

Utility of Human Immunodeficiency Virus Type 1 Envelope as a T-Cell Immunogen^{∇†}

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Human immunodeficiency virus (HIV)-specific CD8 T lymphocytes are important for the control of viremia, but the relative utility of responses to the various HIV proteins is controversial. Immune responses that force escape mutations that exact a significant fitness cost from the mutating virus would help slow progression to AIDS. The HIV envelope (Env) protein is subject to both humoral and cellular immune responses, suggesting that multiple rounds of mutation are needed to facilitate viral escape. The Gag protein, however, has recently been shown to elicit a more effective CD8 T-cell immune response in humans. We studied 30 pigtail macaques for their CD8 T-lymphocyte responses to HIV-1 Env and simian immunodeficiency virus (SIV) Gag following prime/boost vaccination and intrarectal challenge with simian-human immunodeficiency virus SHIV_{mn229}. Eight CD8 Env-specific T-cell epitopes were identified and mapped in 10 animals. Animals that generated Env-specific CD8 T-cell responses had equivalent viral loads and only a modest advantage in retention of peripheral CD4 T lymphocytes compared to those animals without responses to Env. This contrasts with animals that generated CD8 T-cell responses to SIV Gag in the same trial, demonstrating superior control of viral load and a larger advantage in retention of peripheral CD4 T cells than Gag nonresponders. Mutational escape was common in Env but, in contrast to mutations in Gag, did not result in the rapid emergence of dominant escape motifs, suggesting modest selective pressure from Env-specific T cells. These results suggest that Env may have limited utility as a CD8 T-cell immunogen.

Although antiretroviral drug therapies have slowed the progression to AIDS in patients from developed countries, issues of side effects, resistance, adherence, and availability in developing countries highlight the need for a vaccine against human immunodeficiency virus (HIV) (9). Cytotoxic T lymphocytes (CTL) are crucial in controlling viremia in HIV-infected humans and simian immunodeficiency virus (SIV)-infected macaques (3, 20, 32). Several current strategies are evaluating T-cell-based HIV vaccines in large clinical trials (17).

HIV type 1 (HIV-1) and SIV are composed of nine proteins. The most useful proteins to target by vaccination are not known. HIV-1 envelope protein (Env) is highly variable between strains (15) but is the only protein targeted by both neutralizing antibodies (NAbs) and T-cell immune responses. The Env protein must serially mutate to escape NAbs, such that antibodies in serum can neutralize past viral isolates but not contemporaneous isolates (14, 30). It is theoretically possible that, if the Env protein needs to mutate multiple times to escape both effective CD8 T-cell responses as well as NAb responses, this could potentially exact a significant fitness cost on the virus (26). This may result in slower progression to disease and lower transmission rates.

Indeed, several earlier studies have suggested the importance of Env-specific T-cell immunity in HIV control. CTL responses to HIV-1 Env early in infection limited viremia to

one-third the level found in patients without Env-specific CTL responses (25). The inclusion of Env in vaccine regimes has proved beneficial in controlling viremia in macaque studies (1, 8, 27). However, determining the utility of T-cell responses to Env in these macaque studies may be confounded by the induction of effective antibody responses to the closely related whole Env proteins expressed by both the vaccine constructs and challenge viruses.

In contrast to the aforementioned studies, Kiepiela and colleagues (19) recently found that broad Env-specific CTL responses correlated with higher levels of viremia in a study of 578 untreated patients with chronic infection. Gag-specific CD8 T-cell responses strongly correlated with improved viremia. The potential utility of Gag-specific CD8 T-cell responses may in part be explained by the high fitness costs associated with changes in this structural gene. Mutation in HIV-1 and SIV Gag CD8 T-cell epitopes commonly revert rapidly to wild type (WT) upon transmission to major histocompatibility complex-mismatched hosts (12, 13, 23).

We studied 30 pigtail macaques for their T-lymphocyte responses to HIV-1 Env and SIV Gag following prime/boost vaccination and intrarectal challenge with simian-human immunodeficiency virus SHIV_{mn229} (7). Vaccines expressed a heterologous, truncated HIV-1 Env which precluded the stimulation of NAb. Anti-Env T-cell responses were identified in one-third of animals. To address the utility of Env as a T-cell immunogen, we mapped multiple Env epitopes and compared the outcomes of animals with Env-specific CD8 T-cell responses to outcomes of those without Env-specific T-cell immunity. Also, similar comparisons were made between animals with and without Gag-specific T-cell responses. To gain insights into the mechanisms of viral control, we studied the

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TABLE 1. SHIV vaccination and challenge regimes

Vaccine regime	<i>n</i> ^a	Animal no.	Subtype AE SHIV immunization ^b	Animal(s) and response after challenge (CD8 T-cell epitope[s]) ^c	
				Env response	Gag response
Control	6	5350, 5620, 6167, 6264, 6352, 6376	Control	5350 (SP9, NL9), 6167 (SP9)	6167 (KP9), 6352 (KP9)
DNA/FPV (high dose)	6	5085, 5396, 5616, 5912, 6177, 6279	SHIV-DNA (2×), FPV-SHIV (3 × 10 ⁸ PFU)	5085 (QL8), 6279 (RY8)	5912 (CM8, AF9, GL8), 5396 (KP9, KW9), 5616 (KP9, KW9), 6279 (KP9)
DNA/FPV (low dose)	6	5023, 6263, 6276, 6284, 6353, 6370	SHIV-DNA (2×), FPV-SHIV (5 × 10 ⁷ PFU)	6263 (MW9)	5023 (GL8, KW9), 6263 (CM8, KW9), 6276 (KP9), 6370 (KP9)
DNA only	6	5614, 5618, 6173, 6351, 6364, 6371	SHIV-DNA (3×)	6173 (SL9), 6364 (QW9)	6364 (CM8), 6371 (KW9), 5614 (KP9), 6351 (KP9)
VV/FPV	6	6259, 6262, 6349, 6363, 6368, 6377	VV-SHIV, FPV-SHIV	6259 (RY8), 6349 (SP9, NL9), 6363 (SP10)	6262 (KW9), 6349 (KP9), 6377 (KP9), 6259 (KP9)

^a *n*, number of animals.

^b All immunizations were intramuscular and spaced 4 weeks apart. The DNA dose was 1 mg, the FPV dose was 3 × 10⁸ PFU, and the VV dose was 10⁸ PFU unless otherwise shown.

^c All animals received the same challenge dose: SHIV_{mn229} (subtype B Env derived from HIV_{IIB}), 10⁵ 50% tissue culture infective doses administered intrarectally. See Table 2 for detail of epitopes.

patterns of mutational escape at both Env and Gag CD8 T-cell epitopes.

MATERIALS AND METHODS

Monkeys. Experiments on pigtail macaques (*Macaca nemestrina*) were conducted with approval from the University of Melbourne and the CSIRO Livestock Industries Animal Experimentation and Ethics committees. Animals were sedated with 10 mg/kg of body weight ketamine intramuscularly prior to all procedures.

Vaccinations. Macaques were vaccinated with combinations of DNA, recombinant fowlpox virus (FPV), and vaccinia virus (VV) as previously described (7) and as summarized in Table 1. All vaccine constructs contained homologous inserts of SIV_{mac239} gag and pol, HIV-1_{93TH253} tat and rev, and the first and last third of subtype AE HIV-1_{93TH253} env. The DNA vaccines contained these genes inserted into the vector pHIS-64 (Coley Pharmaceutical Group) behind the human cytomegalovirus immediate early promoter. The single recombinant FPV expressed SIV gag/pol, an HIV-1_{93TH253} tat/rev fusion product, and HIV-1_{93TH253} env mutated to remove the middle third of the gene. VV recombinants were constructed encoding either WT SIV gag/pol, an HIV-1_{93TH253} tat/rev fusion product, or the mutated HIV-1_{93TH253} env (Table 1).

SHIV challenge of macaques. All macaques were inoculated intrarectally with SHIV_{mn229} (5 × 10⁴ 50% tissue culture infective doses in 0.5-ml doses over 2 days, the equivalent of 500 monkey infective doses) 14 weeks after the last vaccinations. The challenge stock contained a heterologous X4-tropic subtype B HIV-1 Env derived from HIV-1_{IIB}. Real-time PCR on an ABI 7700 machine was used to quantify SHIV plasma RNA as described previously (6). Depletion of peripheral CD4 T cells was monitored by flow cytometry as described elsewhere (6).

Epitope mapping by intracellular cytokine IFN-γ staining. Antigen specificity of CD8 T cells was determined by the expression of gamma interferon (IFN-γ) after stimulation with peptide as assessed by flow cytometry. The antigen peptides were 1 μg/ml/peptide of Env or Gag peptide pools of 15-mers overlapping by 11 amino acids, either obtained from the NIH AIDS Reagent Repository (subtype B HIV-1 MN Env, 212 peptides; catalog 6451) or from Auspep (156 peptides representing only the truncated vaccine subtype AE Env from HIV-1_{93TH253}, as described previously [7]). To map minimal epitopes, peptides of various lengths (GL Biochem; China) were purchased. For the intracellular cytokine staining (ICS) assay, peptides were dissolved in pure dimethyl sulfoxide and added to 200 μl of whole blood together with anti-CD28 and anti-CD49d antibodies (BD Biosciences Pharmingen) for 2 h at 37°C in 5% CO₂. Brefeldin A (10 μg/ml; Sigma) was then added to the wells and incubated for the remaining 5 h at 37°C. Anti-CD8β-phycoerythrin surface phenotyping antibodies were added to each well and incubated for 30 min at room temperature in the dark. Fluorescence-activated cell sorter lysing solution (BD Biosciences) was used for lysis of red blood cells before cells were washed with a wash buffer (5g/liter bovine serum albumin [Sigma], 2 mM EDTA in phosphate-buffered saline at pH 8). Cells were then permeabilized with fluorescence-activated cell sorter perme-

abilizing solution 2 (BD Biosciences), washed, and then incubated with anti-IFN-γ-allophycocyanin for 50 min at room temperature in the dark. Cells were then washed again with phosphate-buffered saline and fixed with 10% formaldehyde. Acquisition was done on an LSR II flow cytometer (BD Biosciences), and data were analyzed with CellQuest Pro software, version 5.1.1 (BD Biosciences).

Viral sequencing. Plasma was separated from EDTA anticoagulated blood, and SHIV RNA was extracted using a QIAamp Viral RNA kit (QIAGEN). cDNA was made using a reaction mixture of 1.25 μM random hexamers (Invitrogen), a 500 μM concentration of each deoxynucleoside triphosphate (Promega), 5 mM dithiothreitol (Invitrogen), 40 μM RNasin (Promega), 5× first-strand buffer, and Superscript III RNase H reverse transcriptase (Invitrogen). Cycle conditions were 65°C for 5 min, ice for 2 min, 25°C for 10 min, 42°C for 45 min, and 70°C for 15 min. Two units of RNase H was then added for 20 min at 37°C. Cycling was performed on a GeneAmp 9700 (Applied Biosystems) instrument.

PCR amplification of approximately 500-bp env fragments was performed using SHIV_{mn229} env-specific primer pairs (a description of conditions is available upon request). PCR conditions using Phusion high-fidelity DNA polymerase (Finnzymes) were 98°C for 30 s, followed by 35 cycles of 98°C for 10 s, annealing for 20 s, and 72°C for 20 s. A final phase of 72°C for 7 min completed the cycle. Adding Taq polymerase (Promega) to amplicons at 72°C for 10 min facilitated “A” tailing. PCR products were cleaned using a QIAGEN PCR spin kit. Amplicons were ligated into pGEM-T Easy Vector (Promega) and transformed into *Escherichia coli* JM109 (Promega) competent cells. Individual clones were sequenced by a BigDye Terminator, version 3.1 (Applied Biosystems), and sequence analysis was performed with Sequencher, version 4.1, software (Gene Codes Corp.).

Statistics. Means of viral loads (VLs) and CD4 counts were obtained by averaging week 15 postinfection levels. *P* values comparing VL and CD4 levels of responders versus nonresponders were determined by a two-tailed Mann-Whitney U Test from the area under the curve from week 4 until week 20 postinfection. Some animals were euthanized with incipient AIDS during weeks 15 to 20 after challenge, and the last observation of VL and CD4 count were carried forward until week 20 in this instance. *P* values for escape rates and mutation numbers were determined by a Mann-Whitney U test.

RESULTS

Macaque T-cell responses to Env postvaccination. To evaluate the comparative utility of Env-specific T-cell immunity, we first detected and mapped a series of T-cell responses to Env in a large, recently published SHIV vaccine study (7). Env-specific CD8 T-cell responses were identified by IFN-γ expression using ICS on fresh whole blood. The 30 macaques in the study were vaccinated with SHIV DNA and poxvirus constructed to express a truncated AE subtype HIV-1 Env and

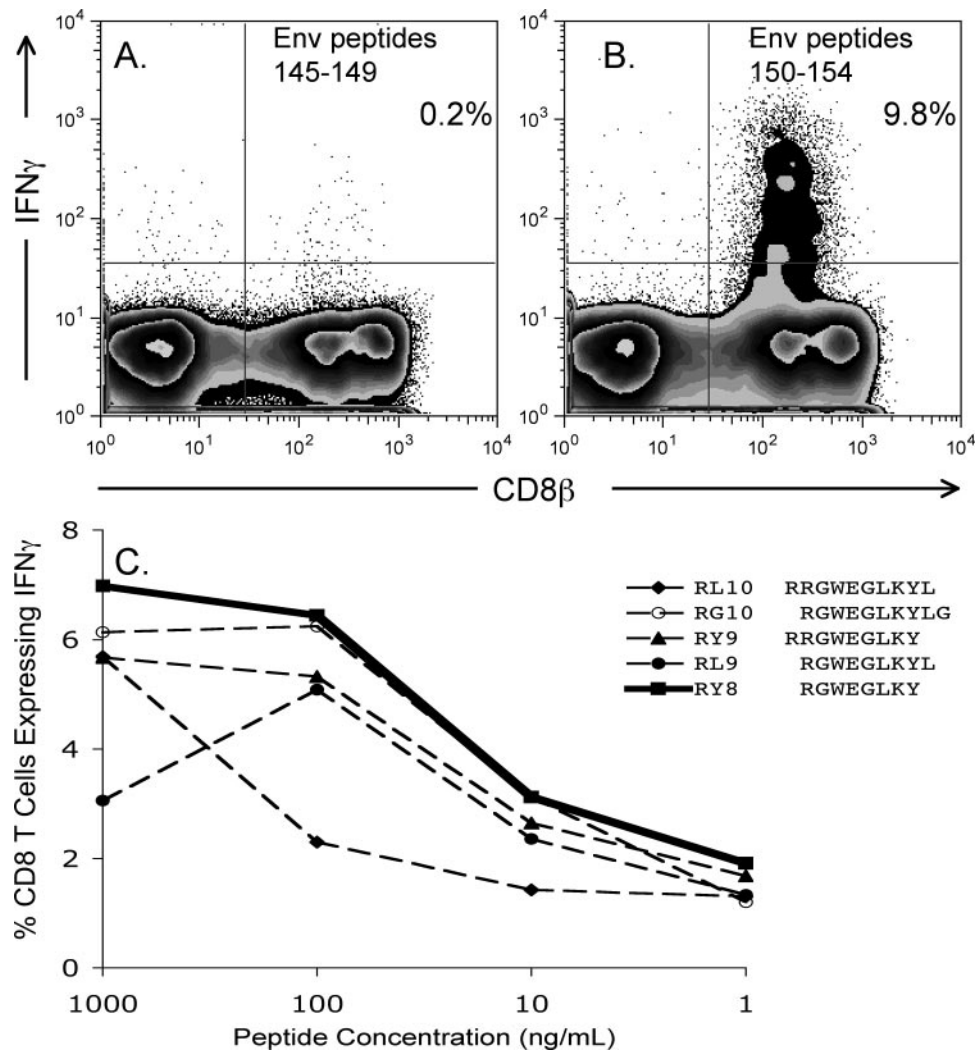


FIG. 1. The identification of an Env-specific T-cell epitope using ICS. (A and B) Pools of 25 15-mer Env peptides overlapping by 11 amino acids were used to identify Env-specific T-cell responses on whole fresh blood. The pool of interest was then reduced to five pools of five 15-mers, and antigen specificity was measured. The pool of five 15-mer peptides eliciting a positive result (peptides 150 to 154; 9.8% antigen specificity) was further reduced to single 15-mer peptides, of which peptides 150 and 151 both had positive responses in this example. (C) All possible combinations of the overlapping 11 amino acids (one 11-mer sequence, two 10-mer sequences, three 9-mer sequences, and four 8-mer sequences) were manufactured and tested on fresh whole blood. Candidate peptides eliciting a positive response were RL10, RG10, RY9, RL9, and RY8. These peptides were then titrated against each other to identify the minimal epitope, i.e., the smallest peptide that gives the largest or equal largest responses down the concentration gradient, in this case RY8.

were subsequently challenged with SHIV_{mn229} expressing a B subtype Env (Table 1). Both AE and B subtype Env overlapping 15-mer peptide pools were used to identify as many epitopes as possible. The Env-specific T-cell responses identified were then mapped early after SHIV challenge (when responses were greater than after vaccination) (7) using diminishing numbers of peptides per pool (Fig. 1A and B). Likely minimal epitope peptides (between 8 and 11 amino acids for CD8 responses) were then manufactured and titrated on fresh blood. The identification and mapping of the Env RY8 CD8 T-cell epitope are illustrated in Fig. 1, with a large proportion of CD8 T cells (9.8%) (Fig. 1B) responding to a small pool of five Env peptides early after SHIV challenge (Fig. 1A). The response was subsequently titrated to smaller peptides to elucidate the minimal 8-mer epitope (Fig. 1C).

From the 30 macaques involved in this trial, eight CD8 T-cell epitopes were identified in 10 animals (Table 2). No Env-specific CD8 T-cell responses could be identified or mapped in the other 20 animals. Three of the CD8 T-cell epitopes, RY8 residues 786 to 793 (RY8₇₈₆₋₇₉₃), SP9₁₁₀₋₁₁₈, and NL9₆₆₉₋₆₇₇, were identified in two or more animals each. Although the responses were mapped after SHIV challenge, the epitopes were located in the outer thirds of the Env protein (expressed by the vaccines) (Table 2), and where tested the majority of epitopes were cross-reactive with both the AE and B subtype peptides used (Fig. 1 and data not shown), suggesting that most of the responses detected in the vaccinated animals were primed by vaccination.

VLs and CD4 T-cell levels in Env responders. VL and peripheral CD4 T-cell levels are critical indicators of modula-

TABLE 2. Mapped Env- and Gag-specific CD8 T-cell epitopes identified in the vaccine study

CD8 T-cell epitope	Amino acid sequence	Residue nos.	Location in protein (domain)	Animal(s) (identifier)
Env specific				
MW9	MHEDIISLW	104–112	C1 gp120	6263
SP9	SLWDQSLKP	110–118	C1 gp120	5350, 6167, 6349
SP10	SLKPCVKLTP	115–124	C1 gp120	6363
SL9	SSNITGLLL	444–452	C4 gp120	6173
QW9	QHLLQLTVW	561–569	Ectodomain gp41	6364
QL8	QEKNEQEL	651–658	Ectodomain gp41	5085
NL9	NWFDITNWL	670–677	Ectodomain gp41	5350, 6349
RY8	RGWEALKY	786–793	Cytoplasmic domain gp41	6259, 6279
Gag specific				
KW9	KYMLKHVVW	25–33	p17	5023, 6262, 6263, 6371
GL8	GNYVHLPL	137–145	p24	5023, 5912
KP9	KKFGAEVVP	161–169	p24	5396, 5614, 5616, 6167, 6259, 6276, 6279, 6349, 6351, 6352, 6370, 6377
AF9	ALAPVPIPF	365–373	p24	5912
CM8	CGKMDHVM	413–420	p7	5912, 6263, 6364

tion of infection following viral challenge. To determine the relative impact of Env-specific and Gag-specific CD8 T-cell responses, VL and CD4 T-cell levels were determined for each animal at serial time points after challenge. Infection with the X4-tropic SHIV_{mn229} reliably results in high VLs and rapid depletion of CD4 T cells. We analyzed serial samples until week 20 after challenge since many animals were euthanized with incipient AIDS thereafter. Ten animals in this trial had significant Env-specific CD8 T-cell responses (Table 2) that were confirmed and mapped to minimal T-cell epitopes. These 10 Env-responding animals were spread evenly across the five vaccine groups of six animals each in the trial (three animals in DNA/FPV [prime/boost] groups, two in DNA-only group, three in the VV/FPV group, and two animals in the control group) (Table 1). The mean VLs of these 10 animals were compared to the mean VLs of the other 20 animals in the trial that did not have detectable Env-specific CD8 T-cell responses. The mean VL of the Env responders at week 15 postinfection was $5.19 \pm 0.28 \log_{10}$ copies/ml compared to $5.38 \pm 0.16 \log_{10}$ copies/ml for animals without CD8 T-cell responses to Env. A comparison of Env responders with Env nonresponders found no significant difference between the weighted averages of the postacute VLs between weeks 4 and 20 postinfection ($P = 0.38$) (Fig. 2A). Levels of peripheral CD4 T cells detected in Env-responders and nonresponders at week 15 postinfection were $4.67\% \pm 1.24\%$ and $2.31\% \pm 0.90\%$, respectively (Fig. 2B). There was a significant benefit shown when the weighted averages of the CD4 counts over weeks 4 to 20 postinfection were compared in Env responders versus Env nonresponders ($P = 0.02$). Two of the animals in which Env-specific CD8 T cells were identified were control animals, potentially confounding the analysis. However, when only vaccinated animals were included in the analysis, there was still no difference in mean VLs between Env responders and nonresponders (not shown).

Impact of Gag-specific CD8 T-cell responses. All 30 animals in this study were also studied for responses to seven common Gag CD8 T-cell epitopes previously identified in pigtail macaques (7, 12). The 12 animals in the trial responding to the Gag KP9 CD8 T-cell epitope were excluded from analysis as

the SHIV_{mn229} challenge virus was already mutated at this epitope, and we recently showed that this results in a poor outcome from challenge (11). Excluding the KP9 responders, six animals responded to the four previously mapped SIV Gag-specific CD8 T-cell epitopes CM8, AF9, GL8, and KW9 (two animals in the DNA/FPV low-dose group, two animals in the DNA/FPV high-dose group, one in the DNA-only group, and one in the VV/FPV group) (Table 1). The SHIV_{mn229} virus is WT at these epitopes (24; also data not shown). The average VL of animals with a Gag-specific CD8 T-cell response at week 15 postinfection was $4.63 \pm 0.26 \log_{10}$ copies/ml versus $5.40 \pm 0.24 \log_{10}$ copies/ml in the Gag nonresponders.

Similar comparisons of VL and CD4 T-cell levels were done on Gag responders versus nonresponders between weeks 4 to 20 postchallenge. Gag responders had a significant advantage over Gag nonresponders in terms of the weighted averages of the postacute VLs between weeks 4 and 20 postinfection ($P = 0.01$) (Fig. 2C). Retention of peripheral CD4 T cells in Gag responders was $6.44\% \pm 2.69\%$ while the nonresponders' level was $2.43\% \pm 0.99\%$ at week 15 postinfection. There was a significant benefit shown in the time-weighted average CD4 T-cell counts over weeks 4 to 20 postinfection in Gag responders compared to Gag nonresponders ($P = 0.02$).

Mutations in Env and Gag CD8 T-cell epitopes. Mutations that evolve at CD8 T-cell epitopes suggest that significant immune pressure is being applied. Further, if the epitopes lie within sufficiently functionally constrained proteins, immune escape mutants may have reduced viral fitness. A comprehensive cloning and sequencing study was therefore conducted to identify mutations in the Env-specific CD8 T-cell epitopes mapped in this study. Cloning and sequencing were done sequentially after viral challenge to establish the timing and rate of mutation since we previously proposed that the rate of mutation reflects the efficiency of clearance of the WT epitope by CTL (12, 18, 24). We concentrated our analysis on the three Env-specific CD8 T-cell epitopes common to multiple animals, RY8, SP9, and NL9, in an attempt to identify common pathways of mutational escape.

Multiple diverse mutations were observed within these three shared Env CTL epitopes. A complete list of mutations ob-

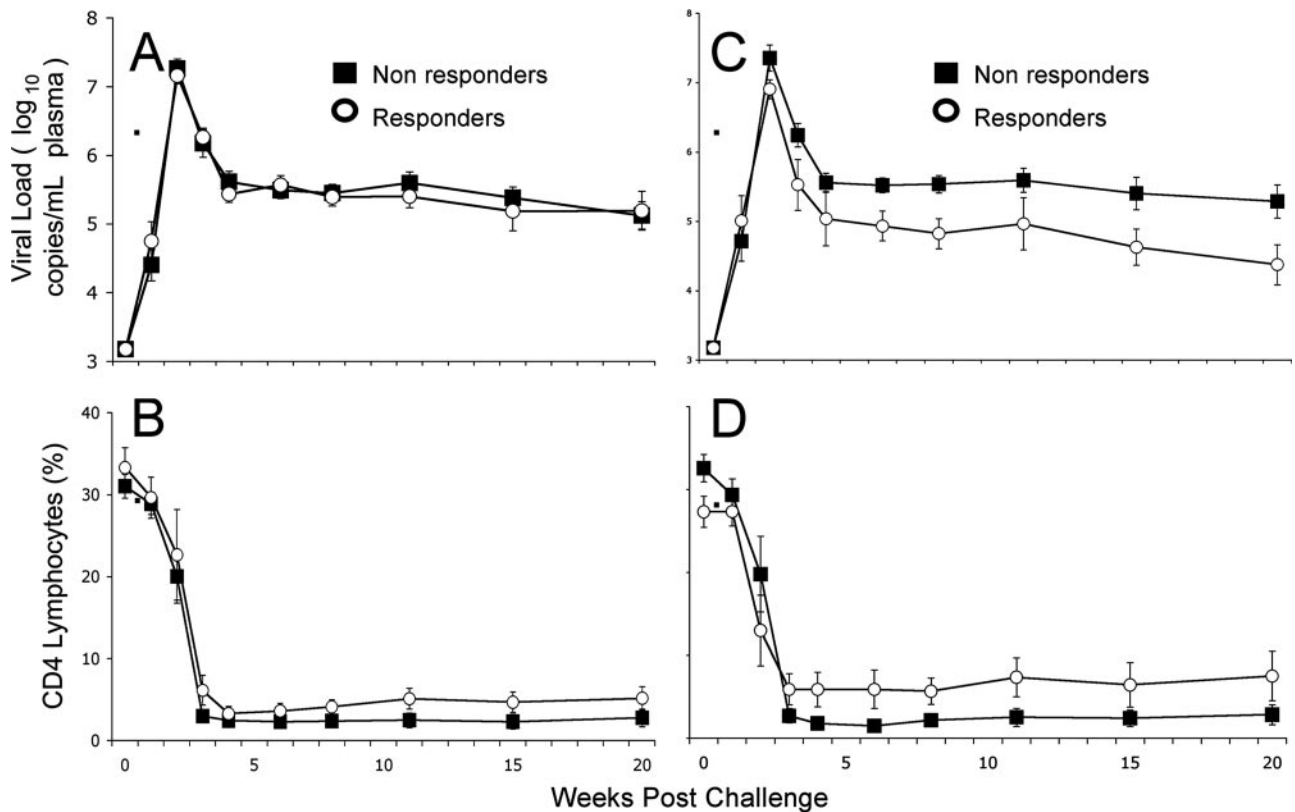


FIG. 2. Outcome of Env and Gag CTL responses on outcome of SHIV challenge. Panels A and B show comparisons of mean (\pm SE) VLs and peripheral CD4 T-cell counts between animals with no Env-specific CD8 T-cell response (20 animals) and those with mapped Env-specific CD8 T-cell responses (eight epitopes in 10 animals). Panels C and D show comparisons of VLs and peripheral CD4 T-cell counts between animals with no Gag-specific CD8 T-cell response (12 animals) and those with mapped Gag-specific CD8 T-cell responses (four epitopes in 6 animals). None of the 12 KP9 Gag epitope responders is included in these two graphs since the SHIV_{mn229} challenge virus was not WT at this epitope.

served is given in Table S1 in the supplemental material. No shared mutational pathways were observed. For example, among the three animals responding to the SP9_{110–118} CD8 T-cell epitope, 18 different viral quasiespecies were identified up until week 15 postinfection. Within the epitope, only one of the nine amino acids (position 5, Q114) did not mutate in any viral clones.

Viral immune escape implies that the virus has mutated the CD8 T-cell epitope to evade the targeted CD8 T-cell attack. Evidence for this Darwinian selective force was first sought by examining the genome sequences flanking the identified epitope. WT sequence was largely maintained in the other 400 nucleotides sequenced for each clone, suggesting that there is selective pressure on the virus by CD8 T cells at these epitopes. Additionally, sequencing across Env epitopes in animals not responding to epitopes did not reveal mutations within these sites. We also constructed peptides from some of the more dominant mutant epitopes and showed diminished recognition of the mutant peptides compared to WT peptides from frozen peripheral blood mononuclear cells. Using a concentration of peptide of 10 μ g/ml, there was a 100% loss of recognition of the W788R mutation within RY8 and of the S110G mutation within SP9 and an 82% loss of recognition of the D673N mutation within NL9.

Although SIV Gag CTL epitopes undergo mutational escape in pigtail macaques, they generally focus on a single common escape motif (12, 24). The CD8 T-cell Gag epitope KP9_{164–172} exhibits only one mutation (K165R) after 8 weeks of infection with SHIV_{SF162P3}, which is WT at KP9. Similarly, at the AF9_{371–379} and KW9_{28–36} Gag CD8 T-cell epitopes, only a single escape motif dominates after challenge. AF9 mutated by a two-amino-acid deletion in 45/51 clones tested between weeks 8 and 15 postinfection, and KW9 mutated only at K28R in all clones tested during the same time period (24). The significant difference between viral escape diversity at CD8 T-cell epitopes in Gag and Env at week 8 postinfection is illustrated in Fig. 3A (number of Env mutants, 3.14 ± 0.40 ; number of Gag mutants, 1.25 ± 0.25 ; values are means \pm standard error [SE]; $P = 0.03$). This difference in numbers of mutant quasiespecies between Env and Gag epitopes was more marked when the epitopes were examined between weeks 8 to 15 after infection. Of the four animals studied for escape at the three common Gag CD8 T-cell epitopes (KP9, AF9, and KW9), very few mutants were identified between weeks 8 and 15. In contrast, of the five animals studied for escape at the three shared Env CD8 T-cell epitopes in the same time frame, 22 mutants were identified. The mean (\pm SE) number of Env

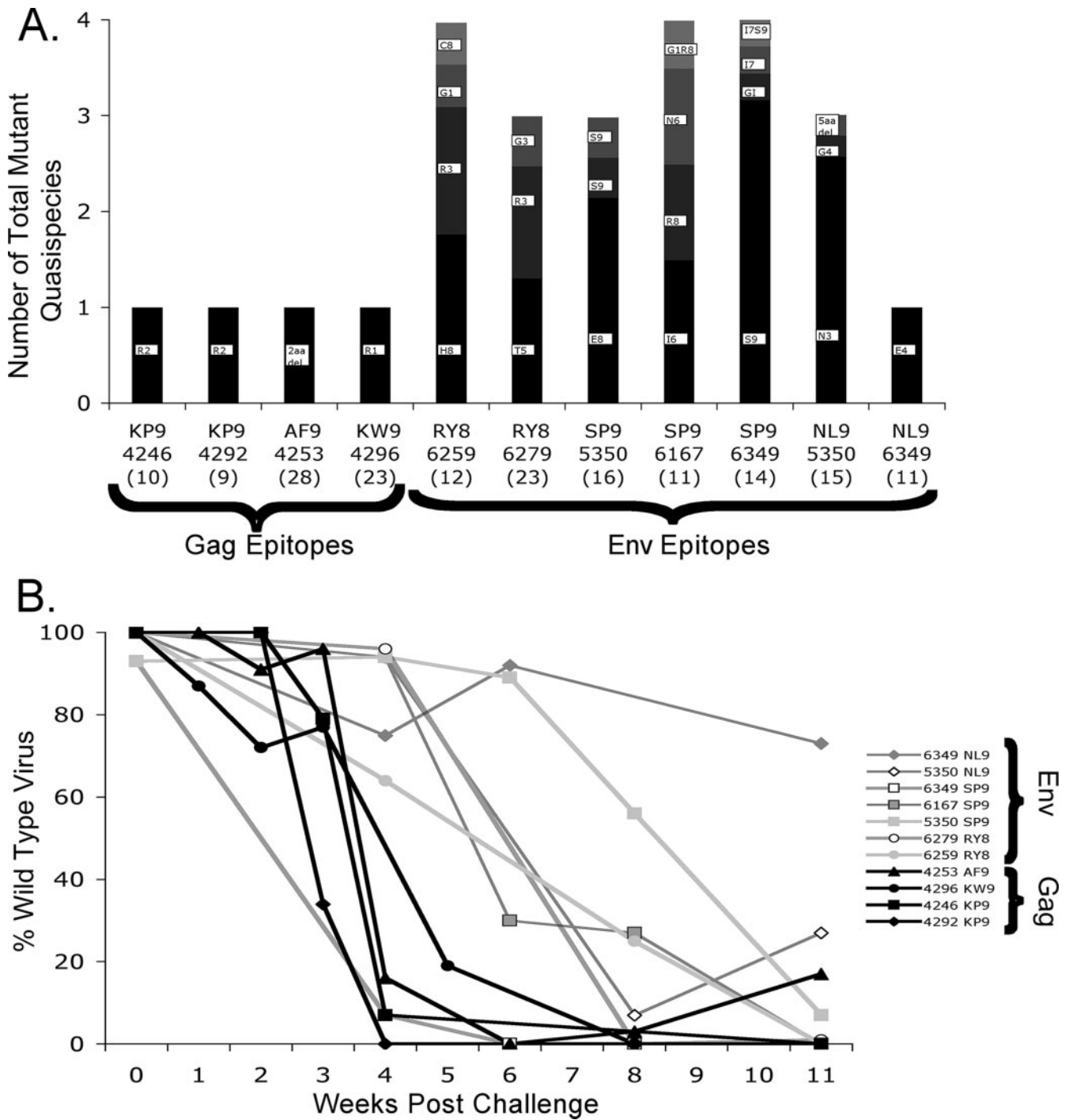


FIG. 3. Mutational pathways of Env and Gag CTL epitopes. (A) The number of ways in which a particular epitope can mutate to escape a CD8 T-cell response may indicate the level of constraint on mutation before viral fitness is intolerably compromised. The total number of different mutations is shown on the y axis, and clone numbers sequenced are in brackets. The relative proportion of each mutant is shown by the height of the shaded bar. R2 indicates a mutation to the amino acid R at position 2 within the epitope. Gag-specific epitopes KP9, AF9, and KW9 (identified in a previous trial; animal numbers are below epitope identifiers) mutated invariably to a single dominant mutant quasispecies at week 8 postchallenge. In contrast, Env-specific epitopes generally mutated to several different viral quasispecies, as indicated by the numerous different shaded bands representing different mutant quasispecies. (B) The rate of mutation in an epitope may indicate the effectiveness of the CD8 T-cell response. The rate of mutation in the Gag-specific CD8 T-cell epitopes KP9, AF9, and KW9 (black lines) was rapid and early in infection compared to the predominantly slower and later mutational kinetics exhibited by the Env-specific CD8 T-cell epitopes RY8, SP9, and NL9 (gray lines).

mutants was 5.4 ± 0.9 from weeks 8 to 15 versus 1.43 ± 0.2 for Gag mutants ($P = 0.01$).

Kinetics of mutations in Env and Gag T-cell epitopes. Mutations at CD8 T-cell epitopes may produce viral populations with reduced replication fitness. Rapid selection of escape mutant virus implies effective elimination of WT virus by CD8 T cells (18). Cloning and sequencing serial plasma samples established the kinetics of mutations at the Env-specific CD8 T-cell epitopes identified. Mutation of all clones at the SP9 epitope occurred by week 6 postchallenge in animal 6349 and by week 11 in animal 6167 and failed to occur in animal 5350 as it maintained a WT viral population at SP9 throughout the trial. RY8 in animals 6259 and 6279 mutated steadily from challenge to 0% WT by weeks 11 and 8 postchallenge, respectively. Epitope NL9 also exhibited highly divergent mutation kinetics, as highlighted at week 8 postchallenge. Animal 6349 had approximately 90% WT, and animal 5350 had 7% WT at this time point. The mutation kinetics of SP9, RY8, and NL9 are illustrated in Fig. 3B (gray lines). Total viral mutation at epitope SL9 in animal 6173 took until 20 weeks postinfection to complete. No WT virus at epitope MW9 was present in animal 6263 by 6 weeks postchallenge (data not shown). Other Env-specific CD8 T-cell epitopes identified (SP10, QW9, and QL8) failed to mutate. These kinetics of development of escape mutations within Env CD8 T-cell epitopes contrast markedly with the total and more rapid mutation rates of the Gag-specific CD8 T-cell epitopes AF9, KW9, and KP9 (Fig. 3B, black lines). Mutations occurred rapidly between weeks 2 and 4 postchallenge in AF9 and KP9. KW9 exhibited a slower rate of mutation, but total escape at this epitope had occurred by week 8 postinfection. Figure 3B shows the much slower development of mutations within Env-specific CD8 T-cell epitopes than in Gag-specific epitopes between weeks 4 and 11 postchallenge. The Gag escape rate between weeks 2 and 8 postinfection ($0.45 \pm 0.17/\text{day}$) is significantly faster than that of Env ($0.07 \pm 0.03/\text{day}$; $P = 0.042$).

If the gradual development of mutations in CD8 T-cell epitopes within Env was likely to significantly reduce viral fitness, we might expect a reduction in VLs as these mutations are selected. Between weeks 4 and 15 postchallenge, the average percentage of mutant viral clones at the Env-specific CD8 T-cell epitope RY8 in two animals increased, but no reduction in VLs ensued (Fig. 4A). Conversely, an average decrease in mutant viral clones at NL9 in two animals accompanied an increase in VLs (Fig. 4B). Changes in average numbers of mutants at epitope SP9 in three animals correlated with a static VL (Fig. 4C). When mutation numbers were averaged over these three Env-specific epitopes identified in seven animals, no overall modulation of average VL was evident between weeks 4 and 15 postchallenge (Fig. 4D).

DISCUSSION

We evaluated a large SHIV vaccine study to analyze the relative abilities of Env- and Gag-specific CD8 T-cell responses to force viral escape mutations that might contribute to controlling infection in pigtail macaques. We first identified and mapped a novel series of Env CD8 T-cell responses generated by 10 of the 30 animals in the study. Animals generating CD8 T-cell responses to Env exhibited no better control

of VL than animals without Env-specific responses. In contrast, Gag-responders had reduced VLs compared to Gag nonresponders. Mutation within Gag-specific CD8 T-cell epitopes beyond 8 weeks after infection was restricted to one or two quasispecies, whereas mutation at Env-specific CD8 T-cell epitopes produced multiple, varied quasispecies. Further, the kinetics of escape at Gag-specific CD8 T-cell epitopes showed a rapid and total conversion to a dominant quasispecies early after infection. The kinetics of escape at Env-specific CD8 T-cell epitopes showed variable and delayed kinetics of mutations away from WT, implying less effective selection pressure.

Overall, Gag-specific CD8 T-cell responses elicited a modest virologic and immunologic benefit to the test animals in this study that Env-specific CD8 T-cell responses did not match. The advantage of the Gag-specific responses is more significant, given the modest protection observed overall in this study (7). Our findings accord with a recent human observational cohort study showing that Env-specific CD8 T-cell responses (and also increasing breadth of Env-specific responses) were associated with higher viremia in humans than in subjects with Gag-specific responses (19). Notably, Env-specific CD8 T-cell responses generated in this pigtail macaque SHIV trial were of little benefit to the animal but did not hasten progression to disease. This is in contrast to Staprans et al., who found that Env-expressing vaccines had the potential to worsen outcomes in a macaque vaccine study (33).

The relatively uniform manner in which viral mutations are selected at Gag-specific CD8 T-cell epitopes in these pigtail macaques suggests that mutation is constrained in this gene and results in viral populations of reduced fitness. The multiple mutations identified in Env-specific CD8 T-cell epitopes would suggest that Env has weaker limitations to its mutational maneuvers. This “mutational robustness,” i.e., the ability to maintain a constant phenotype despite genomic mutation (21), coupled with findings that viral diversity in HIV-1 Env correlates with replicative fitness (35), raises reservations regarding the suitability of Env as a T-cell vaccine target. These are critical topical issues, since a current DNA prime/adenovirus boost HIV-1 vaccine regimen primarily induces Env-specific T-cell immunity, and this vaccine regimen is moving toward large clinical trials (4, 5, 16).

There are, however, several limitations to our study. The overall protection of animals in this vaccine study was poor, given the high pathogenicity and the heterologous nature of the X4-tropic challenge virus used as the infective agent. X4-tropic SHIV strains, paradoxically, can be easily controlled by the immune system with modest levels of preexisting immunity (10) although this was not observed in this study. Further studies on the utility of Env as a T-cell immunogen using an R5-tropic challenge virus would likely be more predictive of the effect of vaccine strategies on human infection (15). R5-tropic studies of HIV-1 Env vaccines are currently difficult to conduct rigorously in macaques since common R5-tropic SHIV strains such as SHIV_{SF162P3} are naturally controlled (without prior vaccination) in up to half of either rhesus or pigtail macaques (2, 29). We are presently mapping an additional series of SIV Env-specific T-cell responses to evaluate the utility of Env-specific T cells in the control of the R5-tropic SIV_{mac251}.

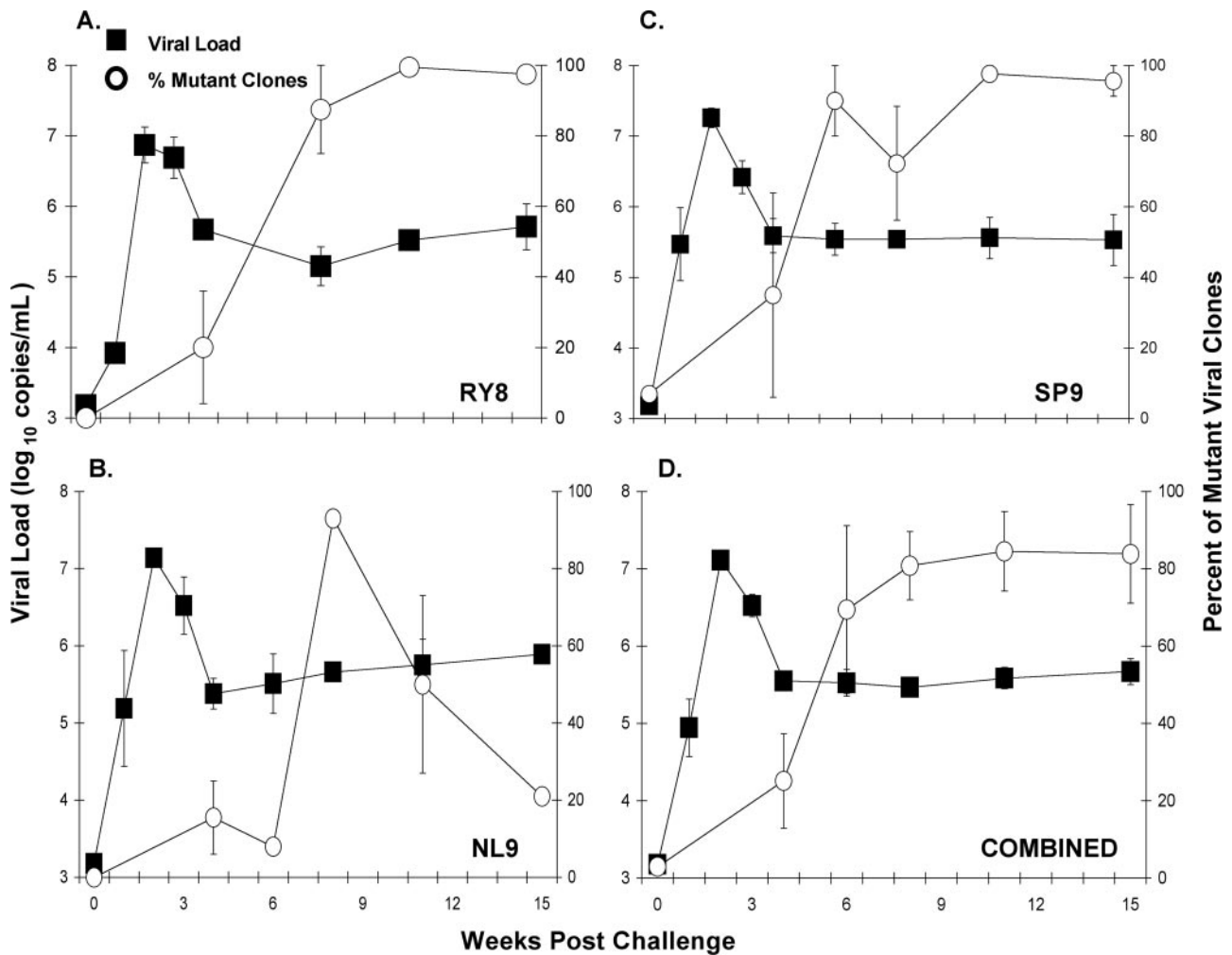


FIG. 4. Impact of Env mutations on VL. (A) Animals 6259 and 6279 had CD8 T-cell responses to the Env-specific epitope RY8. Mutations at this epitope arose such that no WT was evident at week 11 postchallenge. (B) Animals 5350 and 6349 had CD8 T-cell responses to the Env-specific epitope NL9. A dominant mutant quasispecies arose at week 8 postchallenge in 5350 but the viral population was again dominated by WT at week 15 postchallenge. WT NL9 dominated throughout infection in 6349. (C) Animals 5350, 6167, and 6349 had CD8 T-cell responses to the Env-specific epitope SP9. Numerous and varied mutations were evident at this epitope in these three animals. By week 11 postchallenge, 98% of the viral population were SP9 mutants. (D) Average percentages of mutants and VLs of all three epitopes.

An additional limitation to this study is that the Gag responses were stronger than the Env responses we detected (7). The modest Env responses after vaccination necessitated mapping the responses after challenge in this study. Although the responses detected were located within the portions of Env expressed by the vaccines that recognized both AE and B subtype peptides, we cannot exclude the possibility that some Env responses (or Gag responses) among vaccinated animals were primarily generated by infection rather than vaccination. The strength of the T-cell response may be an additional factor controlling viremia and rapidly selecting dominant escape mutations. We are constructing additional Env vaccines to induce stronger and broader Env responses by vaccination and developing major histocompatibility complex tetramers to more sensitively track Env-specific T-cell responses to further probe the utility of Env-specific T-cell immunity.

The HIV-1 Env protein demonstrates a remarkable plasticity in serially escaping NAb responses while apparently main-

taining function (14, 31, 34). Perhaps it should be no surprise that mutational escape from Env-specific CD8 T cells should inflict little fitness cost on the virus. Nonetheless, Env surely cannot be infinitely malleable, and there may yet be combinations of Env-specific immune responses that cannot be escaped without very large fitness costs to the virus (26). It is frequently argued that combinations of T-cell and antibody responses are desirable characteristics of HIV vaccines (9, 17). We are currently evaluating mutations in potential N-linked glycosylation sites within Env adjacent to mapped CD8 T-cell epitopes for clues into the impact of combinations of T-cell and antibody responses against Env.

In summary, although HIV-1 Env is subject to both arms of the adaptive immune system, logically making it an ideal prophylactic and therapeutic vaccine target, we found minimal impact of Env-specific CD8 T cells on the outcome of SHIV infection of pigtail macaques. Env's seemingly unconstrained shifts in "antigen topography" (28) through immune pressure

make Env difficult to target with vaccines (22). Many successful vaccines have targeted conserved surface proteins with minimal capacity to mutate effectively away from the induced immune response. The enormous flexibility of the HIV-1 Env under selective immune pressure may limit the utility of Env as a T-cell immunogen.

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