Antibodies to CD4-induced sites in HIV gp120 correlate with the control of SHIV challenge in macaques vaccinated with subunit immunogens

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Epitopes located in and around the coreceptor binding site of HIV-1 envelope glycoprotein (gp120) exhibit enhanced exposure after attachment to the CD4 receptor and comprise some of the most conserved and functionally important residues on the viral envelope. Therefore, antibody responses to these epitopes [designated as CD4-induced (CD4i)] should be highly cross-reactive and potentially useful for HIV vaccine development. To address this question, rhesus macaques were vaccinated with subunit immunogens designed to raise humoral responses against CD4i epitopes and challenged rectally with $SHIV_{162P3}$, which encodes a heterologous envelope versus the immunogen. We found that animals vaccinated with a rhesus full-length single-chain (rhFLSC) complex exhibited significantly accelerated clearance of plasma viremia and an absence of long-term tissue viremia compared with unvaccinated control animals. Such control of infection correlated with stronger responses to CD4i epitopes in the rhFLSC-vaccinated animals, compared with macaques immunized with gp120, cross-linked gp120–CD4 complexes, or soluble CD4 alone. These responses were strongly boosted in the rhFLSC-vaccinated animals by $SHIV_{162P3}$ infection. The control of infection was not associated with anti-CD4 responses, overall anti-gp120-binding titers, or neutralizing activity measured in conventional assays. Vaccine-naive animals also developed anti-CD4i epitope responses after simian/human immunodeficiency virus (SHIV) challenge, which appeared later than the overall anti-gp120 responses and in concert with the decline of viremia to a low set point. Collectively, these data suggest that antibodies to CD4i epitopes may play a role in controlling SHIV infection and provide insights for HIV vaccine development.

vaccine | infection | immunity

It is generally believed that an effective HIV vaccine will have to elicit antibodies that react against highly conserved domains on the HIV envelope. This view stems from demonstrations that the passive transfer of broadly cross-reactive, neutralizing anti-envelope antibodies affords sterilizing protection in nonhuman primate models for HIV infection (1–3). Moreover, certain vaccine strategies are more effective in suppressing infection in nonhuman primate models when viral envelope is included in the vaccine (4, 5). Recent evidence indicates that anti-envelope antibody-dependent cellular cytotoxicity activity correlates with protection against HIV challenge (6).

Given these findings, CD4-induced (CD4i) epitopes positioned in and around the coreceptor binding site of the HIV envelope glycoprotein (gp120) merit careful consideration for vaccine design. These epitopes exhibit enhanced exposure as a consequence of gp120 attachment to the cell surface CD4 receptor and are essential for the engagement of entry coreceptors. Because CD4i domains comprise some of the most conserved sequences of the HIV envelope (7, 8), cognate antibodies should be highly cross-reactive among viral strains. Moreover, coreceptor binding sequences seem to be immunogenic in humans because HIV-infected persons produce antibodies to CD4i epitopes that can be detected in specific assays (7). In contrast, other conserved/functional envelope domains rarely elicit antibodies in HIV-infected individuals (9, 10) or experimental systems and, for a variety of other reasons, may not be practical for vaccine development (9, 11).

To date, the significance of CD4i epitopes in HIV vaccine development has not been clearly elucidated. Based on computational models (12), it was proposed that CD4i epitopes are occluded from antibodies before and after cell surface CD4 binding and are unlikely to be immunogenic or antigenic *in vivo*. However, more recent cryo-EM images of the HIV envelope trimer may be discordant with the domain topology predicted by computational models and suggest that certain CD4i epitopes could be partially exposed on gp120 before attachment (13, 14). In accordance, certain human anti-CD4i epitope mAbs exhibit some capacity for gp120 binding in the absence of CD4 and possess cross-reactive, cross-clade neutralizing activities *in vitro* (15–21), particularly under conditions that approximate the coreceptor densities found on primary human cells (22). Earlier we showed that some CD4i epitopes become exposed immediately upon completion of the viral fusion process and persist for several hours on freshly infected cells (19). Collectively, these characteristics suggest that antibodies to CD4i epitopes might control infection by direct neutralization or other humoral mechanisms of immunity such as antibody-dependent cellular cytotoxicity if present at the time of infection. In this case, CD4i epitopes and antigens that present them may have utility for HIV vaccine development.

To assess the functions of anti-CD4i epitope responses *in vivo*, we analyzed samples from a study in which rhesus macaques were immunized with antigens that present CD4i epitopes and were then given a heterologous mucosal challenge with a CCR5-tropic simian/human immunodeficiency virus (SHIV).

Results

Groups of four rhesus macaques were immunized four times with either cross-linked BaLgp120–human CD4 complexes (gp120–

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Abbreviations: CD4i, CD4-induced; rhFLSC, rhesus full-length single chain; SHIV, simian/human immunodeficiency virus; SIV, simian immunodeficiency virus; sCD4, soluble CD4; NASBA, nucleic acid sequence-based amplifications.

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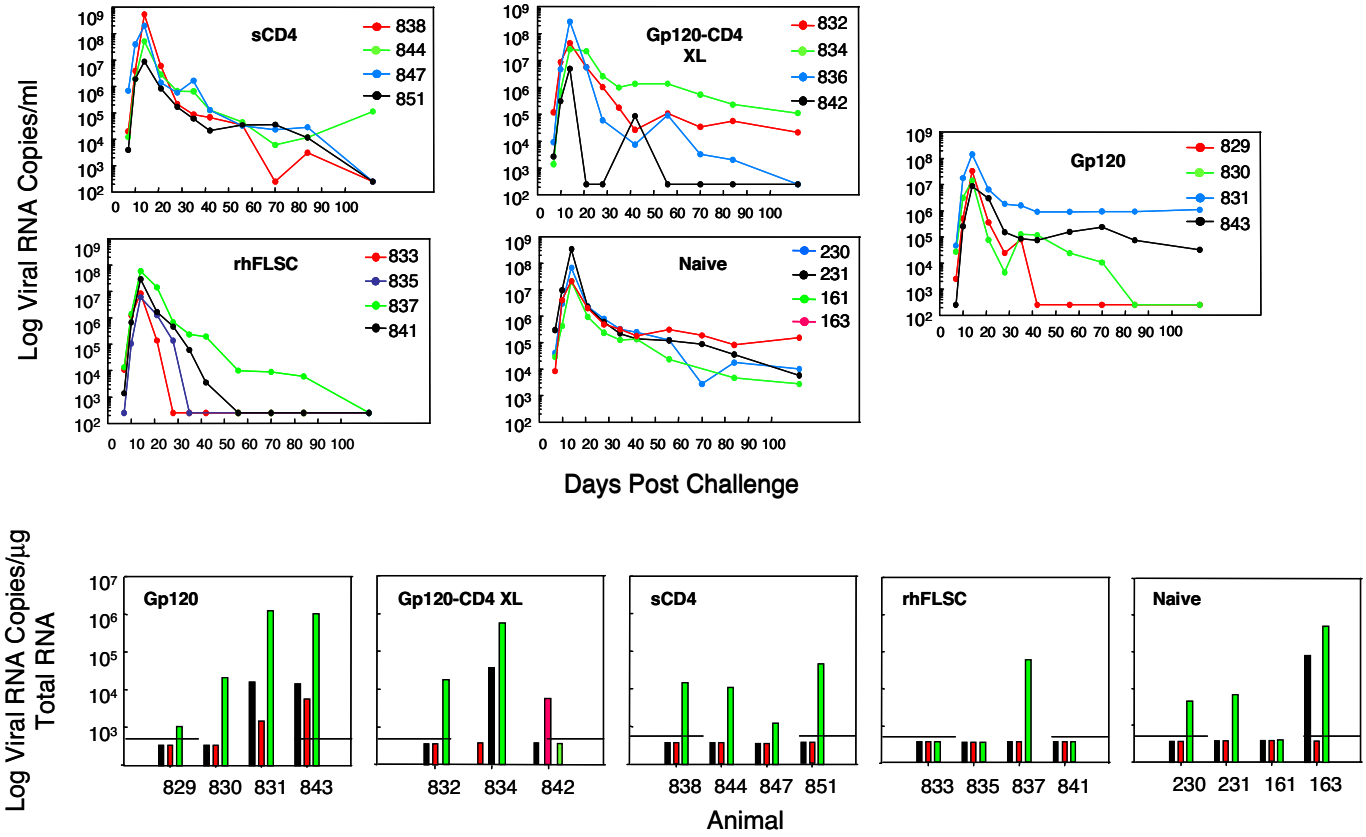

Fig. 1. Postchallenge viral RNA levels. (*Upper*) Plasma viral RNA copies were measured by SIV NASBA with a lower detection limit of 500 copies per ml of plasma (day 0, day of challenge). Undetectable levels below this threshold are marked as 250 copies per ml of plasma. Plasma RNA levels are shown for each animal (color coded). (*Lower*) Viral RNA levels in tissue samples collected 13 months after the challenge were determined by SIV NASBA and expressed as viral RNA copies per microgram of total tissue RNA. The lower limit of detection is marked with a dashed line. Undetectable levels below this threshold are designated as bars falling below the line. Values obtained for pooled lymph nodes (green), jejunum (red), and large intestine (black) are shown. Animals are grouped according to immunogen.

CD4 XL) or a single-chain complex containing BaLgp120 and rhesus macaque CD4 domains 1 and 2 [rhesus full-length single chain (rhFLSC)]. Previously, we showed that cross-linked and single-chain gp120–CD4 complexes present CD4i epitopes, including those located in and around the coreceptor binding site (23, 24). An additional feature of the rhFLSC was that the CD4 moiety was homologous with the CD4 of the vaccinated host, which minimized the chances of raising anti-CD4 responses during vaccination. Control groups of macaques were immunized with either BaLgp120 or soluble human CD4. Vaccine-naive animals were included as challenge controls. All immunogens were formulated with QS21 adjuvant. Four weeks after the final immunization, the animals received a rectal challenge with $SHIV_{162P3}$, which is a pathogenic and neutralization-resistant (25, 26) virus encoding an envelope that is heterologous with respect to the immunogens.

All challenged animals were infected after SHIV challenge (Fig. 1). Viral replication and spread were similar among the four naive animals and produced peak plasma viral loads between 10^7 and 10^9 viral RNA copies per ml by day 14 after challenge (Fig. 1). The plasma viral RNA levels reached a plateau by day 112 after challenge, which ranged between 10^3 and 10^5 viral RNA copies per ml. Replication kinetics in the sCD4-immunized animals closely resembled what was measured in the naive controls. Compared with the naive control animals, the rhFLSC group had a lower mean viral load on day 7 (1.2 log; $P = 0.02$; t test), a lower mean peak viral load on day 14, and an accelerated decline and clearance of postacute viremia. Animal 833 exhibited particularly rapid and extensive clearance of viremia. Although the mean peak viral load was 0.6 log lower in the rhFLSC group versus the naive group, it was not possible to determine statistical significance because the study was not powered to detect a <1.2 log difference in this parameter. Overall, the postpeak decline and clearance of viremia in the rhFLSC group was significantly more rapid compared with the naive group (mean area under the curve postpeak viremia, $P < 0.006$; rate of decline postpeak viremia, $P < 0.0001$; Kaplan–Meier analyses for time to baseline, $P = 0.007$). Combined data for all time points after peak viremia also were significantly lower in the rhFLSC group (t test; $P = 0.0065$). The same comparisons of the other immunization groups (including the sCD4 group) versus the naive group revealed no significant differences in viral replication ($P > 0.05$ in all cases). In accordance with previous studies with $SHIV_{162P3}$ (27, 28), there were no significant changes in the circulating percentage of CD3+CD4+ T cell levels in any of the challenged animals and no significant changes in this parameter between groups over time [supporting information (SI) Fig. 4].

Thirteen months after the challenge, the animals were killed to assess tissue viral loads. Because $SHIV_{162P3}$ plasma viremia declines in naive animals (27, 28), examining the tissue reservoir allowed us to dissect the impact of vaccine-induced effects from spontaneous effects. Viral RNA levels in samples of jejunum, large intestine, and pooled axillary, mesenteric, and periaortic lymph nodes were measured by simian immunodeficiency virus (SIV) nucleic acid sequence-based amplification (NASBA) and scored as viral RNA copies per ug total tissue RNA. One animal in the gp120–CD4 XL group (macaque 836) died of causes

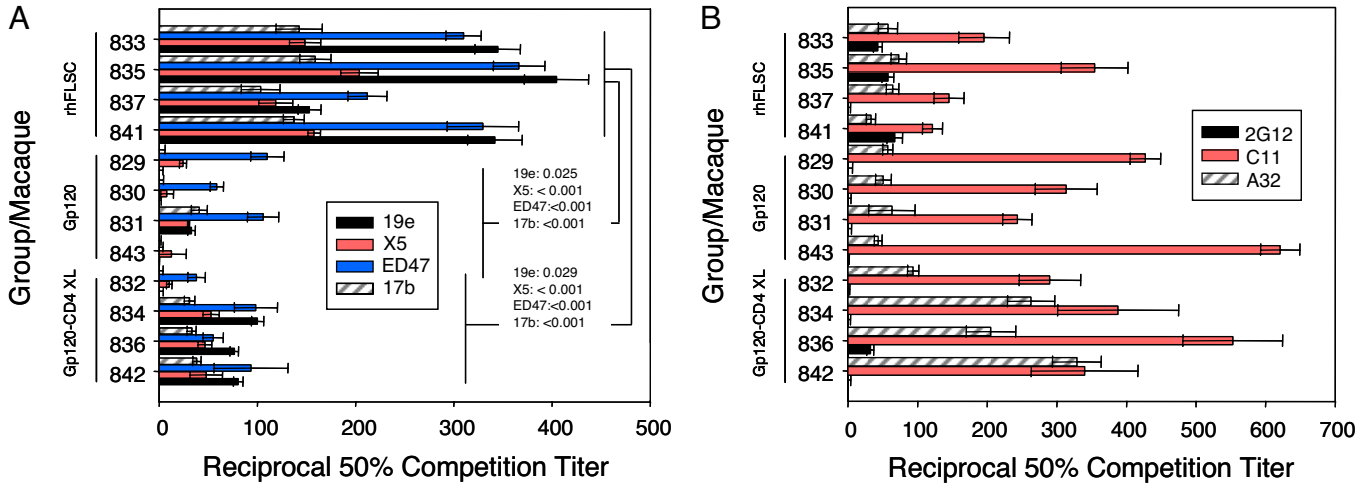

Fig. 2. Competition ELISA with anti-CD4i epitope mAbs. Captured FLSC was reacted with serial dilutions of day of challenge sera in the presence of limiting concentrations of biotinylated human mAbs. Reciprocal 50% competition titers were calculated for each animal and mAb. (*A*) Competition versus mAbs (X5, 17b, ED47, and 19e designated as shown) against CD4i epitopes overlapping the coreceptor binding site. *P* values for group comparisons versus each competing mAb are shown. (*B*) Competition titers versus mAbs against epitopes outside the coreceptor binding site (2G12, C11, and A32 designated as shown). The mean mAb A32 competition titer for the gp120–CD4 XL group was significantly higher than the gp120 ($P = 0.015$) or rhFLSC ($P = 0.017$) groups. Mean competition titers for each animal are shown; error bars indicate standard deviation.

unrelated to SHIV infection before the time of death and therefore was not analyzed. As shown in Fig. 1, three of the four naive animals and all animals in the $gp120_{BaL}$, gp120–CD4 XL, and sCD4 groups had detectable tissue viral loads in one or more tissues. Viral RNA was most often detected in the lymph node tissue, although in some cases it was detected in jejunum and/or large intestine samples. In contrast, three of the four animals vaccinated with the rhFLSC had no detectable viral RNA in any of the tissues. Notably, the animal (macaque 837) that exhibited viral loads in the lymph nodes exhibited the slowest clearance of plasma viral load within the group. The absence of plasma and tissue viral loads (designated as nonsterilizing protection) in three of the four animals in the rhFLSC vaccination group was not seen in any of the other immunization groups. Accordingly, a series of experiments was carried out to determine whether there were humoral responses specific to these animals.

To examine the groups for anti-CD4 autoantibody responses before or after challenge, sera were tested by ELISA with soluble rhesus macaque CD4 adsorbed to the solid phase. None of the sera collected from the rhFLSC or gp120–CD4 XL groups on or after the day of challenge reacted with rhesus CD4 in this assay (SI Fig. 5). Similarly, no reactivity was detected with sera from the naive control and $gp120_{BaL}$ groups (data not shown). Two animals immunized with sCD4 (macaques 847 and 851) exhibited transient postchallenge seroreactivity with rhesus CD4. To corroborate these findings, sera were tested by flow cytometry to assess cell surface reactivity with macaque T cells. To avoid perturbations in surface antigen expression, the cells were not stained with anti-CD4 antibodies, but were instead sorted into CD3+CD8− and CD3+CD8+ (control) subsets. Sera collected on the day of challenge were then tested for cell surface staining at 1:10 dilutions. These sera exhibited equivalent levels of weak, nonspecific reactivity with both subsets of cells. There were no significant differences in reactivity between the animals and no apparent trends indicating group-specific surface reactivity with a particular subset (SI Fig. 6).

Day of challenge sera also were tested for cross-reactive neutralizing activity in a conventional U373 cell line-based assay (23). Sera from three of the four animals in the rhFLSC group exhibited cross-reactive neutralizing titers against primary Clade B X4 and R5 isolates (SI Table 1). The same three sera also neutralized the challenge $SHIV_{162P3}$. In comparison, sera from all animals in the $gp120_{BaL}$ group were not neutralizing in the cell line-based assay; sera from two of the gp120–CD4 XL-immunized animals neutralized all three viruses. High anti-CD4 neutralizing antibody titers were observed in three of the sCD4-immunized macaques; however, such activity was not associated with control of infection (Fig. 1). Notably, nonsterilizing protection in the rhFLSC group did not correlate with neutralizing titers in the conventional assay. This discordance was most evident with animal 833, which extensively controlled viremia (Fig. 1), but did not exhibit neutralizing titers against $SHIV_{162P3}$ in the conventional assay upon or after the challenge (SI Fig. 7). Further, the postchallenge titers again were transient in two other macaques (animals 835 and 837). Only one macaque (animal 841) showed sustained neutralizing titers that increased in accordance with peak viremia.

To examine anti-CD4i epitope responses in the immunized macaques, day of challenge sera were tested for their ability to block single-chain gp120–CD4 complex interactions with four human anti-CD4i epitope mAbs (17b, 19e, ED47, and X5) known to bind epitopes within or proximal to the coreceptor binding site (7, 17, 21). Comparative assays were performed with mAb C11, which is directed against a conserved epitope unrelated to the coreceptor binding site, mAb A32, which recognizes a unique CD4i epitope outside the coreceptor binding site (19), and 2G12, which recognizes a neutralizing carbohydrate epitope (29). As shown in Fig. 2*A*, competition titers versus the four anti-CD4i epitope mAbs were highest in the rhFLSC-immunized macaques. Further, the mean group competition titer of the rhFLSC group was significantly higher than that of any other group for each of these mAbs (Mann–Whitney or *t* test; $P < 0.03$). The differences in competition titers between groups were particularly apparent when data for all mAbs were stacked (SI Fig. 8). Notably, macaque 837, which had the lowest competition titers in the rhFLSC group, exhibited the least control of viral replication (Fig. 1). In comparison, competition titers versus mAb C11 were similar among all immunization groups, whereas mAb A32 was most effectively competed by sera from the gp120–CD4 XL group (Fig. 2*B*). Serum competition versus mAb 2G12 was slightly higher in the rhFLSC-immunized animals versus the other two groups. Day of challenge sera from the naive and sCD4-immunized animals did not compete with any of

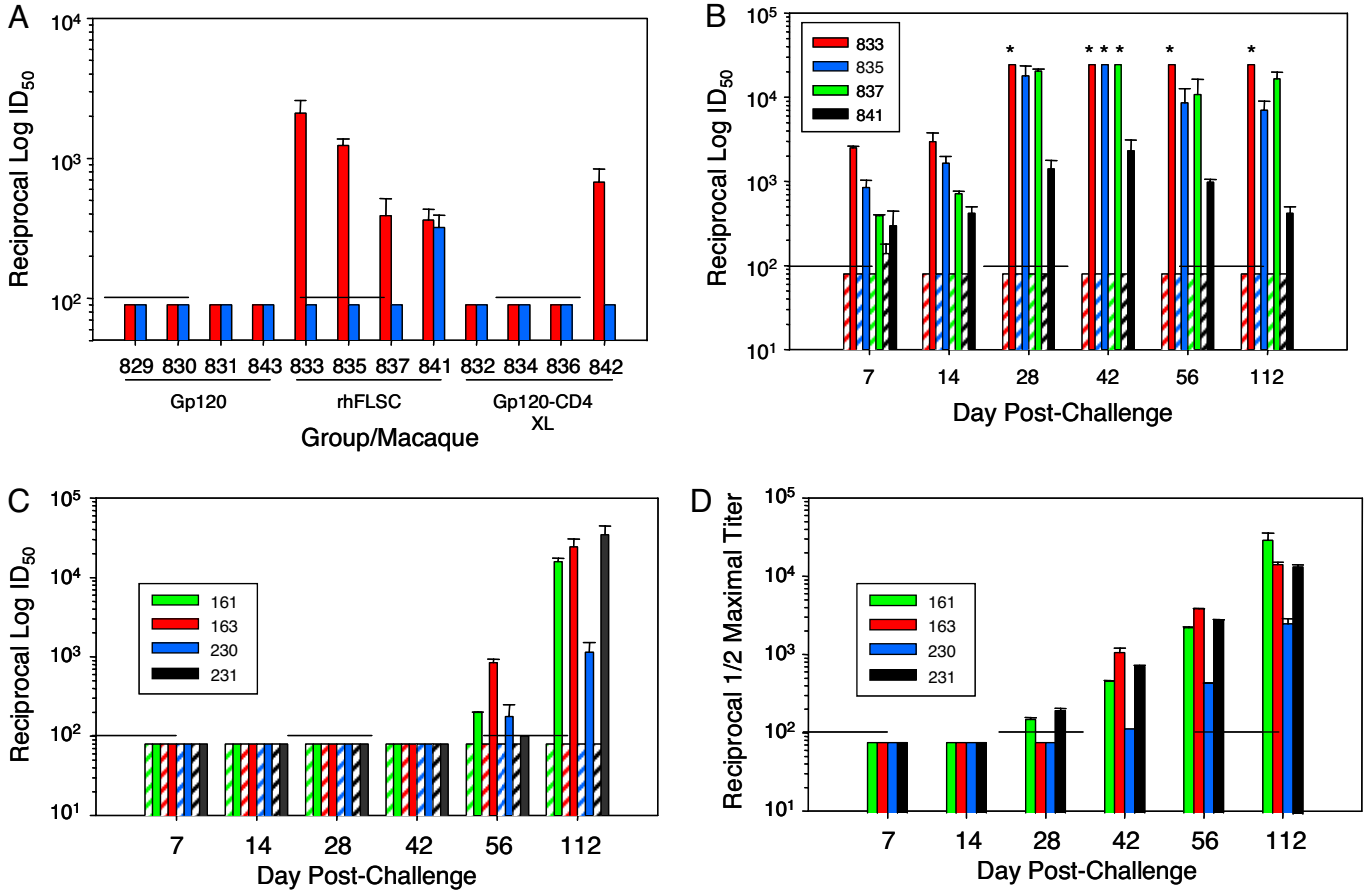

Fig. 3. Neutralizing anti-CD4i antibody responses in rhFLSC-vaccinated and vaccine-naive animals. (*A–C*) Neutralization assays were carried out with TZM-bl target cells and HIV-$2_{7312A/V434M}$ (see *Materials and Methods*). (*A*) Neutralizing activity in day of challenge sera in the presence (red bars) and absence (blue bars) of 9 nM sCD4. (*B*) Neutralizing activity in postchallenge sera collected over time from rhFLSC-vaccinated macaques (color coded) in the presence (solid bars) or absence (hatched bars) of 9 nM sCD4. (*C*) Neutralizing activity in postchallenge sera collected over time from vaccine-naive macaques (color coded) tested in the presence (solid bars) or absence (hatched bars) of 9 nM sCD4. (*D*) For comparison, sera from vaccine-naive macaques were tested in HIV-1_{BaL} gp120 ELISAs to measure total binding titers. Percent neutralization was determined relative to control assays performed with normal macaque sera (*A* and *B*) or no serum (*C*). ID_{50} and ½ maximal binding titers were calculated from binding curves. Mean values are shown; error bars indicate standard deviation. Bars below the dashed line indicate that there was no detectable binding or neutralization at the lowest dilution tested (1:100). Asterisks in *B* indicate values higher than the highest dilution tested (1:24,000).

the mAbs (data not shown). Among the 12 envelope-immunized animals, mAb 19e and ED47 competition titers of >1:200 correlated with the absence of detectable tissue viremia ($P = 0.006$; Fisher exact test).

As an additional measure of anti-CD4i epitope responses, sera collected before the challenge were tested in a neutralization assay that utilizes TZM-bl target cells and a reporter virus containing an HIV-$2_{7312A/V434M}$ envelope (see *Materials and Methods*). This format specifically detects reactivity with highly conserved CD4i epitopes in the coreceptor binding site. Further, such reactivity is revealed by markedly enhanced neutralization of the virus (7) in the presence of subinhibitory quantities of soluble CD4 (sCD4). As shown in Fig. 3*A*, with the exception of one rhFLSC-immunized macaque (animal 841), none of the prechallenge sera was neutralizing in the absence of sCD4. In the presence of 9 nM sCD4, sera from three of the rhFLSC-immunized macaques were neutralizing with titers ranging between 1:300 and 1:2,000. Serum from macaque 841, which neutralized the virus in the absence of sCD4, showed only a modest increase in potency in the presence of sCD4. Of note, this variable pattern of neutralization among the rhFLSC-immunized macaques resembles what was measured in a similar assay of rabbit sera raised against stabilized envelope trimers that present CD4-induced epitopes (30). In comparison, serum from only one animal in the gp120–CD4 XL group (macaque 842) exhibited neutralizing activity in the presence of sCD4 (Fig. 3 *A* and *B*). Sera from the gp120-immunized animals were not neutralizing under any condition. Among the 12 envelope-immunized animals, neutralization titers of >1:100 in the presence of sCD4 correlated with the absence of detectable tissue viremia ($P = 0.045$; Fisher exact test).

The same assay format was used to evaluate the postchallenge evolution of anti-CD4i epitope immune responses in naive and rhFLSC-immunized macaques. As shown in Fig. 3*C*, in the absence of sCD4, no neutralizing activity was detected in serum samples collected from the naive animals at any time after challenge. In the presence of sCD4, serum-neutralizing titers appeared at day 56 after challenge and increased by at least one order of magnitude in each animal by day 112 after challenge. This pattern was discordant with overall anti-$gp120_{BaL}$ ELISA titers, which appeared earlier (28–42 days after challenge) and increased more gradually over time (Fig. 3*D*). In the rhFLSC-immunized animals, neutralizing titers increased substantially by day 28 after challenge (Fig. 3*B*), which suggested that anti-CD4i epitope responses were boosted by infection. The neutralizing responses were diminished by day 112 in concert with the clearance of plasma viremia (Fig. 1).

Discussion

This study used rhesus macaques to investigate whether antigens that present conserved gp120 epitopes raise cognate humoral responses that are effective against a heterologous mucosal challenge with $SHIV_{162P3}$. We found that macaques vaccinated with rhFLSC exhibited an accelerated decline and clearance of plasma viremia and an absence of tissue viremia compared with unvaccinated controls (Fig. 1). The control of viral replication in these macaques correlated with the presence of relatively stronger anti-CD4i epitope responses on the day of the challenge as measured in different assays (Figs. 2 and 3), including one that detected cross-reactive neutralizing activity against HIV-$2_{7312A/V434M}$. Further, the preexisting anti-CD4i epitope responses in the rhFLSC-immunized animals were substantially boosted as a consequence of SHIV challenge (Fig. 3). This effect was interesting insofar as it contrasts with current views that CD4i epitopes are always occluded from the humoral immune system during infection *in vivo* (12). Compared with the rhFLSC group, animals immunized with gp120 or cross-linked gp120–CD4 complexes had lower or no anti-CD4i epitope responses and failed to control infection. Collectively, these findings provide evidence that humoral responses to CD4i epitopes are associated with immunity against $SHIV_{162P3}$ infection. A caveat here is that the $SHIV_{162P3}$ model did not reflect pathogenic changes in CD4+ T cells within the time frame used for analyses. Additional studies are needed to determine whether responses to CD4i epitopes preserve circulating and tissue CD4+ T cell levels. A second potential caveat is that a variant of $SHIV_{162P3}$, which should be present in the challenge stock, is relatively resistant to neutralization by certain anti-CD4i epitope antibodies (25). This characteristic might dampen the impact of anti-CD4i responses on $SHIV_{162P3}$ infection.

Our experiments were not designed to elucidate an immunological mechanism for immunity by anti-CD4i epitope antibodies. However, likely possibilities include direct binding/neutralization of virions, complement-dependent cell lysis, and/or antibody-dependent cellular cytotoxicity of infected cells, which have been correlated with the control of viremia (6). The abilities of human mAbs to anti-CD4i epitopes to bind envelope trimers before and after CD4 engagement (12, 15, 17, 19, 21, 31–33) and to recognize gp120 on the surface of freshly infected cells (19) are consistent with these possibilities. At the same time, it is possible that anti-CD4i responses signal the presence of other antibody specificities or additional modes of immunity (i.e., cellular responses) that may be elicited by vaccination with rhFLSC.

In accordance with our previous findings (23), neutralizing activity was detected in day of challenge sera from three rhFLSC-immunized macaques (SI Table 1) and two of the cross-linked complex-immunized macaques by using conventional *in vitro* assays. However, the conventional neutralizing activity did not correlate with the control of infection and was not boosted by SHIV infection (SI Fig. 7). Such findings are reminiscent of previous vaccine studies in nonhuman primates, which showed that efficacy was improved by the inclusion of envelope antigens, but in a manner that did not correlate with neutralizing antibody titers (4, 5). Nevertheless, we do not dismiss the possibility that higher titers of conventional neutralizing activity at the time of challenge might enhance protection in this model. There also was no association between infection and anti-CD4 responses in any of the immunized macaques. The sCD4-vaccinated animals were not protected, and anti-rhesus CD4 antibodies were absent in the rhFLSC-vaccinated group, which was anticipated because the macaques were expected to be tolerant to homologous antigens. Sera from two of the human sCD4-immunized macaques exhibited reactivity with rhesus CD4 in ELISA, but did not react with rhesus macaque T cells (SI Figs. 5 and 6). Preferential binding to denatured antigen in the ELISA might explain this difference.

Compared with rhFLSC, the cross-linked complexes raised anti-CD4i epitope responses less efficiently. We attribute this detriment to chemical modification of residues in the coreceptor binding site based on our earlier findings that the reactivities of anti-CD4i epitope mAbs to cell surface envelope was strongly reduced after cross-linking (19). Similarly, others have shown that the immunogenicity of gp120–CD4 complexes is altered according to fixative conditions (34). In this study, only one of the four macaques immunized with gp120–CD4 XL (animals 842) exhibited strong conventional neutralizing activity on the day of the challenge (SI Table 1) and apparent anti-CD4i epitope antibody titers (Fig. 3). Thus, gp120 structures constrained by means other than chemical modification may afford a superior method for reliably eliciting anti-CD4i epitope responses.

Longitudinal analyses of samples from the naive animals suggested that relationships between viral replication and anti-CD4i epitope responses may occur during the natural course of SHIV infection. Serum anti-CD4i epitope responses (measured in the HIV-$2_{7312A/V434M}$ neutralization assay) (Fig. 3) did not appear until 56 days after challenge and increased substantially thereafter, in concert with the decline in plasma viremia to a low set point (Fig. 1). In comparison, gross anti-gp120 binding titers were detected in a $gp120_{BaL}$ ELISA (Fig. 3) between 28 and 42 days after challenge. It seems improbable that CD4i epitopes were not immunogenic during SHIV infection because cognate responses were boosted in the rhFLSC group by 28 days after challenge (Fig. 3). A more likely possibility is that anti-CD4i epitope antibodies are preferentially sequestered in immune complexes around the time of peak viremia. This possibility deserves further exploration.

Overall, the data from this study suggest that vaccines capable of generating anti-CD4i epitope responses may have a better chance of achieving prophylactic efficacy against heterologous mucosal challenge. This rationale should provide a basis for experiments to define mechanisms of immunity in the nonhuman primate model and a means for improving vaccine design.

Materials and Methods

Immunogens. Purified soluble human and macaque CD4 (sCD4) was obtained from Biogen (Cambridge, MA). Cross-linked complexes were prepared with HIV_{BaL} gp120 and sCD4 by using bis-sulfosuccinimidyl suberate (Pierce, Rockford, IL) as previously described (35). The rhFLSC was generated from a single-chain HIV_{BaL} gp120–human CD4D1D2 complex (24) by replacing the human D1D2 component with a D1D2 gene fragment from rhesus macaque CD4. The rhesus macaque D1D2 was amplified from pcRhCD4 (AIDS Research and Reference Reagent Program, National Institutes of Health, Bethesda, MD) and subcloned into the NotI/BamHI sites in pEF6-FLSC. The resulting plasmid, pEF6-rhFLSC, was used to express the protein by transient transfection of 293 cells as previously described (24). The rhFLSC was purified by lectin affinity chromatography as previously described. QS-21 adjuvant was provided by Antigenics, and the immunogens were formulated with the adjuvant as described previously (23).

Immunization and $SHIV_{162P3}$ Challenge of Macaques. Groups of four macaques were vaccinated with HIV_{BaL} gp120, rhFLSC, or cross-linked gp120–CD4 complexes. The HIV envelope-containing immunogens were given as 300-μg doses formulated in 100 μg of QS21 adjuvant. A 100-μg molar-equivalent dose of sCD4 was used in a formulation with 100 μg of QS21. The animals were primed on week 0 and boosted on weeks 4 and 26. All animals were given a fourth immunization after a 21-month rest. Four weeks after the final boost, all animals received a rectal challenge with 600 $TCID_{50}$ $SHIV_{162P3}$ (27, 28). At the time of the challenge, four vaccine-naive macaques were added to the experiment as controls. Infection was monitored by SIV NASBA (36) of plasma (lower limit of detection = 500 viral RNA copies per ml of plasma). One year after the challenge, the animals were killed to obtain tissue samples. One

animal in the gp120–CD4 XL group (macaque 836) died before this time, and no tissue samples were collected. Samples of jejunum, large intestine, and axillary, mesenteric, and periaortic lymph nodes were obtained from all animals. Tissue homogenates were prepared with sodium citrate lysis buffer (pH 7.0) containing guanidine thiocyanate, sarcosyl, and β-mercaptoethanol. Lymph nodes were pooled to obtain a single homogenate. Preparations were treated with sodium acetate and extracted with phenol:chloroform, and the RNA was precipitated with isopropanol. Extracted RNA was further purified by reprecipitation with isopropanol. After washing with 70% ethanol, the pellet was dried and dissolved in water, and the RNA content was determined by measuring optical density at 260 nm. Viral RNA in 0.5- to 1-μg sample aliquots of total RNA extract was quantified by SIV NASBA. Tissue viral loads were expressed as SIV RNA copy number per microgram of total tissue RNA.

Immunological and Neutralization Assays. HIV envelope capture ELISAs were performed as previously described (24) by using FLSC or HIV_{BaL} gp120 as target antigens captured by antibody D7324 adsorbed to the solid phase. All assays were carried out in triplicate. For competition ELISAs, serial dilutions of immune sera (from 1:20) were added to assay wells along with limiting concentrations of biotinylated human anti-envelope mAbs [generously provided by J. Robinson (Tulane University, New Orleans, LA) and D. Dimitrov (National Cancer Institute, Frederick MD)] as follows: mAb 2G12 (Polymun, Vienna, Austria), 0.21 μg/ml; mAb A32, 0.66 μg/ml; mAb C11, 0.2 μg/ml; mAb 17b, 0.05 μg/ml; mAb 19e, 0.15 μg/ml; mAb ED47, 0.2 μg/ml; and Fab X5, 0.04 μg/ml. Assay wells were treated with avidin-HRP (KPL, Gaithersburg, MD) and then extensively washed. mAb binding was determined by measuring absorbance at 450 nM. Binding values were plotted against serum concentration to calculate 50% competition titers corresponding to the highest serum dilutions that reduced mAb binding by $\geq$50%, compared with control assays performed in the absence of serum.

Conventional neutralization assays using U373 cells and heterologous primary HIV isolates were carried out as previously described (23). Neutralization assays specific to anti-CD4i epitope antibodies were performed with TZM-bl target cells and a reporter virus [HIV-$2_{7312A/V434M}$; generously provided by George Shaw (University of Alabama, Birmingham, AL)] containing an HIV-2_{7312A} envelope with a mutation in the bridging sheet (V434M) that matches the corresponding primary sequences in HIV-1 envelopes (7). The assays were carried out with serial dilutions of sera in the presence or absence of 9 nM sCD4 according to published methods (7). In our experiments, this amount of sCD4 was not inhibitory. All neutralization assays were carried out in triplicate.

Statistical Analysis. All statistical tests were two sided. The analyses for area-under-curve and rate of decline postpeak viremia were performed by using STATA software (College Station, TX). Repeated-measure regressions were used to compare the rate of decline from peak viremia (log per milliliter per week) between vaccinated groups. To account for the correlation between repeated observations within the same animal, our models included terms representing random intercepts, autocorrelation, and measurement error. Kaplan–Meier analyses for time to undetectable RNA level after vaccination were conducted by using SAS software (Cary, NC).

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