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# Perspectives in HIV Vaccine Development: What we have Learned and how we Proceed Forward

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## Introduction

Thirty years have passed since the first report of Acquired Immunodeficiency Syndrome (AIDS) and the subsequent discovery of its etiological agent, the human immunodeficiency virus (HIV) [1,2]. Never before has a single virus been so thoroughly studied, providing vast amounts of data and insight into the intricate complexities of its innate and adaptive immune responses. Despite this knowledge and the advanced development of antiretroviral drugs that are highly effective in suppressing HIV viremia and preventing progression to AIDS [3,4], an effective vaccine capable of preventing infection from the diversity of the circulating viral variants has lagged behind. Here, we discuss the scientific challenges and review the advances in new vaccine technologies, which may provide the breakthroughs for truly effective HIV vaccine candidates.

## Scientific Challenges

Classical prophylactic vaccines, such as those against measles, polio and Hepatitis A, and before that, smallpox, utilized either whole inactivated or live attenuated virus to generate an immune response similar to that of natural infection to protect the host from subsequent infection. However, inactivated HIV particles have proven to be poorly immunogenic with proportionally little intact envelope glycoprotein (Env) and failed to induce broad or robust neutralizing antibody (nAb) or cytotoxic T lymphocyte (CTL) responses. Indeed, early studies with whole inactivated simian immunodeficiency virus (SIV) in nonhuman primates (NHPs) either failed to confer any protective immunity [5], or could only protect from challenge viruses grown in the same culture system as the vaccine preparations [6]. Further more, protection from challenge appeared to occur only at the peak of vaccine-induced immunity [7]. In the meantime, replication-competent live attenuated viruses with deletions in major accessory viral genes were generated, believing that they would be more immunogenic than the whole inactivated ones. This rationale was partly supported by clinical observations in a cohort of blood transfusion patients infected with *nef*-defective HIV-1 who initially appeared to become long-term non-progressors [8]. While initial studies demonstrated impressive protection from subsequent infection and/or disease in adult macaques immunized with attenuated SIV strains [9-11], further work found that neonates and some adults eventually progressed to AIDS [12,13]. A similar observation was made in the aforementioned patient cohort, in which the CD4 cell count declined over time and antiretroviral therapy was eventually required in half of the individuals [14]. The issue of safety and lack of heterologous efficacy from infection in pre-clinical models, led to a curb in enthusiasm for these vaccine approaches that have been successful for other viral diseases.

Two important differences distinguish HIV from viral infections for which vaccine development has been successful. First, retroviruses replicate through a DNA intermediate, called a provirus, which integrates into the host genome. Infection of CD4 T cells and antigen presenting cells (APCs) allows the virus to quickly establish a reservoir to allow persistent infection. Furthermore, a lack of proof-reading activity of the reverse transcriptase, combined with immune selection pressures, lead to a high mutation frequency and selection for immune escape variants in each generation of the viral progeny, making HIV one of the most variable viruses known. Hence, effective vaccine strategies for HIV must be able to protect against the diverse population of circulating variants by targeting conserved antigenic regions.

The use of a non-human primate (NHP) model with repeated low dose SIV challenge has proven invaluable in evaluating the immunogenicity and efficacy of candidate vaccines pre-clinically, particularly for T cell-based vaccines. However, there remain important differences between the Env of HIV-1 and SIVmac, making it difficult to assess antibody-based vaccines. In response to this, the chimeric simian-human immunodeficiency virus (SHIV) system, which consists of HIV-1 Env and SIVmac backbone, was subsequently developed. However, there are limited strains of SHIV available, most of which are derived from neutralization sensitive subtype B or C isolates that have overt or subtle differences when compared to the transmitting HIV variants [15]. Whether the SHIV/NHP model for vaccine evaluation can predict protection from transmitting HIV variants remains to be seen in the next round of Phase II and III clinical trials. Nonetheless, continued standardization and validation in both animal models and *in vitro* analytical assays are required to better establish any correlates between immunity and protection [16].

## New Tools in Identifying Broadly Neutralizing Antibodies

In order to achieve protection, a vaccine must elicit effector antibodies capable of inactivating the virus. For HIV, the envelope glycoprotein (Env) is the only antigenic target to which nAbs are generated. The phase III AIDSVAX efficacy trials undertook the subunit vaccine approach by employing subtype B monomeric gp120. While the vaccine failed to provoke overall protection from infection, partial protection was found to correlate with a high level of vaccine-induced antibodies [17,18].

Prior to 2009, only four broadly neutralizing antibodies (bnAbs) had been identified. These were: b12, which targets the conserved epitope on the CD4 binding site (CD4bs) [19]; 2G12 which has a unique V(H) domain-exchange domain and targets the carbohydrate moieties of gp120 [20]; and 2F5 and 4E10, both of which recognize the membrane proximal external region (MPER) of gp41, have long

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hydrophobic CDRH3 loops and are polyreactive [21,22]. These antibodies were produced either by selection of antibody-secreting hybridomas or from random cloning of the heavy and light chains in phage display libraries. Comprehensive analysis demonstrated that 4E10 could neutralize viruses from different clades but only with a weak to moderate potency, while b12, 2G12 and 2F5 possessed higher potency but displayed limited breadth against non-clade B viruses [23].

More recently, two approaches have been adopted for the successful identification of a series of bnAbs, which display significantly greater breadth and depth than ever before. The first strategy focused on elite neutralizers, defined as patients who have been infected with HIV-1 for at least 3 years but remained asymptomatic in the absence of antiretroviral therapy and with sera able to neutralize a panel of viruses of different subtypes [24]. Memory B cells were isolated from the top elite neutralizers and screened in high-throughput binding and micro-neutralization assays [24]. Two unique quaternary-specific antibodies that recognize the V2 loop, PG9 and PG16, were first identified and shown to neutralize 80% of the viruses tested [25]. Subsequent analysis further reconstituted an additional panel of bnAbs (PGT series) with even greater potency and/or breadth, many of which recognize either quaternary epitopes similar to those of PG9/16, or epitopes associated to the glycan residues in manners different to that of 2G12 [26]. Recent immunological analysis of the phase III RV144 trial, which itself demonstrated 30% efficacy, found that infection risk is inversely correlated to serum IgG against the V1V2 antigen, generating further incentive for targeting this epitope domain [27]. Several V2-scaffolded antigens are currently being evaluated pre-clinically for their immunogenicity and a passive immunization study employing V2 nAbs in NHPs will be commenced to further determine their protective efficacy (B.F. Haynes, personal communication).

In contrast, the second strategy focused only on the critical functional sites of Env. One example of this is through the use of a resurfaced stabilized core (RSC), which was designed to expose only the CD4bs of the virus by substituting all other regions for either SIV homologues or non-HIV residues, rendering them unrecognizable to HIV antibodies. Using the RSC3 probe, a series of CD4bs-specific bnAbs (VRC01-03 and PGV04) were identified with some able to neutralize more than 90% of all circulating variants [28,29]. Another site-specific probe, the 2CC core, was a gp120ΔV1-V3 core stabilized in the CD4-bound conformation, exposing both the CD4 binding and CD4-inducible co-receptor sites [30]. Among the panel of bnAbs isolated from single B cells captured by the 2CC core, 3BNC117 was shown to have superior breadth and potency to VRC01 and could neutralize up to 96% of the viruses tested [31]. Thus far, a passive immunization study in NHPs has found that a high dose of VRC01 was capable of conferring sterilizing protection from mucosal challenge [32]. A phase I immunization clinical trial is currently under way to better define the specificity and potency of VRC01 in humans.

## Other Strategies in Immunogen Design

Although the RSC and 2CC core have served as useful templates in identifying bnAbs, they displayed poor immunogenicity in vaccine settings and failed to elicit nAbs *in vivo*. Hence, various strategies have been employed to rationally design antigens that are immunogenic and can induce protective immunity. These include a substantial effort in evaluating recombinant envelope glycoproteins, particularly of subtype C, derived from founder/transmitting variants (S.W. Barnett, unpublished data). The hypothesis being that these immunogens can elicit immune responses that can better protect against circulating variants than those derived from laboratory-adapted strains. Alternatively, candidate Env immunogens were selected based on their ability to bind conformational-dependent monoclonal antibodies (mAbs) and their putative reverted unmutated ancestors, arguing that these antigens might better present the normally conserved epitopes. One example is the gp120 antigens used in the ALVAC trial, in which the amino acids at the N-terminal were substituted with glycoprotein D (gD) of herpes simplex virus. Significant increase in mAb binding to the quaternary and other conformational epitopes of the gD+ gp120 was detected, while the effect was marginal for the linear epitopes [33]. Whether this specific increase in antigenicity can confer improved immunogenicity and protective immunity will, however, requires further investigation. More recently, extensive analyses are also being carried out to increase our understanding in the structural and biochemical features of N-linked glycans [34]. The results will provide guidance in designing envelope glycoprotein with specific glycan modification or unusual glycosylation patterns that will enhance its immunogenicity.

Since it is generally difficult to induce specific antibodies to conserved epitopes via immunizations with whole Env spike, site-specific antigens have also been widely examined. For example, the epitope of VRC01 has been grafted onto a Chikungunya virus-like-particle (VLP). While the sera from immunized rabbits could only neutralize tier 1 isolates, the overall antibody response showed increased specificity to CD4bs [35]. Other VLPs presenting the glycan moieties specific for the PGT bnAbs are also being developed. On the other hand, antigen presentation of the variable loop is normally through scaffolding the antigenic site to carrier protein. A series of consensual V1V2 and V3 scaffolds have been constructed and evaluated in small animals and NHPs. While these scaffolds are immunogenic and can elicit target-specific nAbs, their carrier proteins often induce substantial background reactivity after repeated immunization (B.F. Haynes, personal communication). Finally, the linear epitopes on MPER are juxtaposed to the lipid membrane and only transiently exposed post receptor and co-receptor binding. Immunization studies in small animals with peptide mimetics of the gp41 fusion intermediate induced serum IgG that could only weakly neutralize selected variants of tier 1 isolates [36]. A more recent NHP study with gp41 virosomes also failed to induce neutralizing serum IgG, but data suggested that the virosome-induced mucosal IgA antibodies were sufficient to confer partial protection in animals from vaginal challenge [37].

## Limitations of Reverse Vaccinology

By identifying the specific epitopes recognized and resolving the crystal structures of these bnAbs, reverse engineering of the small protein or peptide mimics of these conserved antigenic sites becomes feasible. Nevertheless, several challenges to this strategy must first be overcome (reviewed in [38,39]). First, structural data on HIV-1 Env were elucidated largely based on truncated and/or deglycosylated gp120 monomers in CD4-bound state and little is known about the native trimers and their presentation of both neutralizing and non-neutralizing epitopes [40]. In addition, many of the bnAbs, particularly those targeting V2, recognize discontinuous and/or quaternary epitopes that are conformationally challenging to mimic, synthesize and stabilize [41]. Even for 2F5 and 4E10, which are directed to the linear epitopes on gp41 MPER, their epitope-scaffolds have failed to elicit nAbs in small animals, despite binding strongly to the corresponding bnAbs [42-44].

Furthermore, it has been demonstrated that during chronic HIV-1 infection, extensive somatic hypermutation is required to increase antibody affinity and neutralization breadth and potency [45]. Indeed, analyses of some of the bnAbs showed that they have undergone 19-46% more somatic hypermutation than their weakly or non-

neutralizing counterparts [25,28,46-48]. Many of these mature and hypermutated nAbs are autoreactive and subjected to elimination by B cell tolerance mechanisms (reviewed in [49]). Finally, putative germline ancestor antibodies often divert significantly from their corresponding bnAbs and do not bind to Env antigens [47,50]. Since naïve individuals only have low affinity B cell receptors, immunization with antigens engineered based on these mature and mutated bnAbs may not be capable of inducing the desired nAb responses due to inadequate antigen recognition and binding. Taking these into consideration, the new generation of immunogen design will focus on using unmutated and intermediate ancestor antibodies as templates and their immunogenicity *in vivo* will be compared to the existing antigens [49].

## T Cell-Based Vaccine Strategies for HIV-1

In the last decade, in the absence of Env antigens that could induce bnAbs, efforts were undertaken to develop T cell-based vaccines that could induce potent and broadly reactive CTL responses. In the course of HIV infection, antigen is processed and presented by major histocompatibility complex (MHC) class I or II on the surface of APCs. CD8 T cells recognize short antigen peptides (8-11 amino acids) presented by MHC class I. However, these activated CD8 T cells cannot effectively mediate killing of infected cells without CD4 T cell help. CD4 T cells, on the other hand, recognize longer antigen peptides (10-18 amino acids) presented by MHC-II. Following antigen presentation and CD28 co-stimulation, CD4 T cells become activated and differentiate into type 1 (Th1) or type 2 (Th2) helper cells. The Th1 subset secrets cytokines to drive the proliferation of effector CD8 T cells, which are then licensed to kill infected target cells. The antiviral property of CD8 CTLs has been demonstrated to play an important role in controlling persistent viral infections including HIV [51,52].

Two modalities are typically used for T cell vaccines: plasmid DNA or viral vectors [53]. Immunization with plasmid DNA, either intramuscularly or intradermally, proved to be poorly immunogenic. Several types of modification have been employed to increase the potency, including sequence codon-optimization and the use of strong promoters to enhance expression efficiency [54,55]. Furthermore, plasmid DNA may be enclosed in liposomes or adsorbed onto metal microparticles to increase antigen uptake [56,57]. More recently, delivery of DNA by electroporation has gained preference. Such devices emit short electrical pulse to create temporary pores in the cell membrane to allow antigen uptake and have been shown to improve immunogenicity and enhance immune response [58]. An ongoing phase I clinical trial is set to evaluate the efficacy of DNA vaccine delivered via electroporation compared to standard intramuscular injection [59]. While DNA alone had not provided sufficient potency it was found to be a good priming agent in combination with a variety of viral vector systems.

The first viral vector described as an HIV vaccine candidate in human clinical trials was a live recombinant vaccinia virus for immunocellular therapy in HIV-infected patients [60]. Unfortunately some of the vaccinia viruses had escaped inactivation and caused infection in the immune compromised recipients and three cases of death were reported [61,62]. Subsequent development of pox-based vectors focused on highly attenuated strains such as Modified Vaccinia Ankara (MVA), Copenhagen (NYVAC) and canarypox (ALVAC), all of which are considered to have better safety profiles, as they are replication-deficient in mammalians. While ALVAC was used as the priming regimen (with recombinant gp120 for boosting) in the Phase III RV114 Thai trial, it only induced low level of CD8 responses. Whether these responses contributed to the overall protective efficacy of the vaccine combination is unknown [63]. In contrast to ALVAC, NYVAC was generated from parental vaccinia virus with gene deletions. Recent data has shown that a DNA prime / NYVAC boost regimen is capable of inducing sustained HIV-specific CD4 and CD8 T cells both in blood and at mucosal sites [64-66].

In addition to poxvirus, adenovirus has also been heavily exploited as viral vectors for T cell vaccine. To date, the only T cell vaccine that has been tested in phase III trials (STEP and Phambili) for efficacy was a non-replicating Adenovirus serotype 5 (Ad5) vector expressing HIV-1 Gag, Pol and Nef [67]. Despite the presence vaccine-induced CD8 T cells, both trials were terminated ahead of schedule due to lack of protection and also potential risk of increase infection. While post hoc analyses of the STEP trial indicated that the latter observation was specific to male vaccines that were both uncircumcised and with pre-existing nAbs to the Ad5 vector, this trend was not demonstrated in the Phambili trial [68]. A second T cell vaccine trial is currently being evaluated in a Phase II trial (HVTN 505) to increase our understanding on T cell immunity in protection. The regimen consists of three DNA primes followed by Ad5 vector boosting. Based on the outcome of the STEP trial, only circumcised men without pre-existing immunity to Ad5 were recruited for enrollment [69]. A series of other non-replicating viral vectors, such as rare serotypes of Adenovirus, Adeno-associated virus (AAV), Alphavirus, and Herpesvirus, have also been developed and resulted in varying degree of success [70].

More recently, the field has explored the potential use of replicating viral vectors, including Measles virus, Vesicular stomatitis virus, Cytomegalovirus (CMV), Adenovirus and Poxviruses to express certain HIV genes as antigens [53]. These replicating recombinant vectors are believed to be significantly more immunogenic, capable of stimulating a more robust and sustained T cell responses. Preclinical studies in chimpanzees have demonstrated that replicating Ad5 vector was better at eliciting HIV-specific cellular and humoral responses than its non-replicating counterpart [71]. In addition, immunization with replicating CMV vector was found to induce and maintain a robust HIV-specific CD4 and CD8 effector memory response in NHPs, despite the presence of pre-existing CMV immune responses [72,73]. This T cell response is directly correlated with the 50% protection efficacy observed, as no nAbs were detected.

While T cell-based vaccines, particular those employing replicating recombinant vectors expressing HIV antigens, have shown promise, several caveats must first be overcome. First, the efficacy of the T cell vaccine approach is defined by the efficiency of CTL in recognizing and killing infected cells, which is restricted by the human leukocyte antigen (HLA). Some HLA haplotypes, such as B27 and B57, have been associated with better disease prognosis [74], while the B35 allele is associated with a poorer outcome [75]. Thus, the effectiveness of a T cell vaccine candidate is expected to vary between individuals, depending on the HLA haplotypes of individuals, and the population targeted. Furthermore, HIV can mutate rapidly to escape CTL detection, thus, multiple conversed epitopes must be presented by the vaccines to broaden the array of antigen recognition [76-78].

## Gene Therapy for HIV-1

In addition to the design and development of B or T cell-based vaccines, targeting HIV-1 through gene therapy, or intracellular immunization, has also been actively explored. The notion of intracellular immunization was made popular by David Baltimore in 1988, referring to gene-based expression of inhibitory molecules to render T cells resistant to viral infection [79]. In a proof-of-concept

mice study with AAV vector expressing b12, the mAb remained detectable in the serum for over 6 months post intramuscular immunization [80]. A subsequent NHP study with the same vector expressing immunoadhesins (chimeric molecules containing the functional domain of a binding protein and the immunoglobulin constant domains) protected 6/9 animals from intravenous SIV challenge, demonstrating that virus-inhibiting molecules endogenously synthesized in myofibers can be passively distributed in the circulatory system and induced long-lasting protective immunity [81]. Whether the same degree of protection can be seen at the mucosal level remains to be investigated. Furthermore, the concerns for safety on prolonged exposure to mAbs or immunoadhesins need to be addressed.

## Conclusion

The development of an effective HIV vaccine is perhaps one of the greatest global health and biotechnological challenges in the history of infectious diseases. Classical vaccine approaches that have successfully induced protective immunity to many pathogens and eradicated small pox, have been tried and failed. The global support and funding has facilitated a surge of new enabling technologies. While the humoral (B) and T cell-based vaccine approaches each have their own caveats, the recent success in identifying and producing bnAbs, new generation viral vectors, and the immunotechnology to detect highly specific responses, have greatly facilitated our understanding in vaccinology that will ultimately be required to develop an effective HIV vaccine and meet the challenge of new and re-emerging pathogens.

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