Correction

MICROBIOLOGY

Correction for "Balance of cellular and humoral immunity determines the level of protection by HIV vaccines in rhesus macaque models of HIV infection," by Timothy R. Fouts, Kenneth Bagley, Ilia J. Prado, Kathryn L. Bobb, Jennifer A. Schwartz, Rong Xu, Robert J. Zagursky, Michael A. Egan, John H. Eldridge, Celia C. LaBranche, David C. Montefiori, Hélène Le Buanec, Daniel Zagury, Ranajit Pal, George N. Pavlakis, Barbara K. Felber, Genoveffa Franchini, Shari Gordon, Monica Vaccari, George K. Lewis, Anthony L. DeVico, and Robert C. Gallo, which appeared in issue 9, March 3, 2015, of *Proc Natl Acad Sci USA* (112:E992–E999; first published February 13, 2015; 10.1073/pnas.1423669112).

The authors note that Fig. 3 appeared incorrectly. The corrected figure and its legend appear below.

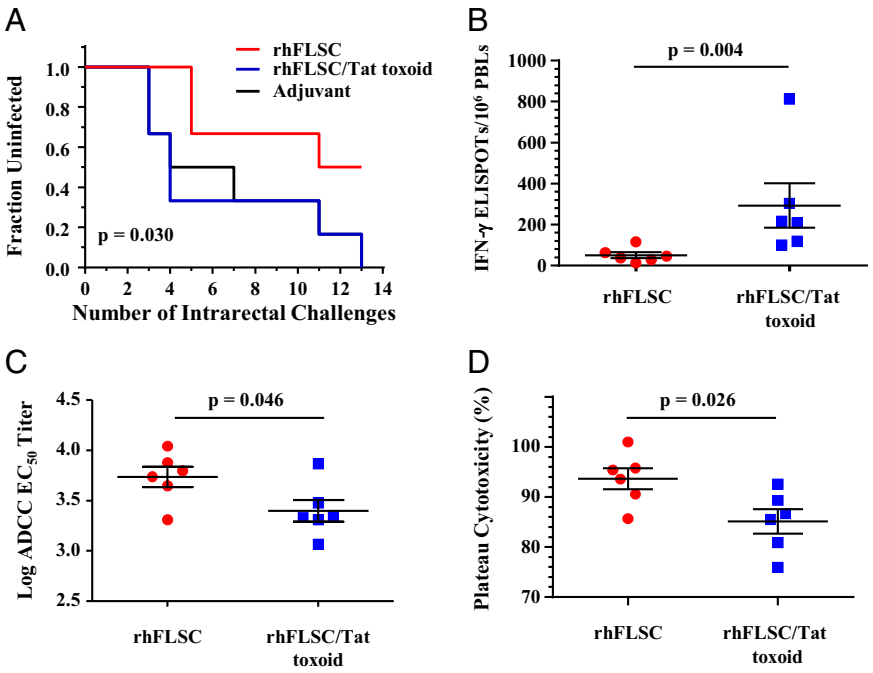

Fig. 3. (*A*) Acquisition rates for the groups in study 2. There was a significant ($P = 0.03$, log-rank test) reduction in the number of SHIV162P3 challenges required for infection in the rhesus macaques vaccinated with rhFLSC compared with the rhesus macaques vaccinated with rhFLSC plus Tat toxoid or rhesus macaques vaccinated with adjuvant alone. (*B*) Comparisons of IFN-γ ELISPOTS in rhesus macaques vaccinated with rhFLSC plus Tat toxoid versus those immunized with rhFLSC. Statistical comparisons were made by Mann–Whitney–Wilcoxon test; the *P* value is shown. (*C*) Comparisons of log ADCC EC_{50} titers in the group vaccinated with rhFLSC alone versus rhFLSC plus Tat toxoid. Statistical comparisons were made by *t* tests; the *P* value is shown. (*D*) Comparisons of ADCC plateau cytotoxicity in animals vaccinated with rhFLSC alone versus rhFLSC plus Tat toxoid. Statistical comparisons made by Mann–Whitney–Wilcoxon test; the *P* value is shown. In *B*–*D*, mean values are shown with the wide line; bars indicate SEM.

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CORRECTION

Balance of cellular and humoral immunity determines the level of protection by HIV vaccines in rhesus macaque models of HIV infection

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Contributed by Robert C. Gallo, January 13, 2015 (sent for review October 10, 2014)

A guiding principle for HIV vaccine design has been that cellular and humoral immunity work together to provide the strongest degree of efficacy. However, three efficacy trials of Ad5-vectored HIV vaccines showed no protection. Transmission was increased in two of the trials, suggesting that this vaccine strategy elicited CD4+ T-cell responses that provide more targets for infection, attenuating protection or increasing transmission. The degree to which this problem extends to other HIV vaccine candidates is not known. Here, we show that a gp120-CD4 chimeric subunit protein vaccine (full-length single chain) elicits heterologous protection against simian-human immunodeficiency virus (SHIV) or simian immunodeficiency virus (SIV) acquisition in three independent rhesus macaque repeated low-dose rectal challenge studies with SHIV162P3 or SIVmac251. Protection against acquisition was observed with multiple formulations and challenges. In each study, protection correlated with antibody-dependent cellular cytotoxicity specific for CD4-induced epitopes, provided that the concurrent antivaccine T-cell responses were minimal. Protection was lost in instances when T-cell responses were high or when the requisite antibody titers had declined. Our studies suggest that balance between a protective antibody response and antigen-specific T-cell activation is the critical element to vaccine-mediated protection against HIV. Achieving and sustaining such a balance, while enhancing antibody durability, is the major challenge for HIV vaccine development, regardless of the immunogen or vaccine formulation.

HIV vaccine | FLSC | SIV challenge | protection | ADCC

There are formidable difficulties for developing a vaccine against a retrovirus such as HIV because of the integration of its genes into the DNA of the host target cells upon infection. For HIV, this problem is compounded by HIV-induced immune suppression and the development of variants that escape immune control. Consequently, an effective preventive vaccine against HIV must work early to block HIV infection and quickly kill HIV-infected cells, or both. To date, only antibodies to the HIV envelope glycoprotein (Env) fit this requirement. Available evidence suggests that such antibodies must recognize highly conserved domains and could inhibit infection by direct neutralization or by Fc receptor-dependent effector mechanisms including antibody-dependent cellular cytotoxicity (ADCC) (1, 2). The ideal result would be sterilizing immunity or, at a minimum, a major restriction of the infection (3). Another challenge stems from evolutionary pressures that abrogate the immunogenicity of conserved, functional epitopes on the envelope spike that are potential targets for cross-reactive antibodies. Large areas are masked by a "glycan shield" of carbohydrate molecules and extensive conformational flexibility (sometimes termed "conformational masking") that dampen immunogenicity of the conserved functional domains (4, 5). The remaining immunogenic domains ("variable" or "V" loops) tolerate a high degree of sequence variability and generate "type-specific" neutralizing antibodies that are not cross-reactive and that limit the efficacy of vaccines that use conventional gp120 monomeric protein.

An emerging concern for HIV vaccine development centers on the quantitative and qualitative aspects of T-cell activation elicited by various immunization regimens (6). Although HIV-specific T

Significance

Our candidate HIV vaccine, a single-chain gp120-CD4 chimera, elicits protection against acquisition of simian-human immunodeficiency virus (SHIV)/simian immunodeficiency virus (SIV) in rhesus macaques. Antibody-dependent cellular cytotoxicity was an inverse correlate of infection risk. However, it is attenuated when antigen-specific T-cell responses exceed a threshold, presumably due to the generation of CD4+ CCR5+ T cells, the preferred cellular targets of SHIV/SIV. Multiple studies strongly suggest that HIV/SIV-specific T-cell responses are a double-edged sword. On one hand, they are required for T-cell help in the protective antibody response. On the other hand, they appear to mitigate protection by creating new targets for viral replication. Determining the balance between protective antibody responses and attenuating T-cell responses is a key challenge confronting HIV vaccine development.

Author contributions: T.R.F. designed and supervised studies 1, 2, and 3, and compiled the data and performed statistical analysis, wrote the manuscript; K.B. designed and supervised study 3; I.J.P. designed, performed, and analyzed ELISA assays; K.L.B. designed, performed, and analyzed ELISA assays; J.A.S. performed Tat binding studies; R.X. performed and analyzed ELSPOT and polyfunctional flow cytometry studies; R.J.Z. designed and supervised study 1, prepared and formulated immunogens for study 1; M.A.E. designed and supervised ELSPOT and polyfunctional flow cytometry studies; J.H.E. designed and supervised study 1; C.C.L. performed neutralization assays; D.C.M. designed and supervised neutralization assays; H.L.B. performed Tat neutralization assays; D.Z. supervised Tat neutralization assays, provided Tat-toxoid; R.P. prepared and titered the SHIV(162P3) challenge stock and provided the SIVmac251 challenge stock; G.N.P. provided historical control macaque data for study 3; B.K.F. provided historical control macaque data for study 3; G.F. provided historical control macaque data for study 3; S.G. provided historical control macaque data for study 3; M.V. provided historical control macaque data for study 3; G.K.L. supervised ADCC assays, analyzed results and wrote the manuscript; A.L.D. compiled the data and performed statistical analysis, wrote the manuscript; R.C.G. designed and supervised study 2, wrote the manuscript.

T.R.F., K.B., I.J.P., J.A.S., R.X., M.A.E., J.H.E., G.K.L., A.L.D., and R.C.G. own stock in Profectus Biosciences.

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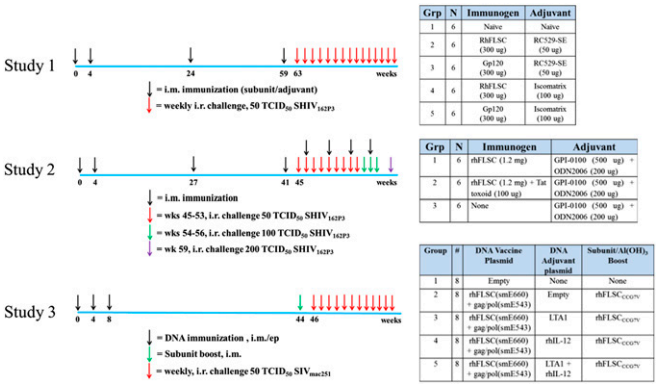
cells might potentially combat infection, certain patterns of T-cell activation (e.g., involving CD4+ CCR5+ T cells) have the potential to promote HIV replication. The latter possibility is emphasized by the HIV vaccine-associated increased risk of infection seen in two large human clinical trials that selectively generated HIV-specific T-cell responses (7). Similar associations between increased risk of infection and T-cell responses of various sorts have been reported in the nonhuman primate model (8–10). Thus, the ideal HIV vaccine strategy is likely to be one that generates antiviral humoral responses without incurring T-cell activation profiles that promote infection and/or overcome the protective benefits of antibodies. Insights for such an approach can be gained by comparative analyses of nonhuman primate models of HIV infection.

The vaccine concept that we have been testing is designed to overcome some of these challenges by stably expressing a highly conserved transition state structure that is exposed on gp120 during a key step in viral entry, exposure of the coreceptor-binding domain consequent to CD4 binding. The prototype immunogen [full-length single chain (FLSC)] is a chimeric protein composed of gp120 from the HIV-$1_{Ba\text{-}L}$ isolate fused to the N terminus of the two outer domains of CD4 by a flexible polypeptide linker (11). For studies of rhesus macaques, the construct is modified to contain "self" rhesus macaque CD4 sequences (rhFLSC) to avoid anti-CD4 responses. The rhFLSC elicits antibody responses to highly conserved epitopes, including the coreceptor-binding domain epitopes (CoRBS) and the C1 regions implicated as a potent ADCC target (12). In an earlier study (12), we showed that rhesus macaques vaccinated with rhFLSC formulated with QS21, a saponin adjuvant derived from the soap-bark tree *Q. saponaria*, exhibited accelerated clearance of plasma viremia and an absence of long-term tissue viral loads compared with unvaccinated controls after a single high-dose rectal challenge with heterologous SHIV162P3. Postinfection control correlated with stronger responses to CD4i epitopes in the rhFLSC-vaccinated animals (CD4i titers > 1:100), compared with macaques that received control immunogens including gp120, soluble CD4, or chemically cross-linked gp120-CD4. Postinfection control did not correlate with anti-CD4 responses, overall anti-gp120–binding titers, or neutralizing activity measured in conventional assays (12), although it did correlate with neutralizing titers in the soluble CD4-triggered assay using HIV-$2_{7312A/V434M}$ that selectively detects responses to highly conserved epitopes in the coreceptor-binding site (13). Taken together, this study showed that rhFLSC elicits antibody responses to highly conserved CD4i epitopes that correlate with postinfection control of viremia after a high-dose rectal challenge with SHIV162P3, but it left open the question of whether rhFLSC can elicit antibodies that block acquisition. Acquisition is typically blocked only in high-dose challenge studies when the vaccine and challenge stock are matched (14), which is not the case for rhFLSC and SHIV162P3. For this reason, we performed three independent studies using different rhFLSC immunization schemes and a repeat low-dose rectal challenge model that is thought to be more reflective of sexual HIV transmission (15). These studies were designed in part as a hypothesis-generating exercise with respect to protective immunity. We consistently found (*i*) inverse correlations between acquisition of infection and certain aspects of humoral immunity and (*ii*) direct relationships between acquisition of infection and vaccine-elicited T-cell responses. Importantly, in certain test groups the apparent protective benefit of humoral responses is absent when T-cell responses are comparatively high. These results strongly suggest that a successful HIV vaccine will need to elicit protective antibody responses without eliciting attenuating levels of vaccine-elicited T-cell responses.

Results

Study Designs. Fig. 1 schematically depicts the three repeat low-dose challenge studies using SHIV162P3 (studies 1 and 2), a pathogenic, CCR5-tropic simian-human immunodeficiency virus (SHIV) (16), or simian immunodeficiency virus (SIV) (study 3). The three study designs are quite similar overall with intramuscular (i.m.) immunizations at weeks 0 and 4 (all studies), week 8 (study 3) followed by i.m. boosts at week 24 (study 1) or week 27 (study 2), and final i.m. boosts at weeks 59 (study 1), 41 (study 2), or 44 (study 3) before initiation of repeat low-dose rectal challenges with SHIV162P3 (studies 1 and 2) or SIVmac251 (study 3). In all cases, the immunization protocol was parenteral and the SHIV/SIV challenges were rectal; in addition, the gp120 components of the vaccines were heterologous with respect to the challenge viruses, both of which are neutralization-resistant (*Materials and Methods*). Although these three studies were performed independently at different times and at different animal facilities, they model the likely scenario that a successful HIV vaccine will be administered parenterally to elicit protection against heterologous, difficult-to-neutralize viruses. The three challenge studies differed significantly in terms of both adjuvants and immunogen composition (*Materials and Methods*), although all included an immunogen based on the rhFLSC-constrained immunogen concept. Studies 1 and 2 used rhFLSC. In this construct, the HIV-$1_{Ba\text{-}L}$ gp120 moiety is heterologous to the SHIV162P3 envelope, which served as the challenge virus. Study 3 is an SIV analog of FLSC in which the gp120 of SIVsmE660 was fused with the two N-terminal domains of rhesus macaque CD4. The two SHIV162P3 challenge studies used soluble subunit rhFLSC protein formulated in RC529 (study 1), Isocomatrix (study 1), GPI-0100/CpG (study 2), or alum (study 3). Study 1 also included parallel groups immunized with HIV-$1_{Ba\text{-}L}$ gp120 in RC529 or Iscomatrix. Study 2 included one group coimmunized with rhFLSC plus an inactivated toxoid of the HIV-1 Tat regulatory protein. Study 3 included a single boost with rhFLSC-SIV in alum at week 44 that was preceded by three intramuscular immunizations (by electroporation) with rhFLSC-SIV + SIVsmE543 gag/pol DNA vaccines. The genetic adjuvant LTA1, a derivative of the enzymatically active A chain of *Escherichia coli* heat-labile enterotoxin, was included on a separate expression plasmid in groups 3 and 5, whereas groups 4 and 5 included a plasmid encoding rhesus IL-12. Because the three trial designs were conceived and executed independently, they represent an unusual, albeit fortuitous, opportunity to identify key factors that contribute commonly but independently to vaccine-elicited protection against SHIV/SIV infection in the nonhuman primate model. The following sections provide data and analyses specific to each study.



Grp	N	Immunogen	Adjuvant
1	6	Naive	Naive
2	6	RhFLSC (300 ug)	RC529-SE (50 ug)
3	6	Gp120 (300 ug)	RC529-SE (50 ug)
4	6	RhFLSC (300 ug)	Isocomatrix (100 ug)
5	6	Gp120 (300 ug)	Isocomatrix (100 ug)

Grp	N	Immunogen	Adjuvant
1	6	rhFLSC (1.2 mg)	GPI-0100 (500 ug) + ODN2006 (200 ug)
2	6	rhFLSC (1.2 mg) + Tat toxoid (100 ug)	GPI-0100 (500 ug) + ODN2006 (200 ug)
3	6	None	GPI-0100 (500 ug) + ODN2006 (200 ug)

Group	#	DNA Vaccine Plasmid	DNA Adjuvant plasmid	Subunit/Al(OH)3 Boost
1	8	Empty	None	None
2	8	rhFLSC(smE660) + gag/pol(smE543)	Empty	rhFLSC$_{CCGV}$
3	8	rhFLSC(smE660) + gag/pol(smE543)	LTA1	rhFLSC$_{CCGV}$
4	8	rhFLSC(smE660) + gag/pol(smE543)	rhIL-12	rhFLSC$_{CCGV}$
5	8	rhFLSC(smE660) + gag/pol(smE543)	LTA1 + rhIL-12	rhFLSC$_{CCGV}$

Fig. 1. Summary of vaccines, immunization schedules, and repeat low-dose SHIV162P3 or SIVmac251 challenges. In all studies, subunit immunizations were intramuscular and the challenges were intrarectal.

Study 1. In this study, animals were repeatedly immunized (Fig. 1) with 300 μg of rhFLSC formulated in RC5290-SE (group 3) or

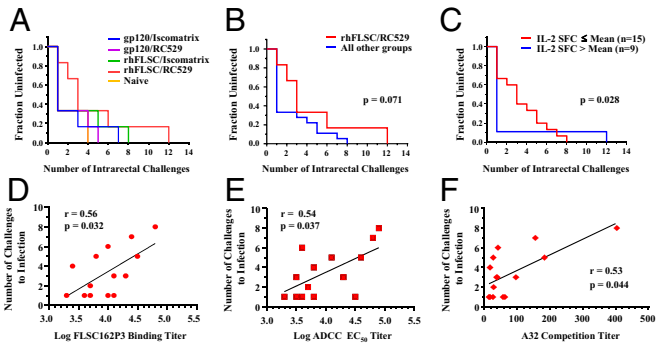


Fig. 2. (*A*) Acquisition rates (intrarectal SHIV162P3 challenge) for the different experimental groups in study 1. (*B*) Acquisition rates for animals that received rhFLSC formulated in RC529-SE compared with all other groups in the study ($P = 0.071$, log-rank test; $P = 0.04$, Wilcoxon–Mann–Whitney *U* test). (*C*) Acquisition rates in study 1 animals dichotomized based on the mean IL-2 ELISPOT responses in the study (spot-forming cells; SFC). A significant difference ($P = 0.028$, log-rank test) was noted between susceptibility to infection and the magnitude of the response. *D*, *E*, and *F* show, respectively, correlations between the number of challenges required for infection and the rhFLSC162P3-binding antibody response (*D*), ADCC EC_{50} titer (*E*), and A32-competition titer (*F*). Correlations were tested using Spearman tests; *P* values and correlation coefficients are shown.

Iscomatrix (group 4) adjuvants. The same amount of HIV-$1_{Ba\text{-}L}$ gp120 was formulated in RC5290-SE (group 5) or Iscomatrix (group 6). Four weeks after the final immunization, all animals received weekly intrarectal challenges with 50 $TCID_{50}$ SHIV162P3. As shown in Fig. 2 *A* and *B*, among all immunization groups there was an apparent trend toward slower acquisition in the group 2 rhesus macaques immunized with FLSC in RC529 adjuvant. The trend was not statistically significant by log-rank test ($P = 0.072$) but was significant in a test that accommodates relatively small group sizes ($P = 0.04$; Wilcoxon–Mann–Whitney *U* test). Although the difference between groups was not significant in all tests, the general trend raised the hypothesis that significant protection might have been seen with larger groups of animals.

Apart from this trend, across the study it was evident that the animals exhibited a wide range of antibody and T-cell response magnitudes. Examination of HIV envelope-specific T-cell responses (using peptides from the HIV-$1_{Ba\text{-}L}$ isolate) at the time of first challenge (Fig. S1*A*) revealed strong Enzyme Linked Immunospot (ELISPOT) responses in groups 4 and 5, receiving rhFLSC or gp120 in Iscomatrix, respectively. By contrast, the ELISPOT responses were much lower for groups 2 and 3, receiving rhFLSC or gp120 in RC529, respectively. Polyfunctional FACS analysis [intracelluar cytokine staining (ICS)] of the envelope specific CD4+ and CD8+ responses revealed that the T-cell responses were primarily single function (IFN-γ or IL-2) or two function (CD107α + IFN-γ) but little-to-no-evidence of T cells with three or more functions (Fig. S1*B*). Cellular responses were too low to generate meaningful ICS data in two animals immunized with RC529 formulations.

The range of ELISPOT responses afforded the opportunity to examine relationships between T-cell responses versus acquisition in all animals. The animals were first dichotomized according to whether they fell above or below the mean IL-2 ELISPOT count 2 wk after the fourth inoculation and number of challenges required for infection (Fig. 2*C*). Interestingly, animals that exhibited IL-2 ELISPOT counts above the mean exhibited a higher rate of infection than those below the mean. All but one animal with higher ELISPOT responses was infected after the first challenge; an outlier animal resisted infection for 12 challenges. The differences between the two groups were significant ($P = 0.028$, Gehan–Breslow–Wilcoxon test; $P = 0.019$, Wilcoxon–Mann–Whitney *U* test). Similar partitioning was performed based on mean IFN-γ ELISPOT responses (Fig. S1*C*). The differences between groups were not significant by any statistical test although a trend of higher acquisition was apparent in the group with higher-than-mean ELISPOTs. Overall, the data indicate that certain qualitative and quantitative aspects of T-cell responses were associated with increased risk of infection against the background of ongoing humoral responses. Specifically, high IL-2 ELISPOT response did not engender a protective benefit against repeated challenges with SHIV162P3 and may have negated any modest protective efficacy provided by the humoral response to rhFLSC.

Both neutralizing antibody activity and ADCC activity (17–22) have been repeatedly associated with protection in high-dose and multiple low-dose nonhuman primate challenge models. Accordingly, direct neutralizing activity and ADCC were examined in serum collected 2 wk after the third boost. Neutralizing responses (Fig. S1*D*) raised under this study protocol were limited to tier 1 viruses (23); there were no neutralizing responses effective against tier 2 viruses or SHIV162P3 at a minimum of 1:20 serum dilutions. In comparison, ADCC activity measured against target cells coated with SF162p3 gp120 using standard methods (24, 25) was detected in all animals. As with the antigen-specific T-cell responses, ADCC EC_{50} titers were significantly higher in animals that received immunogens formulated in Iscomatrix compared with RC529 ($P < 0.001$, Mann–Whitney–Wilcoxon test). Among all immunized animals, there was no significant association between the ADCC EC_{50} titers and the number of challenges required for infection as assessed by Cox proportional hazards regression or Spearman rank correlations.

Viewed in aggregate, the array of humoral and T-cell response data suggested that the impact of vaccination on challenge outcome involved an interconnected relationship between vaccine-elicited T-cell and humoral responses. This is illustrated in Fig. S1*E*, where ADCC activity is plotted versus corresponding ELISPOT data for each animal. The number of challenges to infection for each animal is color-coded. In general, animals with greater resistance to infection exhibit relatively higher ADCC responses and relatively lower ELISPOT responses. A similar pattern was seen when IFN-γ ELISPOT data were used. Such patterns suggested several hypothetical relationships between risk of infection, T-cell responses, and humoral responses, which were evaluated further.

An examination of all vaccinated animals revealed direct correlations between ADCC EC_{50} titers and both IFN-γ ($r = 0.78$, $P = 0.000006$; Spearman test) and IL-2 ELISPOTs ($r = 0.53$, $P = 0.0074$; Spearman test). However, examination of the animals with lower-than-mean ELISPOT responses (Fig. 2*C*) and lower rates of acquisition revealed unique relationships. A significant correlation ($r = 0.54$, $P = 0.037$; Spearman test) emerged between ADCC EC_{50} titer and the number of challenges to infection when we excluded animals with IL-2 ELISPOT counts above the mean (Fig. 2*D*). In the same group of animals, a correlation was also apparent (Fig. 2*E*) between the number of challenges to infection versus binding titers to a variant of rhFLSC (rhFLSC162P3) containing gp120 sequences derived from the SHIV162P3 challenge virus ($r = 0.56$, $P = 0.032$; Spearman test). Notably, there was no significant relationship between acquisition and binding titers to the rhFLSC vaccine containing the HIV-$1_{Ba\text{-}L}$ gp120 sequence or to monomeric HIV-$1_{Ba\text{-}L}$ gp120 itself. This suggested that protection was most closely linked with antibodies that cross-reacted with the challenge virus. To examine this further, we tested immune sera (collected 2 wk before challenge) from the same subset of animals for reactivity with the anti-CD4i epitope antibody A32, which is a highly conserved epitope presented during viral entry (5, 24, 26) and also a potent target for Fc-mediated antiviral activity (27) on both bound and infected target cells (24, 27, 28). These analyses were carried out by cross-competition ELISAs as previously described (12, 29). In the group

of animals with below-the-mean ELISPOT values, there was a significant relationship between A32 competition titers and number of challenges to infection ($r = 0.53$, $P = 0.044$; Spearman test) (Fig. 2*F*). Taken together, these data suggest the hypothesis that protective efficacy is defined by reciprocal relationships between potentially protective antibody responses and T-cell responses. Furthermore, such relationships are apparent even in studies where the overall levels of protection are either nil or marginal, prompting similar analyses in the two additional challenge studies shown in Fig. 1 and described below.

Study 2. Study 2 was used to further test the above hypothesis. This study was independent from study 1 and involved three groups of animals tested under a different vaccine regimen (Fig. 1, *Middle*). For the vaccine groups, 1,200 μg rhFLSC was formulated in GPI-0100, an adjuvant similar to QS-21, also derived from the soap-bark tree *Q. saponaria* along with ODN2006, a type B CpG specific for mouse TLR9 that was also found to activate primate immune responses (30). Antigens formulated in GPI-0100 have induced potent B-cell responses in small animal studies with antigen-specific T-cell responses comparable in magnitude to rhFLSC and gp120 formulated in RC529 (Fig. 1) (31–33). Our previous dose-escalation studies (Fig. S2) in rhesus macaques showed that binding antibody responses to rhFLSC or HIV-$1_{Ba\text{-}L}$ gp120 were poor unless the antigen and GPI-0100 were coformulated with ODN2006. The highest responses, comparable to those seen using RC529 (study 1), were obtained using 1,200 μg antigen (Fig. S2 *A* and *B*). Cross-competition ELISAs (Fig. S2) (12) further showed that this dose formulation raised the highest responses to the A32, 17b, and C11 CD4i epitopes on gp120 (Fig. S2*B*). These responses were similar to what we reported in a previous study of rhFLSC using QS21 adjuvant (12) and to those generated by 300 μg rhFLSC in RC529 (study 1). Accordingly, 1,200 μg of rhFLSC coformulated with GPI-0100 + ODN2006 was used in this study. In one group of animals, rhFLSC was further coformulated with "Tat Toxoid," a biologically inactive but highly immunogenic form of Tat (34) in the same adjuvants. Tat toxoid is expressed from *E. coli* and is inactivated by reduction and carboxamidated so that it does not transduce cells and transactivate the HIV LTR (34). We hypothesized that the inactivated Tat toxoid would improve the protection offered by rhFLSC by inducing antibodies that would neutralize extracellular Tat and reduce the T-cell hyperactivation and dysfunction that exacerbates viral expansion that is induced by native extracellular Tat (35). Combinations of envelope and inactivated mutants of Tat have been reported to protect against homologous SHIV infection in cynomolgus macaques (36, 37). Notably, Tat toxoid physically associated with rhFLSC in solution (Fig. S3) analogous to what was observed with mutant Tat and trimeric gp140 (37). A third group of animals received the adjuvants alone.

Intrarectal challenges with SHIV162P3 began 4 wk after the fourth immunization. A distinguishing aspect of this protocol was that the animals were repeatedly boosted with vaccine formulations every 4 wk through the challenge period. The challenge dose was incrementally increased from 50$TCID_{50}$ to 200 $TCID_{50}$ (Fig. 1, *Middle*) until all group 3 animals were infected.

Overall, envelope antigen-binding titers (measured 2 wk after the fourth immunization) in rhFLSC-vaccinated animals were somewhat lower than what was seen in other studies, although they more extensively favored binding rhFLSC over HIV-$1_{Ba\text{-}L\ gp120}$ (Fig. S2*A*). In accordance, the study 2 animals showed relatively higher competition titers against A32, 17b, and C11 epitopes (Fig. S2*B*). Tat-neutralizing antibody titers were also raised in all Tat-vaccinated animals, but these had no apparent impact on other measures (see below). Following cues from study 1, anti-envelope ELISPOT responses and ADCC activities were also measured in samples collected at the time of first challenge and compared.

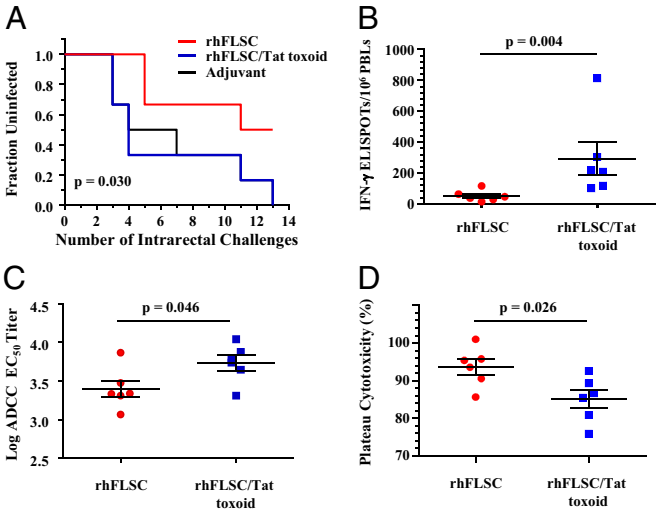


Fig. 3. (*A*) Acquisition rates for the groups in study 2. There was a significant ($P = 0.03$, log-rank test) reduction in the number of SHIV162P3 challenges required for infection in the rhesus macaques vaccinated with rhFLSC compared with the rhesus macaques vaccinated with rhFLSC plus Tat toxoid or rhesus macaques vaccinated with adjuvant alone. (*B*) Comparisons of IFN-γ ELISPOTS in rhesus macaques vaccinated with rhFLSC plus Tat toxoid versus those immunized with rhFLSC. Statistical comparisons were made by Mann–Whitney–Wilcoxon test; the *P* value is shown. (*C*) Comparisons of log ADCC EC_{50} titers in the group vaccinated with rhFLSC alone versus rhFLSC plus Tat toxoid. Statistical comparisons were made by *t* tests; the *P* value is shown. (*D*) Comparisons of ADCC plateau cytotoxicity in animals vaccinated with rhFLSC alone versus rhFLSC plus Tat toxoid. Statistical comparisons made by Mann–Whitney–Wilcoxon test; the *P* value is shown. In *B–D*, mean values are shown with the wide line; bars indicate SEM.

As shown in Fig. 3*A*, immunization with rhFLSC in GPI-0100/ODN2006 elicited significant protection against SHIV162P3 acquisition compared with the rhFLSC/Tat toxoid or adjuvant alone groups (group 3) ($P = 0.030$; log-rank test). Similar to study 1, protection was inversely related to the magnitude of HIV envelope-specific IFN-γ ELISPOT responses (Fig. 3*B*). Marginal ELISPOT responses were seen in the rhFLSC group; substantially higher responses occurred in the rhFLSC/Tat toxoid group where no protection was evident. Conversely, the ADCC responses (Fig. 3 *C* and *D*) as measured by log EC_{50} ($P = 0.046$, *t* test) or plateau cytotoxicity ($P = 0.026$, Mann–Whitney–Wilcoxon test) were elevated in the rhFLSC group compared with the rhFLSC/Tat toxoid group. Taken together, the overall outcome of this study is remarkably similar to that of study 1. The rhFLSC formulated in GPI-0100/ODN2006 reduced rectal SHIV162P3 acquisition in a manner that was related to lower HIV envelope-specific T-cell activation and to higher ADCC activity. It is notable that this scenario remained consistent in a completely independent challenge study using different immunogen formulations and challenge conditions compared with those used in study 1.

Study 3. The two independent SHIV162P3 challenge studies described above consistently showed that acquisition correlates inversely with ADCC responses but directly with vaccine-elicited T-cell responses. We therefore asked whether this scenario could be extended to a heterologous challenge system using the repeat low-dose rectal SIVmac251 challenge model. Accordingly, study 3 used an rhFLSC variant immunogen [rhFLSC (smE660)] containing the gp120 of SIVsmE660, which is heterologous with respect to the SIVmac251. Based on results of the two

SHIV162P3 challenge studies, we investigated additional adjuvant/immunogen combinations that might enhance humoral responses while maintaining relatively low T-cell activities.

To this end, we used a DNA prime/subunit boost regimen in combination with two different genetic adjuvants in the DNA-priming inoculations. Based on our prior studies (38–44), we used DNA constructs encoding rhesus macaque IL-12 or the enzymatically active A1 domain of *E. coli* heat-labile enterotoxin (LTA1) as genetic adjuvants. Others and we have shown that IL-12 is an excellent adjuvant for the induction of Th1 responses (38–44) as well as for augmenting ADCC activity in situ (45–47). We have also shown that LTA1 and the related enzymatically active A1 domain of cholera toxin (CTA1) are potent genetic adjuvants capable of augmenting antibody responses in mice (48–50).

Doses of vaccine and adjuvant plasmids were selected based on published results in macaques (40, 41). As shown in Fig. 1, *Bottom*, groups of eight rhesus macaques were immunized intramuscularly by electroporation (51) of 1 mg rhFLSC(smE660) DNA plus 1 mg DNA encoding SIVsmE543 gag and pol. SIVsmE660 and SIVsmE543 are two closely related viruses (52, 53). These plasmids were coformulated with 300 μg empty vector ("DNA alone"); 50 μg LTA-1 vector; 300 μg rhesus macaque IL-12 (rhIL-12) vector; or both the IL-12 and LTA-1 vectors. Another group received 1 mg empty plasmid and no adjuvant (group 1; naive controls). DNA was delivered on weeks 0, 4, and 8 for all groups. On week 44, all groups except the naive group were boosted with 300 μg subunit protein in alum adjuvant. This protein was another rhFLSC variant ($rhFLSC_{CCG7V}$) containing gp120 from the SIVsmCCG7V, a transmitted/founder variant of SIVsmE660 (54). Two weeks after the final immunization, animals received weekly low-dose rectal challenges with 50 $TCID_{50}$ SIVmac251. The SIVmac251 challenge stock was a neutralization-resistant swarm with a genetic diversity of 2.1% (55). The genetic divergence of SIVmac251 from SIVsmE543 and SIVsmE660 is sufficient to represent a cross-clade challenge relative to the rhFLSC-SIV vaccines. Eight rhesus macaques per group provided 80% power to detect 75% vaccine efficacy.

As shown in Fig. 4 *A* and *B*, the rhesus macaques immunized with antigen with IL-12 showed significantly slower acquisition rates compared with all other groups ($P = 0.024$; log-rank test, Fig. 4*B*) or with naive controls ($P = 0.022$, log-rank test; data not shown). The lower rate of acquisition in this group was even more apparent in comparisons that included the naive animals along with 51 historical controls ($P = 0.002$, log-rank test; Fig. 4*C*) that received intrarectal challenge with the same SIVmac251 virus stock, at the same dose, administered by same technical staff in the same animal facility (Advanced Bioscience Laboratories) within 1 y of the start of our study. Using a Cox proportional hazards regression model, vaccine efficacy in the antigen with rhIL-12 group was 74% and 66% versus the DNA alone group or the naive group, respectively. In contrast, acquisition was not significantly different between the DNA alone group versus the naive group or between the groups that received antigen with either LTA-1 vector or IL-12 and LTA-1. These results follow the general outcomes of study 1 and study 2, which is that that certain formulations and deliveries of FLSC-based immunogens protect against stringent heterologous virus challenges in settings relevant to the application of an HIV vaccine. Because protection varied among immunization groups (as in studies 1 and 2), it was possible to evaluate all animals to define whether protection was linked directly with the magnitude of ADCC responses and inversely with the magnitude of antigen-elicited T-cell responses.

In accordance with studies 1 and 2, analyses of study 3 focused on ELISPOT responses and ADCC activity using peptides corresponding to SIVmac239 gp120 and gp120 from SIVmac239, respectively, as described in *Materials and Methods*. However, neutralizing activity was assessed in sera collected 2 wk after

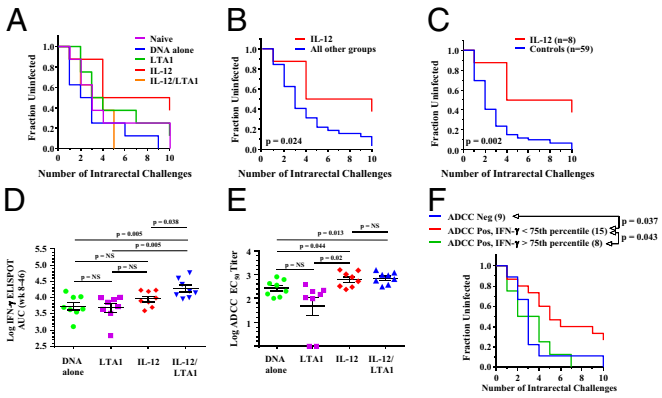


Fig. 4. (*A*) Acquisition rates for the groups in study 3. (*B*) The group that received IL-12 genetic adjuvant showed a significantly lower rate of acquisition compared with all other groups combined ($P = 0.024$, log-rank test). (*C*) The group that received IL-12 genetic adjuvant also showed a significantly lower rate of acquisition ($P = 0.002$, log-rank test) compared with 8 naive control animals in study 3 combined with 51 unimmunized historical controls intrarectally challenged with the same SIVmac251 stock. (*D*) Pairwise comparisons of study 3 groups for IFN-γ ELISPOT values over weeks 8–46 in the protocol. Anti-SIVmac239 Env responses were plotted over time and used to derive an AUC value for each animal. Statistical comparisons were made by *t* tests; *P* values are shown. (*E*) Pairwise comparisons of study 3 groups for log ADCC EC_{50} titers measured on the day of first challenge. Statistical comparisons were made by *t* tests; *P* values are shown. In *D* and *E*, mean values are shown with the wide line; bars indicate SEM. (*F*) All animals in study 3 were divided based on the presence or absence of ADCC activity on the day of challenge. Animals with ADCC activity were further dichotomized based on whether IFN-γ ELISPOT responses on the day of first challenge fell above or below the 75th percentile of all study 3 animals. A significantly lower rate of SIVmac251 acquisition was seen in the animals exhibiting ADCC activity along with ELISPOT responses lower than the 75th percentile ($P = 0.043$, log-rank test). Such animals also exhibited acquisition rates lower than macaques with no ADCC activity on the day of first challenge ($P = 0.037$, log-rank test).

the subunit boost. All vaccine groups had neutralizing titers against tier 1 SIV strains; detectable but significantly lower titers were observed with a tier 2 strain (Fig. S4*B*). There was no relationship between these titers and challenge outcome. T-cell responses in this study were periodically assessed by IFN-γ ELISPOT assays on samples collected between weeks 8 and 46 of the immunization protocol. Areas under the curves (AUC) derived from plots of ELISPOT counts versus time are shown in Fig. 4*D* for each immunization group. The group that received antigen plus IL-12 and LTA-1, which exhibited no protection, had significantly higher IFN-γ ELISPOT responses over time during the protocol ($P < 0.05$, *t* test) versus all other groups. As shown in Fig. 4*E*, ADCC titers 2 wk after the subunit boost were significantly higher ($P < 0.05$, *t* test) in the group that received antigen with IL-12 versus the DNA alone group or the group that received antigen with LTA1, suggesting a potential correlate of infection risk. Notably, there was no significant difference in ADCC titers between the groups that received antigen with rhIL-12 versus antigen with rhIL-12 plus LTA1. These data are compatible with the hypothesis that the vaccine effect correlates with ADCC but is attenuated by the magnitude of the antigen-elicited T-cell response in this group.

As shown in Fig. 4*F*, this hypothesis was tested by stratifying the rhesus macaques from all groups into three categories. One category consisted of rhesus macaques that lacked both ADCC or IFN-γ ELISPOT responses; a second category included rhesus macaques mounting ADCC responses but with IFN-γ ELISPOT values in the lower 75th percentile of responsiveness; and a third category comprised rhesus macaques mounting ADCC responses

but with IFN-γ ELISPOT values in the upper 25th percentile of responsiveness. Rhesus macaques in the second category had significantly reduced acquisition rates compared with those with no ADCC response ($P = 0.037$, log-rank test) or with ADCC responses and IFN-γ ELISPOT values in the upper 25th percentile ($P = 0.043$, log-rank test). Thus, protection against acquisition occurred in animals that mounted an ADCC response, but only when the corresponding IFN-γ ELISPOT response was relatively low. These results are compatible with the hypotheses raised in the two SHIV162P3 challenge studies. In every case, acquisition rates are inversely related to ADCC potency and directly related to the magnitude of the vaccine-elicited T-cell response. Polychromatic flow analyses of the vaccine elicited T-cell responses in studies 1 and 3 (Figs. S1*B* and S4, respectively) strongly suggest that these responses are largely mediated by monofunctional T cells. T-cell responses were too low to perform similar analyses for study 2.

Discussion

Three major conclusions emerge from our studies. First, we show, to our knowledge for the first time, that rhFLSC immunogens, in either an HIV or SIV format, elicit conditional, partial protection against acquisition of two independent heterologous, difficult-to-neutralize challenge viruses, SHIV162P3 and SIVmac251. Furthermore, this protection is elicited by systemic immunization against repeat low-dose rectal challenges with these viruses. These characteristics meet the likely challenges confronting the deployment of a successful HIV vaccine. Moreover, our results inform the future use of rhesus macaque models in proof-of-concept studies for HIV vaccine development. Previously, we showed that rhFLSC formulated in QS-21 did not protect against a high-dose rectal challenge with SHIV162P3; however, it did elicit postinfection control of viremia (12). This is a common observation for high-dose SHIV/SIV challenge studies using viruses that are heterologous to the immunogen (18, 56–62). In contrast, the repeat low-dose challenge SHIV/SIV models used here consistently showed that it is possible to protect against acquisition using rhFLSC immunogens, although the levels of protection varied among the three independent studies and depended on a balance of humoral and T-cell response magnitudes. The ability of a vaccine to protect against acquisition is clearly underestimated by the high-dose challenge models where the challenge dose is typically in the range of 10^8 viral genomes (discussed in ref. 63). This is two to three logs higher than the copy number in ejaculate that is associated with a heterosexual per-act transmission probability of ∼0.01, which is in the upper range of transmission probabilities (64). Due to practical considerations, repeat low-dose challenge studies are typically designed for a per-exposure transmission probability of 0.25 and use challenge doses well above those associated with the highest transmission probability in heterosexual transmission (64), but they are still well below those used for high-dose SHIV/SIV challenges. Thus, a positive result in independent repeat low-dose challenge studies indicates that the responses raised by rhFLSC are relevant to protection against acquisition in humans at risk for HIV infection. In this regard, it should be noted that, by virtue of containing an autologous CD4 moiety, rhFLSC did not elicit anti-CD4 auto-reactive antibodies or cause perturbations in CD4+ T-cell levels during the immunization periods in any of the animals in these studies.

Second, ADCC activity was suggested as a correlate of decreased infection risk in all three studies, which is consistent with the neutralization resistance of the challenge viruses. We recognize that correlation does not establish causality and that it is possible that ADCC is a surrogate for some other Fc-mediated effector function that mediates protection against acquisition. Clarification of this issue requires passive immunization studies using monoclonal antibodies representative of those elicited by rhFLSC that mediate ADCC. In this regard, supplemental evidence is presented suggesting that the potentially protective antibodies recognize CD4i epitopes (Fig. 2*F*). CD4i epitopes include both the classical CoRBS and epitopes in the C1 region of gp120, the latter two of which we have denoted as epitope cluster A (24). Cluster A epitopes are potent ADCC targets during viral entry (24) and viral budding (27). In addition, we have shown that CoRBS mAbs also mediate ADCC during viral entry, although they are less potent than cluster A mAbs (24). Passive immunization studies are underway to determine whether these specificities mediate protection against SHIV162P3 acquisition.

Third, the magnitude of the antigen-elicited T-cell response correlated inversely with acquisition in all three studies, even when the levels of antibody should have been protective. Attenuation of protection by vaccine-elicited T cells is one of two major obstacles confronting HIV vaccine development, the other being poor antibody persistence. We identify antibody persistence in other studies (see ref. 6 for a review), but this report is the first, to our knowledge, to provide evidence that T-cell responses to subunit immunogens can attenuate infection. Although the studies here focused on the FLSC antigen, the general relationships that we define will likely extrapolate to other envelope-based vaccine designs. For example, these relationships were evident in study 1, where gp120- and rhFLSC-immunized animals were considered in aggregate. It is noteworthy that immunization with Tat was associated with the increased T-cell responses that were associated with higher rates of acquisition. The design of study 2 did not allow us to define the precise mechanism(s) for this characteristic or whether it may be due to the physical association of Tat with the HIV envelope (Fig. S3). Nevertheless, these findings suggest that the design of certain vaccines to contain other viral antigens such as Tat along with the HIV envelope might be approached with caution.

The increased rates of acquisition seen in the presence of stronger T-cell activity may come as no surprise in that vaccine-elicited CD4+ CCR5+ T cells appear to be at the root of increased transmission in the Step/Phambili trials (reviewed in ref. 7). There is precedence in the SIV literature as well for increased SIV replication instead of protection that is associated with vaccine-elicited CD4+ T cells (8–10). Thus, a successful HIV vaccine not only will need to elicit protective antibodies but also must do so without eliciting CD4+ T-cell responses that attenuate their ability to protect. This is likely to be a delicate balance as the protective antibody response is nearly certain to require CD4+ T-cell help, particularly by T-follicular helper cells (Tfh), which are themselves reservoirs for HIV (65). Achieving this balance for a vaccine is probably not going to be a trivial pursuit. This is because Tfh apparently lack CCR5, so the infection must occur in one or more of the CD4+ T-cell subsets that are precursors for Tfh (66) as indicated in ref. 67. Because we did not anticipate the problem of "T-cell balance" as a determinant of protection, our studies are hypothesis-generating in this regard. They were not designed to identify the vaccine-elicited T-cell subset most associated with loss of efficacy. At the moment, our extant data suggest only that this subset is CD4+; however, our protocols set the stage for the precise identification of this subset in future studies.

In summary, our data show clearly that an rhFLSC immunogen can elicit protection against SHIV/SIV acquisition under experimental conditions that mimic those likely to confront the deployment of an effective HIV vaccine. These include transmitting viruses that are heterologous to the vaccine and difficult to neutralize. They also show that parenteral immunization can protect against mucosal acquisition, which is a probable requirement for an effective HIV vaccine. In addition, our data strongly implicate an Fc-mediated effector function as a direct correlate of protection and the magnitude of vaccine-elicited CD4+ T cells as an inverse correlate of protection. This sets the stage for refinement of our FLSC vaccine strategy to elicit protective antibody responses involving balanced CD4+ T-cell responses that favor antibody production without attenuating protection.

Materials and Methods

Antigen Preparation and Formulation. rhFLSC, HIV-$1_{Ba\text{-}L}$ gp120, and rhFLSC$_{CCG7V}$ were prepared from HEK-293 cells as previously described (11). Study 1 used adjuvants RC529-SE, a stable emulsion of squalene, glycerol, phosphatidyl choline, and a synthetic monophosphoryl lipid A (a TLR-4 agonist) (68) and Iscomatrix, a saponin-containing lipid iscom (69). For study 2, Tat Toxoid (Neovac SA) was prepared as described (34). GPI-0100 (Hawaii Biotech) is an adjuvant derived from the soap-bark tree, *Q. saponaria* (Hawaii Biotech) (31–33). The adjuvant was mixed with ODN2006 (InvivoGen), a TLR agonist. ODN2006 is a type B CpG ODN specific for mouse TLR9 that was also found to activate primate immune responses (InvivoGen) (30). For study 3, DNA vaccines expressing gag/pol fusion protein derived from neutralization-resistant SIVsmE543 (70) were prepared as previously described for an HIV vaccine (40–42).

ELISAs. Envelope-capture ELISAs to assess serum-binding titers to HIV-$1_{Ba\text{-}L}$ gp120 or rhFLSC were performed as previously described (71). Target antigens were captured by antibody D7324 adsorbed to the solid phase. All assays were carried out in triplicate. Cross-competition ELISAs were performed as previously reported (12, 29) using captured FLSC as the target antigen. Serial dilutions of immune sera were added to assay wells along with limiting concentrations of biotinylated human anti-gp120 antibodies. Assay wells were treated with avidin–HRP and then extensively washed. Antibody 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 50%, compared with control assays performed in the absence of serum.

Tat/FLSC Cross-Linking Assay and Immunoblot. One microgram of rhFLSC was mixed with 1 μg of rhFLSC, Tat, or Tat Toxoid protein and cross-linked with 1 mM of BS3 [bis(sulfosuccinimidyl) suberate] according to the manufacturer's instructions (Thermo Scientific). Each cross-linked reaction and 1 μg of each protein, under reducing or nonreducing conditions, as indicated, was loaded onto Bis·Tris NuPAGE gels, electrophoresed, and either stained with Coomassie blue dye or subjected to Western blot analysis. Proteins were detected using the following antibodies: for FLSC, mouse monoclonal antibodies BHAT-23 and BHAT-32 (72) and for Tat and Tat Toxoid, anti-Tat mouse monoclonal antibody to HIV-1 TAT (Advanced Bioscience Laboratories).

Tat Neutralization Assay. The capacity of the immune sera to neutralize native Tat activation of HIV LTR was assessed as previously described (34). Controls included wells with naive antisera and no antibody.

ADCC Assay. ADCC activity of the immune sera was assessed as described (24, 25).

IFN-γ ELISPOT and Intracellular Cytokine Assays. The ELISPOT assay was performed as described previously (69, 73). The multicolor Cytokine Flow Cytometry (ICS) assay is an adaptation of the method published by Betts et al. (74) and has been described previously (38).

HIV Neutralization Assays. Viral neutralization assays were performed using pseudoviruses and TZM-bl target cells according to published procedures (23, 75).

Animals. In study 1, animals with MAMU A01 major histocompatibility complex (MHC) haplotype were excluded; however, screening for MAMU B08, B17, and Trim5α genotypes was not available when this experiment was performed. There is currently no evidence that the Trim5α genotype impacts SHIV162P3 acquisition as the gag component of this virus is derived from SIVmac239 (76), an SIV strain unaffected by restrictive Trim5α genotypes (77). In study 2, all animals were tested for MAMU haplotypes A01, A02, A08, A11, B01, B03, B04, B08, B17, and B29. Three animals were found to be MAMU A01+ and were randomized one to each group. Animals were also screened for their Trim5α haplotype and randomized accordingly. None had a controlling Trim5α genotype. For study 3, all animals were tested for MAMU haplotypes A01, A02, A08, A11, B01, B03, B04, B08, B17, and B29 as well as for their Trim5α haplotype and randomized accordingly. None had a MAMU A01 haplotype or controlling Trim5α genotypes. After screening, the animals were randomized into their respective groups. Immunized and control animals were challenged rectally with SIVmac251 or with SHIV162P3 as previously described (12). Infection was determined by quantitative SIV NASBA assay with a 50-copy lower limit of sensitivity (78).

Statistical Analyses. Acquisition rates for randomized vaccine groups, or for groups of vaccinated animals categorized by immune response, were compared using log rank or the Gehan–Breslow–Wilcoxon tests. Comparisons were validated against 100,000 random samples. The Gehan–Breslow–Wilcoxon test weights earlier acquisition events more heavily than the log-rank statistic and was applied as indicated by experimental outcomes. Wilcoxon–Mann–Whiney *U* tests were used to compare groups for the numbers of challenges to infection in cases where all animals in a group were infected at the end of the experiment. The test accommodates small group sizes and mitigates the effects of single outlier animals that exhibit unusual resistance to infection. Vaccinated animals were dichotomized for the above tests as indicated in the text. Vaccine efficacy, or efficacy in one category of immune response relative to another, was estimated from proportional hazards (Cox) regression models. Mann–Whitney–Wilcoxon tests were used to compare median values of an immune response in two vaccinated groups or in two subgroups of vaccinated animals defined by level of immune response. Normally distributed data or log-transformed data sets were compared by *t* test. Spearman tests were used to assess correlations between the number of challenges given before infection and an immune response. Given the exploratory nature of the studies, and to optimize the chances of detecting possibly important differences between parameters, most of the analyses involved pairwise comparisons.

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