Protection of rhesus macaques against disease progression from pathogenic SHIV-89.6PD by vaccination with phage-displayed HIV-1 epitopes

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The antigenic polymorphism of HIV-1 is a major obstacle in developing an effective vaccine. Accordingly, we screened random peptide libraries (RPLs) displayed on phage with antibodies from HIV-infected individuals and identified an array of HIV-specific epitopes that behave as antigenic mimics of conformational epitopes of gp120 and gp41 proteins. We report that the selected epitopes are shared by a collection of HIV-1 isolates of clades A–F. The phage-borne epitopes are immunogenic in rhesus macaques, where they elicit envelope-specific antibody responses. Upon intravenous challenge with 60 MID$_{50}$ of pathogenic SHIV-89.6PD, all monkeys became infected; however, in contrast to the naive and mock-immunized monkeys, four of five mimotope-immunized monkeys experienced lower levels of peak viremia, followed by viral set points of undetectable or transient levels of viremia and a mild decline of CD4$^+$ T cells, and were protected from progression to AIDS-like illness. These results provide a new approach to the design of broadly protective HIV-1 vaccines.

A number of studies in animal models have shown a protective role of antibodies (Abs) against acute infection with HIV-1 and simian HIV (SHIV) strains. Adoptive immunotherapy with anti-HIV envelope monoclonal antibodies (mAbs) and HIV-specific immunoglobulins (Igs) has resulted in protection from HIV-1 and SHIV challenge in nonhuman primates. Sterilizing immunity was obtained by passive transfer of monoclonal antibodies and serum immunoglobulins (Igs) from HIV-1 infected subjects or monkeys chronically infected with HIV-1 isolates[1–4]. These results show that protective antibodies are elicited in the course of natural HIV/SHIV infection and indicate that an effective vaccine should elicit, among other effects, an antibody response to HIV-1 envelope proteins[5]. Efforts to induce a broadly protective antibody response by immunization with gp120 protein or peptides have been frustrated, however, by the high antigenic polymorphism of HIV-1 envelope, a consequence of the high rate of mutation in the constant and variable regions of HIV-1 envelope protein[6] and the complex envelope structure of gp120 as an oligomer associated with gp41 (refs. 7,8). The degree of antigenic polymorphism is increased by the continuous *in vivo* evolution of the virus as a result of Ab affinity maturation and viral escape[9], and is compounded by interclade recombinations and by the emergence of highly divergent new viral isolates[10]. Thus, the antigenic polymorphism of HIV-1 is a major obstacle in developing an effective vaccine. In this regard, although monkeys immunized with the envelope of a given HIV-1 isolate might be

protected against a subsequent challenge with a viral strain carrying a homologous envelope, little or no protection is observed in monkeys challenged with heterologous viruses harboring a different gp160 (ref. 11). This lack of significant cross-protection raises further concerns about the capacity of envelope-based vaccines to afford substantial protection against field isolates[12,13]. These difficulties could be overcome by identifying pools of epitopes shared by different HIV-1 clades and quasispecies and using them as immunogens to induce a broad antibody response.

Combinatorial peptide libraries express a large collection of peptide sequences (10^8 or more) that mimic linear or conformational epitopes of folded protein domains, and even carbohydrate structures that contribute to immunological escape[14–16]. Such libraries offer a good method to manage the complexity of the HIV-1 epitope repertoire by allowing the selection of HIV-specific epitopes shared by different HIV clades and quasispecies. We screened two random peptide libraries (RPL) using serum antibodies from subjects infected with HIV-1 clade B viruses and identified a pool of phage-displayed peptides that function as antigenic and immunogenic mimics of discrete domains of the HIV-1 envelope[17]. Phage-displayed peptides can be expressed in multiple copies (up to 2,700 copies when inserted in the N-terminal region of the pVIII coat protein of filamentous bacteriophage fd) and are able to induce specific Ab responses in immunized mice[18,19]. Furthermore, bacteriophage promote rapid

immunoglobulin class switching both in mouse and human subjects[18,20] and are well tolerated when injected in immunocompromised individuals, including those infected with HIV-1 (ref. 20). The phage-borne peptides seem to be processed intracellularly and presented in the context of MHC class I and II to elicit both cytolytic and T helper activity[21]. HIV-specific mimotopes are immunogenic in mice, where they elicit an antibody response that neutralized HIV-1 isolates in an *in vitro* infection assay[17]. These results indicate that the selected phage-displayed epitopes might induce a protective immune response when injected in nonhuman primates. To test this possibility, we immunized rhesus macaques with a selected pool of phage-displayed epitopes and challenged them with pathogenic SHIV-89.6PD. We report that immunizing monkeys with phage-borne epitopes resulted in reduced levels of viremia and protected the monkeys from progression to AIDS-like disease.

Immunization of rhesus macaques with phage-displayed epitopes

By screening RPLs with antibodies of HIV-infected individuals, we identified a series of phage-displayed epitopes that behaved as antigenic mimotopes of discrete regions of HIV-1 gp120 and gp41[17]. In particular, epitope p195 shares sequence homology with the gp120 V1 region (residues 112–120) of HIV-1–U16374, a primary isolate from an acute HIV seroconverter[22]; the p217 sequence matches the C2 region (residues 198–205) of HIV-1–U11607, a primary isolate from an AIDS patient[23]. These regions correspond to the first α helix and the third β sheet of the gp120 crystal structure, respectively, and are immunologically accessible by selected monoclonal antibodies as deduced by X-ray crystallography structure[24,25]. Epitope 197 mapped to a region of gp41 (residues 599–608) of the primary HIV-1 isolate HIVANT7O (ref. 26); this region is conserved among primary isolates of subtypes A–G and defines a disulfide-bonded structure

recognized by a human monoclonal antibody[27] (Table 1). No obvious sequence homology with HIV proteins was detected in p287 and p335. However, previous studies indicated that these epitopes are antigenic mimics of gp160 epitopes[17]. When sera from individuals from diverse geographic regions who were documented to be infected with different HIV-1 clades (A–F) were analyzed for their ability to recognize the phage-displayed epitopes, a considerable degree of shared reactivity was seen (Table 2).

As antigenic mimics of HIV-1 envelope, the phage-borne epitopes would be expected to bind to antibodies from SHIV-infected monkeys. We tested this possibility by analyzing the antibody reactivity of monkeys infected with a series of SHIV strains carrying envelope proteins from different primary isolates. As shown in Table 3, antibodies from SHIV-infected monkeys reacted strongly with the pool of HIV-1 mimotopes, whereas antibodies from SIVmac239-infected monkeys did not. This study included rhesus monkeys infected with pathogenic SHIV-89.6PD that developed detectable amounts of anti-envelope antibodies (Table 3). Thus, by screening RPLs with HIV-1 antibodies, we identified an array of epitopes shared by a substantial percentage of primary isolates of different subtypes. These results are consistent with other studies in which peptide pools defined specific HIV-1 immunotypes shared by HIV-1 isolates of different subtypes[28].

As antigenic mimotopes of SHIV isolates, the selected epitopes are expected to induce an immune response to SHIV envelope and might prevent or inhibit SHIV infection when injected in nonhuman primates. We tested this possibility by immunizing a group of five rhesus macaques with a pool of five phagotopes (195, 197, 217, 287 and 335; 4×10^{13} physical particles each) in QS21 adjuvant; a second group of four rhesus macaques received equal amounts of wild-type phage in QS21 (2×10^{14} physical particles for each monkey) on the same immunization schedule (Fig. 1a). These epitopes were selected for their broad reactivity against antibodies from SHIV-infected monkeys (Table 3) and for their immunogenicity in mice[17]. We detected epitope-specific antibodies in monkeys 481, 485, 490 and 493 after the second mimotope immunization, and increasing levels thereafter. Monkey 480 had little epitope-specific Ab. We detected substantial amounts of Ab to oligomeric gp140-89.6 after the third immunization in all the immunized monkeys except monkey 480 (Web Fig. A). At the end of the immunization schedule, four phagotope-immunized monkeys (481, 485, 490 and 493) had raised substantial amounts of antibodies specific to each of the injected epitopes, with end-point titers ranging from 800 to
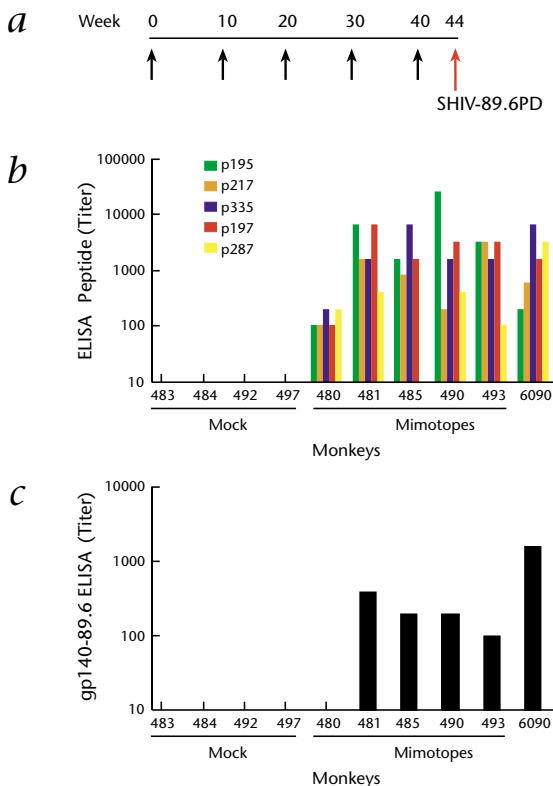

Fig. 1 Vaccine schedule and prechallenge antibody responses. *a*, Immunization and challenge schedule. A pool of 5 epitopes (p195, p197, p217, p287 and p335) was injected into each quadricep of rhesus macaques 480, 481, 485, 490 and 493 (4×10^{13} physical particles each) with 100 µg QS21 adjuvant. In parallel, rhesus macaques 483, 484, 492 and 497 were immunized intramuscularly with f.11.1 wild-type phage (2×10^{14} physical particles each) with 100 µg QS21 adjuvant. Monkeys were immunized as shown at weeks 0, 10, 20, 30 and 40. *b*, Antibody responses to the HIV-specific epitopes were determined by using short peptides whose primary sequence was engineered to function as surrogate mimotopes of the phage-displayed epitopes (see Methods). ELISA reactivities with linear dilutions of monkey plasma were assayed as described[42]. *c*, Antibody responses to HIV-1 envelope were determined in ELISA by using a purified preparation of oligomeric gp140 (89.6 strain) as reported[42]. Panels *b* and *c* show the results from monkey plasma collected two weeks after the last immunization.
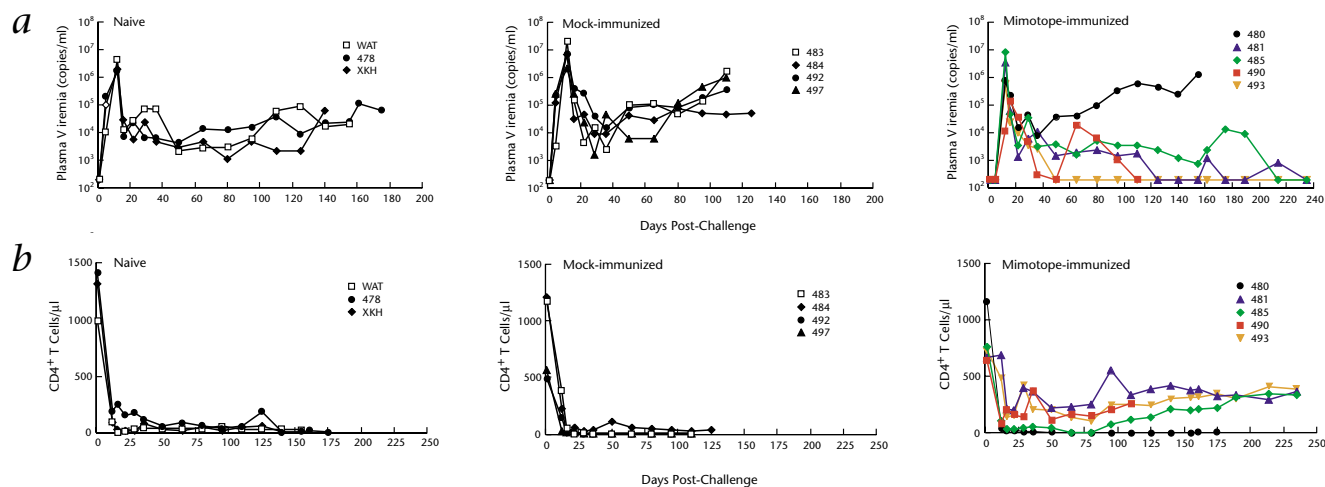
Fig. 2 Post-challenge levels of viremia and CD4+ T lymphocytes. Immunization and challenge schedule was as in Fig. 1a. **a**, Post-challenge viral loads. 4 wk after the last immunization, a group of 3 naive rhesus macaques together with the group of monkeys injected with wild-type phage (mock-immunized) and the group of HIV mimotope-immunized monkeys (mimotope-immunized) were challenged by i.v. injection with 60 monkey infectious doses (MID$_{50}$) of cell-free SHIV-89.6PD. Plasma viremia was determined by branched DNA amplification with a detection limit of 200 copies per ml as reported[31]. **b**, Post-challenge levels of CD4+ T lymphocytes. Peripheral CD4+ T cells were determined by multiplying the absolute lymphocyte counts by the percentages of CD3+CD4+ T cells detected by FACS analysis. Consistent results were obtained by determining the percentages of CD2+CD4+ and CD29+CD4+. No significant differences were observed in the case of CD3+CD8+ and CD2+CD20+ (data not shown).

24,000 (Fig. 1b). These titers were comparable to the peptide binding of serum 6090, obtained from a patient with good control of viremia, which was used in the initial screening of the RPLs (ref. 17).

Next, we tested the phagotope-immunized monkeys for antibody responses to HIV-1 envelope proteins. Four monkeys (481, 485, 490 and 493) that expressed high titers of antibodies to peptides tested positive in ELISA for antibody to HIV-gp140-89.6 (Fig. 1c). This reactivity was lower than that of serum 6090, which probably includes numerous antibody specificities. No ELISA reactivity was detected in the case of monkey 480. To assess whether antibodies to the immunodominant gp120 V3 loop could be elicited by mimotope immunization, we tested prechallenge plasma from monkey 493 for antibodies to peptide sequence corresponding to the V3 sequence of 89.6P. We did not detect antibodies to V3. In addition, increasing concentrations of V3 peptides did not interfere with the binding of plasma antibodies to the single epitopes, whereas binding was efficiently displaced by similar concentrations of peptides 195, 197, 217, 287 and 335 (Web Fig. B).

Viral challenge with SHIV-89.6PD

We next determined whether the mimotope-immunized monkeys are protected from viral challenge. We chose as a challenge virus SHIV 89.6PD, which was derived from cloned SHIV-89.6, carries a R5/X4 envelope and acquired pathogenic properties after *in vivo* passages; it consists of a swarm of several quasi-species and induces a rapid decline of CD4+ T cells associated with rapid onset of an AIDS-like disease[3,29,30]. Four weeks after the final immunization boost, we challenged the groups of mimotope-immunized and wild-type–injected monkeys, together with three naive rhesus macaques, by intravenous injection with 60 monkey infectious doses (MID$_{50}$) of cell-free SHIV-89.6PD (Fig. 2).

We determined post-challenge viral loads in plasma by a real-time polymerase chain reaction with a detection limit of 200

virus RNA copy equivalents per ml (ref. 31). All of the monkeys became infected; the naive monkeys and those injected with wild-type control phage (mock-immunized) showed detectable viremia as early as 5 days after challenge, with peak viremia at day 12 (Fig. 2a). Naive monkeys exhibited peaks of viremia ranging from $1.6–4.5 \times 10^6$ RNA copies per ml (mean 2.6×10^6) at day 12. This was followed by partial control of the acute infection, characterized by viral copy numbers of 0.3–to 1×10^5 copies per ml throughout the chronic infection period (Fig. 2a). Mock-immunized monkeys experienced high peaks of viremia (range $2.1 \times 10^6–20 \times 10^6$ copies per ml; mean value 9.3×10^6 copies per ml) followed by partial clearance of viremia at week 4 and then by a steady increase in the plasma viral copies over the observation time, with final viral titers of $0.5–16.5 \times 10^5$ copies per ml (Fig. 2a). The mimotope-immunized monkeys had variable amounts of plasma viremia (Fig. 2a). Monkey 490 had a modest viremia at day 12 after infection: 1×10^4 copies per ml, with a delayed peak of 1.3×10^5 copies per ml at day 19, substantially lower than the peaks in naive and mock-immunized monkeys. This monkey's viremia cleared rapidly and virus was undetectable at day 50 after infection (<200 viral copies per ml); the virus titer rebounded at day 65 and then spontaneously declined again to undetectable by day 110. Monkey 493 had peak viremia of 0.5×10^6 copies per ml at week 2 after infection, followed by a complete clearance of viremia at week 5 and undetectable virus titer for the remainder of the observation period (Fig. 2a). In this monkey, no viral copies were detected in inguinal lymph node cells by *in situ* hybridization, and by cocultivation of peripheral blood mononuclear cells (PBMC) with phytohemagglutinin-activated PBMC from uninfected monkeys (Web Fig. C). Monkeys 480, 481 and 485 showed viral peaks within the range of the values observed in naive and wild-type phage–immunized monkeys. Monkeys 481 and 485 showed low or undetectable viremia after primary infection, with virus titers ranging from 1.9×10^4 copies per ml to undetectable. In monkey 480, the monkey with the poorest response to immunogen, the peak of viremia was fol-

lowed by a steady increase in plasma viral copies up to 4.9×10^6 per ml at week 22, at which time the monkey was euthanized. Statistical analyses of the viral loads on day 12 showed no significant difference between the two control groups (naive and mock-immunized monkeys) and between the naive or the mock-immunized group and the mimotope-immunized monkeys (P = 0.20, two-tailed Wilcoxon rank sum test). To take into account the variability among monkeys, we carried out statistical analyses of the viral set points by measuring the median of three consecutive plasma viremia levels at days 80, 95 and 110 for each monkey. No significant difference was observed between the control groups (P = 0.057), indicating that the two groups could be pooled together for a statistical analysis. We therefore compared the viral loads in the group of mimotope-immunized monkeys consisting of 481, 485, 490 and 493 (with monkey 480, the single mimotope-immunized monkey that did not mount an antibody response, omitted) to those in the pool of control monkeys. The antibody response elicited by the immunogen correlated significantly with the low plasma viremia at viral set points, in that the viral loads in the mimotope-immunized monkeys were significantly lower than those in the pooled control monkeys (P = 0.012).

Infection with SHIV-89.6P and its derivative SHIV-89.6PD induces a profound decline of peripheral CD4+ T lymphocytes[3,30]. Accordingly, both naive and mock-immunized monkeys had a rapid depletion of CD4+ T cells at day 12 after infection (Fig. 2b), concomitant with the high peaks of plasma viremia (Fig. 2a). With the exception of naive monkey 478 and mock-immunized monkey 484, which showed transient detectable titers of CD4+ T cells, these monkeys had a profound and irreversible depletion of peripheral CD4+ T lymphocytes throughout the observation time (Fig. 2b). In the mimotope-immunized group, monkeys 490, 481 and 493 had a significant decrease in their CD4+ T cells during the acute phase of viremia, followed by a

partial recovery to 250–500 CD4+ T cells per µl (40–60% of the prechallenge titers; Fig. 2b). Similar titers of peripheral CD4+ T cells are associated with absence of AIDS-like illness in HIV-1–infected subjects[32,33]. Monkey 485 showed a prolonged decline of peripheral CD4+ T lymphocytes through week ten, followed by increasing numbers of CD4+ T cells that resulted in final CD4+ T-cell titers similar to those observed in monkeys 490, 481 and 493. Monkey 480 had an irreversible loss of peripheral CD4+ T lymphocytes to less than 1% of the initial titer (Fig. 2b). We carried out statistical analyses of the peripheral CD4+ T cell counts after acute infection as described in the case of viral loads, by analyzing the median values of three consecutive CD4+ T cell-determinations. The CD4+ T cell-counts of the group of mimotope-immunized monkeys (monkeys 481, 484, 490 and 493) were significantly higher than those of the pooled control monkeys (P = 0.0061).

Immune response after challenge

Because of the profound depletion of CD4+ T cells, few or no HIV-specific antibodies are detected in unprotected SHIV-89.6PD–infected monkeys[3,30]. Consistent with this pattern, we found that two naive monkeys (WAT and XKH) and three mock-immunized monkeys (483, 492 and 497) did not show a detectable antibody response (Fig. 3a). Naive monkey 478 and mock-immunized monkey 484 showed an envelope-specific antibody response at week seven after challenge and declining titers thereafter (Fig. 3a). In contrast, the mimotope-immunized monkeys 481, 485, 490 and 493 showed a rapid increase from prechallenge levels of antibody to oligomeric gp140-89.6, with titers rising as high as 2.5×10^5 (Fig. 3a). One monkey (480) showed a dramatic decline in CD4+ T cells and did not show a detectable Ab response to the challenge virus (Fig. 3a).

We analyzed neutralizing antibodies in cultures of monkey PBMCs infected with a stock of SHIV-89.6PD expanded in PBMCs from rhesus macaques. Antibodies from prechallenge sera of mimotope-immunized monkeys showed neutralization titers of 1:10 in monkeys 481 and 485 and of 1:30 in monkeys 490 and 493; these mimotope-immunized monkeys manifested increased titers of neutralizing antibodies against the challenge virus starting at week four after challenge (Fig. 3b). No neutralizing activity was detected in monkey 480 (Fig. 3b). Within the control groups, low titers of neutralizing antibody were transiently detected in naive monkey 478 and mock-immunized monkey 484 (Fig. 3b). These results indicate that monkeys primed by mimotope immu-
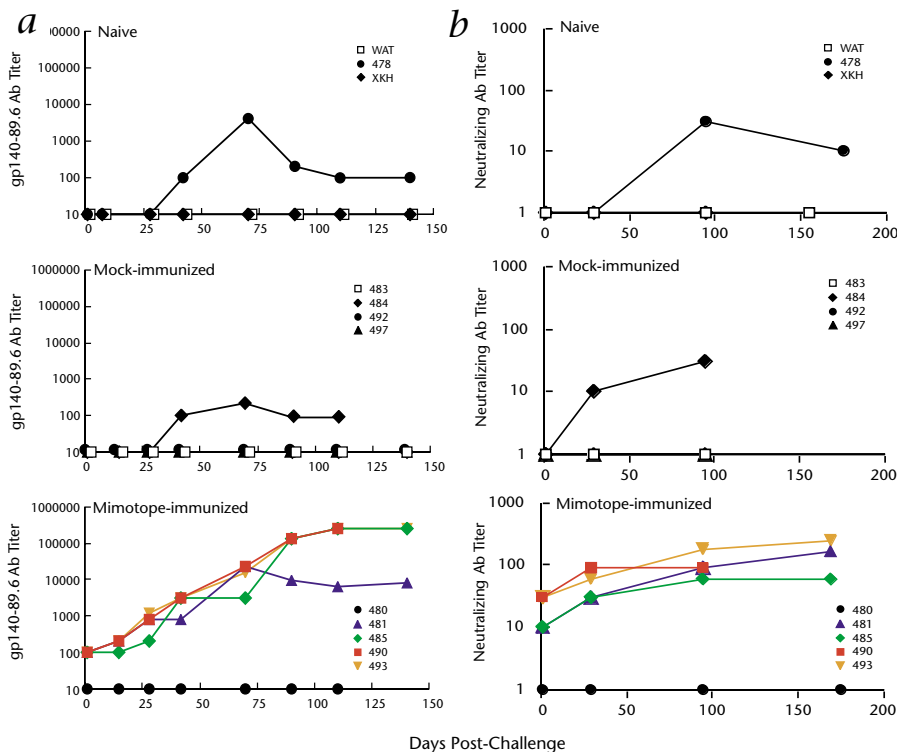

Fig. 3 Post-challenge antibody response. *a*, Antibody titers were determined at the indicated time points by ELISA. Linear dilutions of monkeys' sera were tested for binding to a preparation of oligomeric gp140-89.6 as reported[42]. *b*, Analysis of neutralizing antibody response. Virus neutralizing antibodies were determined by using rhesus macaque PBMCs infected with SHIV-89.6PD (see Methods). Neutralization titers were defined as the plasma dilution that resulted in >90% reduction of viral production.

Table 1 Amino acid sequences of the HIV-1 epitopes

Epitope[a]	Amino acid sequence	Assignment to regions of gp160
p195	KSSGKLISL	gp120 V1[b]
p217	CNGRLYCGP	gp120 C2[c]
p197	GTKLVCFAA	gp41[d]
p287	CAGGLTCSV	Undetermined[e]
p335	SGRLYCHESW	Undetermined

[a], Epitopes were selected by screening random peptide libraries as reported[17]. [b], Epitope p195 shares sequence homology with the gp120 V1 region (residues 112–120) of HIV-1–U16374, a primary isolate from an acute HIV seroconverter[22]. [c], p217 sequence matches the C2 region (residues 198–205) of HIV-1–U11607, a primary isolate from an AIDS patient[23]. [d], Epitope 197 maps to a region of gp41 (residues 599–608) of the primary HIV-1 isolate HIVANT7O (ref. 26). [e], Although no obvious sequence homology with HIV proteins was detected in the case of p287 and p335, previous studies have indicated that these epitope are antigenic mimics of gp160 epitopes[17].

nization have a rapid and durable antibody response to the challenge virus.

As phage-displayed peptides have been reported to stimulate specific T cell responses[21], we analyzed PBMC obtained from the mimotope-immunized monkeys on the day of viral challenge for the presence of T cells capable of producing intracellular IFN-γ upon stimulation with peptide mimotopes, a sensitive assay of epitope-specific immune responses[34]. In these experiments, no intracellular IFN-γ production was induced by pooled peptide mimotopes, indicating that the selected peptides do not function as T cell epitopes (Web Table A).

Clinical events associated with viral challenge

Naive and mock-immunized monkeys experienced severe illness starting at week six after challenge. This included untreatable anemia, fever, multiple infections, hemorrhagic diarrhea and weight loss; the degree of illness required euthanasia. Among the mimotope-immunized monkeys, monkey 480, which showed no immune response to the immunogen before challenge and high viremia and dramatic loss of CD4+ T cells after challenge, developed an AIDS-like illness similar to that observed in naive and wild-type phage–immunized monkeys. Monkey 490 developed acute glomerulonephritis followed by anuria and heart failure, along with low viremia, conserved CD4+ T cell titer and a robust antibody response to SHIV-89.6PD. Histological analysis of tissues revealed a focal necrosis of kidney tubules and glomeruli, with massive deposits of proteins; lymph nodes showed numerous macrophages and prominent erythrophagocytosis. This histopathologic pattern is not characteristic of disease resulting from SHIV-89.6PD infection and resembles the immune-complex disease associated with aberrant immune responses[35]. In addition, monkey 490 experienced a focal subendocardial myocyte necrosis with neutrophilic inflammation that might have contributed to the heart failure. No abnormalities were detected in other tissues, including liver, lymph nodes, spleen and lung (Web Figs. D,E). These findings indicate that the cause of death in monkey 490 was probably unrelated to poor control of SHIV-89.6PD infection. The remaining three mimotope-immunized monkeys (481, 485 and 493) have remained healthy, with no reported clinical events for a period of 270 days at the time of this writing.

Discussion

To cover the high antigenic polymorphism of HIV-1 isolates, an effective HIV-1 vaccine should include immunogenic epitopes shared by different clades and quasispecies from distant geographic regions. Vaccine formulations that deliver envelope proteins from a single virus strain, or a pool of envelopes, have failed to induce a significant degree of protection against challenge with heterologous viruses[11,36] and might well afford little or no protection against field isolates[12,13]. To address this difficulty, we isolated a pool of phage-displayed epitopes from RPLs by taking advantage of the HIV-specific antibody repertoire induced by natural infection[17]. The selected phage-borne peptides are antigenic mimics of conformational epitopes of gp120 and gp41 generated *in vivo* in the course of natural infection[17] and are shared by a substantial percentage of HIV-1 strains of non-B clades, including clades A, C, D, E, and F (Table 1). These findings indicate that the selected epitopes might induce broad antibody responses in primates without the constraint of a predefined envelope with narrow specificity. In support of this concept, we have shown that bacteriophage displaying epitope determinants of HIV-1 gp160 are immunogenic in nonhuman primates and are endowed with protective properties. In this regard, four of five mimotope-immunized rhesus monkeys raised anti-envelope antibody responses during the immunization followed by a rapid increase in antibody titers after challenge with SHIV-89.6PD. In contrast to control monkeys, these monkeys showed reduced peaks of plasma viremia, with low or undetectable viremia set points during the chronic phase of infection. They retained substantial titers of CD4+ T lymphocytes and remained free of illness related to SHIV-89.6PD infection. These findings are consistent with human studies, in which low viral loads are associated with a favorable prognosis[37]. In addition, low or undetectable viremia in the absence of AIDS-like illness might result in reduced rates of viral transmission[38,39] and could be advantageous in populations affected by high rates of HIV-1 infection.

The results of this study are comparable with those recently obtained in monkeys using a combination of DNA vaccination with administration of an IL-2 plasmid[40] or DNA priming followed by recombinant modified vaccinia Ankara (rMVA) booster[41]. In these studies, protection against disease progression was correlated with cell-mediated immune responses against SHIV. Here we have not detected a contribution of SHIV-specific cell-mediated immune responses in the observed effects of immunization. Indeed, the observed protection was probably medi-

Table 2 ELISA reactivities of HIV-1 epitopes with HIV-positive sera

	HIV-infected subjects		
Clade[a]	Number of tested samples[b]	Number of positive samples	% of positive samples
B	120	119	99.1
A	25	22	92.0
D	26	25	96.1
C	22	20	90.1
E	19	13	68.4
F	8	6	75.0

[a], Serum samples from USA, Uganda, Nigeria, India, Thailand and Peru were assigned to specific clades as reported[44]. [b], ELISA reactivities of HIV-1–specific epitopes were detected as reported[17]. Serum antibodies were tested for binding to each of the phage-displayed epitopes; values were considered positive when at least 4-fold ± 2 s.e.m. higher than the background signal of wild-type phage was detected. A detailed representation of ELISA reactivities is shown in Web Fig. F.

Table 3 ELISA reactivities of HIV-1 epitopes with SHIV-positive sera

Virus strain[a]	SHIV-infected monkeys	
	Number of tested monkeys[b]	Number of positive monkeys
SHIV-DH12	17	17
SHIV-89.6	9	9
SHIV-89.6PD	21	19
SHIV-W6.1D	4	4
SHIV-Han2	2	2
SIVmac239	12	0

[a], Rhesus macaques were infected with the indicated SHIV chimeric strains carrying envelopes from primary HIV-1 isolates as reported[3]. [b], antibodies from infected monkeys were tested at different times after infection for reactivity with a pool of ten HIV-1 specific epitopes displayed on phage as described[17]. Values were considered positive when at least 4-fold ± 2 s.e.m. higher than the background signal of wild-type phages. No ELISA reactivity was detected in the case of antibodies from SIV-infected monkeys. A detailed representation of ELISA reactivities is shown in Web Fig. G.

ated by the antibody responses to SHIV-89.6PD envelope induced by the immunogen. In fact, the favorable outcome was restricted to the group of four monkeys (481, 485, 490,. 493) that manifested a prechallenge antibody response to oligomeric gp140-89.6 envelope followed by a rapid increase in the antibody response (Fig. 3a). Consistently, antibodies from these monkeys effectively neutralized the challenge SHIV-89.6PD virus in an *in vitro* infection assay (Fig. 3b). The protection observed was achieved upon infection with the highly virulent SHIV-89.6PD strain, raising the possibility that the degree of protection afforded by mimotope immunization might be greater with a less virulent challenge. Notably, monkeys were immunized with conformational epitopes shared by a substantial number of virus isolates of different subtypes. Thus, immunization with HIV-1 mimotopes overcomes the constraint of vaccination regimens that rely on predetermined virus strains, and might afford protection from infection with heterogeneous field isolates.

Methods

Vaccine trial. Rhesus macaques were maintained in accordance with the *American Association for Accreditation of Laboratory Animal Care Standards* and housed in a biosafety level 2 facility. The envelope-specific epitopes used as immunogen were isolated from RPLs displayed on filamentous f.11.1 as reported[17]. A pool of five epitopes (p195, p197, p217, p287 and p335) was injected into each quadricep of juvenile rhesus macaques 480, 481, 485, 490 and 493 at 4×10^{13} physical particles each with 100 μg of QS21 adjuvant (Aquila Biopharmaceuticals, Framingham, MA). In parallel, rhesus macaques 483, 484, 492 and 497 were immunized intramuscularly with 2×10^{14} physical particles of f.11.1 wild-type phage with 100 μg of QS21 adjuvant. Monkeys were matched for gender and body weight and inoculated five times at ten-week intervals. Viral challenge was performed at week 44 by intravenous inoculation of 60 MID_{50} (as previously determined) of a SHIV-89.6PD preparation (3,500 $TCID_{50}$ per ml) expanded on monkey PBMCs as reported[3]. After challenge, monkeys were monitored for plasma viral load by detecting SHIV RNA using a real-time PCR method[31], with minor modifications. Briefly, 500 μl of plasma was added to 1 ml of Dulbecco PBS and spun for 1 h at 10,000g. The viral pellet was lysed using RNASTAT-60 (Tel-Test 'B', Friendwood,TX) according to the manufacturer's directions. The samples were then amplified by using the following primers and probe: SIV-F, 5'-AGTATGGGCAGCAAATGAAT-3'; SIV-R, 5'-TTCTCTTCTGCGTGAATGC-3'; SIV-P, 5'-6FAM-AGATTTGGATTAGCA-GAAAGCCTGTTGGA-TAMRA-3'. If plasma viremia was not detected, PBMCs from infected monkeys (1×10^6 per ml) were stimulated with 10 μg/ml PHA (Sigma-Aldrich) for two days and cocultured with equal titers of PHA-activated PBMCs from uninfected rhesus macaques. Reverse transcrip-

tase activity and p27 production were determined every three days over a three-week infection time. Titers of peripheral lymphocytes positive for CD2, CD3, CD4, CD8, CD29 and CD20 were determined by flow cytometry by using the following mAbs: CD2-FITC, CD4-PE, CD8-PE, CD20-PE (Becton Dickinson, San Jose, California), CD3-FITC and CD29-PE (Pharmingen, San Diego, California). Monkeys were monitored clinically, by routine hematological testing and by blood-chemistry measurements. Monkeys were then subjected to necroscopy followed by histological analysis of tissues, including brain, kidney, large and small intestine, lung, liver, lymph nodes, myocardium and spleen.

Antibody detection. Titers of antibodies to peptide mimotopes and to oligomeric envelope proteins were determined by ELISA as described[17,42]. In brief, Immunolon-2 (Dynex Technology, Chantilly, VA) 96-well U-bottom plates were coated overnight with 5 μg/ml of either peptide mimotopes or oligomeric gp140 (89.6 strain). Twofold serial dilutions of monkey plasma were incubated overnight at 37 °C in blocking buffer containing 5% goat serum and 0.02% (w/v) sodium azide. After washing, alkaline phosphatase–conjugated anti-monkey Ab (Fc-specific) was added for 1 h and the plates were developed. Results were expressed as the difference between OD_{405} and OD_{620}. Assays were performed in duplicate. The following peptides were used: p195, EGEFCKSSGKLISLCGDPA; p197, EGE-FCQTKLVCFAAAGDPA; P217, EGEFCCNGRLYCQPCGDPA; p287, EGE-FCCAGQLTCSVCGDPA; p335, EGEFCSGRLYCHESWCGDPA. Antibody reactivities to V3 loop were detected by ELISA using the peptides RPNTRERLSIGPGRAFYA (89.6P V3) and KSIHIGPGRAFYTTG (V3 consensus subtype B) together with scrambled control peptides.

The neutralizing properties of monkey plasma were assayed using monkey PBMC as reported[43]. The virus stock consisted of the SHIV-89.6PD inoculum used for the monkey's challenge and expanded on PHA-activated PBMCs from naïve rhesus macaques. Triplicate plasma dilutions were incubated for 1.5 h at 37 °C with 20 μl of previously titrated virus aliquots (1.1×10^5 reverse transcriptase-cpm per 15 ng of p27 per 100 $TCDI_{50}$) in a total volume of 100 μl. Preimmunization plasma was used as negative control. The mixtures were added to rhesus macaque PBMCs (4×10^5 per 200-μl culture) previously activated with PHA (10 μg/ml) for 36 h. After a 24-h incubation, medium was removed by centrifugation and replaced. Cultures were monitored every three days by reverse transcriptase assay. End-point titrations were calculated at day seven as the plasma dilution that resulted in > 90% virus inhibition.

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