compared with macrophages, dendritic cells are more potent antigen-presenting cells.[152,153]

These data suggest that following intranasal immunisation with DNA adsorbed onto PLG microparticles, monocyte lineage cells, macrophages and/or dendritic cells, are involved in the uptake and expression of gag DNA, since we detected both CD11b+ and CD11c+ gag-expressing cells. Whether these cells also actively present gag peptides to neighbouring naive T cells *in vivo* is an important question that needs further investigation. Our previous *in vitro* data showed that bone marrow-derived dendritic cells can take up PLG DNA encoding HIV-1 gag and present it to a gag-specific T-cell hybridoma.[154] The prolonged expression of DNA following intranasal immunisations with PLG DNA may be in part due to protection of DNA from damage by tissue DNAse, previously reported *in vitro*.[155] Interestingly, comparison of intranasal and intramuscular immunisations with DNA encoding HIV antigens together with MPL revealed that while both routes of immunisation induce similar levels of T-cell response, only intranasal immunisation induced intestinal SIgA antibody responses.[156] PLG microparticles have also been used for delivery of QS21 adjuvant in combination with gp120.[157]

9. Mucosal RNA-Based Vaccines against HIV

Several RNA-based delivery systems have been invented that result in the infection of target cells and delivery of RNA encoding the gene of interest. In general, such an approach has a clear advantage over the more popular plasmid DNA immunisation, as RNA, unlike DNA, does not require access to the nucleus and thus minimises the possibility of chromosomal integration.[158] A number of RNA delivery systems as potential vaccine candidates have been described, including purified complementary DNA-transcribed RNA, neuraminidase-deficient influenza A virus, tick-borne encephalitis virus, *Listeria monocytogenes*, non-transmissible Sendai virus, liposome-entrapped messenger RNA, and alphaviruses.[158-166] Of these, the most popular RNA delivery systems for an anti-HIV vaccine have been alphaviruses, either as replicating or non-replicating replicon particles.

Alphaviruses, including Sindbis virus (Sin), Semliki Forest virus (SFV), and Venezuelan equine encephalitis virus (VEE), are enveloped RNA viruses that have been developed into replication-defective 'suicide' vectors.[167,168] Alphavirus replicon RNA vectors maintain the nonstructural protein gene and *cis* replication sequences required to drive abundant expression of heterologous antigens from the viral subgenomic 26S promoter but are devoid of any alphaviral structural protein genes required for propagation and spread. These vectors also offer the prospect of natural adjuvanticity and stimulation of the innate immune response, in addition to the antigen-specific adaptive response arising from the cytoplasmic amplification of these vectors through double-stranded RNA intermediates.[169] Replicon vectors have been widely evaluated as vaccine immunogens, both as plasmid DNA replicon vaccines and as virus-like replicon particles.[170]

Replicating VEE expressing HIV-1 matrix/capsid were used to inject mice subcutaneously, which induced IgA as well as CTL responses.[171] Cynomolgus macaques immunised parenterally with SFV RNA vectors expressing HIV-IIIB gp160 and challenged parenterally with SHIV-4 were not protected against high viral loads,[172] even though a mouse

study suggested that compared with a plasmid DNA encoding HIV envelope, SFV expressing HIV env induced the highest serum anti-env antibodies.[173] The level of biosafety manufacturing may play an important role in selecting the type of alphavirus-based replicon particles for an HIV vaccine. The VEE-based replicon particles will require biosafety level (BSL)-3 manufacturing facilities and may not be as attractive as for example Sindbis virus-based replicon particles that require BSL-2 Good Manufacturing Practice production facilities.[174]

10. Mucosal Live Attenuated Virus-Based Vaccines against HIV

In general, live attenuated viruses as antigen delivery systems offer relatively high potency. It is important to note that parenteral immunisations with live attenuated-based vaccines can often result in mucosal responses as a result of the traversing of the replicating vaccines to both mucosal and systemic compartments. However, most have the problem of inducing high antivirus vector immunity, thus making their multiple or even subsequent use obsolete. In this regard, a report using a mouse model indicated that mucosal vaccination (which in this case was intrarectal) overcomes the barrier (i.e. immunity against the vaccinia vector) to recombinant vaccinia virus immunisation caused by pre-existing poxvirus immunity.[175] There have been clear examples of reverting to the wild type phenotype/genotype and causing severe disease if the live attenuated virus is the infectious virus with attenuations to eliminate or reduce disease.[176-179] The level of attenuation appears to inversely correlate with potency since the more attenuated strains are less immunogenic.[180,181] This section provides some examples of live attenuated viruses given intranasally or by other routes to induce anti-HIV immune responses.

Vaccinia virus-based live attentuated vaccines have been extensively used to induce anti-HIV responses as intranasal immunisations alone or as priming or boosting immunisations. A popular approach has been DNA priming followed by vaccinia boosting, in effect avoiding strong anti-vaccinia immune responses. A DNA prime/vaccinia boost immunisation containing multiple HIV genes controlled viral loads following intrarectal challenge.[182] Intranasal priming with HIV env-expressing influenza virus and intranasal boosting with HIV env-expressing vaccinia virus in mice induced systemic cellular responses in the spleen and local responses in the genitorectal draining lymph nodes.[183] Intranasal priming with DNA and intranasal boosting with vaccinia virus expressing HIV env induced mucosal and systemic humoral and cellular responses.[184]

In a non-human primate model, intranasal, intramuscular and intrarectal immunisations with a live attenuated pox virus (NYVAC) expressing an immunodominant CD8+ CTL gag-epitope induced CTL responses in the small intestine.[185] An IL-2-augmented DNA intranasal prime/vaccinia virus intranasal boost induced mucosal and systemic humoral and cellular responses and protected from disease, i.e. all animals became infected but maintained CD4 cell counts and did not develop AIDS.[186] Other studies in the rhesus macaque model have also demonstrated the immunogenicity and partial protective efficacy of the combination of parenteral and mucosal immunisations with a combination of DNA prime and vaccinia virus boosts.[187-189]

Recently, the safety and immunogenicity of an HIV recombinant canarypox vaccine (ALVAC), expressing HIV gag and env proteins, was tested in infants of HIV-infected women. The vaccine was administered three times parenterally. While the vaccine appeared safe in the short term, as expected, it did not successfully induce mucosal IgA responses.[190] However, in another study, it was found that a canarypox virus-based vaccine expressing both HIV gag and env proteins did not induce any

immune responses when given mucosally, i.e. through the nose, vagina, rectum or orally.[191] In yet another contradicting study, intramuscular immunisations with a canarypox-based vaccine induced rectal and systemic CTL responses.[192] These data indicate that vaccinia virus-based mucosal vaccines as a boosting regimen can induce significant local and systemic CTL responses. However, overall, because of serious issues with induction of vaccinia virus-specific immunity and the safety issues, the widespread use of vaccinia virus-based vectors in HIV vaccine development seems limited.

Live attenuated vesicular stomatitis virus (rVSV) was shown to prevent AIDS in rhesus macaques.[193] A comparison of intranasal and intramuscular routes of immunisation with rVSV in rhesus macaques demonstrated that the intranasal route induced higher cellular as well as nasal and saliva responses, but both routes of immunisation conferred protection against vaginal SHIV challenge.[194] The cellular responses were not measured in the vaginal mucosa and the humoral responses at this site were low after intranasal immunisations, making deciphering the correlates of protection unallowable. However, intranasal immunisation was as good as or perhaps better than intramuscular immunisations for protection against AIDS. However, in another study, intraperitoneal (another systemic route of immunisation) versus intranasal immunisations with rVSV expressing HIV gag and env polyproteins induced far higher CD8+ CTL responses in spleens.[195] Although these studies indicate that rVSV may serve as a vaccine delivery system for a potential anti-HIV vaccine, serious potential hazards exist with regard to this virus. rVSV belongs to the family of Rhabdoviridae and as an important zoonotic pathogen can cause disease in humans.[196,197] Therefore, the use of this vector as an ultimate human vaccine delivery system against HIV seems unlikely.

Adenovirus-based vaccines have been considered as a vaccine delivery system for anti-HIV vaccines for over a decade.[198-202] In early reports, adenoviruses expressing HIV env induced serum HIV-neutralising antibodies following intratracheal immunisations of dogs.[198] This study was soon followed by two chimpanzee studies using intranasal immunisations as stand-alone or as boosting modality, with adenoviruses expressing HIV gag and env inducing variable mucosal and systemic humoral responses.[199,200] To avoid pre-existing immunity, it has been suggested that DNA priming followed by adenovirus boosting would enhance the responses.[203,204] Also, priming with adenoviruses and boosting with HIV env have been reported to reduce acute viraemia following challenge.[205,206] Several reports have also demonstrated immunogenicity of replication-incompetent adenoviruses vectors as anti-HIV vaccine vectors.[202,206,207] Thus, in general, although replication-competent adenovirus vectors pose some safety concerns, they can induce mucosal and systemic humoral responses following intranasal immunisations as a stand-alone modality and they can induce strong CTL responses following parenteral immunisations as a stand-alone or as a boosting modality. The use of replication-incompetent adenovirus vectors might circumvent the safety issues, but may result in significantly reduced immune responses that may, nonetheless, be sufficient to reduce viraemia.

11. Mucosal Live Attenuated Bacteria-Based Vaccines against HIV

Immunisation with invasive bacterial systems has been explored. Examples include the use of live attenuated bacterial gene delivery systems using *Shigella, Salmonella and Listeria* as vector systems, generating strong responses to HIV antigens after intranasal immunisations. A single intranasal dose of Shigella/HIV-1 gp120 vaccine vector in mice induced a strong CD8+ T-cell response comparable to systemic immunisation with a vaccinia-env vector.[208] In addition to this, single immunisation was

Protein-based vaccines	Inactivated virus, VLP, peptides, recombinant proteins, all with or without adjuvants and/or delivery systems
DNA-based vaccines	Plasmid DNA with or without delivery systems; live attenuated DNA viral or bacterial vectors
RNA-based vaccines	Alphavirus-based replicon particles, live attenuated RNA viruses

Fig. 3. General experimental HIV vaccine approaches in small animals, non-human primates and humans. **VLP** = virus-like particles.

also effective in affording protection against a vaccinia-env challenge and this vector was also effective given orally in mice.[209] *Shigella* and *Salmonella* delivering HIV-1 gp120 were administered intranasally to mice,[210] showing that *Shigella* was more efficient than *Salmonella* in generating CD8+ T-cell responses. Furthermore, the intranasal route was more effective than the intramuscular route for generating IgA responses in vaginal washes. Another study reported that a single intranasal dose of *Shigella fleneri* 2a mutant encoding for HIV-1 SF2 gag was effective in inducing gag-specific T-cell responses both in the spleen and the lung.[211] The live bacterial delivery as an approach is currently being explored more through the oral route than the intranasal route for ease of administration and safety reasons.

12. Discussion

There is general agreement that mucosal vaccination best affords immunity at mucosal surfaces. Most HIV transmissions occur through the female genital tract and rectum and then spread systemically, targeting CD4+ T cells at all mucosal effector sites, including the intestine and the lungs, regardless of the route of transmission. Thus, immunity induced by B cells and CTL against HIV will be required at the site of virus entry, draining lymph nodes, distant mucosal tissues and systemically. The combination of mucosal and parenteral vaccinations may achieve this goal. For mucosal priming or boosting immunisations, various protein-, DNA- and RNA-based experimental vaccines have been tested. Moreover, attenuated viral or bacterial vectors have also been investigated. These widely different approaches have taught us that it is unlikely that a single vaccine modality will be effective against HIV infection and that there is a fine balance between achieving immunity and being safe on the one hand and inducing B versus CTL responses on the other. Moreover, while intranasal immunisation may prove effective at induction of vaginal responses, it may not readily induce immunity in the intestine, a major site of rapid HIV replication and CD4+ T-cell depletion. However, rectal immunisation may induce immunity, not only in the rectum, but also in the vaginal mucosa, which may alleviate some safety concerns regarding the intranasal route of immunisation. Whether the rectal route will result in immunity in the small intestine is not well established, although it is assumed that this is likely to be the case.

Figure 3 lists the experimental approaches described in this review. The International AIDS Vaccine Initiative lists those approaches that have reached clinical trials, including recombinant proteins, and live attenuated viruses.[212]

13. Conclusion

The literature reviewed in this paper suggest that future vaccinations against HIV may include combinations of mucosal and systemic routes to induce both B- and T-cell responses, using optimal adjuvants and delivery systems. This may be necessary if mucosal sites of HIV entry as well as systemic tissues, including the brain, should be protected from HIV pathogenesis. Thus far, published data on the combinations of mucosal and systemic immunisations show promise, although significantly more experiments are yet to be performed.

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References

1. Girard MP, Osmanov SK, Kieny MP. A review of vaccine research and development: the human immunodeficiency virus (HIV). Vaccine 2006; 24 (19): 4062-81. Epub 2006 Feb 28
2. Haase AT, Henry K, Zupancic M, et al. Quantitative image analysis of HIV-1 infection in lymphoid tissue. Science 1996 Nov 8; 274 (5289): 985-9
3. Spira AI, Marx PA, Patterson BK, et al. Cellular targets of infection and route of viral dissemination after an intravaginal inoculation of simian immunodeficiency virus into rhesus macaques. J Exp Med 1996 Jan 1; 183 (1): 215-25
4. Heise C, Vogel P, Miller CJ, et al. Distribution of SIV infection in the gastrointestinal tract of rhesus macaques at early and terminal stages of AIDS. J Med Primatol 1993 Feb; 22 (2-3): 187-93
5. Mazzoli S, Trabattoni D, Lo CS, et al. HIV-specific mucosal and cellular immunity in HIV-seronegative partners of HIV-seropositive individuals. Nat Med 1997 Nov; 3 (11): 1250-7
6. Lehner T, Wang Y, Cranage M, et al. Protective mucosal immunity elicited by targeted iliac lymph node immunization with a subunit SIV envelope and core vaccine in macaques. Nat Med 1996 Jul; 2 (7): 767-75
7. Bomsel M, Heyman M, Hocini H, et al. Intracellular neutralization of HIV transcytosis across tight epithelial barriers by anti-HIV envelope protein dIgA or IgM. Immunity 1998 Aug; 9 (2): 277-87
8. McGhee JR, Lamm ME, Strober W. Mucosal immune responses. In: Ogra PL, Mestecky J, Lamm ME, et al., editors. Mucosal immunology. San Diego (CA): Academic Press, 1999: 485-506
9. Gallichan WS, Rosenthal KL. Specific secretory immune responses in the female genital tract following intranasal immunization with a recombinant adenovirus expressing glycoprotein B of herpes simplex virus. Vaccine 1995; 13 (16): 1589-95
10. Staats HF, Montgomery SP, Palker TJ. Intranasal immunization is superior to vaginal, gastric, or rectal immunization for the induction of systemic and mucosal anti-HIV antibody responses. AIDS Res Hum Retroviruses 1997; 13 (11): 945-52
11. Wu HY, Abdu S, Stinson D, et al. Generation of female genital tract antibody responses by local or central (Common) mucosal immunization. Infect Immun 2000; 68 (10): 5539-45
12. Johansson EL, Rask C, Fredriksson M, et al. Antibodies and antibody-secreting cells in the female genital tract after vaginal or intranasal immunization with cholera toxin B subunit or conjugates. Infect Immun 1998 Feb; 66 (2): 514-20
13. Di Tommaso A, Saletti G, Pizza M, et al. Induction of antigen-specific antibodies in vaginal secretions by using a nontoxic mutant of heat-labile enterotoxin as a mucosal adjuvant. Infect Immun 1996; 64 (3): 974-9
14. Bergquist C, Johansson EL, Lagergard T, et al. Intranasal vaccination of humans with recombinant cholera toxin B subunit induces systemic and local antibody responses in the upper respiratory tract and the vagina. Infect Immun 1997; 65 (7): 2676-84
15. van Ginkel FW, Jackson RJ, Yuki Y, et al. Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. J Immunol 2000 Nov 1; 165 (9): 4778-82
16. Duverger A, Jackson RJ, van Ginkel FW, et al. Bacillus anthracis edema toxin acts as an adjuvant for mucosal immune responses to nasally administered vaccine antigens. J Immunol 2006 Feb 1; 176 (3): 1776-83
17. Eriksson AM, Schon KM, Lycke NY. The cholera toxin-derived CTA1-DD vaccine adjuvant administered intranasally does not cause inflammation or accumulate in the nervous tissues. J Immunol 2004 Sep 1; 173 (5): 3310-9
18. Lycke N. From toxin to adjuvant: basic mechanisms for the control of mucosal IgA immunity and tolerance. Immunol Lett 2005 Mar 15; 97 (2): 193-8
19. Boyaka PN, Tafaro A, Fischer R, et al. Therapeutic manipulation of the immune system: enhancement of innate and adaptive mucosal immunity. Curr Pharm Des 2003; 9 (24): 1965-72
20. Fujimura Y. Evidence of M cells as portals of entry for antigens in the nasopharyngeal lymphoid tissue of humans. Virchows Arch 2000 Jun; 436 (6): 560-6
21. Pope M. Mucosal dendritic cells and immunodeficiency viruses. J Infect Dis 1999 May; 179 Suppl. 3: S427-30
22. Moore KL, Dalley AF. Clinically oriented anatomy. 4th ed. Baltimore (MD): Lippincott Williams & Wilkins, 1999
23. Neutra MR, Frey A, Kraehenbuhl JP. Epithelial M cells: gateways for mucosal infection and immunization. Cell 1996 Aug 9; 86 (3): 345-8
24. Kutteh WH, Moldoveanu Z, Mestecky J. Mucosal immunity in the female reproductive tract: correlation of immunoglobulins, cytokines, and reproductive hormones in human cervical mucus around the time of ovulation. AIDS Res Hum Retroviruses 1998; 14 Suppl. 1: S51-5
25. Crowley-Nowick PA, Bell MC, Brockwell R, et al. Rectal immunization for induction of specific antibody in the genital tract of women. J Clin Immunol 1997 Sep; 17 (5): 370-9
26. Mestecky J, Fultz PN. Mucosal immune system of the human genital tract. J Infect Dis 1999 May; 179 Suppl. 3: S470-4
27. Vajdy M, Veazey RS, Knight HK, et al. Differential effects of simian immunodeficiency virus infection on immune inductive and effector sites in the rectal mucosa of rhesus macaques. Am J Pathol 2000 Aug; 157 (2): 485-95
28. Pavli P, Maxwell L, van de Pol E, et al. Distribution of human colonic dendritic cells and macrophages: functional implications. Adv Exp Med Biol 1995; 378: 121-3
29. Yeaman GR, White HD, Howell A, et al. The mucosal immune system in the human female reproductive tract: potential insights into the heterosexual transmission of HIV. AIDS Res Hum Retroviruses 1998 Apr; 14 Suppl. 1: S57-62
30. Johansson EL, Rudin A, Wassen L, et al. Distribution of lymphocytes and adhesion molecules in human cervix and vagina. Immunology 1999 Feb; 96 (2): 272-7

31. Hu J, Gardner MB, Miller CJ. Simian immunodeficiency virus rapidly penetrates the cervicovaginal mucosa after intravaginal inoculation and infects intraepithelial dendritic cells. J Virol 2000; 74 (13): 6087-95
32. Lohman BL, Miller CJ, McChesney MB. Antiviral cytotoxic T lymphocytes in vaginal mucosa of simian immunodeficiency virus-infected rhesus macaques. J Immunol 1995 Dec 15; 155 (12): 5855-60
33. McChesney MB, Collins JR, Miller CJ. Mucosal phenotype of antiviral cytotoxic T lymphocytes in the vaginal mucosa of SIV-infected rhesus macaques. AIDS Res Hum Retroviruses 1998 Apr; 14 Suppl. 1: S63-S6
34. Challacombe SJ, Rahman D, O'Hagan DT. Salivary, gut, vaginal and nasal antibody responses after oral immunization with biodegradable microparticles. Vaccine 1997 Feb; 15 (2): 169-75
35. Ugozzoli M, O'Hagan DT, Ott GS. Intranasal immunization of mice with herpes simplex virus type 2 recombinant gD2: the effect of adjuvants on mucosal and serum antibody responses. Immunology 1998; 93 (4): 563-71
36. Parr MB, Parr EL. Immunohistochemical localization of immunoglobulins A, G and M in the mouse female genital tract. J Reprod Fertil 1985; 74 (2): 361-70
37. Thaparr MA, Parr EL, Bozzola JJ, et al. Secretory immune responses in the mouse vagina after parenteral or intravaginal immunization with an immunostimulating complex (ISCOM). Vaccine 1991; 9 (2): 129-33
38. O'Hagan DT, Rafferty D, McKeating JA, et al. Vaginal immunization of rats with a synthetic peptide from human immunodeficiency virus envelope glycoprotein. J Gen Virol 1992; 73 (Pt 8): 2141-5
39. O'Hagan DT, Rafferty D, Wharton S, et al. Intravaginal immunization in sheep using a bioadhesive microsphere antigen delivery system. Vaccine 1993; 11 (6): 660-4
40. Vajdy M, Gardner J, Neidleman J, et al. Human immunodeficiency virus type 1 Gag-specific vaginal immunity and protection after local immunizations with sindbis virus-based replicon particles. J Infect Dis 2001; 184 (12): 1613-6
41. Wira CR, Richardson J, Prabhala R. Endocrine regulation of mucosal immunity: effect of sex hormones and cytokines on the afferent and efferent arms of the immune system in the female reproductive tract. In: Ogra PL, Lamm ME, Mcghee JR, et al., editors. Handbook of mucosal immunology. San Diego (CA): Academic Press, 1994: 705-18
42. Kaul R, Trabattoni D, Bwayo JJ, et al. HIV-1-specific mucosal IgA in a cohort of HIV-1-resistant Kenyan sex workers. AIDS 1999 Jan 14; 13 (1): 23-9
43. Devito C, Hinkula J, Kaul R, et al. Cross-clade HIV-1-specific neutralizing IgA in mucosal and systemic compartments of HIV-1-exposed, persistently seronegative subjects. J Acquir Immune Defic Syndr 2002; 30 (4): 413-20
44. Vajdy M, Veazey RS, Knight HK, et al. Differential effects of simian immunodeficiency virus infection on immune inductive and effector sites in the rectal mucosa of rhesus macaques. Am J Pathol 2000 Aug; 157 (2): 485-95
45. Sissons JG, Oldstone MB. Killing of virus-infected cells by cytotoxic lymphocytes. J Infect Dis 1980 Jul; 142 (1): 114-9
46. Kohl S. Role of antibody-dependent cellular cytotoxicity in defense against herpes simplex virus infections. Rev Infect Dis 1991 Jan-Feb; 13 (1): 108-14
47. Kohl S, Strynadka NC, Hodges RS, et al. Analysis of the role of antibody-dependent cellular cytotoxic antibody activity in murine neonatal herpes simplex virus infection with antibodies to synthetic peptides of glycoprotein D and monoclonal antibodies to glycoprotein B. J Clin Invest 1990 Jul; 86 (1): 273-8
48. Sawyer LA, Katzenstein DA, Hendry RM, et al. Possible beneficial effects of neutralizing antibodies and antibody-dependent, cell-mediated cytotoxicity in human immunodeficiency virus infection. AIDS Res Hum Retroviruses 1990 Mar; 6 (3): 341-56
49. Ahmad A, Menezes J. Antibody-dependent cellular cytotoxicity in HIV infections. FASEB J 1996 Feb; 10 (2): 258-66
50. Brenner BG, Gryllis C, Wainberg MA. Role of antibody-dependent cellular cytotoxicity and lymphokine-activated killer cells in AIDS and related diseases. J Leukoc Biol 1991; 50: 628-40
51. Tyler DS, Lyerly HK, Weinhold KJ. Anti-HIV-1 ADCC. AIDS Res Hum Retroviruses 1989 Dec; 5 (6): 557-63
52. Kozlowski PA, Cu-Uvin S, Neutra MR, et al. Mucosal vaccination strategies for women. J Infect Dis 1999 May; 179 Suppl. 3: S493-S8
53. Kozlowski PA, Cu-Uvin S, Neutra MR, et al. Comparison of the oral, rectal, and vaginal immunization routes for induction of antibodies in rectal and genital tract secretions of women. Infect Immun 1997 Apr; 65 (4): 1387-94
54. Eriksson K, Quiding-Jarbrink M, Osek J, et al. Specific antibody-secreting cells in the rectums and genital tracts of nonhuman primates following vaccination. Infect Immun 1998 Dec; 66 (12): 5889-96
55. Giuliani MM, Del Giudice G, Giannelli V, et al. Mucosal adjuvanticity and immunogenicity of LTR72, a novel mutant of Escherichia coli heat-labile enterotoxin with partial knockout of ADP-ribosyltransferase activity. J Exp Med 1998 Apr 6; 187: 1123-32
56. Asanuma H, Aizawa C, Kurata T, et al. IgA antibody-forming cell responses in the nasal-associated lymphoid tissue of mice vaccinated by intranasal, intravenous and/or subcutaneous administration. Vaccine 1998 Aug; 16 (13): 1257-62
57. Lowell GH, Kaminski RW, VanCott TC, et al. Proteosomes, emulsomes, and cholera toxin B improve nasal immunogenicity of human immunodeficiency virus gp160 in mice: induction of serum, intestinal, vaginal, and lung IgA and IgG. J Infect Dis 1997 Feb; 175 (2): 292-301
58. Gupta S, Janani R, Bin Q, et al. Characterization of HIV-gag-specific IFNg-expressing cells following protective mucosal immunization with alphavirus replicon particles. J Virol 2005; 79 (11): 7135-45
59. Imaoka K, Miller CJ, Kubota M, et al. Nasal immunization of nonhuman primates with simian immunodeficiency virus p55gag and cholera toxin adjuvant induces Th1/Th2 help for virus-specific immune responses in reproductive tissues. J Immunol 1998 Dec 1; 161 (11): 5952-8
60. Moldoveanu Z, Clements ML, Prince SJ, et al. Human immune responses to influenza virus vaccines administered by systemic or mucosal routes. Vaccine 1995 Aug; 13 (11): 1006-12

61. Enose Y, Ui M, Miyake A, et al. Protection by intranasal immunization of a nef-deleted, nonpathogenic SHIV against intravaginal challenge with a heterologous pathogenic SHIV. Virology 2002 Jul 5; 298 (2): 306-16
62. Keren DF, McDonald RA, Carey JL. Combined parenteral and oral immunization results in an enhanced mucosal immunoglobulin A response to Shigella flexneri. Infect Immun 1988 Apr; 56 (4): 910-5
63. Koster FT, Pierce NF. Parenteral immunization causes antigen-specific cell-mediated suppression of an intestinal IgA response. J Immunol 1983 Jul; 131 (1): 115-9
64. Bruhl P, Kerschbaum A, Eibl MM, et al. An experimental prime-boost regimen leading to HIV type 1-specific mucosal and systemic immunity in BALB/c mice. AIDS Res Hum Retroviruses 1998 Mar 20; 14 (5): 401-7
65. Zolla-Pazner S, Lubeck M, Xu S, et al. Induction of neutralizing antibodies to T-cell line-adapted and primary human immunodeficiency virus type 1 isolates with a prime-boost vaccine regimen in chimpanzees. J Virol 1998 Feb; 72 (2): 1052-9
66. Vajdy M, Singh M, Kazzaz J, et al. Mucosal and systemic anti-HIV responses in rhesus macaques following combinations of intra-nasal and parenteral immunizations. AIDS Res Hum Retroviruses 2004; 20 (11): 1269-81
67. Vajdy M, Singh M, Ugozzoli M, et al. Enhanced mucosal and systemic immune responses to *Helicobacter pylori* antigens through mucosal priming followed by systemic boosting immunizations. Immunology 2003 Sep; 110 (1): 86-94
68. Herremans TM, Reimerink JH, Buisman AM, et al. Induction of mucosal immunity by inactivated poliovirus vaccine is dependent on previous mucosal contact with live virus. J Immunol 1999 Apr 15; 162 (8): 5011-8
69. Cyster JG. Homing of antibody secreting cells. Immunol Rev 2003 Aug; 194: 48-60
70. Kunkel EJ, Butcher EC. Plasma-cell homing. Nat Rev Immunol 2003 Oct; 3 (10): 822-9
71. Johansson C, Ahlstedt I, Furubacka S, et al. Differential expression of chemokine receptors on human IgA+ and IgG+ B cells. Clin Exp Immunol 2005 Aug; 141 (2): 279-87
72. Williams IR. Chemokine receptors and leukocyte trafficking in the mucosal immune system. Immunol Res 2004; 29 (1-3): 283-92
73. King NJ, Parr EL, Parr MB. Migration of lymphoid cells from vaginal epithelium to iliac lymph nodes in relation to vaginal infection by herpes simplex virus type 2. J Immunol 1998 Feb 1; 160 (3): 1173-80
74. Andrew DP, Ruffing N, Kim CH, et al. C-C chemokine receptor 4 expression defines a major subset of circulating nonintestinal memory T cells of both Th1 and Th2 potential. J Immunol 2001 Jan 1; 166 (1): 103-11
75. Campbell DJ, Butcher EC. Rapid acquisition of tissue-specific homing phenotypes by CD4 (+) T cells activated in cutaneous or mucosal lymphoid tissues. J Exp Med 2002 Jan 7; 195 (1): 135-41
76. Krieg C, Maier R, Meyerhans A. Gut-homing (alpha (4)beta (7) (+)) Th1 memory responses after inactivated poliovirus immunization in poliovirus orally pre-immunized donors. J Gen Virol 2004 Jun; 85 (Pt 6): 1571-9
77. Rappuoli R. Reverse vaccinology, a genome-based approach to vaccine development. Vaccine 2001 Mar 21; 19 (17-19): 2688-91
78. Mora M, Veggi D, Santini L, et al. Reverse vaccinology: identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. From toxin to adjuvant: the rational design of a vaccine adjuvant vector, CTA1-DD/ISCOM. Drug Discov Today 2003 May 15; 8 (10): 459-64
79. Rappuoli R, Pizza M, Scarlato V, et al. Reverse vaccinology. Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. Curr Opin Microbiol 2000 Oct; 3 (5): 445-50
80. Pizza M, Scarlato V, Masignani V, et al. Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. Science 2000 Mar 10; 287 (5459): 1816-20
81. Clements JD, Finkelstein RA. Isolation and characterization of homogeneous heat-labile enterotoxins with high specific activity from *Escherichia coli* cultures. Infect Immun 1979; 24 (3): 760-9
82. Clements JD, Yancey RJ, Finkelstein RA. Properties of homogeneous heat-labile enterotoxin from *Escherichia coli*. Infect Immun 1980; 29 (1): 91-7
83. Pizza M, Domenighini M, Hol W, et al. Probing the structure-activity relationship of *Escherichia coli* LT-A by site-directed mutagenesis. Mol Microbiol 1994; 14 (1): 51-60
84. Pizza M, Fontana MR, Giuliani MM, et al. A genetically detoxified derivative of heat-labile *Escherichia coli* enterotoxin induces neutralizing antibodies against the A subunit. J Exp Med 1994; 180 (6): 2147-53
85. Partidos CD, Pizza M, Rappuoli R, et al. The adjuvant effect of a non-toxic mutant of heat-labile enterotoxin of *Escherichia coli* for the induction of measles virus-specific CTL responses after intranasal co-immunization with a synthetic peptide. Immunology 1996 Dec; 89 (4): 483-7
86. Neidleman JA, Vajdy M, Ugozzoli M, et al. Potent non-toxic mutant heat-labile enterotoxins as adjuvants for induction of HIV-1_{SF2} p55 gag-specific cytotoxic responses through mucosal immunization. Immunology 2000; 101: 154-60
87. Ugozzoli M, Santos G, Donnelly J, et al. Potency of a genetically detoxified mucosal adjuvant derived from the heat-labile enterotoxin of *Escherichia coli* (LTK63) is not adversely affected by the presence of preexisting immunity to the adjuvant. J Infect Dis 2001; 183 (2): 351-4
88. Staats HF, Nichols WG, Palker TJ, et al. Mucosal immunity to HIV-1: systemic and vaginal antibody responses after intranasal immunization with the HIV-1 C4/V3 peptide T1SP10 MN (A). J Immunol 1996 Jul 1; 157 (1): 462-72
89. Holmgren J, Adamsson J, Anjuere F, et al. Mucosal adjuvants and anti-infection and anti-immunopathology vaccines based on cholera toxin, cholera toxin B subunit and CpG DNA. Immunol Lett 2005 Mar 15; 97 (2): 181-8
90. Bird AP. CpG islands as gene markers in the vertebrate nucleus. Trends Genet 1987; 3: 342-7
91. Hemmi H, Takeuchi O, Kawai T, et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000; 408 (6813): 740-5
92. Klinman DM, Barnhart KM, Conover J. CpG motifs as immune adjuvants. Vaccine 1999; 17 (1): 19-25

93. Broide D, Schwarze J, Tighe H, et al. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. J Immunol 1998; 161 (12): 7054-62
94. Pulendran B. Modulating vaccine responses with dendritic cells and Toll-like receptors. Immunol Rev 2004 Jun; 199: 227-50
95. Jiang JQ, Patrick A, Moss RB, et al. CD8+ T-cell-mediated cross-clade protection in the genital tract following intranasal immunization with inactivated human immunodeficiency virus antigen plus CpG oligodeoxynucleotides. J Virol 2005 Jan; 79 (1): 393-400
96. Dumais N, Patrick A, Moss RB, et al. Mucosal immunization with inactivated human immunodeficiency virus plus CpG oligodeoxynucleotides induces genital immune responses and protection against intravaginal challenge. J Infect Dis 2002 Oct 15; 186 (8): 1098-105. Epub 2002 Sep 30
97. Kang SM, Guo L, Yao Q, et al. Intranasal immunization with inactivated influenza virus enhances immune responses to coadministered simian-human immunodeficiency virus-like particle antigens. J Virol 2004 Sep; 78 (18): 9624-32
98. Horner AA, Datta SK, Takabayashi K, et al. Immunostimulatory DNA-based vaccines elicit multifaceted immune responses against HIV at systemic and mucosal sites. J Immunol 2001; 167 (3): 1584-91
99. Clark MA, Hirst BH, Jepson MA. Lectin-mediated mucosal delivery of drugs and microparticles. Adv Drug Deliv Rev 2000 Sep 30; 43 (2-3): 207-23
100. Manocha M, Pal PC, Chitralekha KT, et al. Enhanced mucosal and systemic immune response with intranasal immunization of mice with HIV peptides entrapped in PLG microparticles in combination with Ulex Europaeus-I lectin as M cell target. Vaccine 2005; 23 (48-49): 5599-617
101. Miyake A, Akagi T, Enose Y, et al. Induction of HIV-specific antibody response and protection against vaginal SHIV transmission by intranasal immunization with inactivated SHIV-capturing nanospheres in macaques. J Med Virol 2004 Jul; 73 (3): 368-77
102. Okada H, Toguchi H. Biodegradable microspheres in drug delivery. Crit Rev Ther Drug Carrier Syst 1995; 12 (1): 1-99
103. Putney SD, Burke PA. Improving protein therapeutics with sustained-release formulations. Nat Biotechnol 1998 Feb; 16 (2): 153-7
104. Eldridge JH, Staas JK, Meulbroek JA, et al. Biodegradable and biocompatible poly (DL-lactide-co-glycolide) microspheres as an adjuvant for staphylococcal enterotoxin B toxoid which enhances the level of toxin-neutralizing antibodies. Infect Immun 1991 Sep; 59 (9): 2978-86
105. O'Hagan DT, Rahman D, McGee JP, et al. Biodegradable microparticles as controlled release antigen delivery systems. Immunology 1991 Jun; 73 (2): 239-42
106. O'Hagan DT, Jeffery H, Roberts MJ, et al. Controlled release microparticles for vaccine development. Vaccine 1991 Oct; 9 (10): 768-71
107. O'Hagan DT, Jeffery H, Davis SS. Long-term antibody responses in mice following subcutaneous immunization with ovalbumin entrapped in biodegradable microparticles. Vaccine 1993; 11 (9): 965-9
108. Maloy KJ, Donachie AM, O'Hagan DT, et al. Induction of mucosal and systemic immune responses by immunization with ovalbumin entrapped in poly (lactide-co-glycolide) microparticles. Immunology 1994 Apr; 81 (4): 661-7
109. Moore A, McGuirk P, Adams S, et al. Immunization with a soluble recombinant HIV protein entrapped in biodegradable microparticles induces HIV-specific CD8+ cytotoxic T lymphocytes and CD4+ Th1 cells. Vaccine 1995 Dec; 13 (18): 1741-9
110. Nixon DF, Hioe C, Chen PD, et al. Synthetic peptides entrapped in microparticles can elicit cytotoxic T cell activity. Vaccine 1996 Nov; 14 (16): 1523-30
111. Randolph GJ, Inaba K, Robbiani DF, et al. Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. Immunity 1999 Dec; 11 (6): 753-61
112. Lutsiak ME, Robinson DR, Coester C, et al. Analysis of poly (D,L-lactic-co-glycolic acid) nanosphere uptake by human dendritic cells and macrophages in vitro. Pharm Res 2002; 19 (10): 1480-7
113. Newman KD, Elamanchili P, Kwon GS, et al. Uptake of poly (D,L-lactic-co-glycolic acid) microspheres by antigen-presenting cells in vivo. J Biomed Mater Res 2002; 60 (3): 480-6
114. Kazzaz J, Neidleman J, Singh M, et al. Novel anionic microparticles are a potent adjuvant for the induction of cytotoxic T lymphocytes against recombinant p55 gag from HIV-1. J Control Release 2000; 67 (2-3): 347-56
115. Alving CR. Immunologic aspects of liposomes: presentation and processing of liposomal protein and phospholipid antigens. Biochim Biophys Acta 1992 Dec 11; 1113 (3-4): 307-22
116. Gregoriadis G. Immunological adjuvants: a role for liposomes. Immunol Today 1990 Mar; 11 (3): 89-97
117. VanCott TC, Kaminski RW, Mascola JR, et al. HIV-1 neutralizing antibodies in the genital and respiratory tracts of mice intranasally immunized with oligomeric gp160. J Immunol 1998 Feb 15; 160 (4): 2000-12
118. Ambrosch F, Wiedermann G, Jonas S, et al. Immunogenicity and protectivity of a new liposomal hepatitis A vaccine. Vaccine 1997 Aug; 15 (11): 1209-13
119. Sakaue G, Hiroi T, Nakagawa Y, et al. HIV mucosal vaccine: nasal immunization with gp160-encapsulated hemagglutinating virus of Japan-liposome induces antigen-specific CTLs and neutralizing antibody responses. J Immunol 2003 Jan 1; 170 (1): 495-502
120. Lian T, Bui T, Ho RJ. Formulation of HIV-envelope protein with lipid vesicles expressing ganglioside GM1 associated to cholera toxin B enhances mucosal immune responses. Vaccine 1999 Nov 12; 18 (7-8): 604-11
121. Barr IG, Sjolander A, Cox JC. ISCOMs and other saponin based adjuvants. Adv Drug Deliv Rev 1998; 32: 247-71
122. Hu KF, Lovgren-Bengtsson K, Morein B. Immunostimulating complexes (ISCOMs) for nasal vaccination. Adv Drug Deliv Rev 2001 Sep 23; 51 (1-3): 149-59
123. Lovgren-Bengtsson K, Morein B. The ISCOM™ technology. In: O'Hagan D, editor. Vaccine adjuvants: preparation methods and research protocols. Totowa (NJ): Humana Press, 2000: 239-58
124. Bradney CP, Sempowski GD, Liao HX, et al. Cytokines as adjuvants for the induction of anti-human immunodeficiency

virus peptide immunoglobulin G (IgG) and IgA antibodies in serum and mucosal secretions after nasal immunization. J Virol 2002; 76 (2): 517-24

125. Albu DI, Jones-Trower A, Woron AM, et al. Intranasal vaccination using interleukin-12 and cholera toxin subunit B as adjuvants to enhance mucosal and systemic immunity to human immunodeficiency virus type 1 glycoproteins. J Virol 2003 May; 77 (10): 5589-97
126. Ahlers JD, Belyakov intramuscular, Berzofsky JA. Cytokine, chemokine, and costimulatory molecule modulation to enhance efficacy of HIV vaccines. Curr Mol Med 2003 May; 3 (3): 285-301
127. Akagi T, Kawamura M, Ueno M, et al. Mucosal immunization with inactivated HIV-1-capturing nanospheres induces a significant HIV-1-specific vaginal antibody response in mice. J Med Virol 2003 Feb; 69 (2): 163-72
128. Kawahara M, Matsuo K, Nakasone T, et al. Combined intrarectal/intradermal inoculation of recombinant *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) induces enhanced immune responses against the inserted HIV-1 V3 antigen. Vaccine 2002 Dec 13; 21 (3-4): 158-66
129. Golding B, Eller N, Levy L, et al. Mucosal immunity in mice immunized with HIV-1 peptide conjugated to *Brucella abortus*. Vaccine 2002 Jan 31; 20 (9-10): 1445-50
130. Deml L, Speth C, Dierich MP, et al. Recombinant HIV-1 Pr55gag virus-like particles: potent stimulators of innate and acquired immune responses. Mol Immunol 2005 Feb; 42 (2): 259-77
131. Yao Q, Bu Z, Vzorov A, et al. Virus-like particle and DNA-based candidate AIDS vaccines. Vaccine 2003 Jan 30; 21 (7-8): 638-43
132. Yao Q. Enhancement of mucosal immune responses by chimeric influenza HA/SHIV virus-like particles. Res Initiat Treat Action 2003 Spring; 8 (2): 20-1
133. Gronowski AM, Hilbert DM, Sheehan KC, et al. Baculovirus stimulates antiviral effects in mammalian cells. J Virol 1999 Dec; 73 (12): 9944-51
134. Donnelly JJ, Ulmer JB, Liu MA. DNA vaccines. Life Sci 1997; 60 (3): 163-72
135. Donnelly JJ, Friedman A, Martinez D, et al. Preclinical efficacy of a prototype DNA vaccine: enhanced protection against antigenic drift in influenza virus. Nat Med 1995; 1 (6): 583-7
136. Wang R, Doolan DL, Le TP, et al. Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. Science 1998; 282 (5388): 476-80
137. Roy MJ, Wu MS, Barr LJ, et al. Induction of antigen-specific CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. Vaccine 2000; 19 (7-8): 764-78
138. Haas J, Park E, Seed B. Codon usage limitation in the expression of HIV-1 envelope glycoprotein. Curr Biol 1996; 6 (3): 315-24
139. zur Megede J, Chen MC, Doe B, et al. Increased expression and immuunogenicity of sequence-modified human immunodeficiency virus type 1 gag gene. J Virol 2000 Mar; 74 (6): 2628-35
140. Andre S, Seed B, Eberle J, et al. Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. J Virol 1998 Feb; 72 (2): 1497-503
141. Uchijima M, Yoshida A, Nagata T, et al. Optimization of codon usage of plasmid DNA vaccine is required for the effective MHC class I-restricted T cell responses against an intracellular bacterium. J Immunol 1998; 161 (10): 5594-9
142. Sasaki S, Tsuji T, Asakura Y, et al. The search for a potent DNA vaccine against AIDS: the enhancement of immunogenicity by chemical and genetic adjuvants. Anticancer Res 1998; 18 (5D): 3907-16
143. Wang X, Kochetkova I, Haddad A, et al. Transgene vaccination using Ulex europaeus agglutinin I (UEA-1) for targeted mucosal immunization against HIV-1 envelope. Vaccine 2005 May 31; 23 (29): 3836-42
144. Okada E, Sasaki S, Ishii N, et al. Intranasal immunization of a DNA vaccine with IL-12- and granulocyte-macrophage colony-stimulating factor (GM-CSF)-expressing plasmids in liposomes induces strong mucosal and cell-mediated immune responses against HIV-1 antigens. J Immunol 1997 Oct 1; 159 (7): 3638-47
145. Wierzbicki A, Kiszka I, Kaneko H, et al. Immunization strategies to augment oral vaccination with DNA and viral vectors expressing HIV envelope glycoprotein. Vaccine 2002; 20 (9-10): 1295-307
146. Xin KQ, Lu Y, Hamajima K, et al. Immunization of RANTES expression plasmid with a DNA vaccine enhances HIV-1-specific immunity. Clin Immunol 1999; 92 (1): 90-6
147. Wang X, Hone DM, Haddad A, et al. M cell DNA vaccination for CTL immunity to HIV. J Immunol 2003 Nov 1; 171 (9): 4717-25
148. Hamajima K, Kojima Y, Matsui K, et al. Chitin micro-particles (CMP): a useful adjuvant for inducing viral specific immunity when delivered intranasally with an HIV-DNA vaccine. Viral Immunol 2003; 16 (4): 541-7
149. Singh M, Vajdy M, Gardner J, et al. Mucosal immunization with HIV-1 gag DNA on cationic microparticles prolongs gene expression and enhances local and systemic immunity. Vaccine 2001; 20 (3-4): 594-602
150. Yan C, Rill WL, Malli R, et al. Intranasal stimulation of long-lasting immunity against aerosol ricin challenge with ricin toxoid vaccine encapsulated in polymeric microspheres. Vaccine 1996 Aug; 14 (11): 1031-8
151. Kuper CF, Koornstra PJ, Hameleers DM, et al. The role of nasopharyngeal lymphoid tissue. Immunol Today 1992 Jun; 13 (6): 219-24
152. Alpar HO, Almeida AJ, Brown MR. Microsphere absorption by the nasal mucosa of the rat. J Drug Target 1994; 2 (2): 147-9
153. Marx PA, Compans RW, Gettie A, et al. Protection against vaginal SIV transmission with microencapsulated vaccine. Science 1993 May 28; 260 (5112): 1323-7
154. Denis-Mize KS, Dupuis M, MacKichan ML, et al. Plasmid DNA adsorbed onto cationic microparticles mediates target gene expression and antigen presentation by dendritic cells. Gene Ther 2000; 7 (24): 2105-12
155. Singh M, Briones M, Ott G, et al. Cationic microparticles: a potent delivery system for DNA vaccines. Proc Natl Acad Sci U S A 2000; 97 (2): 811-6

156. Sasaki S, Hamajima K, Fukushima J, et al. Comparison of intranasal and intramuscular immunization against human immunodeficiency virus type 1 with a DNA-monophosphoryl lipid A adjuvant vaccine. Infect Immun 1998; 66 (2): 823-6
157. Cleland JL, Barron L, Daugherty A, et al. Development of a single-shot subunit vaccine for HIV-1. 3. Effect of adjuvant and immunization schedule on the duration of the humoral immune response to recombinant MN gp120. J Pharm Sci 1997; 85 (12): 1350-7
158. Cannon G, Weissman D. RNA based vaccines. DNA Cell Biol 2002 Dec; 21 (12): 953-61
159. Martinon F, Krishnan S, Lenzen G, et al. Induction of virus-specific cytotoxic T lymphocytes in vivo by liposome-entrapped mRNA. Eur J Immunol 1993; 23 (7): 1719-22
160. Polo JM, Gardner JP, Ji Y, et al. Alphavirus DNA and particle replicons for vaccines and gene therapy. Dev Biol (Basel) 2000; 104: 181-5
161. Li HO, Zhu YF, Asakawa M, et al. A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression. J Virol 2000 Jul; 74 (14): 6564-9
162. Schoen C, Kolb-Maurer A, Geginat G, et al. Bacterial delivery of functional messenger RNA to mammalian cells. Cell Microbiol 2005 May; 7 (5): 709-24
163. Michiels A, Tuyaerts S, Bonehill A, et al. Electroporation of immature and mature dendritic cells: implications for dendritic cell-based vaccines. Gene Ther 2005 May; 12 (9): 772-82
164. Yoshii K, Hayasaka D, Goto A, et al. Packaging the replicon RNA of the Far-Eastern subtype of tick-borne encephalitis virus into single-round infectious particles: development of a heterologous gene delivery system. Vaccine 2005 Jun 10; 23 (30): 3946-56
165. Shinya K, Fujii Y, Ito H, et al. Characterization of a neuraminidase-deficient influenza a virus as a potential gene delivery vector and a live vaccine. J Virol 2004 Mar 7; 78 (6): 3083-8
166. Ahlquist P, Schwartz M, Chen J, et al. Viral and host determinants of RNA virus vector replication and expression. Vaccine 2005 Mar 7; 23 (15): 1784-7
167. Rayner JO, Dryga SA, Kamrud KI. Alphavirus vectors and vaccination. Rev Med Virol 2002 Sep-Oct; 12 (5): 279-96
168. Schlesinger S. Alphavirus vectors: development and potential therapeutic applications. Expert Opin Biol Ther 2001 Mar; 1 (2): 177-91
169. Schlesinger S, Dubensky TW. Alphavirus vectors for gene expression and vaccines. Curr Opin Biotechnol 1999 Oct; 10 (5): 434-9
170. Leitner WW, Hwang LN, deVeer MJ, et al. Alphavirus-based DNA vaccine breaks immunological tolerance by activating innate antiviral pathways. Nat Med 2003 Jan; 9 (1): 33-9
171. Caley IJ, Betts MR, Irlbeck DM, et al. Humoral, mucosal, and cellular immunity in response to a human immunodeficiency virus type 1 immunogen expressed by a Venezuelan equine encephalitis virus vaccine vector. J Virol 1997 Apr; 71 (4): 3031-8
172. Berglund P, Quesada-Rolander M, Putkonen P, et al. Outcome of immunization of cynomolgus monkeys with recombinant Semliki Forest virus encoding human immunodeficiency virus type 1 envelope protein and challenge with a high dose of SHIV-4 virus. AIDS Res Hum Retroviruses 1997 Nov 20; 13 (17): 1487-95
173. Brand D, Lemiale F, Turbica I, et al. Comparative analysis of humoral immune responses to HIV type 1 envelope glycoproteins in mice immunized with a DNA vaccine, recombinant Semliki Forest virus RNA, or recombinant Semliki Forest virus particles. AIDS Res Hum Retroviruses 1998; 14 (15): 1369-77
174. Davis NL, West A, Reap E, et al. Alphavirus replicon particles as candidate HIV vaccines. IUBMB Life 2002 Apr-May; 53 (4-5): 209-11
175. Belyakov intramuscular, Moss B, Strober W, et al. Mucosal vaccination overcomes the barrier to recombinant vaccinia immunization caused by preexisting poxvirus immunity. Proc Natl Acad Sci U S A 1999 Apr 13; 96 (8): 4512-7
176. Hofmann-Lehmann R, Vlasak J, Williams AL, et al. Live attenuated, nef-deleted SIV is pathogenic in most adult macaques after prolonged observation. AIDS 2003 Jan 24; 17 (2): 157-66
177. Ruprecht RM. Live attenuated AIDS viruses as vaccines: promise or peril? Immunol Rev 1999; 170: 135-49
178. Baba TW, Liska V, Khimani AH, et al. Live attenuated, multiply deleted simian immunodeficiency virus causes AIDS in infant and adult macaques. Nat Med 1999; 5 (2): 194-203
179. Baba TW, Jeong YS, Pennick D, et al. Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques. Science 1995; 267 (5205): 1820-5
180. Wyand MS, Manson K, Montefiori DC, et al. Protection by live, attenuated simian immunodeficiency virus against heterologous challenge. J Virol 1999; 73 (10): 8356-63
181. Johnson RP, Lifson JD, Czajak SC, et al. Highly attenuated vaccine strains of simian immunodeficiency virus protect against vaginal challenge: inverse relationship of degree of protection with level of attenuation. J Virol 1999 Jun; 73 (6): 4952-61
182. Amara RR, Villinger F, Altman JD, et al. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. Science 2001; 292 (5514): 69-74
183. Gherardi MM, Najera JL, Perez-Jimenez E, et al. Prime-boost immunization schedules based on influenza virus and vaccinia virus vectors potentiate cellular immune responses against human immunodeficiency virus Env protein systemically and in the genitorectal draining lymph nodes. J Virol 2003 Jun; 77 (12): 7048-57
184. Gherardi MM, Perez-Jimenez E, Najera JL, et al. Induction of HIV immunity in the genital tract after intranasal delivery of a MVA vector: enhanced immunogenicity after DNA prime-modified vaccinia virus Ankara boost immunization schedule. J Immunol 2004 May 15; 172 (10): 6209-20
185. Stevceva L, Alvarez X, Lackner AA, et al. Both mucosal and systemic routes of immunization with the live, attenuated NYVAC/simian immunodeficiency virus SIV (gpe) recombinant vaccine result in gag-specific CD8 (+) T-cell responses in mucosal tissues of macaques. J Virol 2002 Nov; 76 (22): 11659-76
186. Bertley FM, Kozlowski PA, Wang SW, et al. Control of simian/human immunodeficiency virus viremia and disease progression after IL-2-augmented DNA-modified vaccinia virus An-

kara nasal vaccination in nonhuman primates. J Immunol 2004 Mar 15; 172 (6): 3745-57

187. Wang SW, Bertley FM, Kozlowski PA, et al. An SHIV DNA/MVA rectal vaccination in macaques provides systemic and mucosal virus-specific responses and protection against AIDS. AIDS Res Hum Retroviruses 2004 Aug; 20 (8): 846-59

188. Kent SJ, Dale CJ, Ranasinghe C, et al. Mucosally-administered human-simian immunodeficiency virus DNA and fowlpox-virus-based recombinant vaccines reduce acute phase viral replication in macaques following vaginal challenge with CCR5-tropic SHIVSF162P3. Vaccine 2005 Oct 10; 23 (42): 5009-21

189. Makitalo B, Lundholm P, Hinkula J, et al. Enhanced cellular immunity and systemic control of SHIV infection by combined parenteral and mucosal administration of a DNA prime MVA boost vaccine regimen. J Gen Virol 2004 Aug; 85 (Pt 8): 2407-19

190. Johnson DC, McFarland EJ, Muresan P, et al. Safety and immunogenicity of an HIV-1 recombinant canarypox vaccine in newborns and infants of HIV-1-infected women. J Infect Dis 2005 Dec 15; 192 (12): 2129-33

191. Wright PF, Mestecky J, McElrath MJ, et al. Comparison of systemic and mucosal delivery of 2 canarypox virus vaccines expressing either HIV-1 genes or the gene for rabies virus G protein. J Infect Dis 2004 Apr 1; 189 (7): 1221-31

192. Musey L, Ding Y, Elizaga M, et al. HIV-1 vaccination administered intramuscularly can induce both systemic and mucosal T cell immunity in HIV-1-uninfected individuals. J Immunol 2003 Jul 15; 171 (2): 1094-101

193. Rose NF, Marx PA, Luckay A, et al. An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. Cell 2001; 106 (5): 539-49

194. Egan MA, Chong SY, Rose NF, et al. Immunogenicity of attenuated vesicular stomatitis virus vectors expressing HIV type 1 Env and SIV Gag proteins: comparison of intranasal and intramuscular vaccination routes. AIDS Res Hum Retroviruses 2004 Sep; 20 (9): 989-1004

195. Haglund K, Leiner I, Kerksiek K, et al. High-level primary CD8 (+) T-cell response to human immunodeficiency virus type 1 gag and env generated by vaccination with recombinant vesicular stomatitis viruses. J Virol 2002 Mar; 76 (6): 2730-8

196. Schmitt B. Vesicular stomatitis. Vet Clin North Am Food Anim Pract 2002 Nov; 18 (3): 453-9, vii-viii

197. Letchworth GJ, Rodriguez LL, Del C Barrera J. Vesicular stomatitis. Vet J 1999 May; 157 (3): 239-60

198. Natuk RJ, Chanda PK, Lubeck MD, et al. Adenovirus-human immunodeficiency virus (HIV) envelope recombinant vaccines elicit high-titered HIV-neutralizing antibodies in the dog model. Proc Natl Acad Sci U S A 1992 Aug 15; 89 (16): 7777-81

199. Natuk RJ, Lubeck MD, Chanda PK, et al. Immunogenicity of recombinant human adenovirus-human immunodeficiency virus vaccines in chimpanzees. AIDS Res Hum Retroviruses 1993 May; 9 (5): 395-404

200. Lubeck MD, Natuk RJ, Chengalvala M, et al. Immunogenicity of recombinant adenovirus-human immunodeficiency virus vaccines in chimpanzees following intranasal administration. AIDS Res Hum Retroviruses 1994 Nov; 10 (11): 1443-9

201. Barouch DH, Nabel GJ. Adenovirus vector-based vaccines for human immunodeficiency virus type 1. Hum Gene Ther 2005 Feb; 16 (2): 149-56

202. Shiver JW, Emini EA. Recent advances in the development of HIV-1 vaccines using replication-incompetent adenovirus vectors. Annu Rev Med 2004; 55: 355-72

203. Santra S, Seaman MS, Xu L, et al. Replication-defective adenovirus serotype 5 vectors elicit durable cellular and humoral immune responses in nonhuman primates. J Virol 2005 May; 79 (10): 6516-22

204. Mascola JR, Sambor A, Beaudry K, et al. Neutralizing antibodies elicited by immunization of monkeys with DNA plasmids and recombinant adenoviral vectors expressing human immunodeficiency virus type 1 proteins. J Virol 2005 Jan; 79 (2): 771-9

205. Gomez-Roman VR, Patterson LJ, Venzon D, et al. Vaccine-elicited antibodies mediate antibody-dependent cellular cytotoxicity correlated with significantly reduced acute viremia in rhesus macaques challenged with SIVmac251. J Immunol 2005 Feb 15; 174 (4): 2185-9

206. Casimiro DR, Bett AJ, Fu TM, et al. Heterologous human immunodeficiency virus type 1 priming-boosting immunization strategies involving replication-defective adenovirus and poxvirus vaccine vectors. J Virol 2004 Oct; 78 (20): 11434-8

207. Gomez-Roman VR, Robert-Guroff M. Adenoviruses as vectors for HIV vaccines. AIDS Rev 2003 Jul-Sep; 5 (3): 178-85

208. Shata MT, Hone DM. Vaccination with a Shigella DNA vaccine vector induces antigen-specific CD8 (+) T cells and antiviral protective immunity. J Virol 2001; 75 (20): 9665-70

209. Devico AL, Fouts TR, Shata MT, et al. Development of an oral prime-boost strategy to elicit broadly neutralizing antibodies against HIV-1. Vaccine 2002 May 6; 20 (15): 1968-74

210. Vecino WH, Morin PM, Agha R, et al. Mucosal DNA vaccination with highly attenuated Shigella is superior to attenuated Salmonella and comparable to intramuscular DNA vaccination for T cells against HIV. Immunol Lett 2002 Jul 3; 82 (3): 197-204

211. Xu F, Hong M, Ulmer JB. Immunogenicity of an HIV-1 gag DNA vaccine carried by attenuated Shigella. Vaccine 2003 Jan 30; 21 (7-8): 644-8

212. International AIDS Vaccine Initiative [online]. Available from URL: http://www.iavi.org [Accessed 2006 Aug 16]

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