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Which Vaccination Strategies and Immune Responses are More Effective Against HIV Infections?

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Abstract

Vaccination is one of the most successful approaches for controlling various viral diseases. Novel approaches will be needed to develop highly effective vaccines to prevent infectious diseases such as HIV. There are many aspects of HIV-1 biology that make the development of an HIV vaccine difficult, including viral diversity, effective type of immune response, and suitable experimental model for preclinical trials. In spite of these challenges, recent published results showed that a vaccine regimen could reduce HIV infection rates by 31% in Thailand. This vaccine named as RV144 is composed of a recombinant canarypox vector expressing three HIV-1 proteins as a prime and two different recombinant HIV-1 gp120 envelope glycoproteins with alum adjuvant as a boost. In addition, a subunit vaccine constructed from the viral envelope protein could be efficiently developed using new techniques available through genetic engineering. The current HIV-1 vaccine development focuses on antibody-based approaches. It was shown that immunization with the viral envelope glycoprotein, gp120, should generate neutralizing antibodies that would prevent infection, thereby yielding protective immunity. However, HIV could develop many pathways to escape from antibodies that bind to the different parts of the viral envelope molecules. Thus, the generation of neutralizing antibodies is very difficult after viral infection or immunization protocols. Indeed, the viral envelope molecules (Env) possess glycosylated residues that cover surface epitopes for binding and neutralizing antibodies, even if the antibodies are produced. Furthermore, the trimeric structures of envelope molecules show rapid conformational changes due to the interaction with viral cell surface receptors, CCR5/CXCR4 and CD4; thus the transition state is very poor to be recognized by the immune system. Currently, studies focus on generating stable trimeric envelope molecules (gp120/gp41) as immunogens that can induce neutralizing antibodies that can compete for binding to the cell surface receptors. Altogether, it is clear that the design of a vaccine to elicit HIV-neutralizing antibodies is not straightforward, and it causes major challenges in structural biology and immunology, several other studies strongly suggest cytotoxic T-lymphocyte (CTL)-based immune responses against HIV infections. Indeed, CD8⁺ T cells play a major role in controlling viral replication during primary HIV infections and in maintaining a stable viral load during the chronic phase. In this line, live-attenuated vaccines could elicit more potent and durable pathogen-specific immune responses than inactivated or subunit vaccines. Gen-

erally, DNA vaccines are poorly immunogenic alone, and viral vector vaccines are ineffective due to vector-specific immune responses if used repeatedly; hence, the two approaches have often been tested in combination as prime-boost vaccination strategies. Indeed, the prime-boost vaccination has been considered as an efficient strategy against HIV infections. In this chapter, we will represent challenges to determine the best vaccine strategies against HIV infections.

Keywords: HIV infection, Immune responses, Vaccination, DNA vaccine, Prime-boost vaccine, Adjuvant, Challenges for HIV vaccine

1. Introduction

1.1. HIV infection and vaccination

According to recent reports, 35 million people were living with HIV-1 at the end of 2013, the considerable majority being in Sub-Saharan Africa, with dynamic epidemics in Asia [1]. HIV infection results in gradual loss of CD4⁺T lymphocytes, containing immune competence, and progression to AIDS. Effective treatment with combined antiretroviral drugs decreases viral load below detectable levels but cannot eliminate the virus from the body. Furthermore, the success of combined antiretroviral drugs is hindered by accumulating drug toxicities and chronic immune activation leading to increased risk of several non-AIDS disorders, even when viral replication is inhibited. Therefore, there is a major need for therapeutic strategies as an alternative to the combined antiretroviral drugs [2]. HIV vaccine strategies are expected to be a critical component for controlling the HIV epidemic [3, 4]. Immunotherapy, or therapeutic vaccination, aims to enhance existing immune responses against HIV or stimulate immune responses. These immune responses should provide an efficient cure by controlling viral replication and preventing disease progression in the absence of combined antiretroviral drugs [2]. The cost-effective and different HIV-1 vaccine approaches have recently attracted a special interest. Both antibodies and cell-mediated immune responses are considered to be important to prevent HIV-1 infection in the mucosal compartment, *i.e.*, the entry point for sexual transmission [1]. A great majority of HIV-1 infections occur at mucosa during sexual contact. Therefore, it is important to provide mucosal barrier protection against this entry by mucosal vaccination. A number of mucosal routes of vaccination such as enteric oral or intranasal vaccines have significant barriers that limit vaccine efficacy or cause safety risks. In contrast, the sublingual region of the mouth could provide a simple route for mucosal vaccination with immunogens, but this site does not always induce strong immune responses, especially when protein antigens are used [5].

Currently, antibody-inducing vaccines are a major focus in the preventive HIV vaccine field [6]. In addition, T-cell-based therapeutic vaccines have focused on three strategies: a) to increase the levels of vaccine-induced responses, b) to enhance the responses targeting only conserved regions of the virus, and c) to use replication-competent viral vectors [1]. Generally, antiviral T-cell and B-cell responses play a crucial role in suppressing HIV replication during chronic infection [7, 8]. Novel approaches of HIV treatment include both conventional

therapeutic vaccines (*i.e.*, active immunization strategies using HIV-derived immunogens) and the use of checkpoint blockers such as anti-PD-1 antibodies. These complex therapeutic strategies appear as promising approaches against HIV infection [7].

The biggest barrier for many vaccines is the pathogen's variability. Thus, studies should be further focused on the functionally most conserved regions of proteins common to many variants, including escape mutants inducing both antibody and T-cell immune responses. For vector-based vaccines, the "universal" subunit immunogens are efficiently delivered using heterologous prime-boost regimens, which can be further improved using adjuvants and delivery approaches [9–11]. Several studies have described the development of vaccine strategies, including improved envelope proteins formulated with potent adjuvants, DNA and vectors expressing mosaics or conserved sequences, capable of inducing strong relevant immune responses, such as neutralizing antibodies (NAb)s and non-neutralizing antibodies, CD4⁺ and CD8⁺ cell-mediated immune responses, mucosal immune responses, and immunological memory. The type of immune response elicited by different immunogens can also correlate with the risk of HIV infections. For example, IgG antibodies against the V2 loop of gp120 are associated with a decreased risk of HIV infection, while Env-specific IgA antibody is directly related to increased risk [12]. Generally, a combination of two independent approaches, containing the induction of broadly neutralizing antibodies (bNAb)s to prevent or reduce acquisition of infection and stimulation of effective CTL responses, is the currently used technique to slow disease progression in advance infections [13].

Briefly, more than 20 years after the discovery of HIV, researchers are trying to design a protective AIDS vaccine. The problem is the lack of basic knowledge about the immunological requirements for the protection against HIV. Virus diversity and escape from immune responses are the most important challenges to the development of an effective HIV vaccine. In this chapter, we will represent the challenges to vaccine design against HIV biologically and immunologically. Moreover, different vaccine strategies will be described to determine the best strategies already focused on HIV infections. In this line, the relationship between HIV biology and immunity will be demonstrated for the first time.

2. Immunogen-induced Neutralizing Antibodies (NAb)s

The most common tests for HIV infections rely on detecting antibodies against virus. Thus, these tests can also detect antibodies induced by a candidate HIV vaccine. The detection of vaccine-induced antibodies to HIV by serological tests is referred to as vaccine-induced seroreactivity (VISR) [6]. Neutralizing antibodies are useful in identifying the neutralizing epitopes of vaccine and for understanding the mechanism of potent and broad cross-neutralization, thereby providing a modality of preventive and therapeutic value [14, 15]. It has been shown that some NAb)s confer protection toward neonatal HIV-1 infection [16].

Various types of HIV-1 Env immunogens were developed that express epitopes for broadly neutralizing antibodies and their precursors. There are three new structures proposed for the HIV-1 Env trimer, which will be more immunogenic than previously used immunogens: a)

minimal immunogens that are fragments of HIV-1 Env-neutralizing epitopes, b) intermediate Env immunogens (*e.g.*, monomeric Env gp120), and c) various forms of Env trimers [17]. To date, these structures have not been capable of inducing the immune system to generate bNAbs after vaccination. Thus, a successful vaccination for HIV-1 and induction of bNAbs will need repetitive immunizations for a long time [17].

The current studies of HIV-positive patients with strongly neutralizing sera indicated that the immune system is able to produce antibodies neutralizing up to 90% of HIV strains [18]. The neutralizing antibodies bind to conserved gp120 sites, and the identification of these sites can help to design effective vaccines. Glycosylated residues (or carbohydrates) have a key role because of binding broadly neutralizing antibodies to carbohydrates and combining carbohydrate and peptide elements on gp120. However, carbohydrates partially cover some peptides on envelope surface recognized by bNAbs. Thus, the use of engineered glycoproteins as vaccines for the stimulation of bNAbs is a subject of interest in HIV vaccine design [18]. Antibody responses to the HIV-1 envelope glycoproteins can be classified into two: a) non-neutralizing responses directed to peptide epitopes expressed on isolated envelope glycoproteins but not on the native envelope trimers responsible for mediating the entry of virus into target cells; b) broadly neutralizing antibody responses targeting epitopes expressed on the native envelope trimers. Currently, many potent broadly neutralizing antibodies have been isolated to stimulate prophylactic and therapeutic activities in animal models. These antibodies help us to improve vaccine design and therapeutic strategies for HIV-1 [19]. The recent characterization of new epitopes for stimulating broadly neutralizing antibodies has encouraged studies in the synthesis of novel antigenic constructs for the development of HIV-envelope-directed vaccines [20]. Thus, an important step in vaccine design is the determination of antibodies and epitopes associated with broad HIV neutralization. Indeed, immunogens and/or immunization protocols should be designed to increase antibody affinity maturation [1].

Regarding the studies, HIV-1 envelope gp120 is the target for neutralizing antibodies against the virus. HIV-1 envelope gp120 exhibits a great degree of variability that causes a major challenge for the development of vaccines against HIV/AIDS. Different approaches have been used to improve immunogenicity of broadly neutralizing epitopes on HIV-1 gp120 with limited success [21]. For example, immunogenicity of gp120 and its V3 epitopes was enhanced when gp120 was co-injected with monoclonal antibodies (mAb) to the CD4-binding sites (CD4bs). Indeed, the gp120/CD4bs complex was a potent immunogen for eliciting cross-reactive functional NAb against V3 epitopes [21]. In contrast, the membrane proximal external region (MPER) of the gp41 subunit of the HIV-1 envelope glycoprotein (Env) includes epitopes for recognizing bNAb as an important region in vaccine development. However, designing an immunogen for the induction of bNAbs to MPER is difficult because of the relative inaccessibility of the MPER in the native conformation of Env [22]. Therefore, a group of oligomeric gp41 immunogens was designed to further expose MPER in a suitable conformation. The immunogens comprised different gp41 N-heptad lengths and insertion of extra epitopes and flexible C-termini. These immunogens were used in two different immunization strategies, including gp41/gp140 proteins and gp41/gp160 DNA associated with various

adjuvants and modalities. It was observed that the gp41 immunogens elicit higher levels of MPER than the gp140 immunogens. In prime-boost strategies, the best MPER responses were shown in the groups receiving gp41 DNA followed by gp41 protein. Several agents may influence MPER immunogenicity such as the immunization route, dose, or adjuvant. Generally, these data encourage the researchers for designing MPER immunogens with optimized immunization protocols [22]. Furthermore, the aggregation of HIV-1 virions was detected by antibodies (IgG) to the viral envelope glycoprotein (Env). Neutralizing antibodies directed to a V3-base- and glycan-dependent epitope on gp120 and to the apex of the Env trimer, as well as non-neutralizing antibodies to the epitope cluster I on the gp41-ectodomain, could aggregate virions, but the neutralizing antibody 2G12, which is specific for a glycan-dependent monovalent epitope on gp120, could not aggregate. These data can potentially open the ways for the development of HIV-1 vaccine [23].

3. Preventive HIV vaccines

The studies indicated that a successful vaccine candidate needs to elicit broad antibodies targeting the Env protein. Immunogens targeting gp120 have been developed, which block infection in monkeys. Attempts to induce antibody persistence were complicated by increasing the number of HIV-target cells [24]. RV144 consisting of canary pox vector vaccine ALVAC-HIV (vCP1521) prime and AIDSVAX®gp120 B/E boost was the first vaccine against HIV-1 infection achieved in clinical trial [1, 25]. The analysis of vaccine-induced immune responses in vaccinated-infected and vaccinated-uninfected volunteers indicated that IgG specific for the V1V2 region of gp120 was related to the decreased risk of HIV-1 infection, and plasma Env IgA was directly associated with infection risk. Thus, RV144 studies indicated that Env is essential and possibly sufficient to stimulate protective antibody responses against mucosally acquired HIV-1. Efficacy trials were planned in heterosexual populations in southern Africa and Thailand [1]. Generally, the studies of nonhuman primates suggest that Env is a necessary component for a successful protection against viral infections. Two approaches are being followed to induce Env-specific-antibody-mediated protection: a) vaccines that elicit potent and broadly reactive neutralizing antibodies against viruses which are common in human transmission, b) vaccines that induce antibody neutralizing only in less commonly transmitted HIV strains, but that block HIV-1 infection by non-neutralizing (nNAbs) mechanisms [26, 27]. Monomeric gp120 HIV-1 envelope proteins alone failed to protect high-risk individuals against infection. In fact, the level and breadth of elicited NAb were not sufficient for protection [1]. Furthermore, the results indicated that IgG to linear epitopes in the V2 and V3 regions of gp120 is part of a complex interaction of immune responses that contribute to the protection in RV144 [28]. In RV144, Env IgG3 was correlated with decreased risk of HIV infection, a response that declined rapidly compared to overall IgG responses. Indeed, the rates of Env-specific IgG3 and V1/V2 IgG3 responses were high, and conversely IgG4 responses were considerably low in recipients of the RV144 vaccine. These findings indicated that V2 IgG plays a role in protection against HIV-1 infection. Generally, an increase in magnitude, affinity, breadth, and importantly in frequency and durability of V2- and V3-specific antibodies of IgG3

and IgG1 subclasses may confer a higher and more durable rate of protection against HIV-1 infection. The induction of cross-reactive V1V2-specific IgG raises the hypothesis of cross-clade protection. Additional booster vaccinations may increase the antibody levels. Residual antibody responses against gp120 were detected 6–8 years post vaccination in RV144 vaccinees. Additional boosts increased plasma IgG gp120 and gp70 V1/V2 antibodies at titers higher than those in RV144, while weak gp120 IgA responses were induced. These HIV-specific IgG antibodies were also detected in rectal secretions while IgAs were undetectable [29–34].

Regarding the studies, HIV antibodies capable of preventing mucosal cell-free or cell-to-cell HIV transmission are critical for the development of effective prophylactic and therapeutic vaccines. The interactions between antigen-presenting cells (APCs) and HIV result in cell-to-cell transmission of HIV. In the experimental macaque model, data indicated that the broadly neutralizing antibodies are capable of neutralizing an extensive range of HIV strains, preventing cell-to-cell transfer, and protecting from infection [35]. In addition, IgG Fcγ receptor (FcγR)-mediated inhibition of antibodies at the mucosal site may play a role in protection against HIV mucosal transmission. On the contrary, mucosal IgA antibodies may be effective in protection against HIV sexual transmission. Thus, the determination of inhibitory effects of antibodies is critical for evaluating protection in HIV vaccines [35]. Furthermore, the majority of the antibodies against different viral proteins described a marker (shared idiotope) that is recognized by the monoclonal antibody 1F7. This shared idiotope on antibodies induced by HIV-1 was involved in the immune memory mechanism linking the early and late antibodies, the so-called back-boost effect. This finding was supported by auto-antibodies that bind to the 1F7 idiotope in sera of HIV-1-infected individuals. The expression of a shared idiotope in antibodies could provide a strategy to stimulate B cells selected to produce antibodies against HIV-1 and HCV, suggesting their implications in vaccine design [36].

The use of potent adjuvants may also enhance antigen-specific antibody responses. Several adjuvants have been tested in nonhuman primates and humans indicating a significant benefit of HIV envelope proteins formulated with MF59 and AS01 adjuvants. A study indicated that alum protected macaques from simian immunodeficiency virus (SIV)mac251 infection, while MF59 did not protect despite its ability to elicit higher systemic T-cell and antibody responses. Adjuvant-associated differences in the homing of plasmablasts and induction of key cellular signaling pathways may explain these effects. The formulation of HIV-1 gp120 with MPLA and alum induced significantly higher levels of neutralizing antibodies and T-cell lymphoproliferation compared to alum, MF59, or MPLA alone. Importantly, antibodies to gp70 V1V2 (subtypes B, C, and CRF01 AE) were induced more rapidly, to a higher magnitude and with a greater durability than alum-adjuvanted gp120 [37–42]. Also, the formulation of antigens with solid nanoparticles may prolong the duration of antibody responses by increasing antigen retention locally in the tissues driving B-cell responses, enhancing dendritic cell (DC) antigen presentation, and the development of CD4⁺ Th cells that provide cytokines and signals that are required to initiate somatic hypermutation and affinity maturation for effective B-cell memory [1]. Furthermore, the ability of two mucosal adjuvants, including α-galactosylceramide (α-GalCer) as a potent stimulator of natural killer (NK) T cells and CpG-oligodeoxynucleotide (CpG-ODN) as a TLR9 agonist, was evaluated to enhance immune responses against

clade C gp140 HIV-1 envelope protein antigen. The results showed that CD4⁺ and CD8⁺ T-cell responses in systemic and mucosal tissues were significantly higher in mice immunized with gp140 in the presence of either α -GalCer or CpG-ODN and further enhanced when both adjuvants were used. Also, the use of two adjuvants and especially their combination effectively increased gp140-specific serum IgG and vaginal IgA antibody levels. Memory T-cell responses detected at 60 days after immunization revealed that α -GalCer is more potent than CpG-ODN, and the combination of α -GalCer and CpG-ODN adjuvants was more effective than either alone [5]. Another approach is called B-cell lineage vaccine design. In this line, the recombinant antibodies belonging to bNAb members were used to determine HIV-1 envelope constructs as immunogens in a prime-boost strategy. These envelope constructs utilize the naïve B-cell repertoire residing in bone marrow and secondary lymphoid tissues *in vivo* and subsequently stimulate B-cell evolution until bNAb-producing cells are elicited [43, 44].

The nNAb B-cell progenitors become activated and internalize Env compared with bNAb B-cell progenitors. The reports showed that rational immunogen modifications can reduce the activation of naïve B cells that lead to such nNAbs, while promoting the activation of naïve B cells that result in germline-reverted bNAbs [43, 44]. A number of potent broadly neutralizing antibodies have been identified from HIV-infected individuals although the generation of bNAbs using traditional vaccine approaches has been obscure. The researchers tested a single dose of 3BNC117 or 10-1074 (mAb specific for the CD4-binding site and the V3 region, respectively) and also a combination of both antibodies. The data showed a total decline in viral loads post infusion in simian-human immunodeficiency virus (SHIV)-infected macaques. Another approach to the generation of bNAbs is to circumvent “normal” immune responses and direct non-lymphoid cells to produce bNAbs *in vivo* using gene therapy. Vectored immunoprophylaxis (VIP) is a gene therapy method in which transgenes encoding bNAbs are delivered directly into muscle tissue where bNAbs are produced. Two recent animal studies demonstrated that VIP could generate modest titers of NAb that can effectively prevent *in vivo* HIV infection in a humanized bone marrow–thymus–liver (BLT) HIV infection model and a simian immunodeficiency macaque infection model [7]. Recent findings showed that adeno-associated virus (AAV)-delivered broadly neutralizing antibodies can inhibit HIV replication. Indeed, a single injection of AAV could generate long-term antibody responses as a therapeutic approach in the lack of antiretroviral drugs. Induction of vector-mediated antibodies could inhibit cell-to-cell transmission and replication of HIV. This result represented an alternative to immunogen-based vaccine design and a novel therapeutic intervention by enabling particular manipulation of humoral immunity [45, 46].

A challenge for HIV-1 immunogen design is the induction of neutralizing antibodies against neutralization-resistant (Tier-2) viruses that control human transmissions. A soluble recombinant HIV-1 envelope glycoprotein trimer possessing a native conformation (BG505 SOSIP.664) could induce NAbs potently against the sequence-matched Tier-2 virus in rabbits but weaker and similar responses in macaques. The trimer also stably stimulated cross-reactive NAbs against more sensitive (Tier-1) viruses. Tier-2 NAbs recognized conformational epitopes that differed between animals and in some cases overlapped with those recognized by broadly neutralizing antibodies, whereas Tier-1 responses targeted linear V3 epitopes. A second trimer,

B41 SOSIP.664, also induced a strong autologous Tier-2 NAb response in rabbits. Thus, native-like trimers may represent a promising starting point for developing HIV-1 vaccines directed at inducing bNAbs [47].

The goal of an HIV vaccine is to generate robust and durable protective antibody. Thus, it is important to induce CD4⁺ T-follicular helper (TFH) cells. However, very little is known about the TFH response to HIV vaccination and its relative contribution to magnitude and the quality of vaccine-elicited antibody titers [48]. In this line, a DNA/modified *vaccinia* virus Ankara SIV vaccine with and without gp140 boost in aluminum hydroxide was administered in rhesus macaques. The studies indicated that booster immunization with modified *vaccinia* virus Ankara induces a distinct and transient accumulation of proliferating CXCR5⁺ and CXCR5⁻ CD4 T cells in blood at day 7 post-immunization, and the frequency of the former but not the latter correlated with TFH and B-cell responses in germinal centers of the lymph node. Furthermore, gp140 boost elicited a skewing toward CXCR3 expression on germinal center TFH cells, which was strongly associated with longevity, avidity, and neutralization potential of vaccine-elicited antibody response. However, CXCR3⁺ cells preferentially expressed the HIV co-receptor CCR5, and vaccine-induced CXCR3⁺CXCR5⁺ cells showed a moderate positive association with peak viremia following SIV251 infection. These data demonstrated that vaccine regimens eliciting CXCR3-biased TFH cell responses favor antibody persistence and avidity but may prompt higher acute viremia in advance infections [48, 49].

Generally, broadly neutralizing antibodies specific for conserved epitopes on the HIV-1 envelope (Env) are believed to be essential for the protection against multiple HIV-1 clades. Recently, an HIV vaccine incorporating the molecular adjuvants B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) was designed with the potential to facilitate the maturation of polyreactive and autoreactive B cells as well as to enhance the affinity and/or avidity of Env-specific antibodies. The results indicated that mice immunization with a DNA vaccine encoding BAFF or APRIL multimers, together with IL-12 and membrane-bound HIV-1 Env gp140, induced neutralizing antibodies against Tier-1 and Tier-2 viruses. The APRIL-containing vaccine was especially effective at generating Tier-2 neutralizing antibodies following a protein boost. Notably, BAFF and APRIL did not cause B-cell expansion or an increase in total IgG. Thus, BAFF and APRIL multimers were proposed as promising molecular adjuvants for inducing bNAbs against HIV-1 infection [50].

4. Stimulation of cell-mediated immune responses

A successful HIV vaccine must either completely prevent infection or eliminate the first round of infected CD4 T cells before the latent pool of HIV-infected cells is established. Thus, an effective HIV vaccine requires high levels of protective immunity at the time of virus contact with the host, and it cannot rely on memory immune responses to occur. CD8 T cells can effectively kill HIV-infected T cells, but in most cases of acute HIV infection, the virus rapidly escapes. For example, anti-HIV CD8 CTL activity is capable of eliminating virus-infected T cells in the setting of vaccination with an attenuated rhesus cytomega-

lovirus (rhCMV) containing simian immunodeficiency virus genes, but in the setting of acute HIV infection, the transmitted/founder virus usually escapes from CD8 T-cell control [51]. CTL responses targeting specific HIV proteins (*e.g.*, Gag) have been associated with relative control of viral replication *in vivo*. In a SIVmac251 intravenous challenge model, breadth of Gag CTL epitope recognition correlated with control of viremia peak [1]. These data have demonstrated that CD8⁺ T cells are associated with the control and eradication of early retrovirus infections [17].

The vaccine trials were focused on whether cell-mediated immune-response-inducing vaccines can prevent infection or reduce post-infection plasma viral load [1]. Vaccinees with HLA alleles associated with HIV-1 control had a significantly lower mean viral load over time. Interestingly, the most highly conserved epitopes were detected at a lower frequency, suggesting that stronger responses to conserved sequences may be as important as breadth for protection [52, 53]. Interestingly, heterologous vector prime-boost regimens enhanced immunity by increasing the magnitude, onset, and multi-functionality of the insert-specific cell-mediated immune responses compared to homologous regimens [54]. New progress has been made in overcoming HIV-1 diversity through induction of cross-reactive T-cell responses to HIV-1 by vaccines designed *in silico* (called conserved and mosaic vaccines) [17]. Polyvalent mosaic immunogens derived by the recombination of natural HIV-1 strains were designed to induce cellular immune responses that recognize genetically diverse circulating virus isolates. Increasing the breadth and depth of epitope recognition may contribute to the protection against infection by genetically diverse viruses and also to the control of variant viruses that emerge as they mutate away from recognition by CTLs. For example, mosaic HIV-1 Gag, Pol, and Env antigens expressed by Ad26 vectors markedly augmented both the breadth and depth without compromising the magnitude of antigen-specific T-lymphocyte responses as compared with consensus or natural sequence HIV-1 antigens in rhesus monkeys [55, 56]. An alternative to multivalent wild-type or mosaic vaccines is the use of conserved element immunogens as a novel and effective strategy to broaden responses against highly diverse pathogens by avoiding decoy epitopes, while focusing responses to critical viral elements for which few escape pathways exist. Priming with conserved elements boosted with the complete immunogen induced broad cellular and humoral immunity focused on the conserved regions of the virus. In contrast, full-length HIV-1 immunogens elicited greater magnitude and comparable breadth of T-lymphocyte responses to conserved HIV-1 regions compared with conserved-region only HIV-1 immunogens in rhesus monkeys [57, 58].

An important point is the use of replicating vector. A replication-competent rhesus cytomegalovirus vaccine expressing SIV proteins induced and maintained high frequency of SIV-specific CD4⁺ and CD8⁺ T-cell effector memory responses at extra-lymphoid sites without measurable antibody responses to SIV. Half of vaccinated monkeys showed a severe control of three routes of SIVmac239 transmission including intrarectal, intravaginal, and intravenous. The conservation of particular cytotoxic epitopes does make them good candidates for a global HIV-1 vaccine [59, 60].

5. Therapeutic HIV vaccines

Recent studies have focused on the improvement of effective prophylactic and therapeutic approaches to combat persistent viral infections. Therapeutic vaccines for HIV infection should aim to elicit antiviral CD8 T cells (CTLs), CD4 T cells, and neutralizing antibody since these immune responses control viral replication [7, 61]. It is critical to generate broad cellular responses as HIV mutates very rapidly to escape immune system. In addition, recent studies determined that T-follicular helper cells constitute a significant source of virus production and contribute to the total viral reservoir. Since these cells reside in B-cell follicles/germinal centers, it may be critical to generate CD8 T cells that can home to B-cell follicles and exert immune response on these cells. The HIV-specific CD4 T-cell response is also important for maintaining the functional CD8 T-cell and B-cell responses. However, these HIV-specific CD4 T cells could also serve as potential targets for virus replication [7]. Interestingly, CD4 T cells with cytolytic function have been shown to be associated with enhanced viral control, although it is demonstrated whether these responses can be primed by vaccination. The function of dendritic cells may be also critical for generating a protective cellular and humoral immune response, as chronic HIV infections are associated with impaired DC function. Thus, therapeutic vaccines may also need to use strategies such as adjuvants to enhance the function of innate immunity [7].

Several therapeutic vaccine strategies have already been used such as live-attenuated microbes, viral vectors, and dendritic cell-based vaccines that led to suppress and/or clear infections. Among them, improved DNA vaccines have emerged as a promising candidate for the treatment of infectious diseases especially HIV infections [61–63]. Some strategies have been considered to improve immune responses stimulated by DNA vaccines such as *in vivo* efficient DNA delivery systems, co-delivery with molecular adjuvants as well as the development of potent heterologous prime-boost regimens [61, 62, 64]. DNA vaccines have been utilized as candidate HIV vaccines because of their ability to generate cellular and humoral immune responses, the lack of anti-vector response allowing for repeat administration, and their ability to prime the response to viral-vectored vaccines. Because the HIV epidemic has unreasonably affected the developing world, the favorable thermostability profile and relative ease and low cost of manufacture of DNA vaccines offer additional advantages. *In vivo* electroporation (EP) has been utilized to improve immune responses to DNA vaccines as candidate HIV-1 vaccines alone or prime-boost regimens with both proteins and viral-vectored vaccines in several animal models, and recently, in human clinical trials [65]. In addition, intradermal electroporation of HIV DNA was well tolerated. Strong cell- and antibody-mediated immune responses were elicited by the HIV-DNA prime and HIV-Modified Vaccinia Ankara (MVA) boosting regimen, with or without intradermal electroporation use [66]. DNA vaccines have an intrinsic bias toward generating cellular immunity against intracellular pathogens. By manipulating the DNA formulation and delivery, effective antibody responses can also be induced. For instance, studies showed that the immunized monkeys with DNA vaccine developed HIV-specific T-cell immune responses that persisted for months [62].

The use of live-attenuated invasive bacteria as a carrier for DNA-based vaccines has previously been reported [67]. Immunization with recombinant invasive bacteria including *Shigella*, *Salmonella*, and *Listeria* carrying plasmid DNA (pDNA) vaccines has been shown to induce protective immune responses in mice. The use of human enteric bacteria is especially useful due to their ability to infect human colonic mucosa, and their tropism for the activation of dendritic cells and macrophage of internal mucosa. Thus, they are very efficient for the delivery of DNA vaccines to APCs in the mucosa resulting in stimulation of potent systemic and local immune responses. Such responses may be critical for the development of an effective prophylactic HIV vaccine, because a large number of HIV transfer through human mucosal routes [67]. For instance, a live-attenuated strain of *Salmonella typhimurium* was used to deliver plasmid orally and showed an adjuvant role through the release of various cytokines [68]. In addition, intranasal immunization of mice with live recombinant *Shigella* cells induced an HIV Gag-specific cellular immune response similar to that observed by intramuscular injection of naked DNA. Importantly, a strong boosting effect was obtained in mice primed with DNA, suggesting the efficacy of bacterial vectors in prime-boost vaccination regimens [67]. The studies indicated that a novel vaccine delivery system using bacterial ghosts (BGs) can be considered as an efficient and nontoxic delivery system for DNA vaccines *in vitro* and *in vivo*. In this line, a new strategy of HIV vaccine delivery was designed using *Salmonella typhi* Ty21a bacterial ghosts. The data showed that Ty21a BGs loaded with an HIV gp140 DNA vaccine (Ty21a BG-DNA) are easily taken up by murine macrophage cells (RAW264.7), and gp140 is efficiently expressed in these cells. Peripheral and intestinal mucosal anti-gp120 antibody responses in mice vaccinated with BGs-DNA vaccine were significantly higher than those in mice immunized with naked DNA vaccine. The enhancement of antibody responses was associated with BG-induced production of IL-10 through TLR4 pathway [69].

Attenuated virus vaccines have traditionally been potent and relatively easy to produce and deliver. Vaccination with a live virus results in high intracellular synthesis of viral proteins. This high-level expression stimulates strong cellular and humoral immune responses and results in the production of long-lasting memory B and T cells. However, attenuated HIV vaccines replicate strongly in animal models to retain residual virulence. Recent studies indicated that priming with a DNA vaccine induces a Th1 response that can be boosted by the subsequent administration of a viral vector encoding the same gene. This prime-boost strategy elicited strong protective immunity in several primate models [70]. Currently, immunogenicity of a highly attenuated *vaccinia* virus with low neuro-virulence, LC16m8 strain, was studied as an HIV vaccine vector. The data showed that the recombinant *vaccinia* virus-based vaccine (vLC-Env) combined with DNA vaccine expressing the HIV *env* gene (pCAG-Env) produces a protective immune response against HIV infection in BALB/c mice. Vaccination of vLC-Env alone induced much higher HIV-specific humoral and cellular immune responses than that of pCAG-Env. Priming with pCAG-Env further enhanced vLC-Env-induced immune responses, especially cell-mediated immune response. In addition, administration of vLC-Env-infected dendritic cells to mice generated a high cellular immune response. These results demonstrated that priming with pCAG-Env and boosting with vLC-Env represents a potential candidate for vaccination against HIV infection [70]. A few studies have used DC presenting either the autologous virus or virus-derived peptides as therapeutic vaccines (DC-based vaccines) in

macaques and humans. They also showed that a similar approach could successfully control HIV replication in humans. Similarly, a recent study showed that an efficient HIV-1-specific immune response could be generated using an autologous monocyte-derived DC (MDDC) transfer. Thus, HIV-specific immune responses could be elicited by DC-based therapeutic vaccinations. In general, therapeutic vaccinations should be explored as a combination therapy with other immune modulators to achieve a functional cure (*i.e.*, long-term control of virus replication in the absence of antiretroviral therapy) [7].

There is growing interest in the role of anti-HIV antibody-dependent cellular cytotoxicity (ADCC) antibodies in the prevention and control of HIV infection. Passive transfer studies in macaques supported a role for the Fc region of antibodies in the prevention of simian–human immunodeficiency virus infection. The Thai RV144 HIV-1 vaccine trial induced anti-HIV ADCC antibodies that may play a role in the partial protection observed. Several studies showed a role for ADCC antibodies in slowing HIV disease progression. However, HIV evolves to escape ADCC antibodies, and chronic HIV infections cause the dysfunction of effector cells such as natural killer cells that mediate the ADCC functions. Furthermore, four recent studies showed that the HIV-1 *Vpu* protein, by promoting release of virions, reduces the capacity of ADCC antibodies to recognize HIV-infected cells [71]. On the contrary, The HIV-1 transactivator of transcription (Tat) is a key HIV virulence factor, which plays critical roles in virus gene expression, replication, transmission, and disease progression. The results indicated that Tat-induced immune responses are necessary to restore immune homeostasis, to block the replenishment and to reduce the size of the viral reservoir. Anti-Tat antibodies are uncommon in natural infection and, when present, correlate with the asymptomatic state and lead to lower or no disease progression. Hence, targeting Tat represents a pathogenesis-driven intervention [72].

6. Adjuvants

Although the importance of DNA vaccines, especially as a priming immunization, has been well proved in different HIV vaccine studies, the immunogenicity of DNA vaccines is generally moderate. Novel adjuvant is necessary for improving the immunogenicity of DNA vaccine [73]. Multiple groups have demonstrated the potential of co-administering plasmid DNA that express cytokines, chemokines, or co-stimulatory molecules together with plasmids encoding target viral antigens [74]. The potency of DNA vaccines can be developed by the co-delivery of plasmid-encoded molecular adjuvants. The pDNAs encoding granulocyte-macrophage colony-stimulating factor (GM-CSF), hematopoietic factor fms-like tyrosine kinase 3 ligand (Flt-3L), and interleukin-12 (IL-12) could markedly enhance cell-mediated immune responses elicited by an HIV-1 *env* pDNA vaccine in BALB/c mice [74]. Plasmid GM-CSF also increased the immune responses elicited by DNA vaccines expressing HIV-1 Gag and Nef-Tat-Vif. In addition, the use of pGM-CSF as a vaccine adjuvant appeared to increase antigen-specific proliferative responses and the percentage of polyfunctional memory CD8⁺ T cells. Co-delivery of pFlt-3L with pGM-CSF did not result in a further increase in adjuvant activity. However, the co-administration of pGM-CSF with pIL-12 significantly enhanced Env-specific prolifera-

tive responses and vaccine efficacy in the murine *vaccinia* virus challenge model relative to mice immunized with the *env* pDNA vaccine adjuvanted with either pGM-CSF or pIL-12 alone [74]. In another study, co-administration of the HIV-1 DNA vaccine with pIL-12 and pGM-CSF by topical application to the skin enhanced the levels of both the HIV-specific cytotoxic T-lymphocyte response and delayed-type hypersensitivity (DTH). Indeed, the skin is accessible for generating immune responses by both intradermal injection and topical use of gene delivery vectors [75]. In addition, co-administration of plasmids encoding the codon-optimized GM-CSF sequence with the HIV-1 Gag DNA vaccine resulted in a strong antibody and CTL response and a protective immune response against infection with recombinant *vaccinia* virus expressing HIV-1 Gag [76]. On the contrary, researchers strongly support the use of IL-6 or IL-15 as a cytokine adjuvant in HIV DNA vaccination. The data indicated that intranasal administration of DNA vaccine and pIL-15 can enhance Th1-dependent HIV-1-specific cell-mediated immunity. However, co-injection of pIL-15 with pIL-2 or pIL-12 did not show any synergistic effect on the immune responses induced by DNA vaccine *in vivo* [77]. Furthermore, the immunogenicity of HIV-1 DNA vaccine expressing the chimeric gene Gag-gp120 (pVAX1-Gag-gp120) was increased by co-inoculating pVAX1-IL6 in BALB/c mice [78]. The studies demonstrated that in-frame fusion of tumor necrosis factor alpha (TNF- α), DNA to DNA, encoding a large fragment of HIV gp120 could enhance Th1 immune responses against gp120 antigen. Also, in-frame fusion of IFN γ -encoding DNA at the 5' end of the chimeric molecule, to create a tripartite fusion, had no additional effect on immunogenicity [79]. A number of studies have shown that α -galactosylceramide, a natural killer T-cell (NKT) ligand, was applied as an adjuvant for various vaccines, including viral, parasite, and protein-based vaccines. The α -GalCer was able to enhance HIV-specific antibody responses. Furthermore, co-administration of α -GalCer with suboptimal doses of DNA vaccines greatly increased antigen-specific CD4⁺ and CD8⁺ T-cell responses. The level of cell-mediated immune responses in mice vaccinated with 5 μ g of DNA in the presence of α -GalCer was similar to that of mice vaccinated with 50 μ g of DNA in the absence of α -GalCer [80].

Recombinant adjuvants composed of a fusion between surfactant protein-D (SP-D) and either CD40 ligand (CD40L) or GITR ligand (GITRL) were previously shown to enhance HIV-1 Gag DNA vaccines. It was demonstrated that similar fusion constructs composed of the TNF superfamily ligands (TNFSFL) including 4-1BBL, OX40L, RANKL, LIGHT, CD70, and BAFF can also enhance immune responses to an HIV-1 Gag DNA vaccine. Importantly, the SP-D-4-1BBL, SP-D-OX40L, and SP-D-LIGHT constructs enhanced CD8⁺ T-cell avidity and CD8⁺/CD4⁺ T-cell proliferation 7 weeks after vaccination [81]. Also, the SP-D-OX40L, SP-D-LIGHT, and SP-D-BAFF constructs increased Gag-specific IL-2 secretion in memory T cells, suggesting their potency to elevate the number of self-renewing Gag-specific CD8⁺ and CD4⁺ T cells. Finally, the SP-D-OX40L and SP-D-CD70 adjuvants augmented IgG2a but not IgG1 antibody responses in the immunized animals. Interestingly, the B-cell-activating protein BAFF did not enhance anti-Gag antibody responses when administered as an SP-D fusion adjuvant, but augmented CD4⁺ and CD8⁺ T-cell responses. Indeed, various SP-D-TNFSFL fusion constructs can enhance immune responses following DNA vaccination with HIV-1 Gag expression plasmid [81]. Several studies indicated that 4-1BB and 4-1BB ligand (4-1BBL) interactions are important for inducing robust CTL responses and also long-lived memory T cells. Recently,

plasmid DNAs expressing either the membrane bound or soluble form of 4-1BBL were designed to enhance the Gag DNA vaccine as an adjuvant. The data showed that 4-1BBL DNA increased the Gag-specific IgG and cellular immune responses. Importantly, the expression of Gag and 4-1BBL from the same plasmid was critical for the adjuvant activity [82].

To improve the immunogenicity of DNA vaccines, some studies were focused on the immunoglobulin (Ig) fusion antigen. These reports showed that cytokine-coding plasmids fused with Ig have higher expression efficiency and better adjuvanticity. Furthermore, these plasmids have features that make them useful such as augmentation of half-life *in vivo*, formation of a multivalent antigen, and solubilization of hydrophobic proteins [83]. The possibility of increasing HIV gp120-specific cellular immune responses was determined in mice using a DNA vaccine encoding a mouse Ig fragment fused with gp120 in two directions (gp120-Ig or Ig-gp120). *In vitro* expression analysis revealed that the efficiency of HIV gp120 protein expression was higher in cells transfected with the gp120-Ig-coding plasmid (pGp120Ig) than in those transfected with the gp120 and Ig-gp120 expression plasmids (pGp120 and pIgGp120, respectively). The gp120-Ig-coding plasmid elicited more HIV-specific CD8⁺ T cells and effector memory CD8⁺ T cells than pGp120 in immunized mice. Furthermore, pGp120Ig significantly reduced the viral load after challenge with an HIV Env gp160-expressing *vaccinia* virus. These results represented that covalent antigen modification with an Ig sequence can modulate antigen-specific cellular immune responses [83].

Conversely, polysaccharide and nucleic acid fraction extracted from *Mycobacterium bovis bacillus Calmette–Guérin* (BCG-PSN) could be used as a novel adjuvant of DNA vaccine to elicit potent cellular and humoral immune responses against the HIV-1 Env antigen in BALB/c mouse model. In this experiment, the BCG-PSN was mixed with 10 µg or 100 µg of DNA vaccine and injected intramuscularly two or three times. BCG-PSN co-immunization with 10 µg DNA vaccine could elicit cellular and humoral immune responses which were comparable to that induced by 100 µg DNA vaccine alone. Moreover, BCG-PSN could activate TLR signaling pathways and induce Th1-type cytokine secretion. These findings suggested that BCG-PSN can be applied as a new and effective adjuvant for DNA vaccination [73].

Chemokines are largely bioactive inflammatory molecules which play a major role in a variety of immune and inflammatory responses, acting primarily as chemoattractants and activators of various leukocytes. In addition, some chemokines play a critical role in the transmission and progression of HIV-1 and HIV-2 viruses responsible for AIDS. Recent studies have indicated that chemokines and their receptors may play an important role in the differentiation and expansion of T cells in response to immune activation. These regulatory properties of chemokines make them suitable as molecular adjuvants [84]. For example, the modulation and regulation of immune responses were evaluated from the co-delivery of two β-chemokines as gene expression cassettes, MIP-1α or RANTES, along with HIV-1 DNA immunogen constructs. The data showed that MIP-1α had the greatest effect on antibody responses. In addition, co-expression of MIP-1α also modulated the shift of immune responses to Th2-type (*i.e.*, the increase of IgG1/IgG2a ratio). RANTES co-immunization also enhanced the levels of antigen-specific Th1 and CTL responses. The use of chemokine adjuvanted vaccines as HIV vaccine modulators may be important due to the interesting relationship between HIV cell entry and

the receptors for β -chemokines. Indeed, β -chemokines as vaccine adjuvants increased β -chemokine production in an antigen-specific manner [84].

7. Heterologous prime-boost strategies

Most of the current DNA vaccines utilize CMV, β -actin, or muscle-specific desmin promoters to potentiate expression of one or two fused genes of HIV-1 including the Env, Gag, Pol, and Tat. DNA vaccines comprising multiple plasmids encoding different HIV-1 proteins have been used to obtain a broader spectrum of immunity than individual plasmids expressing single proteins. The use of these plasmid DNA vaccines proved to be safe and immunogenic in macaques; however, these constructs needed to be boosted with viral proteins expressed by various vector systems including recombinant *pox virus*, modified *vaccinia virus Ankara*, and *adenovirus* for enhancing their efficiency in preventing AIDS [85].

The heterologous prime-boost regimen uses the ability of the immune system to generate large numbers of secondary antigen-specific T cells following an initial priming step. The same antigen is delivered subsequently using different vectors. Following a priming immunization, the antigen-specific T-cell populations develop to modest levels and then reduce. Indeed, a percentage of these cells transform into antigen-specific memory T cells. In a heterologous boost, because the priming and boosting vectors are different, T cells that specifically target the viral vector are not boosted and do not activate cell number control mechanisms, therefore allowing for greater development of the disease antigen-specific T-cell populations [82]. Several groups have now established that heterologous prime-boost regimens are the most potent strategies to induce cellular immune responses [86, 87]. In a plasmid DNA vaccine priming and viral vector-boosting regimen, the order of DNA followed by recombinant virus is important, as the reverse order did not induce higher levels of antigen-specific CD8⁺ T cells. It seems that the cytokine microenvironment created by a local virus infection during boosting is responsible for the efficient expansion of effector T cells [86]. In 2004, a consecutive immunization strategy involving priming with DNA and boosting with recombinant fowlpoxvirus (rFPV) vaccines encoding multiple common HIV-1 antigens was evaluated in 30 macaques. The vaccines were well tolerated, and a significant enhancement of DNA-vaccine primed HIV-1-specific T-lymphocyte responses was observed following rFPV boosting. Co-expression of IFN γ or IL-12 by the rFPV vaccines did not further enhance immune responses [88]. In addition, a subtype A or B HIV gp160 plasmid DNA and Env gp140 trimeric glycoprotein co-immunization was superior to immunization with glycoprotein alone by enhancing neutralizing antibodies. These data showed that co-delivering DNA and protein can increase antibody responses to Env. Hence, this approach has the potential to simplify vaccine regimens by inducing higher antibody responses using fewer vaccinations, an advantage for a successful HIV vaccine design [89].

The reports showed that co-immunization of a DNA vaccine encoding HIV-1P24-Nef with GM-CSF in DNA priming and peptide boost strategy increases the immunogenicity of the

candidate vaccine. Cytokine profile studies showed that both IL-4 and IFN- γ levels were increased. Also, co-immunization with GM-CSF resulted in a higher level of total IgG, comprising approximately equal levels of both specific IgG1 and IgG2a subtypes. Taken together, the results suggested that GM-CSF is able to induce long-term memory for the HIV-1 P24-Nef vaccine candidate [90]. Recent studies have used DNA/protein or DNA/adeno-vector regimens for HIV immunization. The essential mechanisms of heterogeneous prime/boost regimens are not well understood, but DNA priming results in much lower antigen expression compared to protein vaccines, and this may prime T-helper cell responses with the humoral response subsequently being boosted by the high-dose protein or viral vector (*e.g.*, RV 144 tested in clinical trials) [87].

8. Clinical trials

Several vaccine candidates were used in different phases of clinical trials. DNA prime-viral vector boost regimens have become the primary choice for stimulation of T-cell immune responses. For example, *Poxvirus* vector-based vaccines including the *Modified Vaccinia Ankara* and the genetically modified NYVAC-based vaccines appeared to be efficient in inducing the immune responses and could be evaluated in combination with DNA priming in clinical trials [91]. In addition, the safety and immunogenicity of several *Canarypox*-based vaccines with multiple HIV-1 gene inserts have been studied in humans. A phase III trial, RV144, using *Canarypox* (vCP1521) prime and AIDSVAX B/E boost has demonstrated modest protective efficacy in Thailand. The protection in RV144 trial was short-time and needed to use the additional boosters in participants for improving recall responses and continuing protection among them. AIDSVAX was also a component of the prime-boost (ALVAC/AIDSVAX) RV 144 vaccine in Thailand that showed successful results. In both cases, the vaccines targeted gp120 and were specific for the geographical regions. Among the adenoviral vector vaccine candidates, replication-defective Ad5 candidate indicated high immunogenicity in phase I clinical trials and reduced viral load in the SHIV/NHP model. However, this strategy failed to prevent new infections as well as reduce post-infection viral RNA levels in the vaccinated individuals in phase IIb. Furthermore, participants with preexisting antibodies against Ad5 vector showed increased HIV infection rates [91]. The heterologous prime-boost strategy using DNA prime and Ad5 boost was considered to avoid the problem of preexisting immunity. It has been shown that the preexisting Ad5-neutralizing antibodies did not affect the levels of cell-mediated responses in the DNA/rAd5 prime-boost recipients, as compared to participants who received rAd5 alone. However, in spite of robust immune responses induced by DNA/Ad5 strategy in phase I and phase II trials, the strategy failed to show protection from new infections in phase IIb [91]. Generally, prime-boost vaccination is an efficient approach compared to other strategies, but it still needs to develop against HIV infections in future.

9. Challenges for HIV vaccines

There are some major challenges against HIV vaccine design as described below. Figure 1 shows these challenges briefly.

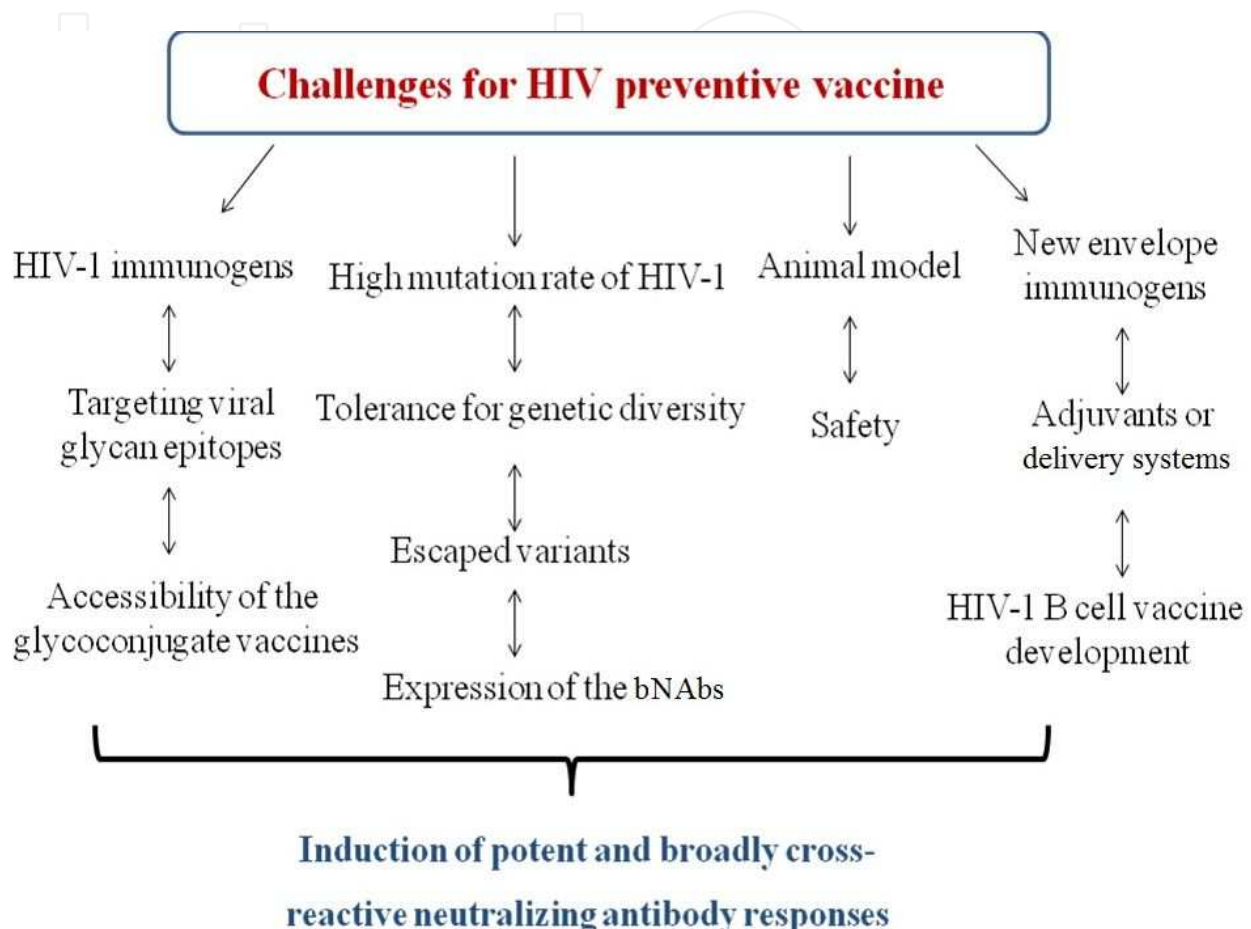


Figure 1. Challenges for HIV-preventive vaccines

9.1. Designing trimeric HIV-1 envelopes

The challenge remains to develop HIV-1 immunogens that will elicit protective immunity [13]. The challenge is to design, engineer, and produce a pure stable envelope immunogen that mimics the antigenic profile of the functional envelope spike. The engineered trimeric envelope was unable to induce bNAb in animals. Modification of the trimers, including removal of individual glycans proximal to CD4-binding region, elimination of the glycosylation site near the gp41 loop, linker-stabilized gp140 trimeric envelopes, have resulted in improved immunogenicity but have not yielded the desired bNAb. A combination of mosaic envelopes increased the magnitude of NAbs but not the breadth of the response in macaques. Therefore, no trimeric envelope induces bNAb in humans [1]. Some studies showed that HIV-1 bNAbs

identifies four conserved Env targets for HIV neutralization. To date, more than 30 bNAbs specific for conserved neutralizing Env epitopes have been characterized [17].

9.2. Why does vaccination with HIV envelope not induce bNAbs?

Whether bNAbs will effectively confer protection against HIV infection in humans remains unknown. An alternative to inducing bNAbs by vaccination with immunogens is to deliver these bnMAbs with viral vectors (*e.g.*, adeno-associated virus (AAV) gene transfer vector expressing antibodies or antibody-like immunoadhesins). This approach generated a long-lasting neutralizing activity in serum of macaques conferring complete protection against intravenous challenge with virulent SIVmac316. Similarly, full protection against intravenous HIV-1 challenge was observed in humanized mice receiving AAV carrying b12, while those receiving AAV carrying 2G12, 4E10, and 2F5 were partially protected [1]. A recent study has demonstrated that up to 50% of HIV-infected individuals will make cross-reactive antibodies that neutralize 50% of HIV primary strains. However, when bNAbs develop in HIV infection, they only occur after 2–4 years of infection. In contrast, no vaccine immunizations to date have induced high levels of bNAbs. The bNAbs are targeted to one of five conserved sites on the HIV Env trimer: the CD4 binding site, the membrane proximal gp41 region, the V3-glycan site, the V1V2-glycan site, and gp41-gp120 bridging regions [51]. Each of these sites is protected by surrounding glycans, and each one of these sites is restricted in access, such that relatively few antibody variable heavy (VH) and variable light (VL) combinations may be used to bind these Env sites. Example of restricted VH/VL usage is the use of VH1-2 paired with a 5 aa VL complementarity-determining region 3 (LCDR3) for the VRC01 type of CD4 binding site bNAbs, and the use of VH1-69, Vk3-20 for 4E10-like gp41 bNAbs. Moreover, all bNAbs have one or more unusual features, including high levels of somatic mutations, and poly- or autoreactivity that can result in immune tolerance control for bNAbs. However, in the simian-human immunodeficiency virus rhesus macaque challenge model, passive infusion of the new bNAbs could potentially protect against SHIV challenge [51].

9.3. Effective adjuvants

Adjuvants are important for the use of recombinant envelope immunogens, since these proteins by themselves generate only weak immune responses. For potent vaccine formulations delivered by mucosal routes, incorporation of adjuvants that controls the potential of innate immune modulators is important for overcoming immune tolerance and enhancing the immunogenicity of co-administered antigens [25]. The RV144 trial used alum as an adjuvant, which was then the only licensed vaccine adjuvant. However, alum is not believed to support robust cellular immune responses. Also, bacterial toxins are the most potent mucosal adjuvant candidates but concerns remain regarding their safety even when mutated to reduce toxicity. In contrast, ligands for TLRs 7/8 and 9 serve as potent adjuvants for parenteral and mucosal vaccines based on plasmid DNA, viral vectors, and recombinant proteins [25]. In particular, CpG-containing synthetic oligodeoxynucleotides (CpG-ODN) that activate TLR9 on dendritic cells appear potent in stimulating antigen presentation and induction of antigen-specific

immune responses. The synthetic glycolipid α -galactosylceramide has been tested primarily in cancer immunotherapy studies because of its capacity to serve as a ligand and potent activator of invariant natural killer T cells. In addition, the repeated mucosal delivery of α -GalCer adjuvant was done in primary and booster immunizations that resulted in repeated activation of NKT cells and DC to progressively increase adaptive immune responses [25]. The parallel development of adjuvants along with better HIV-1 immunogens will be needed for a successful AIDS vaccine. Additional comparative testing will be required to determine the optimal adjuvant and immunogen regimen that can elicit antibody responses capable of blocking HIV-1 transmission [92].

Both flagellin (fliC) and IL-18 (interferon- γ -inducing factor) have been developed as adjuvants to improve immunogenicity in DNA-vaccinated hosts. An HIV-1 Gag plasmid encodes a protein harboring broad epitopes for CTLs [93]. The immunogenicity of BALB/c mice immunized with an HIV-1 Gag plasmid (pVAX/Gag), combined with a chimeric plasmid encoding IL-18 fused to flagellin (pcDNA3/IL-18_fliC) or a single plasmid encoding IL-18 (pcDNA3/IL-18) and/or flagellin (pcDNA3/fliC), was studied. The IL-18 and flagellin fusion protein effectively induced IFN- γ by lymphocytes. During a 12-week immunization, both Gag-specific IgG in sera and spleen cell proliferation were elevated in all murine groups. However, the IgG2a/IgG1 ratio, Th1 cytokine (IL-2 and IFN- γ) production, and the proportion of Gag-specific CD3⁺ CD8⁺ IFN- γ -secreting cells were significantly increased in the murine group co-immunized with the pVAX/Gag plasmid and pcDNA3/IL-18_fliC compared with the mice immunized with the pVAX/Gag plasmid combined with either the pcDNA3/fliC or pcDNA3/IL-18 plasmid or both of them. The data suggested that the chimeric plasmid encoding IL-18 fused to flagellin can be used as an adjuvant-like plasmid to improve the Th1 immune response, particularly for the induction of CD3⁺ CD8⁺ IFN- γ -secreting cells in Gag plasmid-vaccinated mice [93].

9.4. High mutation rate of HIV-1

The high mutation rate of HIV-1 and tolerance for genetic diversity represented central challenges for vaccine design. Because the immune response is itself adaptive, the optimal HIV-1 sequence within an individual also differs over time. HIV-1 develops specific mutations within its genome that allow it to escape detection by human leukocyte antigen (HLA) class I-restricted immune responses, notably those of CD8⁺ CTLs. HLA thus represents a major force driving the evolution and diversity of HIV-1 within individuals [94].

9.5. Escaped variants

A major challenge is how to induce effective immune responses against escaped variants. It is important that a CTL-based vaccine stimulate effective cellular responses across the range of HLA class I alleles expressed in a host population. These observations have led to the idea that immune-mediated control of HIV-1 replication to levels that slow disease progression might be feasible through the design of vaccines that focus CTL responses against viral regions where escape cannot occur [94]. To date, adenovirus vector prime and pox vector boost vaccines have

been one among the most immunogenic vaccines for inducing HIV CD8 T-cell responses in humans. Efforts continue to overcome HIV diversity for T-cell epitope recognition by the *in silico* design of centralized consensus or mosaic HIV gene inserts based on optimizing the coverage of T-cell epitopes in HIV strains in the Los Alamos HIV Sequence Database, or based on conserved epitopes in the vaccine [51].

9.6. Expression of the bNAbs is limited by host tolerance mechanisms

The studies showed that two human recombinant bNAbs, called 2F5 and 4E10, that bind near the virion membrane to Env gp41 were reactive in human autoantibody assays. In a subsequent study, 2F5 was shown to avidly bind the human protein kynureninase (KYNU), and 4E10 was shown to react with the mammalian RNA splicing factor 3B3. The nominal gp41 epitope of the 2F5 bNAb is the linear peptide ELDKWAS and an identical 6-residue sequence is present in KYNU (ELDKWA). This ELDKWA motif in KYNU is conserved in nearly all mammalian species and absent in all proteins other than the HIV Env. Thus, the autoantigens for these two bNAbs, 2F5 and 4E10, have been identified, suggesting that expression of these bNAbs is limited by host tolerance mechanisms [17].

9.7. Challenges for developing vaccines targeting viral glycan epitopes

Generation of antibodies to glycans has several challenges: a) due to the inherent weakness of carbohydrate–protein interactions, binding affinities must be enhanced through avidity effects. For example, lectins are able to overcome this problem using interaction of multiple carbohydrate binding domains with arrays of glycan ligands; b) glycoproteins usually always exist as a number of different glycoforms where the same protein backbone is glycosylated with different glycan structures. This microheterogeneity weakens the antigenic response to the individual glycan structures. These multiple conformations may be presented to the immune system further weakening the response; c) glycosylation is ubiquitous to all mammalian cells, thus the host may display tolerance toward these sugars. Generally, these effects result in glycans are poorly immunogenic. The major concern of generating antibodies against self-glycan structures is their potential autoreactivity *in vivo* [95].

9.8. Animal model for preclinical studies

There are few nonhuman primate models of enhanced HIV susceptibility [96]. Animal model research during the past years has focused on the development of models in order to explore key questions about HIV entry, immune control, and persistence and also their use for testing therapeutic vaccines [97].

9.9. Design of new envelope immunogens

A major challenge for HIV-1 B-cell vaccine development is the design of new envelope immunogens that can trigger the selection and expansion of germline precursor and intermediate memory B cells associated with the maturation of a broadly neutralizing antibody response. The identification of delivery systems, prime-boost strategies, and synergistic

adjuvant combinations is important to induce the magnitude and quality of antigen-specific T-follicular helper cell responses needed to induce somatic hypermutation (SHM) and B-cell maturation against heterologous primary virus envelopes [98].

9.10. Safety of vaccines

Safety of vaccines is one of the most important subjects for the design of vaccines that should be determined in clinical trials [99].

9.11. Accessibility of the glycoconjugate vaccines

Accessibility of these glycoconjugate vaccines in resource-poor regions which bear the highest disease burden from these pathogens remains challenging largely due to high vaccine pricing [100].

9.12. Induction of potent and broadly cross-reactive neutralizing antibody responses

Induction of potent and broadly cross-reactive neutralizing antibody responses remains a major challenge for the development of HIV vaccines because of the high diversity of gp120. The high glycosylation, large conformational changes, and steric restriction of the epitopes in gp120 during receptor binding and membrane fusion processes prevent the access of antibodies to these sites [101].

9.13. Challenges associated with antigen immunogenicity

The failure to date of Env-based antigens to stimulate bNAb is likely to result from several specific reasons that influence BCR recognition of unusual structural antigenic elements:

- a. **Incorrect presentation of the vaccine antigen:** Immunization with linear peptides of MPER failed to re-elicited neutralizing responses because the MPER peptide mimics adopted an inappropriate conformation in solution and failed to present the correct surface for B-cell recognition [5].
- b. **Cross-reactivity with self:** The 4E10 mAb, and to a lesser extent the 2F5 mAb, binds lipid as part of their epitope by using an array of hydrophobic residues. This binding appears to make them autoreactive, resulting in B-cell tolerance mechanisms [5].
- c. **Epitopes with steric constraints for BCR recognition:** The CD4bs is an apparent target for eliciting NAb as it requires conservation for function, and needs to be exposed for CD4 binding. In contrast, most infected individuals do not make CD4bs-specific bNmAbs. The main reason is the intrinsic immunorecessive nature of the conserved segments of CD4bs [5].
- d. **Unique antigenic features for BCR recognition:** The 2G12 bNmAb has an epitope composed entirely of oligomannose groups. The PG and PGT series of bNmAbs have complex glycan-peptide binding surfaces in which the glycans are heterogeneous.

Preparation of such epitopes will require powerful synthetic chemistry related to scaffolded peptide design. Both MPER bNmAbs 2F5 and 4E10 require a lipid component to their epitopes and up to now this has not been incorporated into a successful immunogen [5].

- e. **Germline BCR recognition and requirement for extensive antibody affinity maturation:** There are two possible consequences of the steric constraints imposed on BCRs during the recognition of structurally unusual antigens: a) the frequency of germline BCRs available to recognize such complex antigens will be low, thus an extensive degree of affinity maturation will be required to generate a high-affinity bNAbs recognizing structurally “difficult” epitopes; b) the germline BCR affinity for a bNmAb epitope may be undetectable. A feasible outcome of these constraints is that the host will require long-term antigen exposure to select and clonally expand the rare B cells with appropriate BCRs and to affinity mature them into bNAbs, because most bNmAbs arise in individuals after chronic HIV-1 infection [5].
- f. **Conceptual concerns relating to epitope recognition by BCRs:** There are concerns that isolating an epitope from its antigenic context will not lead to re-elicitation of the same type of antibody against the epitope, which is a reasonable concern. However, although an epitope mimic may not re-stimulate an antibody identical to the template bNmAb, it may be sufficiently balance between elicited Ab and epitope mimic to allow specific binding to trimeric Env. If this is achieved, trimeric Env may be used to boost and affinity mature those B cells reactive with the epitope mimetic [5].
- g. **Responders and non-responders:** The finding that among large cohorts of HIV-1-infected individuals only a minor percentage makes a bNmAb response suggests that this may apply also to responses to vaccination [5].

Finally, the key difficulty in the development of an HIV vaccine is our ignorance of the immune responses that control viral replication, how these responses can be elicited, and how they can be monitored [2]. The question of whether to focus on induction of antibody or CTLs continues to be discussed in the HIV-1 field. However, evidence from many other vaccine-preventable infectious diseases indicates that antibody titers correlate with protection from infection, but CTL-mediated immune responses are required for protection against disease. This suggests that a dual approach is still necessary. Aspects of CTL vaccine technology such as replicating or persistent vectors may need to express Env-based antigens to allow long-term antigenic exposure for the induction of bNmAb. In contrast, approaches to elicit bNmAbs may need to be immunologically compatible with the generation of a parallel CTL response.

9.14. Conclusions

The development of a safe and effective vaccine for HIV is a major global priority. To date, efforts to design an HIV vaccine have not been successful due to HIV diversity, HIV integration into the host genome, and ability of HIV to consistently evade antiviral immune responses. While the RV144 immunization strategy remains a priority for future efficacy trials, newer

prime-boost mosaic and conserved sequence immunization strategies inducing efficient and long-time immune responses as well as the development of immunogens inducing broadly neutralizing antibodies should be followed and tested in humans. Recent success in isolation of potent broadly neutralizing antibodies, in discovery of mechanisms of bNAb induction and atypical mechanisms of CD8 T-cell killing of HIV infected cells, has opened new ways for HIV vaccine design. Indeed, the most protective HIV vaccine will require the combination of T-cell-inducing and antibody-inducing vaccine candidates with appropriate adjuvant formulations, since the innate and adaptive arms of the immune system cooperate for virus neutralization and pathogen-infected cell elimination. In general, acceleration of vaccine discovery depends on basic research and new technologies. Novel strategies should be safe, but rapidly tested in humans.

10. Key points

- Development of a safe and effective vaccine for HIV is a major global priority
- Efforts to design an efficient HIV vaccine have not succeeded due to HIV diversity, HIV integration into the host genome, and ability of HIV to consistently evade antiviral immune responses
- The cost-effective and different HIV-1 vaccine approaches have recently attracted a special interest
- Both antibodies and cell-mediated immune responses are considered to be important to prevent HIV-1 infection in the mucosal compartment
- Novel prime-boost mosaic and conserved sequence immunization strategies as well as the development of immunogens inducing broadly neutralizing antibodies have attracted a special interest
- Recent success in isolation of potent broadly neutralizing antibodies, in discovery of mechanisms of bNAb induction and atypical mechanisms of CD8 T-cell killing of HIV infected cells, has opened new ways for HIV vaccine design
- Several therapeutic vaccine strategies have already been used such as live-attenuated microbes, viral vectors, and dendritic cell-based vaccines that led to suppress and/or clear HIV infections
- Among different vaccines, improved DNA vaccines have emerged as a promising candidate for the treatment of infectious diseases especially HIV infections
- Some strategies have been considered to improve immune responses stimulated by DNA vaccines such as *in vivo* efficient DNA delivery systems, co-delivery with molecular adjuvants as well as the development of potent heterologous prime-boost regimens

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