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New developments in an old strategy: heterologous vector primes and envelope protein boosts in HIV vaccine design

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Abstract

Prime/boost vaccination strategies for HIV/SIV vaccine development have been used since the early 1990s and have become an established method for eliciting cell and antibody mediated immunity. Here we focus on induction of protective antibodies, both broadly neutralizing and non-neutralizing, with the viral envelope being the key target antigen. Prime/boost approaches are complicated by the diversity of autologous and heterologous priming vectors, and by various forms of envelope booster immunogens, many still in development as structural studies aim to design stable constructs with exposure of critical epitopes for protective antibody elicitation. This review discusses individual vaccine components, reviews recent prime/boost strategies and their outcomes, and highlights complicating factors arising as greater knowledge concerning induction of adaptive, protective immunity is acquired.

Keywords

Vector prime/envelope boost vaccine strategy; DNA; adenovirus; poxvirus vectors; monomeric/trimeric envelope immunogens; neutralizing/non-neutralizing antibody; cellular immunity

Introduction

In spite of continuing progress in HIV treatment and advancement toward a sustainable ‘cure’, a prophylactic vaccine is urgently needed to stop the worldwide HIV pandemic. Given the importance of such a vaccine, it is remarkable that more than 30 years since the identification of HIV as the etiologic agent of AIDS, only six clinical efficacy trials have been conducted. The concepts tested demonstrate broad shifts in overall vaccine strategies, which until recently resulted from repeated failures and the inability of the field to identify clear, protective immune correlates of protection.

Initial vaccine approaches were based on the concept that most vaccines protect via antibody (Ab) responses. Vaxgen conducted two clinical trials of recombinant HIV gp120 protein, which had previously elicited protective neutralizing Abs (nAbs) in chimpanzees [1]. In Vax003, injection drug users in Thailand were immunized with bivalent clade B and E gp120s (AIDSVAX B/E), while in VAX004 US and European men and women at high risk of HIV infection received two clade B gp120s (AIDSVAX B/B). Neither trial elicited potent Ab responses or protective efficacy [2,3]. Consequently the vaccine field shifted, de-emphasizing Ab approaches in favor of cellular immunity.

The Step Study vaccinated an HIV clade B cohort in the Americas, the Caribbean, and Australia, [4] and the Phambili trial vaccinated an HIV clade C cohort in South Africa [5]. The vaccine in both cases consisted of Merck's subtype B Ad5-HIV vaccine (consisting of three Ad5 vectors separately expressing HIV Gag, Pol, and Nef), aiming to generate cell-mediated immune responses. In both cases, this was achieved [5,6] but resulted in no protection, suggesting that the level and/or specificity of cellular immunity induced was insufficient, or that a vaccine lacking an Env component to induce Ab was not able to prevent HIV infection. The interpretation of the data was confounded in the Step trial by a trend toward increased HIV infections in the vaccine group compared to those that received placebos. This outcome was subsequently shown to be significant in uncircumcised men seropositive for Ad5 prior to vaccination [7]. A similar conclusion could not be reached in the Phambili trial as it was stopped prematurely in view of the Step trial results.

In an attempt to enhance induction of cellular immunity and also elicit protective Ab responses, the HVTN 505 trial evaluated priming with DNA expressing HIV clade B Gag, Pol, and Nef as well as clade A, B, and C Env glycoproteins, followed by boosting with four Ad5 vectors (in a 3:1:1:1 ratio) expressing a clade B Gag-Pol fusion protein plus clade A, B, and C Env glycoproteins, respectively. This trial was also halted for futility, showing no apparent protection [8]. The cellular response rates were slightly lower than those seen in the Step and Phambili trials. Anti-HIV Env Abs were elicited but exhibited low response rates and neutralized only tier-1 isolates (the lower the tier, the greater the neutralization sensitivity).

Given these disappointing results, expectations were low following initiation of the RV144 trial in Thailand, which combined ALVAC HIV *env/gag/pol* priming, previously shown in a phase 1/2 trial to elicit CD8 T-cell responses in only 24% of vaccinated individuals, [9] with the previously ineffective AIDSVAX B/E boost [3]. Surprisingly, this phase III trial was the first to demonstrate significant protective efficacy (31.2%) [10]. Anti-Env V1V2 Abs inversely correlated with infection risk [11]. Although Abs elicited were not broadly neutralizing and only able to neutralize tier-1 viruses, [12] robust Ab-dependent cellular cytotoxicity (ADCC) together with low plasma anti-Env IgA levels was inversely associated with infection risk [11]. The ADCC Abs recognized both V2 and C1 regions and synergized in mediating both ADCC and neutralization [13]. Additional studies, including sieve analysis, [14] strengthened the association of anti-V2 Abs with protection [15,16].

Taken together, these findings had a dramatic effect on HIV vaccine design. Passive transfer studies had demonstrated the ability of nAb to confer protection [17]. The RV144 trial widened the scope of potentially protective Abs and illustrated induction of these Abs by a combined vector prime/envelope protein boost strategy, leading to a renewed focus on this 'prime/boost' approach. Innumerable vectors and envelope proteins have been evaluated in preclinical and clinical vaccine studies. Here, we will discuss vector and protein components separately. Subsequently, we will summarize combined prime/boost strategies, the immune responses elicited, and protection achieved, making heavy use of preclinical vaccine studies in nonhuman primates (NHPs), a key model for evaluating candidate vaccines. Overall, we will present an overview of promising strategies incorporating vector priming with envelope protein boosting.

Vaccine vectors

Vaccination with protein antigens leads to Ab induction, whereas vectors are used in vaccine design primarily to introduce and express a vaccine antigen intracellularly. This approach elicits both cytolytic $CD8^+$ T-cell responses and $CD4^+$ T-helper cell responses. Abs can also be induced following recognition of extracellular antigen by B-cells. The vectors that have advanced furthest in vaccine trials include naked DNA and two viral vectors: adenoviruses and poxviruses.

DNA vaccines

DNA vaccines have been utilized in HIV vaccine approaches since the early 1990s [18] and were among the first vaccines utilized in NHPs [19,20] and humans [21]. They have many advantages, including ease of antigen design, safety, stability, no anti-vector immunity, and low production costs [22]. Numerous plasmid DNA designs targeting the breadth of HIV strains have been evaluated. Both centralized ancestral and consensus DNA Env vaccines, as well as mosaic DNA Env vaccines, have elicited a greater breadth of immune response in small animal models compared to strain-specific sequences [23,24]. Immunization of macaques with conserved element HIV Gag DNA vaccines, designed to focus immune responses on critical viral elements, followed by boosting with DNA encoding full-length Gag, was shown to elicit broader cellular and humoral immunity against conserved viral regions [25,26]. Immunization with multiple or polyvalent DNA vaccines, in some cases with protein boosts, has also resulted in a greater breadth of response in both NHPs and humans [27–29].

In spite of optimal DNA designs, a disadvantage of the DNA approach has been a lack of potency, due in part to inefficient uptake of DNA by host cells. This has been addressed by several methods including gene gun injection, jet injection, and *in vivo* electroporation. The latter methodology has been extensively used. It was shown to enhance DNA delivery and immunogenicity in mice [30] and was subsequently applied successfully in numerous vaccine strategies in both NHPs and humans [31–34]. The technique has also been applied in mucosal vaccination, a potentially important route for eliciting mucosal immunity [35]. However, the question of how electroporation technology can successfully be used for global vaccine administration needs to be addressed.

In addition to electroporation, molecular adjuvants have enhanced the immunogenicity of DNA vaccines. Both cytokine and chemokine molecular adjuvants have been used to enhance Th1 and Th2 responses or influence trafficking of induced immune cells [36]. Cytokine adjuvants have been most extensively studied. Among the more promising are IL-12, IL-15, and GM-CSF, which can all potentially enhance Th1 responses, although consistent results have not been obtained in all models. For example, IL-12 and IL-15 enhanced SIV Gag DNA-induced cellular and humoral responses in a NHP model, [37] yet in humans no effect of either cytokine resulted when they were administered with an HIV Gag DNA vaccine [38]. Thus, appropriate clinical trials are necessary to validate pre-clinical findings. Among chemokines tested, mucosal adjuvants have included CCL25, CCL27, and CCL28 (CCR9 and CCR10 ligands), [39] while CCL3 (macrophage inflammatory protein 1-

alpha) has been used to attract antigen presenting cells to the site of immunization [40]. New adjuvants are continually being explored. IL-33 was recently shown to enhance polyfunctional CD8 T-cells in a murine model, [41] while CD40L has been shown to stimulate dendritic cells (DCs) and B-cells [42]. Other novel approaches include the heat shock protein HSP70, a damage-associated molecular pattern that binds and activates DCs by means of the pattern recognition receptor TLR2/4 [43]. Mice vaccinated with DNA encoding HIV Gag plus HSP70 exhibited enhanced T-cell polyfunctionality and proliferation. More conventional approaches, such as use of Vaxfectin, a combination of cationic lipid and neutral lipid formulation, together with plasmid DNA vaccines, have induced potent, sustained Ab responses in NHPs [44]. The number of molecular adjuvant approaches currently being explored for enhancing the immunogenicity of DNA vaccines is a testament to the overall desirability of the DNA vector approach.

Adenovirus vaccines

Adenovirus (Ad) vectors have long been utilized in gene therapy approaches and are increasingly being exploited in HIV vaccine strategies. The ability to safely grow high titers of these double-stranded DNA viruses that target mucosal sites is very appealing. Ads infect both dividing and nondividing cells while expressing large amounts of transgene, leading to robust immune responses. As with other vectors, anti-vector immunity is an issue, particularly with Ad5, but using other Ad serotypes can potentially mitigate this problem [45]. Ad vectors are used in both replication- and non-replication-competent forms, depending on the E1 gene: present in the former, but absent in the latter. Both forms normally lack E3, which is not essential for viral replication, to increase space for the desired transgene. Replicating Ad vectors better mimic a natural viral infection and act as a potent adjuvant to stimulate an initial immune response [46]. While replication-competent Ad has been shown to persist for at least 25 weeks following administration, [47] low levels of transcriptionally active replication-defective Ad vectors have also been reported [48]. Both types of vectors not only elicit innate immune responses, resulting in the production of several cytokines and chemokines, [49] but also due to their persistence, are able to elicit a durable memory $CD8^+$ T-cell response [50]. This has been shown to directly contribute to control of viremia in the SIV rhesus macaque model [51].

Various Ad serotypes are being utilized as nonreplicating vaccine vectors. Most commonly used is human Ad5, which in the Step trial, induced $CD8^+$ T-cell responses [4,6] but failed to confer protection. Preexisting immunity to Ad vectors potentially impinges upon immune efficacy. As a large proportion of the world's population is seropositive for Ad5, 'alternative serotype Ads', such as Ad26, Ad35, and Ad48, which exhibit less seroprevalence, [52] are being developed as vaccine vectors. The latter three vectors utilize CD46 as cellular receptor, in contrast to Ad5's use of CAR. As such, they exhibit enhanced innate immune responses consisting of more antiviral and proinflammatory cytokines [53]. They have been reported to induce long-lived memory $CD8^+$ T-cell responses as opposed to terminally differentiated $CD8^+$ T-cells observed with the Ad5 vector [54] and exhibit greater recall capacity [55]. Other studies, however, have suggested that while Ad26 and Ad35 are less seroprevalent, they may have lessened immunogenicity [56]. Nevertheless, both vectors have moved forward to clinical trials. A multigenic Ad35 vector expressing HIV Gag, INT, RT,

and nef and one expressing HIV Env induced $CD8^+$ and $CD4^+$ T-cell-responses in healthy uninfected humans, [57] while an Ad26 HIV recombinant vaccine induced broadly reactive humoral and cellular anti-Env immune responses in humans [58]. Recombinant Ad26 has also successfully elicited antigen-specific humoral and cellular mucosal immunity in humans, [59] further demonstrating that pursuit of these alternative recombinant Ad vectors is warranted.

In addition to the alternative human Ad serotypes, chimpanzee Ads are being developed due to their lack of seroprevalence in humans and their ability to induce strong immune responses [56]. Using ChAdV63 encoding 14 of the most conserved regions of consensus Gag, Pol, Vif, and Env proteins from the HIV genome in a prime/boost approach with both DNA and Modified vaccinia virus Ankara (MVA) vaccines, significant $CD4^+$- and $CD8^+$-specific T-cell responses were elicited in humans [60]. Heterologous boosting with different chimpanzee Ad vectors (AdC6 and AdC7) was shown to overcome preexisting Ad5 immunity and better elicit T- and B-cell responses compared to sequential immunization with an Ad5 vector [61]. However, such T-cell-based heterologous vaccine regimens failed to protect rhesus macaques from a repeated low-dose SIV challenge [62]. Simian Ads (sAd11 and sAd16) have also been used as vectors, and recently three novel Ads were isolated from rhesus monkeys and vectorized [63]. Like chimpanzee Ads, simian Ads have low seroprevalence in humans [64]. A comparative study ranked several human, simian, and chimpanzee Ad vectors based on immunogenicity and protective capacity. Ad5 and chAd3 provided the most, and sAd16 and rAd35 the least in a murine model [64]. Overall, the Ad system has great flexibility with the ability to combine heterologous vaccine regimens for optimizing immune responses.

While the Ad vectors tested in humans have been primarily replication-defective, a replication-competent Ad4 platform has advanced to clinical trials. This vector was selected based on its long-term use as a wild-type vaccine in the military, in which oral immunization was shown to be safe and effective [65]. Evaluation of Ad4-HIV Env and Gag vaccines administered orally or to the upper respiratory tract is currently ongoing (Clinical Trials.gov, NCT01989533). However, a completed phase I trial of a replicating Ad4-influenza vaccine demonstrated not only safety of the vector platform but also its utility as a priming immunogen. Following oral Ad4-flu priming, administration of a licensed H5N1 subunit vaccine as a boost led to greatly enhanced nAb responses [66]. The hope is that the replicating Ad4-HIV vaccine, by eliciting mucosal immunity and demonstrating longer persistence, will prove useful as an HIV vaccine. Another twist to the use of replicating Ad vectors is the design of single-cycle replicating Ad vaccines [67]. This vector contains the E1 gene but is deleted in the gene for the IIIA capsid cement protein. Thus, it maintains its ability to replicate the transgene, but is unable to package its genome and produce mature virions. Similar to replicating Ad, it elicits amplified and persistent immune responses. The overall diversity of available Ad vectors, both replication-competent and defective, continues to place this vector system in the fore-front of efforts to develop a highly effective HIV vaccine.

Poxvirus vaccines

Following the eradication of smallpox by a vaccination program using the poxvirus vaccinia, the virus was recognized as a potentially useful vector for design of vaccines against other pathogens (for review, see [68]). Due to safety concerns regarding its use, particularly in immunocompromised individuals, a variety of attenuated, modified forms have been developed as vectors, and several poxvirus-based vaccines are now being heavily used in the HIV vaccine field. They possess several advantages that make them amenable for vaccine use, including the ability to be stably freeze-dried and cheaply manufactured [69]. Poxvirus genes are expressed in the cell cytoplasm, and large amounts of foreign DNA can be inserted into their genomes without negatively impacting their infectivity [70]. They induce both $CD8^+$ T-cell and Ab responses long after a single immunization [71]. Additionally, there are increasingly fewer numbers of individuals previously vaccinated against smallpox, minimizing the concern of preexisting immunity seen with other vectors. These attributes combined make poxviruses highly attractive for HIV vaccine design.

MVA is an attenuated vaccinia virus obtained following 570 passages through chicken embryo fibroblasts (CEFs) [72]. As a result, it lost a significant amount of DNA compared to the parent virus, including many virulence and immune evasion genes, resulting in a block in virion assembly and production of immature virus particles [73]. Consequently, the virus does not productively replicate in most mammalian cells, yet expresses inserted foreign genes. MVA thus has an enhanced safety profile while retaining the ability to stimulate immune responses to the desired antigen. A related virus, NYVAC, was derived from a Copenhagen vaccinia vaccine strain by deleting 18 open reading frames affecting virulence, also resulting in an attenuated virus that cannot produce infectious virions in humans. The vector provides a high level of antigen expression and corresponding specific immune responses [74]. Although related, these two vectors behave differently in host cells and induce different immune responses. MVA has been characterized as eliciting preferentially a $CD8^+$ T-cell response and NYVAC more of a $CD4^+$ T-cell response, [74] although MVA has elicited $CD4^+$ T-cell responses in humans [75]. Both viruses have been used in clinical trials, MVA especially, as stand-alone vectors or as boosting immunogens following DNA priming [71]. This prime/boost approach with heterologous vectors is one strategy to enhance the immunogenicity of poxvirus vectors. Other approaches have included use of co-stimulatory molecules, engineering deletion of immunomodulatory genes, optimizing poxvirus promoters, and use of adjuvants [76]. One of the more interesting strategies is enhancing the replicability of the vector, in line with the current belief that more persistent vectors will elicit better immune responses. NYVAC has been engineered by deleting a viral gene that inhibits type I interferon, and by reintroducing two host range genes to restore replication competence while retaining attenuation [77]. *In vitro* studies with this modified NYVAC-HIV recombinant vector showed enhanced expression of interferon and interferon-induced genes along with increased Gag expression and activation of antigen processing and presentation pathways [78]. Priming of rhesus macaques with a replicating NYVAC-HIV vector followed by boosting with synthetic long peptides (SLPs) resulted in a more balanced polyfunctional $CD4^+$ and $CD8^+$ T-cell response, whereas the SLP immunization alone gave a poor $CD8^+$ T-cell response [79].

Avipoxviruses, including canarypox and fowlpox, are naturally attenuated poxviruses that do not replicate in humans. ALVAC, a canarypox vector obtained by 200 passages through CEFs, has been used much more extensively than fowlpox vectors. It has been evaluated in more clinical trials than any other poxvirus vector, [80] perhaps due to concern that immunogenicity of MVA or NYVAC vectors would be compromised by preexisting immunity in people vaccinated against smallpox. Early findings that protein subunit vaccines could elicit significantly high titers of antigen-specific Abs but poor levels of antigen-specific $CD8^+$ T-cells [81–83] led to the concept of first priming with a viral vector. ALVAC has been used extensively in this approach, most notably in the RV144 clinical trial. The modest protection obtained in that trial has appropriately resulted in great interest in ALVAC-based vaccine strategies. As a vector, ALVAC elicits higher levels of proinflammatory and interferon-related cytokines and chemokines immediately following immunization, compared to MVA and NYVAC, [84] possibly due to differences in the function and presence of immunoregulatory genes in the three vectors. MVA and NYVAC vectors also differ from each other, in that MVA elicits a stronger interferon-stimulatory phenotype while NYVAC induces a more proinflammatory phenotype. A direct comparison of ALVAC and NYVAC vector priming when coupled with a subunit Env boost showed that the NYVAC regimen stimulated more potent $CD4^+$ T-cell responses and trends toward enhanced $CD8^+$ T-cell and Ab responses compared to ALVAC, [85], suggesting its candidacy as an alternative to the ALVAC vector. Certainly, improvements to the modestly successful RV144 vaccine regimen are of importance. Ongoing studies are exploring the basis for the protective efficacy induced by the ALVAC component of the vaccine regimen used in the RV144 trial.

Other vaccine vectors

In addition to the predominant vectors discussed above, many others with different advantages are being developed as HIV vaccine candidates and are summarized in Table 1. Among the most interesting are cytomegalovirus (CMV) and self-amplifying mRNA (SAM) vaccines. CMV has moved to the forefront of the field due to the demonstrated ability of a rhesus CMV recombinant vector to prevent and/or clear SIV viremia from ~50% of vaccinated macaques [86,87]. This control was accomplished without an Env protein component in the vaccine. Whether this approach combined with an Env protein subunit would provide protective efficacy for the other 50% of vaccinees has not been evaluated. Further, use of this approach in people will need to address significant safety concerns regarding use of a replicating human CMV vector. The SAM vaccine approach provides the ease of nucleic acid design together with the ability to use a significantly lower dose than plasmid DNA in order to elicit potent immune responses. Using a cationic nanoemulsion delivery system, the safety and immunogenicity demonstrated to date indicate that this approach could provide a cost-effective vaccine strategy needed to combat the global AIDS pandemic [88]. In spite of the progress made to date with DNA, Ad, and poxvirus vectors, the many potentially advantageous features among the variety of vectors available for vaccine exploitation should not be overlooked in developing a vaccine strategy against such a dynamic target as HIV.

Protein design and desired Abs

The shift of the field from a cellular-based HIV vaccine to one focused on Ab induction has led to an emphasis on Env immunogen design. HIV Env binds the CD4 receptor on host target cells, and thus is crucial to establishing infection. However, preventing this interaction is extraordinarily difficult due to numerous Env characteristics, including sequence variability and immune escape, carbohydrate shielding, and conformational masking and complexity. Moreover, since the results of the RV144 trial showed a protective effect of non-neutralizing ADCC-mediating Abs, even the type of vaccine-induced Ab response desired has become less clear. Increasingly, attention is not only being paid to eliciting broadly neutralizing Abs (bnAbs), but also non-neutralizing Fc-mediated effector functions. The choice of envelope immunogen and the epitopes presented impact the functionality of Abselicited [101,102]. bnAbs remain as the gold standard for a protective vaccine, based on their established ability to prevent infection in passive Ab transmission studies, recently estimated to require relatively modest Ab titers that should be achievable by vaccination [103]. However, the easier elicitation of non-neutralizing Abs, which have exhibited protective activities in both human [104,105] and NHP studies, [106,107] has provided another avenue for vaccine exploration. In spite of an inability to illustrate protection in passive transfer experiments, [108,109] non-neutralizing Abs have been shown to limit the number of transmitted founder viruses during SHIV infection of rhesus macaques [110]. It has been suggested that neutralizing and non-neutralizing Abs should be considered complementary or synergistic [111]. In this regard, the seminal study of Hessell et al. [112] showed that non-neutralizing activity mediated by the Fc receptor of the bnAb b12 contributed to protection conferred by this Ab. Ideally, through the induction of both bnAbs and non-neutralizing Abs, virus at the mucosal site can be either neutralized directly, or infected cells and virus can be lysed by complement, phagocytosed, or eliminated by ADCC [111].

Proteins used in vaccines span a range of complexity, from basic gp120-based peptide fragments to complex trimers that seek to emulate the native structure. Many strategies have focused on the generation of Abs targeting a specific epitope, such as one of the variable loops, the CD4 binding site, or gp41 [113]. The gp120 Env component was an early target for vaccine development, but due to its inability to elicit bnAbs it was displaced by more complex protein designs. Even though interest in gp120 has been rekindled due to its elicitation of V2 region Abs and ADCC activity, identified as immune correlates of protection, [11,15] efforts to design Env immunogens able to elicit the desired bnAb response have not waned. A number of approaches addressing Env variability have been based on computer-generated sequences in order to elicit immunity against a broader spectrum of HIV isolates. Consensus sequences are based on the most common amino acid at each Env site, while ancestral sequences are based on the ‘center of the tree’. Both minimize the genetic distance between the Env immunogen and current circulating isolates, thus eliciting broader immune responses [24]. Mosaic immunogens composed of polyvalent sets of proteins assembled from fragments of natural sequences [114] were initially designed to elicit broad T-cell responses but have since been successfully applied to Env immunogens and shown to elicit humoral as well as cellular responses [115,116].

Efforts to target epitopes exposed on native virions, including conformational epitopes, have focused on Env trimers, the glycoprotein spike on the virion composed of three gp120 and three gp41 molecules, weakly linked non-covalently. In comparison to monomeric gp120 immunogens, trimer constructs have elicited more potent nAb responses [117,118]. Recently, a clade C trimer, of interest due to the global prevalence of clade C HIV, was shown to elicit nAb activity against some tier-2 viruses [119]. Moreover, in guinea pigs, a quadrivalent mixture of clade C trimers elicited higher nAb responses against a panel of tier-1A and B viruses compared to a single trimer alone [120]. Trimers are difficult to prepare and lack stability, but significant advances have been made in developing soluble proteins that closely resemble the native trimer. A stable Env trimer termed 'SOSIP' has been engineered by introducing a disulfide bond ('SOS') to covalently link gp120 to the gp41 ectodomain, the latter possessing a point mutation at position 559 (I559P, or 'IP') in the N-terminal heptad repeat region to stabilize intratrimer interactions [121,122]. Following cleavage of the gp160 subunit into gp120 and gp41 components, the stabilized SOSIP trimer exhibits native structure, including quaternary epitopes [123]. Several newly described bnAbs target quaternary epitopes that are not present in conventional gp120 and gp140 protein immunogens. Thus, SOSIP Env trimers provide a method of presenting these difficult-to-replicate epitopes, important for eliciting bnAbs, [124] and have generated great interest in the vaccine field. To date, they have induced an autologous tier-2 nAb response, but no heterologous tier-2 neutralization [125] and generally sporadic and low tier-1 neutralization, suggesting further design changes are warranted.

Numerous structural studies of HIV envelope complexed with bnAbs such as PGT122 and 35O22, [126] VRC01, [127] and PGT128 and 8ANC195 [128] are providing valuable information regarding recognition of key Env epitopes targeted by bnAbs, as well as a detailed understanding of bnAb evolution. It is beyond the scope of this review to cover these approaches in detail, and they have not as yet advanced to definitive candidate immunogens. However, these strategies will mature and continue to be a key focus of future HIV vaccine research. For further information on HIV Env structure, as it relates to approaches to elicit bnAbs, we cite two recent reviews [129,130].

Another novel strategy in HIV vaccine design seeks to elicit bnAb through the use of germ line-targeted immunogens. As the Envs present in contemporaneous HIV populations do not bind well to inferred germ line bnAb precursors, the approach involves engineering Env immunogens that will bind such germ line precursors. The goal is to jumpstart B-cell maturation while simultaneously generating Abs with increased affinity for the desired epitope/antigen, resulting in a significantly broad and potent Ab response [131–133].

Combined vector strategies to elicit potent, functional Ab responses

Vectored vaccine approaches were initially pursued to elicit cellular immune responses, but depending on the design of the inserted gene, Ab responses can also be induced. Single vectors were initially pursued due to their greater ease of production compared to protein production and purification. Subsequently, more complex strategies have attempted to enhance elicitation of protective Abs using adjuvants and combinations of heterologous vectors without resorting to Env protein immunogens, as illustrated by three recent studies in

NHPs. Repeated administrations of DNA encoding $SIV_{mac}239$ *pol* and SIV_{sm} consensus *env* and *gag* adjuvanted with CCR10 ligand, followed by administration of consensus $pSIV_{mac}$ *nef-rev* plasmid elicited mucosal SIV-specific Abs, and neutralization titers correlated with trends in protection against SIVsmE660 challenge [39]. CD40L incorporated into a SIV DNA priming vaccine followed by rMVA boosting resulted in increased magnitude and functional quality of Abs, including low-titer neutralization of tier-2 SIV_{E660}, along with a delay in SIV_{E660} acquisition and better control of viremia [42]. Mucosal priming with replicating modified vaccinia virus Tiantan strain encoding SIVgag/pol/env and boosting with a replication-defective Ad5-SIVgag/pol/env recombinant led to higher titers of nAbs compared to a homologous Ad5-SIV prime/boost regimen and better viremia control [134].

Similar incremental enhancements of humoral immunity have been seen in human clinical trials using combined vaccine vectors. As was shown in the HVTN 505 trial, [8] priming with DNA and boosting with Ad5 recombinant, both encoding HIV clade A, B, and C Env glycoproteins, elicited tier-1 nAbs but resulted in no protective efficacy. A phase I/II clinical trial in Tanzania tested priming with DNA (encoding HIV clade A, B, and C gp160 in addition to other HIV genes) and boosting with MVA expressing HIV CRF01_AE Env plus Subtype A gag/pol [135]. nAbs were elicited in 83% of the vaccinees; however, the activity was significantly reduced by depleting NK cells. Further studies established the presence of potent ADCC activity in 97% of the vaccinees. A phase 1b trial comparing sequential NYVAC-HIV and Ad5-HIV administrations showed that an Ad5-HIV prime followed by the NYVAC-recombinant boost was better than the reverse order; however, nAb responses elicited were still low [136]. Clearly, vectored vaccine regimens can elicit both neutralizing and non-neutralizing Abs; however, the failure of these vector-only strategies to induce potent nAb responses has led to greater interest in boosting vector-elicited immune responses with Env protein immunogens.

Vector prime/Env boost strategies

The vector prime/Env protein boost strategy was introduced in 1987, when an individual was first immunized with recombinant vaccinia virus expressing HIV gp160 Env and subsequently boosted with fixed autologous cells infected with the vaccinia recombinant and then with gp160 Env, resulting in an antigen-specific anamnestic response [137]. In 1991, this approach was systematically investigated in a murine study, which showed that priming with a live vaccinia virus expressing HIV envelope and boosting with Env glycoprotein exhibited significantly higher Ab responses compared to either immunogen alone or the combination in the reverse order [138]. Subsequently, a phase I trial using live vaccinia-HIV_{IIIB} Env recombinant with an HIV gp160 protein boost showed enhanced immunity in people along with detectable nAb in 7 of 13 vaccinees [139]. Since then, innumerable vector prime/protein boost studies have been conducted, most using monomeric Env immunogens. Clearly, improvements are needed in the vector systems and/or the Env protein component in order to obtain potent bnAbs and adequate protective efficacy. Here, we will summarize some of the most recently evaluated prime/boost studies.

Combination vaccine regimens based on DNA priming have been most frequently evaluated, reflecting both the need to enhance DNA vaccine-induced immunity as well as the ease of

manipulating plasmid DNA vaccines. It has long been known that Env protein boosting can augment Env-specific humoral immunity elicited by DNA priming. For example, in rabbits, boosting of DNA encoding HIV gp120 with mono- or polyvalent gp120 protein enhanced both Ab avidity and neutralizing activity [140]. Co-immunization of rabbits with an HIV gp160 DNA vaccine together with gp140 trimeric protein led to more rapid Ab development, higher titers, higher avidity, and neutralizing activity compared to protein alone [141]. In macaques, both sequential and co-immunization DNA/Env protein strategies have been evaluated. Using a DNA prime consisting of pHIV Gag and Pol along with consensus clades A, B, C, D, and A/E Env gp140s, followed by boosting with SF162 gp140 protein, broadly cross-reactive Abs were induced that had both neutralizing and non-neutralizing activities [142]. The utilization of multiple antigenic targets in the priming vector contributed to greater breadth and functionality of induced Abs. Co-immunization with a mixture of SIV Gag, HIV Env, and IL-12 DNAs together with HIV gp120 protein in EM-005 adjuvant (an oil-in-water emulsion containing a TLR-4 agonist) elicited higher Ab levels and broader cross-neutralizing activity to tier-1 isolates, [143] with no detrimental effects on cellular immunity. Using 2,2′-dithiodipyri-dine (aldrithol-2, AT-2)-inactivated SIV viral particles as the protein component, a similar SIV DNA-protein co-immunization regimen elicited better Ab breadth, longevity, and mucosal localization of the SIV Ab response compared to either component alone [144]. This same SIV DNA/AT-2-inactivated SIV strategy partially protected macaques against SIV_{E660} challenge, eliciting Abs with reasonable binding breadth across SIV and rectal Env-specific IgG that correlated with delayed acquisition [145]. While these experiments validated the DNA-protein co-immunization strategy, use of inactivated viral particles as a prophylactic vaccine component has not been approved for human use due to safety concerns regarding potential residual infectivity.

The DNA/Env protein co-immunization strategy has not yet been tested in clinical trials; however, sequential DNA/Env protein vaccination has been evaluated and shown to elicit strong Ab responses while preserving cellular immunity elicited by the DNA component. A phase I trial of DNA encoding HIV Gag and V2-deleted gp140 Env adsorbed onto microparticles followed by boosting with V2-deleted HIV Env in MF59 adjuvant elicited strong nAb responses in the majority of individuals, although not to tier-2 isolates [146]. Multiple Env antigens have been incorporated into vaccine strategies in order to address the problem of variability. In a prime/boost study utilizing DNA encoding multiple clade gp120s and clade C Gag, followed by boosting with a mixture of homologous gp120 proteins in QS-21 adjuvant, anti-HIV gp120 IgG was detected in the majority of the human vaccinees at levels equivalent to those found in chronically HIV-infected people; however, the induced Ab was capable of only tier-1 virus neutralization [29].

Overall, utilization of a DNA prime-Env boost vaccine regimen results in strong cellular responses and significant Ab production, better than strategies making use of only a single component. While there is ample evidence that DNA vaccines used in conjunction with monomeric gp120 or gp140 protein can elicit Abs that exhibit adequate breadth and non-neutralizing activity, this approach to date has not been able to generate Abs with any degree of neutralizing ability beyond tier-1 viruses. Further, while polyvalent vaccine components have increased Ab breadth, the ability to neutralize tier-2 viruses has not been similarly enhanced.

To address these issues, additional vectors have been incorporated into the overall vaccine strategy. An approach combining priming with DNA encoding SIV_{mac} Gag/Pol and HIV clade E gp140 followed by boosting with MVA recombinant containing similar genes plus clade E gp140 in Ras3C adjuvant (a squalene oil-in-water emulsion containing *Mycobacterium phlei* cell wall skeleton and synthetic dicorynomycolate) resulted in enhanced binding Ab titers and breadth of nAbs, together with reduced viral loads following intravenous challenge with SHIV-E virus [147]. The three-component regimen was superior to vaccination with protein only, MVA plus protein, or DNA/MVA only, and highlighted the value of the Env subunit and the SIV Gag/Pol component in viremia control post-challenge. An even more complex study compared the immunogenicity of a transmitted/founder Env, an HIV group M consensus Env (Con-S), and trivalent mosaic Envs in the form of DNA, NYVAC recombinants, and protein [148]. Following two DNA priming immunizations, macaques were boosted twice with the NYVAC recombinants and subsequently boosted with the protein immunogens. While the macaques immunized with the Con-S and mosaic immunogens developed the most potent cellular responses, macaques that received the Con-S immunogens developed the highest nAb titers, but again only to tier-1 viruses. The DNA immunogens described in this study are currently being evaluated for safety and immunogenicity in a clinical trial (NCT02296541) together with a boost of MVA encoding HIV clade CRF01_AE Env/Gag/Pol.

In addition to DNA priming, various Ads have been extensively used in prime/boost strategies. Our group has conducted numerous studies in rhesus macaques, utilizing mucosally administered replication-competent Ad5 host-range mutant recombinants encoding SIV or HIV genes and boosting with subunit gp120 or gp140 proteins. Our results have shown the ability of this vaccine strategy to elicit Abs at mucosal sites [149,150] as well as non-neutralizing Abs [106,107] both correlated with protective efficacy. The availability of Ad vectors of different subtypes has facilitated numerous vaccine studies showing the benefit of heterologous priming and/or boosting. Recently, priming with Ad26 encoding SIV_{smE543} Env, Gag, and Pol and boosting with SIV_{mac32H} gp140 in AS01B adjuvant resulted in complete protection in 50% of vaccinated macaques following repeated intrarectal heterologous challenge with SIV_{mac251} [151]. The Abs elicited in this study neutralized tier-1 but not tier-2 SIV isolates. The protection observed was correlated with induction of polyfunctional Abs able to mediate numerous activities such as ADCC, Ab-dependent complement deposition, and Ab-dependent cellular phagocytosis. This breadth of functional Ab activity has not been seen before using either DNA or poxvirus approaches.

With regard to poxvirus priming, a recent study compared NYVAC and ALVAC encoding HIV clade C trimeric gp140 and Gag/Pol/Nef polyprotein as Gag-induced virus like particles along with boosting with clade C gp120 protein [85]. While nAb and ADCC responses induced were similar for each regimen, priming with NYVAC was found to induce higher IgG binding titers against gp120, gp140, and gp70 scaffolded V1/V2 compared to ALVAC, indicating a potential benefit of NYVAC moving forward. Nevertheless, the success of ALVAC in the RV144 trial continues to stimulate research with this vector. For example, one such study utilized an ALVAC-SIV prime followed by boosting with heterologous papillomavirus pseudovirion-based vaccines and gp120 protein in an attempt to stimulate vaginal immunity [152]. While significant protection was not achieved following

intravaginal SIV challenge, animals that exhibited delayed acquisition had high-avidity V1/V2 Abs, while control of viremia in mucosal tissues was associated with T-cell responses.

Expert commentary

Based on the vector prime/Env protein boost regimens evaluated to date, improvements are needed to obtain sufficient protective efficacy. One approach would be to make use of better adjuvants to enhance the immunogenicity of the Env protein component and improve durability of responses. We have not discussed this topic, but refer readers to recent review articles [153,154]. However, the prime/boost strategy should not be discarded. Here, we have focused on induction of humoral immunity as a critical feature for a successful vaccine, but cellular immune responses are also needed, and are largely induced by the vector component of the vaccine regimen. Moreover, considering the striking results using CMV vectors, leading to initial low-level viremia and subsequent apparent clearance of virus in 50% of immunized macaques, the absence of an Env protein component emphasizes the contribution of cellular immunity [87]. The CMV vector has been shown to elicit $CD8^+$ T-cells that recognize unusual epitopes restricted by MHC class II [99]. Perhaps a change in priming immunogen from the standard DNA, Ad, or poxvirus vectors would lead to enhanced protective efficacy. It would be of interest to add an Env protein boost to the CMV priming regimen to see if protection could be obtained in the other 50% of vaccinees.

Other vaccine vectors are being developed. For example, intramuscular priming of rhesus macaques with chimeric VEE/SIN alphavirus replicon particles encoding SIV Gag/Pol and HIV Env followed by boosting with HIV oligomeric gp140ΔV2 protein led to complete protection following intrarectal SHIV challenge [155]. However, a Phase I trial of VEE alphavirus replicon HIV clade C gag, although confirming safety of the vector, elicited little immune response, illustrating the difficulty of translating new vectors to the clinic [156]. The use of different combinations of heterologous vectors or the increased interest in use of replication-competent vectors may lead to enhanced, more persistent vaccine-induced immunity.

While there has been great activity in the area of Env protein design and development, some of the more promising constructs have yet to be tested in NHP models and even fewer have progressed to human trials, the critical determinant for vaccine efficacy. Moreover, *In vitro* assessment of the ability to broadly neutralize HIV across clades will not necessarily translate into *in vivo* protection. This was illustrated by a recent NHP study in which DNA/MVA regimens together with either GM-CSF or CD40L led to incomplete protection following a SIV_{smE660} challenge. Surprisingly, analysis of breakthrough SIV isolates revealed that they could be neutralized by potent vaccine elicited Abs observed prior to challenge [157]. Several possibilities were offered to explain this observation: (1) the *In vitro* TZM-bl neutralization assay did not appropriately mimic the *in vivo* situation; (2) there was a significant disconnect between the nAb present in sera of the vaccinated macaques and in secretions at the rectal site of exposure; (3) the immunization regimen responsible for inducing high nAb titers might have increased potential $CD4^+$ T-cell targets. With regard to the latter, a DNA/MVA regimen in which the MVA boost was augmented with gp140 protein

in alum induced CXCR5$^+$ CD4$^+$ T-cells skewed toward expression of CXCR3. Although these cells exhibited B-cell helper activity strongly associated with Ab avidity and persistence, [158] this population was also correlated with peak viral load in the challenged rhesus macaques. This suggests that approaches to induce potent, long-lasting Ab, including generation of CD4$^+$ T follicular helper cells, may also provide more target cells susceptible to viral infection. The proportional balance between cell populations that facilitate immune responses yet also serve as viral targets will be an important consideration moving forward.

Many factors can impact the characteristics of vaccine-elicited Abs. Among these is the microbiome, an area of recent intense interest. The microbiome is critically important in shaping mucosal immune responses [159] and also impacts HIV acquisition and pathogenesis [160]. Recent analysis of a non-protective vaccine in clinical trials, consisting of a DNA prime followed by boosting with Ad5 recombinants encoding HIV Gag, Pol, Nef, and clade A, B, and C gp140, revealed that the non-neutralizing Abs elicited were predominantly to gp41 and cross-reacted with the intestinal microbiota [161]. That this B-cell repertoire was already imprinted suggests that neonatal imprinting with HIV Env or slight alteration of gp41 amino acid sequences in future immunogens might be able to overcome this cross-reactive response. Greater appreciation and understanding of the microbiome's influence on adaptive immunity are needed in order to improve vaccine strategies.

In spite of the focus on bnAbs as a protective immune correlate, multiple immune responses, including humoral, cellular, and mucosal, have been shown to contribute to protective efficacy. Although this makes evaluation of vaccine regimens more complex, it is likely that there is not just a single protective immune correlate. Contributing to the complexity is our recent observation of a sex bias in vaccine-induced protection, wherein female but not male macaques exhibited delayed SIV acquisition following vaccination with combined Ad-SIV prime/Env boost regimens [162]. The delay in acquisition was correlated with Env-specific IgA responses in mucosal secretions, rectal Env-specific memory B-cells, and total rectal plasma cells, highlighting the importance of mucosal immunity for protection against HIV/SIV. This sex bias is well known in the pathogenesis of other viral diseases [163] and also in HIV infection [164]. We concur with the importance of Ab induction in a prophylactic vaccine for HIV. Ab evaluations should assess both systemic and mucosal responses, and should encompass more than just neutralizing activity. Non-neutralizing effector functions and other key features such as Ab avidity, isotype, and subtype could well be critical for efficacy.

Five-year view

The next few years should see movement of more of the Env immunogens deduced from structural studies and germ line Ab analyses into preclinical and clinical trials. As it may not be possible to target a single conserved Env epitope to elicit the desired bnAb response, heterologous combinations of Env immunogens will likely be needed together with novel vector combinations as priming immunogens to achieve the right mix of non-neutralizing and nAb responses necessary to prevent infection. We expect significant attention will be paid to non-neutralizing Ab effector functions and that considerable efforts will be placed on

'tuning' the Fc portion of Abs in order to enhance certain activities and achieve desired functionality [165]. The current emphasis on basic studies of B-cell maturation and development will lead to new approaches for eliciting durable Ab responses without invoking an overabundance of $CD4^+$ T_{FH} viral targets. To date, little attention has been placed on mucosal immunity in vaccine trials, but we predict a greater effort will be made to evaluate mucosal responses in human clinical trials in view of continued evidence for its importance in protecting against HIV infection at the site of initial exposure. The need to take the microbiome into account, as with the importance of including both men and women in clinical vaccine trials, will introduce additional layers of complexity into the assessment of vaccine efficacy. Our understanding of the vectors used in prime/boost strategies, and their resultant immuno-logical effects, will continue to evolve as more immunological data is amassed from the various strategies employed. Over the next 5 years, applying lessons learned in the RV144 trial together with novel vector prime/Env protein boost approaches, we expect continual, gradual improvement in vaccine-induced protective efficacy.

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Key Issues

- Among a limited number of clinical efficacy trials, only the RV144 trial, which evaluated an ALVAC prime/bivalent Env boost, provided a modest efficacy of 31%. Despite representing a significant achievement and stimulating the field, significant room for improvement remains.
- DNA vaccines are safe, stable, able to incorporate almost any sequence, lack anti-vector immunity, and provide durable immunity, yet require alternate delivery methods, adjuvants, or heterologous vectors to enhance their potency.
- The large diversity of Ad vectors, both replicating and nonreplicating, combined with their ability to elicit potent innate and adaptive immunity and their proven clinical safety, makes them ideal for usage in HIV prime-boost strategies.
- Based on the successful eradication of smallpox by the vaccinia vaccine, modified and alternate poxviruses have been heavily used as vaccine vectors, including in clinical trials which have established their safety and immunogenicity. The ALVAC vector is currently of greatest interest due to the success of the RV144 trial.
- Various envelope protein immunogens are being used in vaccine strategies, trending toward increasingly complex structures in order to emulate the native glycoprotein spike and elicit protective Ab responses.
- Not only broadly nAbs are the desired result of current prime-boost vaccine strategies, but also non-neutralizing Abs.
- Heterologous vector prime/envelope boost strategies afford an opportunity to effectively elicit the multiplicity of immune responses likely necessary for an efficacious vaccine and especially significant breadth, potency, and longevity in protective Ab responses.
- To achieve greater protective efficacy, prime/boost strategies might need to take advantage of new vector systems paired with newly designed envelope immunogens. Achieving enhanced immune responses while minimizing potential targets for infection will be challenging, as will taking into account effects of the microbiome and sex differences.

Table 1.

Some alternative vaccine vectors in development.

Vector	Features	Reference
Vesicular stomatitis virus	Attenuated, nonpathogenic; cytoplasmic replication	[89]
	Accommodates several inserts	
	Stable over several generations	
	No preexisting immunity	
Sendai virus	Attenuated by gene alterations	[90]
	Easily modified to insert transgenes	
	Cytoplasmic replication, no integration	
	Targets mucosa	
Rubella vaccine	Live, attenuated	[91]
	Durability of response	[92]
	Induction of memory B-cells	
	History of safety	
Yellow fever vaccine	Replication competent	[93]
	Highly efficacious against yellow fever	
	Elicits both CD4 and CD8 responses	
	Low preexisting immunity	
Rabies virus	Nonpathogenic	[94]
	Elicits Th1 responses	
Adeno-associated virus	Approved for use in humans	[95]
	Accommodates up to 5 kb transgenes	
	Preexisting immunity a potential issue	
Norovirus	NoV P particles for expression of conserved epitopes	[96]
	Multiple insertion sites	
	High immunogenicity and stability	
TianTan vaccinia virus	Attenuated, replicating	[97]
	In phase II trials in China	
Cytomegalovirus	Replicating and persistent	[98]
	Elicits strong effector memory CD8+ T-cell responses	[86,99]
	Potent protective efficacy in NHP	
Self-amplifying mRNA	Replicates itself and acts as adjuvant	[100]
	Delivery by cationic nanoparticles	[88]
	Induces potent cellular and humoral immunity	
	Synthetic, no cell culture for production	
	Low-dose administration	