

## HIV-1 vaccine development: tackling virus diversity with a multi-envelope cocktail

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## 1. ABSTRACT

A major obstacle to the design of a global HIV-1 vaccine is viral diversity. At present, data suggest that a vaccine comprising a single antigen will fail to generate broadly reactive B-cell and T-cell responses able to confer protection against the diverse isolates of HIV-1. While some B-cell and T-cell epitopes lie within the more conserved regions of HIV-1 proteins, many are localized to variable regions and differ from one virus to the next. Neutralizing B-cell responses may vary toward viruses with different i) antibody contact residues and/or ii) protein conformations while T-cell responses may vary toward viruses with different (i) T-cell receptor contact residues and/or (ii) amino acid sequences pertinent to antigen processing. Here we review previous and current strategies for HIV-1 vaccine development. We focus on studies at St. Jude Children's Research Hospital (SJCRH) dedicated to the development of an HIV-1 vaccine cocktail strategy. The SJCRH multi-vectored, multi-envelope vaccine has now been shown to elicit HIV-1-specific B- and T-cell functions with a diversity and durability that may be required to prevent HIV-1 infections in humans.

## 2. INTRODUCTION

### 2.1. Tackling the HIV-1 Pandemic: New and Old Vaccination Strategies

It is estimated that over 40 million people are currently infected with HIV-1; over 20 million cumulative deaths are attributable to HIV/AIDS; and each day 16,000 people are newly infected (UNAIDS, (1, 2)). A challenge unique to the design of a vaccine to prevent HIV-1 is the large number of circulating variants. This variability is present both within and between subtypes and is evident within essentially all HIV-1 proteins. The HIV-1 envelope protein has five designated hypervariable regions (V1-V5) and five 'conserved' regions. However, these designations are relative rather than absolute, as there is heterogeneity present throughout the sequence. Internal HIV-1 proteins such as gag and pol, while lacking hypervariable regions, also exhibit significant variability, and their mutations can readily mediate virus escape from a qualitatively weak immune response (3).

The production of a successful HIV-1 vaccine has been a global objective for more than two decades.

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Early studies demonstrated that the immune system was able to elicit a protective immune response toward the simian immunodeficiency virus (SIV). Researchers showed, for example, that when macaques were administered wildtype or attenuated SIV, they were protected from a later challenge with a different SIV (4-6). Furthermore, passively transferred sera from SIV infected animals could protect naïve recipients from challenge, suggesting that antibodies were sufficient to confer complete protection (7-11). These experiments demonstrated the feasibility of preventing immunodeficiency virus infections with an appropriately primed immune system. Nonetheless, the results did not lead to the development of HIV-1 vaccines appropriate for human use, because attenuated immunodeficiency virus vaccines had the capacity to revert to a wildtype phenotype (12, 13) and the attenuated SIV vaccines ultimately caused morbidity and mortality in monkey models (14). Attempts were made by researchers to overcome this potential by creating highly-attenuated viruses, but a severe crippling of virus replication resulted in limited virus evolution in infected animals, yielding a qualitatively weak and unprotective immune response (15).

Another vaccination approach involved the use of recombinant vaccines. In the early 1990s, recombinant vaccines often targeted the envelope protein, the primary target of neutralizing antibodies. This strategy proved to be successful. In fact, when envelope antigens were delivered to naïve macaques in the form of vaccinia virus and protein recombinants, animals were protected from SIV challenge, provided that the envelope proteins in challenge viruses were precisely matched with those in the vaccines (16, 17). Successful vaccination experiments were also conducted with HIV-1 in the chimp model (17). Recombinant vaccines were less successful in non-human primate models when the challenge virus was mismatched for envelope protein (18), and because the challenge virus could not be selected to match the vaccine in humans, clinical trials using the single-envelope (or a dual-envelope) strategy were unsuccessful (19, 20).

The difficulty of developing an effective human vaccine prompted questions concerning the efficacy of conventional delivery vehicles and exploration of alternative vectors. There was also unresolved debate (which continues today (21)) concerning (i) correlates of protection and (ii) interpretations of *in vivo* and *in vitro* assay results. The new vectors that emerged included Semliki Forest virus (22, 23), VEE (24), adenovirus (25), adeno-associated virus (26), Modified Vaccinia Virus Ankara (MVA) (27, 28), canarypox/fowlpox (29-31), yeast (32), and vesicular stomatitis virus (33), as well as naked DNA envelope-expression constructs (34, 35). Numerous adjuvants were also tested to enhance T- and B-cell activities toward recombinant vaccines (36, 37). Each new system proved at least partially effective at eliciting responses toward HIV/SIV viral antigens, but none demonstrated striking improvements compared to previously-tested vaccines. Results suggested that problems surrounding vaccine design were not entirely related to the delivery systems.

Internal proteins were also studied in response to the failures of single-envelope vaccines in humans. Vaccines were developed in which antigens such as gag, pol,tat or vpu were added to, or substituted for envelope proteins (33, 38-41). These vaccines were shown to elicit T-cell responses and were proven effective in impeding disease progression in monkeys challenged with viruses matched for internal protein sequences. However, these vaccines could not prevent viral infections (3, 42).

Regardless of the vaccine target antigen(s), variability among HIV-1 isolates remained a major challenge. Strategies to overcome variability have included (a) the modification of envelope variable regions or glycosylation sites (43, 44), (b) the capture of envelope-CD4 fusion intermediates (45, 46), and (c) the design of consensus or ancestral proteins (47). Still, a gold-standard single-antigen vaccine has not been identified.

### 2.2. Lessons from vaccine successes in primate systems

While no HIV-1 vaccine has yet demonstrated protection in a clinical trial, a number of the research findings described above lend credence to the hypothesis that a preventive vaccine against HIV-1 is well within reach. Perhaps the most telling results were i) macaques infected with attenuated SIV (or wildtype SIV) were protected from subsequent challenge (4, 48), ii) immunoglobulins from SIV-infected (or HIV infected) animals could be passively transferred to naïve recipients to prevent infection (even when the same antibodies scored negatively for neutralization against the challenge virus *in vitro* (7-11, 49, 50)), and iii) recombinant vaccines protected animals from infection provided that the envelopes were matched between the vaccines and the challenge viruses (16, 17).

The following course of events may explain why the primed immune systems of infected macaques are capable of preventing virus infection from an exogenous source when they cannot clear endogenous virus: when virus exposure occurs before immune activation, a portion of the virus quickly sequesters in privileged sites (e.g. brain tissue), and remains hidden from the immune system for the lifetime of the animal. When an immune response is generated in peripheral tissues (51), effector cells and antibodies cannot reach the founder virus, which perpetually sheds escape variants into the periphery. Cycles of immune response, virus mutation and virus escape continue until the peripheral immune system is exposed to many variant epitopes of infectious virus. At this point, a qualitatively and quantitatively robust response confers protection against exogenous viruses, even though the virus in privileged sites cannot be reached.

The feature of virus sequestration and protective immunity is not unique to the immunodeficiency viruses, but is characteristic of numerous pathogens such as varicella-zoster virus (VZV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV). Each of these viruses can activate a protective immune response toward peripheral pathogen exposures, yet will persist asymptomatically for years in an immunocompetent host. If/when the immune system

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becomes compromised, endogenous viruses can overwhelm the host and pose a serious threat to human health. HIV-1 is particularly insidious due to its specific targeting of the CD4+ T cell population. It should be noted that the chronic nature of a viral infection need not thwart vaccine development. For example, in the case of VZV, researchers have successfully developed an effective and fully licensed vaccine (52).

It is likely that an effective HIV-1 vaccine must recapitulate some of the events of natural infection. We suggest that the creation of an antigen cocktail is necessary to capture the antigenic variability of naturally evolving viruses. One may postulate that while the overall protein sequences differ extensively among HIV-1 variants, there are certain restrictions in protein sequence and conformation which are dictated by function. For example, the envelope protein must bind the conserved CD4 molecule as well as a conserved co-receptor molecule (e.g. CCR5 and/or CXCR4 (53, 54)) to mediate fusion and virus entry. Thus, the number of proteins required to represent the envelope structures that are compatible with infection, while greater than one or two, need not be vast. The immune system has evolved precisely to combat variant antigens, in that sophisticated recombination mechanisms (using arrays of V,D,J and C antibody and T-cell receptor genes) are utilized to create billions of unique lymphocytes, each armed with a different antigen receptor (55). The harnessing of such immune potential with an antigen cocktail (as opposed to a single antigen) may well lead to an HIV-1 vaccine capable of eliciting protective immunity in humans. Of note, the cocktail approach has been successful in other fields. For example, the pneumococcus vaccine, comprising 23 distinct components, is currently licensed and highly effective (56).

### 3. THE SJCRH MULTI-ENVELOPE APPROACH TO HIV-1 VACCINE DESIGN

Investigators at SJCRH acknowledged the importance of arming both the B-cell and T-cell populations of the immune system against the diverse antigens of HIV-1 and pioneered a novel envelope cocktail vaccine strategy. The following methods have been used to select gp140 envelope sequences for inclusion: (1) the sampling of envelopes obtained longitudinally from infected individuals, thus representing HIV-1 escape mutants (51), (2) the sampling of envelopes with diverse antibody-antigen binding patterns as recognized by *in vitro* tests (57, 58), and (3) the sampling of envelopes from multiple distinct subtypes (A-E).

#### 3.1. Strengths of a prime-boost vaccine regimen

In order to elicit strong and durable responses toward envelope cocktails, the selection of delivery vehicles was considered as important as the selection of antigens. In the mid-1990s, researchers at SJCRH and the University of Massachusetts Medical School together tested a prime-boost vaccine regimen, in which antigens were delivered by successive immunizations with DNA (D) and vaccinia virus (V). D was specifically chosen for study due to its ease of preparation and ability to elicit both B-cell

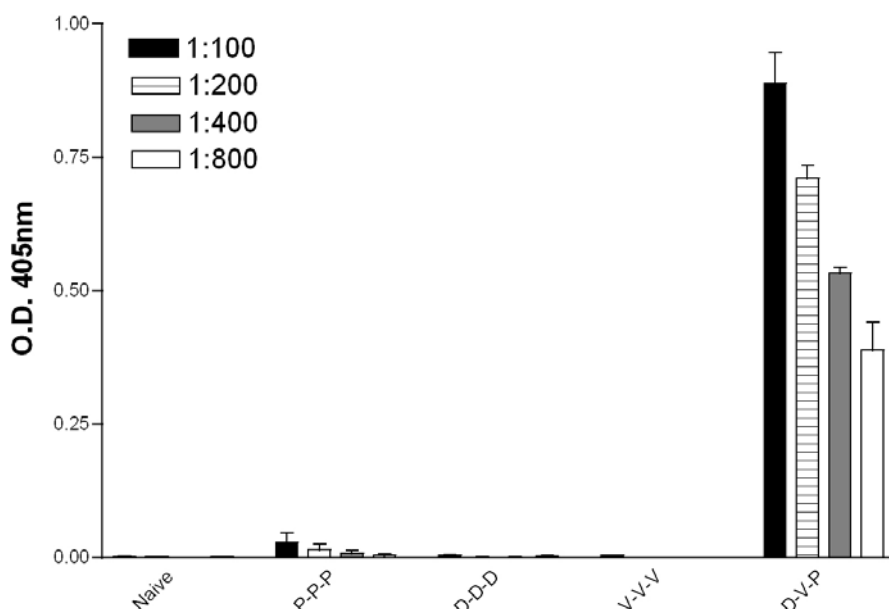
and T-cell activities. V was attractive for the same reasons. In addition, V immunizations were known to elicit durable responses and V was (and remains) the only vaccine associated with complete eradication of a human disease (59-62). Immunizations with alternating vectors (D and V) were compared to immunizations with single vectors (D alone or V alone) and the former were shown to elicit improved immune activity (63). Prime-boost regimens using recombinant D in conjunction with V or other viral vectors have now been adopted by many researchers in the vaccine field (64, 65). The addition of protein (P) as a second booster following D and V inoculations (designated D-V-P) further enhanced responses, yielding significant T-cell activities and antibodies of multiple isotypes (IgG1, IgG2a, IgG2b, IgA), identified at both systemic and mucosal sites (66).

To evaluate the durability of immune responses elicited by the D-V-P regimen, C57BL/6 mice were administered recombinant D (100 micrograms by intramuscular inoculation), followed by recombinant V (approximately  $1 \times 10^7$  plaque forming units (PFU) by intraperitoneal inoculation) followed by recombinant P (1-5 micrograms with complete Freund's adjuvant by intraperitoneal inoculation). Vaccines were administered in succession with one month intervals. Animals were then rested for at least 1 year to determine the longevity of the response. Control mice received the same recombinant vector three times (i.e. three immunizations with D, three immunizations with V or three immunizations with P). As shown in Figure 1, long-lasting anti-envelope antibodies were identified in the D-V-P immunized mice. These antibodies were measured not only in the blood, but also in bronchoalveolar lavage and vaginal washes. Studies clearly showed that the administration of vaccine at the site of a predicted viral exposure was not required for generation of antibodies at that site (66).

Antibody responses correlated with the presence of durable antibody-forming cells (AFC, measured by ELISPOT analyses) in the bone marrow of vaccinated mice (see Figure 2). T-cell responses, like B-cells responses, were also highly durable, and could be measured with T-cell ELISPOT assays for the lifetime of the animals (see Figure 3 and (66)).

#### 3.2. Immune Responses to an Envelope Cocktail

A number of labs have demonstrated that cocktail vaccines provide a greater breadth of activity than single component vaccines (67-71). In one such experiment conducted at SJCRH, cotton rats were administered either a single envelope vaccine (IIIB) or a multi-envelope vaccine (>30 envelopes, including IIIB). In each case, animals received sequential immunizations with D, V and P. IIIB-immunized animals generated potent neutralizing antibodies toward IIIB, but no measurable neutralizing activities toward two other subtype B viruses, 310a or 30e. Sera from animals that received the mixed envelope vaccines neutralized all three viruses, even though viruses 310a and 30e were not matched by sequence with components in the vaccine (68). This predictable outcome was reminiscent of the clinical trials described above, in



**Figure 1.** Highly durable anti-envelope antibody responses toward prime-boost regimens with envelope vaccines D, V and P. C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine) were administered three recombinant envelope vaccines with one month intervals (D, V, P). Control mice received only one vector in the three consecutive inoculations (D-D-D, V-V-V or P-P-P). Inoculations were with approximately 100 micrograms D,  $1 \times 10^7$  plaque forming units (pfu) V and 1-5 micrograms P in complete Freund's adjuvant (CFA; incomplete Freund's adjuvant replaced CFA when P was administered a second and third time). Each vaccine expressed the UG92005 gp140 envelope protein (81). ELISA antibody tests were conducted with UG92005 protein as the target antigen more than 20 months after inoculations were complete, using serially-diluted serum samples. Mice that had received vaccines with D-V-P developed the most durable and robust envelope-specific antibody responses. Methods: To perform the ELISA, 96 well microtitre plates (Becton Dickinson and Company, Franklin Lakes, N.J, USA) were coated overnight at 4°C with 2 micrograms/ml purified CHO cell-derived UG92005 envelope protein (matched with the vaccine) in PBS. The plates were washed three times with 0.05% Tween 20 (Sigma-Aldrich, St Louis, MO, USA) in PBS, blocked with 1% Bovine Serum Albumin (BSA) Fraction V/PBS at room temperature for 1 hr, and then washed an additional three times. Serum samples were diluted in 1% BSA/0.05% Tween 20/PBS to a final volume of 50 microliters and incubated on the coated plates for 2 hrs at room temperature. After three more washes, plates were incubated with alkaline phosphatase-conjugated anti-mouse IgG (50 microliters/well; Southern Biotechnology Associates, Birmingham, AL, USA) diluted 1:1000 in 1% BSA/Tween 20/PBS. After a one hr incubation period at room temperature, three final washes were performed. The assay was then developed with 75 microliters/well of *p*-nitrophenyl phosphate (Sigma-Aldrich) substrate (330 micrograms/ml in diethanolamine buffer) and the optical density (O.D.) was read at 405 nm.

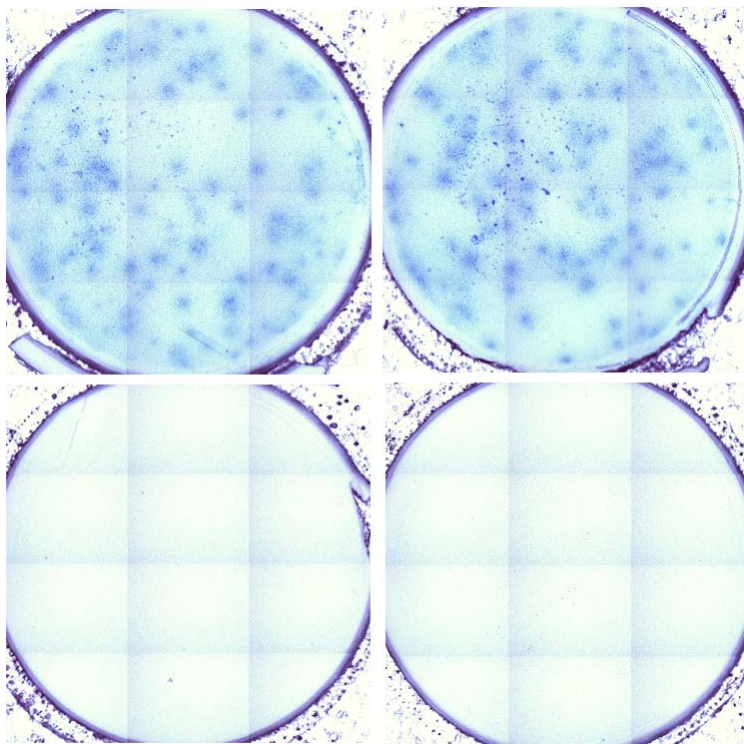
which single-envelope vaccines elicited restricted, type-specific responses and failed to protect humans from diverse HIV-1 (72). In the cotton rat studies, cocktail vaccines were used to present numerous envelope epitopes to immune cells. While the 310a and 30e sequences were not precisely included in the cocktail vaccine, one or more of the envelopes within the cocktail must have shared epitopes with 310a and with 30e proteins. The advantage of immunizing with a multi-envelope vaccine to increase immune breadth toward heterologous viruses was clearly illustrated.

Pertinent to delivering a cocktail of envelopes, SJCRH researchers also conducted experiments to confirm that a minor fraction of a mixed vaccine can be recognized by the immune system. For this purpose, mice received a combination of two different recombinant D vaccines, each expressing a different envelope protein. In some cases one of the components was represented as only 1% of the entire mix. Results showed that when a recombinant D

represented only 1 part per 100 of a vaccine mixture, an antigen-specific immune response toward that minor component could nonetheless be induced (73). Together, these studies have shown that immune cells, which have evolved precisely to combat diverse pathogens, are capable of responding to combination HIV-1 vaccines.

### 3.3. Protection in a Non-Human Primate challenge system

Pre-clinical evaluation of the SJCRH multi-envelope D-V-P vaccine approach included immunizations of six macaques (see (74) for details) with dozens of envelope proteins, delivered by successive inoculations with recombinant D, recombinant V and recombinant P. Of note, the V was administered by the subcutaneous route (74, 75) to show that the induction of an immune response did not require the appearance of a cutaneous skin lesion (the absence of a cutaneous lesion reduces the possibility of rare inadvertent transmissions of V (76), a feature pertinent to advancement of V in the clinical setting). Following

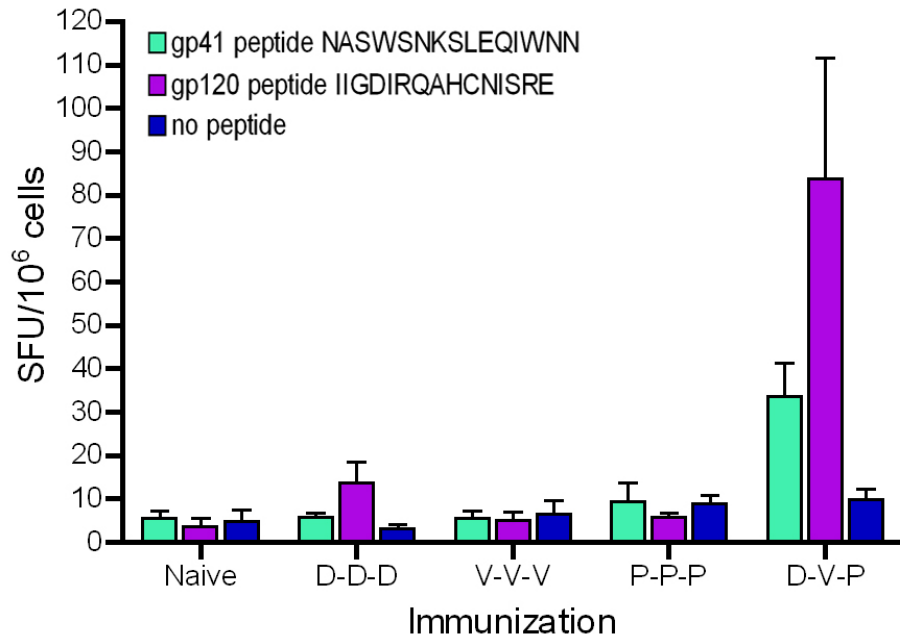


**Figure 2.** Highly durable anti-envelope antibody responses associated with long-term bone marrow-resident plasma cells. C57BL/6 mice received immunizations with D (administered twice), V (administered once), and P (administered twice) with at least one month intervals between inoculations. Vaccine concentrations were as described in the Legend for Figure 1. In this experiment, the D vaccine was a mixture of 20 recombinant plasmids each expressing a different gp140 envelope protein; the V inoculation was a mixture of 22 envelope-recombinant viruses, and the P inoculation was a mixture of four purified envelope proteins derived from recombinant Chinese Hamster Ovary cells (approximately 0.5 micrograms each). After more than 1 year from the time of the last inoculation, mice were sacrificed and bone marrow cells were tested for antibodies toward the 1007 envelope protein (a protein which was included by sequence in the vaccine (82)). Briefly, 96-well nitrocellulose Multiscreen-IP plates (Millipore, Bedford, MA) were coated with 50  $\mu$ l purified 1007 envelope protein at a concentration of 25 micrograms/ml in PBS. After overnight incubation at 4°C, wells were washed with PBS and blocked for 1 hr with 100 $\mu$ l complete tumor medium (CTM (83, 84), a Modified Eagles Medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum, dextrose (500  $\mu$ g/ml), glutamine (2mM), 2-mercaptoethanol ( $3 \times 10^{-5}$  M), essential and non-essential amino acids, sodium pyruvate, sodium bicarbonate and antibiotics) at 37°C, 10% CO<sub>2</sub>. Single cell suspensions were made in CTM and cells were added to the wells ( $10^6$  cells/well) for 3 hr at 37°C, 10% CO<sub>2</sub> followed by thorough washing with PBS. Alkaline phosphatase-conjugated goat anti-mouse Ig antibodies were diluted 1:500 in PBS with 1% BSA and added to wells for overnight incubation at 4°C. After washes with PBS, spots were developed with 1mg/ml 5-bromo-4-chloro-3-indolyl phosphate in diethanolamine buffer for 10-60 min at room temperature. Plates were washed and dried. Shown are photographs of wells plated with the bone marrow from vaccinated (upper panels) and unvaccinated (lower panels) animals.

vaccination, all animals developed diverse binding and neutralizing activities toward a number of heterologous envelope proteins including X4 and R5 viruses. Antibodies were additionally functional in antibody dependent cell-mediated cytotoxicity assays (see Figure 4). T-cell immune responses were also measured by gamma-interferon ELISPOT assays using envelope-derived peptide pools. Specific T-cell responses were identified and responses were shown to include both CD4+ and CD8+ T cell functions (74).

While macaques are not susceptible to HIV infection, artificial HIV-SIV chimeric viruses have been created to provide a challenge model system. When

vaccinated and control macaques were inoculated by the intravenous route with a pathogenic SHIV (89.6P) vaccinated monkeys experienced better maintenance of CD4+ T-cell populations (Figure 5) and low virus titers compared to unvaccinated control animals. Both differences were statistically significant. This was despite the fact that the virus was entirely heterologous to sequences in the vaccine (no 89.6P envelope or internal SIV proteins were present in the vaccine). Virus-specific B-cell and T-cell immune responses observed after SHIV challenge were far superior in vaccinated animals versus control animals. Here was the first instance in which disease control had been achieved in the 89.6P challenge model with a vaccine lacking SIV or homologous envelope

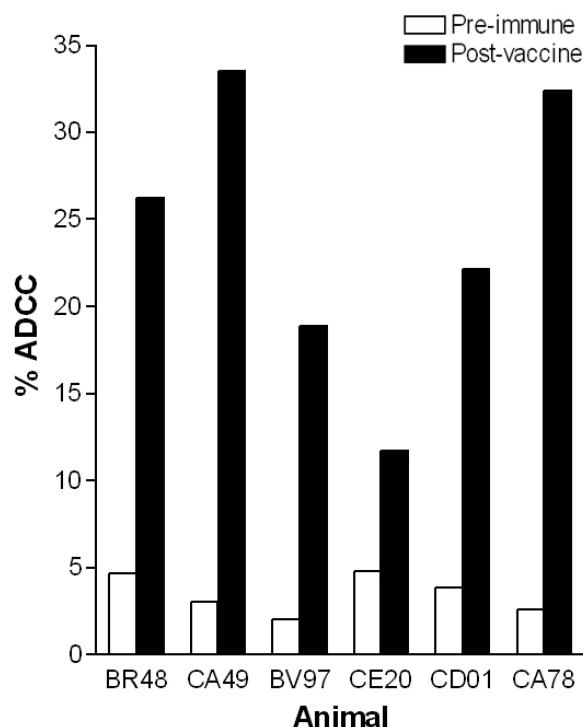


**Figure 3.** CD4<sup>+</sup> T cell populations are sustained in vaccinated animals. C57BL/6 mice received three inoculations, either with different vectors (D-V-P) or with the same vector (D-D-D, V-V-V, or P-P-P) administered at one month intervals. Vaccine concentrations were as described in the legend to Figure 1. In this experiment, the vectors each expressed the 1007 gp140 envelope protein (81). One year later, CD4<sup>+</sup> T cells from vaccinated and control animals were tested using ELISPOT analyses with two previously-defined immunodominant peptides (NASWSNKSLEQIWNN and IIGDIRQAHCNISRE (82)). A statistically significant difference was measured between ELISPOT numbers from test and control animals. Methods: mice were euthanized, spleens were removed aseptically and CD4<sup>+</sup> T cells were enriched for assay (81). Briefly, cells were treated with rat anti-mouse MHC class II (TIB 120 cell supernatants) and rat anti-mouse CD8 (53-6.72 cell supernatants) monoclonal antibodies. Cells were then incubated with sheep anti-mouse and sheep anti-rat IgG coated Dynabeads (Dyna, Oslo, Norway) and applied to a magnet to remove the MHC class II<sup>+</sup>, CD8<sup>+</sup>, and Ig<sup>+</sup> populations. Antigen presenting cells (APCs) were prepared from naïve mouse spleen cells by depleting T cells with an anti-mouse Thy1.2 (AT83) antibody and complement in Hanks Balanced Salt Solution plus 0.1% BSA. Cells were irradiated with 2500 rads. Multiscreen-hemagglutinin filtration plates (Millipore, Bedford, MA) were prepared for ELISPOT assay by coating overnight with 10 micrograms/ml anti-mouse IFN-gamma (BD Biosciences, San Diego, CA, USA) in PBS (0.1 ml/well) at 4°C. The plates were washed four times with PBS and blocked with complete tumor medium (83, 84) containing 10% FCS for 1 hour. Freshly prepared cells were plated at 1x10<sup>6</sup> CD4<sup>+</sup> T cells/well and 5x10<sup>5</sup> APCs/well with or without 10 μM envelope peptide (synthesized by the Hartwell Center for Bioinformatics and Biotechnology at St Jude Children's Research Hospital). The cultures were incubated for 48 hrs at 37°C in 10% CO<sub>2</sub> and then washed four times with PBS/ 0.05% Tween 20 before the addition of 100 microliters/well of 5 micrograms/ml biotinylated rat anti-mouse IFN-gamma (BD Biosciences) in PBS containing 0.05% Tween 20 and 1% FCS. Following overnight incubation at 4°C, the plates were washed five times and streptavidin-conjugated alkaline phosphatase (DAKO, Copenhagen, Denmark) diluted 1:500 was added to each well (0.1 ml/well). After 1 hr at room temperature, plates were washed five times, rinsed four times with water, and developed by the addition of 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium alkaline phosphatase substrate (Sigma-Aldrich). Plates were rinsed in water and air dried before spots were counted on an Axioplan 2 microscope (Carl Zeiss, Munich-Hallbergmoos, Germany). SFU=Spot-forming unit.

antigens. Clearly, while the sequences were non-identical between vaccine and challenge components, at least one component of the vaccine must have presented epitopes similar to those of 89.6P to elicit responsive cells and confer disease protection.

Results from pathogenic SHIV challenges must be cautiously interpreted, since pathogenic SHIVs represent artificial viruses grown in unnatural hosts. Pathogenic variants of the HIV-SIV chimeras, such as the 89.6P stock described here, were obtained only after serial passages of

viruses through monkeys (77). Achieving pathogenicity (i.e. abrupt and persistent profound loss of CD4<sup>+</sup> T cells) was thus associated with the acquisition of mutations in several different viral genes (78). Mutations in the HIV-1 envelope gene acquired during macaque passage may have altered key antigenic determinants, thus precluding an absolute test of HIV-1 vaccine capacity. This phenomenon is exemplified by the fact that immunoglobulin from humans infected with HIV (HIVIg) cannot prevent infection in the 89.6P model, while SIV hyperimmune globulin is fully protective in an SIV challenge model (7,



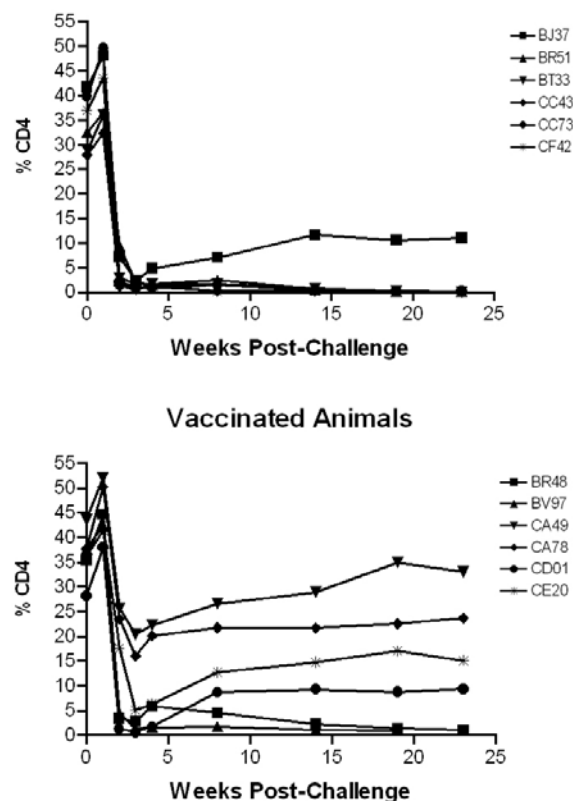
**Figure 4.** Antibody dependent cell-mediated cytotoxicity (ADCC) in vaccinated monkeys. Six macaques (housed in the Tulane Primate Center, Covington, LA) were immunized with vectors D, V and P. In this case, each vector comprised a cocktail of constructs or proteins, so that dozens of envelopes were presented to vaccinated animals. Vectors were given more than once (see (74) for details) with at least 4 weeks between injections. Immunoglobulins from pre-immune sera and sera taken two weeks following the last immunization of each animal were purified using affinity chromatography with protein G sepharose, after which samples were brought to their original serum volume and diluted 1:50 prior to testing. Activity was reproducibly identified in each of the vaccinated animals. Of note, the HIV-1 IIIB envelope which was used for ADCC testing was not a component of the cocktail vaccine. Methodology: The ADCC method has been described previously (85). Briefly, target cells were prepared by mixing  $5 \times 10^6$  CEM-NK<sup>+</sup> (NK-resistant, CD4<sup>+</sup> human T-lymphoblastoid cell line, contributed by Peter Cresswell to the NIH AIDS Research and Reference Reagent Program, Germantown, MD cat#458) cells with 15 micrograms envelope protein (HIV-1<sub>IIIB</sub> gp120 Purified Native Glycoprotein, Advanced Biotechnologies Inc, Columbia, MD, Cat#14-102-050) in 300ul RPMI 1640 (Invitrogen, Carlsbad, CA Cat #22400-089). Cells were labeled for 1 hr at room temperature with intermittent mixing. Target cells were then washed and stained with both PKH-26 (a lipid-associating dye used to mark both viable and non-viable cell membranes, Sigma-Aldrich #MINI-26) and carboxyfluorescein diacetate succinimidyl ester (CFSE, an uncharged fluorescein derivative that permeates the cell membrane and serves to label intact cells, Invitrogen #C34554). Effector cells were human peripheral blood mononuclear cells (PBMCs) from HIV-negative individuals. In a 96 well V bottomed plate, 50 microliters diluted test or control antibody were mixed with 50 microliters containing 5000 target cells (as described above, labeled with gp120, PKH26 and CFSE) for 15 minutes at room temperature. One hundred microliters human PBMC ( $2.5 \times 10^5$ ) were then added to each well and the plates were centrifuged briefly to increase cell-cell contact. Plates were subsequently incubated for 4 hrs at 37°C in a 5% CO<sub>2</sub> incubator. Cells were then transferred to a tube, centrifuged to remove medium, and fixed with 3.7% formaldehyde (Ted Pella Inc, Redding, CA #18505). FACS analyses were performed (using a FACSCan, BD Biosciences) and the percentages of CFSE<sup>low</sup> cells within the PKH26<sup>high</sup> population were calculated.

9). Despite these recognized weaknesses associated with the pathogenic SHIVs, the above-described experiments provided an important demonstration that a multi-envelope vaccine lacking homologous antigens to the challenge virus could protect against disease.

### 3.4. Clinical evaluation of the SJCRH HIV-1 multi-envelope vaccine

Three separate FDA-approved phase I safety trials have now been fully enrolled at SJCRH as a means to evaluate

(i) recombinant D (EnvDNA), (ii) recombinant V (PolyEnv1) and (iii) recombinant P (EnvPro) vaccines in humans. Results demonstrated safety and showed that envelope cocktails were immunogenic (80). Tests also showed that immune activity could be generated in humans toward envelopes that were not represented by sequence in the vaccine. Again, these data provided proof of principle that despite the lack of precise matching of amino acid sequences between vaccine and target antigens, there were components in the vaccine sufficiently similar to



**Figure 5.** Immune responses elicited by the D-V-P vaccine control disease in vaccinated animals. Six macaques (described above) were immunized with vectors D, V and P. Each inoculation comprised a cocktail of constructs or proteins, so that dozens of envelopes were presented to vaccinated animals. Vectors were administered more than once (see Zhan et al. (74) for details) with at least 4 weeks between injections. Animals were challenged 10 weeks following the last immunization and blood samples were collected at various intervals thereafter. The percentages of CD4<sup>+</sup> T cells among lymphocytes were determined using a FACS-Calibur cytometer (BD Biosciences) and Cell Quest software. Assays were conducted at Tulane Primate Center. One additional animal was excluded from this analysis due to an inadvertent, incomplete i.v. delivery of the challenge material. Significant differences were observed between vaccinated and control animals (statistical analyses were conducted using Mann-Whitney tests).

heterologous HIV-1 targets to elicit immune reactivity. An immune response in the EnvPro trial was seen among 100% of the vaccinees, providing much encouragement for vaccine advancement. An FDA-approved D-V-P vaccine trial, a trial expected to elicit enhanced immune strength and durability, is now underway.

#### 4. PERSPECTIVE

This review describes numerous approaches and methodologies for the development of an HIV vaccine, highlighting the multi-envelope approach as a means to capture and target viral diversity. Researchers in other vaccine fields have had enormous successes with the

cocktail approach (56), encouraging continued efforts in this area. Future clinical trials may prove that by harnessing potent and diverse B-cell and T-cell activities with a cocktail vaccine, we may ultimately eliminate the spread of HIV-1 infections in humans.

#### 5. ACKNOWLEDGEMENTS

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