

Macaques vaccinated with live-attenuated SIV control replication of heterologous virus

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An effective AIDS vaccine will need to protect against globally diverse isolates of HIV. To address this issue in macaques, we administered a live-attenuated simian immunodeficiency virus (SIV) vaccine and challenged with a highly pathogenic heterologous isolate. Vaccinees reduced viral replication by ~ 2 logs between weeks 2–32 ($P \leq 0.049$) postchallenge. Remarkably, vaccinees expressing MHC-I (MHC class I) alleles previously associated with viral control completely suppressed acute phase replication of the challenge virus, implicating CD8⁺ T cells in this control. Furthermore, transient depletion of peripheral CD8⁺ lymphocytes in four vaccinees during the chronic phase resulted in an increase in virus replication. In two of these animals, the recrudescing virus population contained only the vaccine strain and not the challenge virus. Alarming, however, we found evidence of recombinant viruses emerging in some of the vaccinated animals. This finding argues strongly against an attenuated virus vaccine as a solution to the AIDS epidemic. On a more positive note, our results suggest that MHC-I-restricted CD8⁺ T cells contribute to the protection induced by the live-attenuated SIV vaccine and demonstrate that vaccine-induced CD8⁺ T cell responses can control replication of heterologous challenge viruses.

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Abbreviations used: Eq, equivalents; p.c., postchallenge; p.i., postinoculation; QRT-PCR, quantitative RT-PCR; SFC, spot-forming cells; SGA, single genome amplification; SIV, simian immunodeficiency virus; vRNA, viral RNA.

The enormous variability of HIV is one of the major hurdles that must be overcome in the development of a successful AIDS vaccine. Accumulated nucleotide changes within the highly mutable *env* gene form the basis for classifying HIV-1 into different groups (M, N, and O) and subtypes (clades A, B, C, D, E, F, G, and K). Evolutionary analysis of nucleotide sequences shows that *env* can vary by up to 35% among different clades (1). Even within clades, this diversity can reach 20% (1). This variability is driven by several factors, including recombination between different strains of HIV and the high rate of mutation associated with HIV RT (2–4).

The online version of this article contains supplemental material.

For this reason, many HIV-1 vaccine designs focused on inducing cell-mediated immunity have abandoned using Env as an immunogen in favor of concentrating on more conserved regions of the virus. However, even relatively minor variations in these proteins may have grave implications for vaccine efficacy, as single-aa differences can negatively affect recognition by vaccine-induced antibodies and CD8⁺ T cells (5, 6). To address this issue, several strategies have been proposed to improve

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vaccine design by including polyvalent formulations or immunogens based on ancestral, center of tree, or consensus HIV-1 sequences (1, 7, 8). Each of these methods seeks to minimize differences between the vaccine sequence and circulating viruses while maximizing cross-reactive immune responses.

Currently, most AIDS vaccine strategies include a prime/boost component to induce antiviral immune responses. Recent results from the HIV Vaccine Trial Network and Merck's STEP trial (<http://www.hvtn.org/media/pr/step111307.html>) have clearly shown that current versions of these approaches fail to either protect against pathogenic infections or reduce viral replication. In contrast, immunization of rhesus macaques with live-attenuated simian immunodeficiency virus (SIV) has consistently induced protective immunity against homologous pathogenic SIV challenge (9–11). However, characterization of the vaccine-induced immune responses accounting for this protection has proven difficult. Antibodies (12, 13), CD4⁺ and CD8⁺ T cells (11, 13–15), NK cells (16, 17) and even viral interference (18–20) have been implicated in mediating live-attenuated SIV-induced protection. Understanding the underlying mechanisms for this protection should facilitate the design of improved HIV vaccines (21).

Despite the effectiveness of live-attenuated SIV vaccination against homologous virus challenge, only a few small-scale studies have addressed the ability of live-attenuated SIV to control heterologous SIV challenge and have had mixed results with regard to vaccine efficacy (9, 22, 23). We therefore sought to determine whether macaques vaccinated with SIVmac239Δnef could effectively control heterologous virus replication in a large-scale study designed to achieve statistical significance. We included MHC-I (MHC class I)-defined macaques to facilitate careful monitoring of CD8⁺ T cell responses. Vaccinated animals, along with ten naive controls matched for the MHC-I alleles of interest, were challenged i.v. with the highly pathogenic heterologous “swarm” virus SIVsmE660, and their mean plasma virus concentrations were compared at different time intervals.

RESULTS

SIVmac239Δnef vaccination

To address the issue of whether an HIV vaccine can ameliorate the pathogenic effects of a heterologous challenge, we used a well-described macaque AIDS model of protective immunity. We induced antiviral immune responses by inoculating ten MHC-I-defined rhesus macaques with the attenuated SIV strain SIVmac239Δnef (24). We included animals expressing Mamu-A*01, -A*02, -A*11, -B*08, or -B*17 ($n = 2$ for each) because all of the SIVmac239 epitopes bound by these MHC-I molecules had previously been defined (25–31). Most animals had a peak of virus replication at 2 wk postinoculation (p.i.), with SIVmac239Δnef ranging between 3.2×10^3 and 9.4×10^5 viral RNA (vRNA) copy equivalents (Eq)/ml of plasma (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20081524/DC1>). 8 of the 10 vaccinated animals controlled SIVmac239Δnef plasma virus replication below 500 vRNA copy Eq/ml between weeks 12 and 26 p.i. (Fig. S1).

All vaccinated macaques developed broad, but low frequency, cellular immune responses. We monitored the development of SIV-specific cellular immune responses using a combination of IFN-γ ELISPOT and MHC-I tetramer staining. We did not conduct such an extensive analysis in the Mamu-A*11⁺ animals because MHC-I tetramers were unavailable. Throughout the vaccine phase of the study, the frequency of tetramer binding cells in PBMC varied from animal to animal and within animals from epitope to epitope (Table S1, available at <http://www.jem.org/cgi/content/full/jem.20081524/DC1>). In most animals, at least one tetramer-specific response attained a frequency of >0.3% of the circulating CD8⁺ T cells. At the time of challenge, 26 wk p.i., the frequency of tetramer binding cells in the PBMC for each specificity ranged from undetectable to 0.2% of the CD8⁺ T cells. In IFN-γ ELISPOT assays, most animals had detectable cellular immune responses to multiple peptide pools from several SIV proteins before challenge (Table S2), ranging between 50 and 850 spot-forming cells (SFC) per million PBMC (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20081524/DC1>).

Table I. Percentage difference in amino acid sequence among different strains of immunodeficiency viruses

Protein	Consensus Clade B ^a versus Clade B isolates ^b	SIVmac239 ^a versus SIVsmE660 ^b	Consensus Clade B ^a versus Clade C isolates ^b
	%	%	%
Tat	12.5	26.0	30.5
Rev	12.1	25.0	35.3
Nef	17.7	21.3	35.2
Vif	6.8	17	18.1
Env	27.4	15.1	53.1
Vpr	9.8	12.0	17.3
Pol	3.2	8.3	10.0
Gag	4.4	8.0	16.4

The mean percentage difference per protein of five clade B and five clade C isolates in comparison to the consensus clade B amino acid sequence. The clade B and C isolates were obtained from the Los Alamos National Laboratory HIV sequence database.

^aVaccine strain.

^bChallenge strain.

To differentiate CD4⁺ and CD8⁺ T cell responses, we performed IFN- γ ELISPOT assays using total PBMC and PBMC depleted of CD8⁺ cells. Most animals made CD4⁺ T cell responses against multiple peptide pools derived from several proteins (Fig. S2). We only measured responses directed against Gag, Tat, Rev, Vif, and the intact portion of Nef using PBMC depleted of CD8⁺ cells because of the limited number of available cells. The CD4⁺ T cell responses that we did detect were primarily directed against Gag (Fig. S2).

Pathogenic heterologous SIV challenge

To assess the protective capacity of SIVmac239 Δ nef-induced immune responses against heterologous SIV infection, we challenged the 10 vaccinated animals, along with 10 naive controls matched for expression of the MHC-I of interest, i.v. with the highly pathogenic heterologous swarm virus SIVsmE660 at 6 mo p.c. with SIVmac239 Δ nef. SIVsmE660 differs from SIVmac239 by \sim 15% of its amino acids (Table I). This difference approximates the variation between single clade-based HIV vaccines and circulating HIV isolates within that clade (1).

During acute infection, the SIVmac239 Δ nef-vaccinated animals reduced plasma virus concentrations by 2–3 logs in comparison to the naive controls (Fig. 1, a and b). At 2 ($P = 0.011$), 3 ($P < 0.001$), and 4 ($P = 0.005$) wk postchallenge (p.c.), the mean plasma virus concentrations in the vaccinated animals were significantly lower than that of the naive controls. The vaccinated animals maintained a reduction in mean plasma virus concentrations through 8 mo p.c. (the point at which some animals began experiencing simian AIDS). Only at week 24 p.c. did the difference in viral loads fall below the level of significance ($P = 0.052$). Despite gradually increasing plasma virus levels in some of the vaccinees, the majority of the time points showed significant difference between vaccines and controls (weeks p.c.: 6, $P < 0.001$; 8, $P = 0.001$; 12, $P = 0.005$; 16, $P = 0.002$; 20, $P = 0.049$; 28, $P = 0.046$; and 32, $P = 0.008$).

Vaccinated macaques expressing the MHC-I alleles *Mamu-B*08* or *Mamu-B*17*, which have previously been associated with spontaneous control of pathogenic SIVmac239 replication in naive macaques (25, 32), manifested highly effective control of acute infection after the heterologous challenge (Fig. 1, c and d). The four animals expressing either of these two protective alleles had plasma virus concentrations of <30 , 40, 240, and 14,000 vRNA copy Eq/ml at 2 wk p.c., which is normally the peak of virus replication. By comparison, the MHC-I-matched naive controls expressing these same protective alleles had more than one million copy Eq/ml plasma. The high level of acute viral replication in *Mamu-B*08*⁺ and *-B*17*⁺ naive macaques was expected because these two alleles only exert their protective effect in the chronic phase in naive animals (25, 32). Differences in mean plasma virus replication between the vaccinated and naive *Mamu-B*08*⁺ or *-B*17*⁺ macaques were significant at 1 ($P = 0.002$; $n = 4$), 2 ($P = 0.014$), 3 ($P = 0.002$), 4 ($P < 0.001$), 6 ($P = 0.002$), and 8 ($P = 0.011$) wk p.c. The two *Mamu-B*08*⁺ vaccinated macaques maintained control of SIV replication below 5,000 vRNA copy Eq/ml plasma out to 11 mo p.c. However, despite low

virus replication in the acute phase peak, virus replication in the vaccinated *Mamu-B*17*⁺ macaques progressively increased during the chronic phase of infection (Fig. 1 c). The *Mamu-B*08*⁺ and *-B*17*⁺ vaccinated animals no longer had significantly different ($P > 0.055$) plasma virus levels in comparison to the naive *Mamu-B*08*⁺ or *-B*17*⁺ animals by week 12 p.c. (Fig. 1, c and d).

Significant reduction in plasma virus concentrations observed in SIVmac239 Δ nef-vaccinated animals was not solely caused by the effect of *Mamu-B*08*⁺ and *-B*17*⁺ on SIVsmE660 replication. Vaccinated *Mamu-A*01*⁺, *-A*02*⁺, and *-A*11*⁺ macaques also showed significantly reduced plasma replication at 3 ($P = 0.004$; $n = 6$), 4 ($P = 0.031$), 8 ($P = 0.044$), 16 ($P = 0.036$), and 32 ($P = 0.037$) wk p.c. in comparison with their MHC-I-matched naive control counterparts (Fig. 1, e and f). However, only two of these animals (one *Mamu-A*01*⁺ and one *Mamu-A*02*⁺) maintained replication below 500 copy Eq/ml plasma at 11 mo p.c.

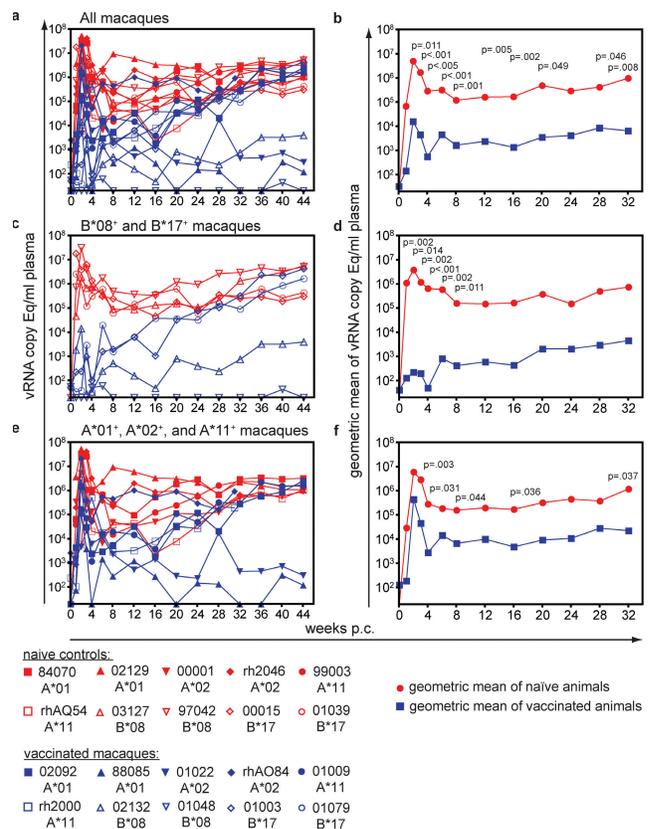


Figure 1. Plasma virus concentrations after SIVsmE660 challenge.

(a) Plasma virus concentrations of the ten SIVmac239 Δ nef-vaccinated macaques and their ten MHC-I-matched naive controls. (b) Geometric means for the ten vaccinated and ten naive controls. (c) Plasma virus concentrations of the vaccinated and naive *Mamu-B*08*⁺ and *-B*17*⁺ macaques. (d) Geometric means for the vaccinated and naive *Mamu-B*08*⁺ and *-B*17*⁺ macaques. (e) Plasma virus concentrations of the vaccinated and naive *Mamu-A*01*⁺, *-A*02*⁺, and *-A*11*⁺ macaques. (f) Geometric means for the vaccinated and naive *Mamu-A*01*⁺, *-A*02*⁺, and *-A*11*⁺ macaques.

Interestingly, we found no evidence that vaccine-induced neutralizing antibodies contributed to the control of viral replication. We assessed the level of preexisting neutralizing antibodies in the plasma of two animals that controlled acute or chronic virus replication (01022 and 01003) and two animals that did not control virus replication p.c. (rhAO84 and rh2000). None of the animals had any neutralizing antibody activity against SIVsmE660 (Fig. 2). This indicates that cellular immune responses likely play the main role for controlling virus replication after heterologous virus challenge.

Cellular immune responses p.c.

Cross-reactive vaccine-primed immune responses recognizing the infecting virus will likely determine the efficacy of HIV-1 vaccines. We therefore monitored cellular immune responses elicited by SIVmac239 Δ nef vaccination to determine which, if any, expanded after challenge with SIVsmE660. In IFN- γ ELISPOT assays using pools of peptides spanning the entire SIVmac239 proteome, we observed varying degrees of expansion in cellular immune responses above prechallenge levels at 2 wk p.c. (Fig. 3). Expanded responses were primarily directed against Gag and Pol, which are the most conserved proteins between SIVmac239 Δ nef and SIVsmE660 (Table I).

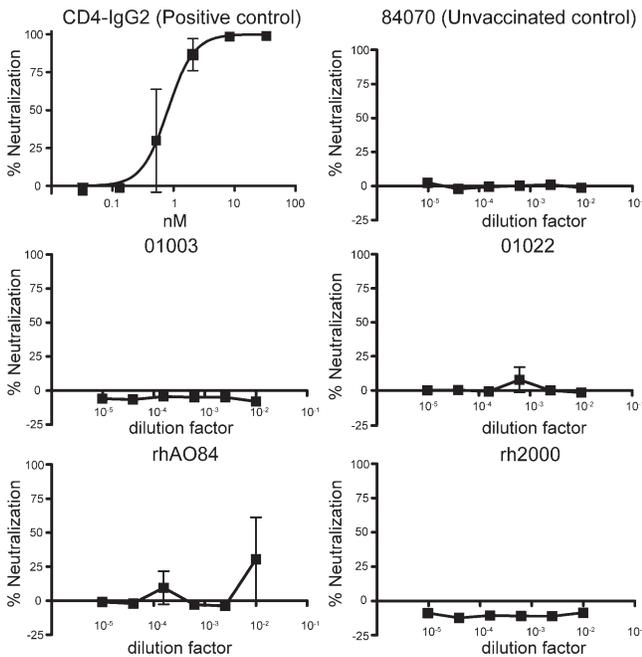


Figure 2. No in vitro neutralization of SIVsmE660 by vaccine-induced antibodies. Percent neutralization of SIVsmE660 replication in rhesus macaque PBMC after 7 d in culture by plasma collected before challenge in SIVmac239 Δ nef vaccinees. The chimeric molecule CD4-IgG2 was used as a positive control for neutralization and plasma collected from an unvaccinated animal, 84070, before challenge was used as a negative control. Percent neutralization is presented as the reduction in the amount of virus at the end of the assay in comparison to cells infected with SIVsmE660 alone. Similar negative plasma neutralization results were observed in a TZM-bl cell neutralization assay (65). Error bars represent SEM of experiments done in triplicate.

To more closely examine the effect that amino acid changes within CD8⁺ T cell epitopes have on p.c. expansion, we used MHC-I-restricted peptides corresponding to the minimal optimal epitopes in SIVmac239 Δ nef. We found that some of the epitopes with 1 or 2 aa differences between SIVmac239 Δ nef and SIVsmE660 stimulated responses above prechallenge levels (Table II and Fig. S3 a, available at <http://www.jem.org/cgi/content/full/jem.20081524/DC1>). Correlating with the IFN- γ ELISPOT assays, some of the vaccinated animals displayed a twofold or more expansion of at least one tetramer binding population during acute infection (Fig. 4). Interestingly, the animals that controlled SIVsmE660 replication at 2 wk p.c. generally had the lowest frequency of p.c. cellular immune responses in the PBMC.

The *Mamu-B*08*⁺ animal 02132 experienced a massive expansion of Mamu-B*08-restricted CD8⁺ T cells after SIVsmE660 challenge during acute infection. CD8⁺ T cells against peptide pools Nef A, Vif C and D (both Vif pools containing the Mamu-B*08-restricted epitope Vif₁₂₃₋₁₃₁RL9), and Env O (containing the Mamu-B*08-restricted epitope Env₅₇₃₋₅₈₁KL9) were the only vaccine-induced T cell responses to expand after heterologous challenge (Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20081524/DC1>). This animal had a peak concentration of plasma virus (14,000 vRNA Eq/ml) at 2 wk p.c. (Fig. 1 and Fig. 4), which was subsequently brought under control. CD4⁺ T cells against two epitopes in Gag (pools B and C) also expanded in this animal (Fig. S4). Thus, in this *Mamu-B*08*⁺ macaque, vaccine-induced Nef-, Vif-, and Env-specific CD8⁺ T cells expanded during the time in which control of virus replication was established. Remarkably, three of the four vaccine-induced CD8⁺ T cell responses were restricted by Mamu-B*08.

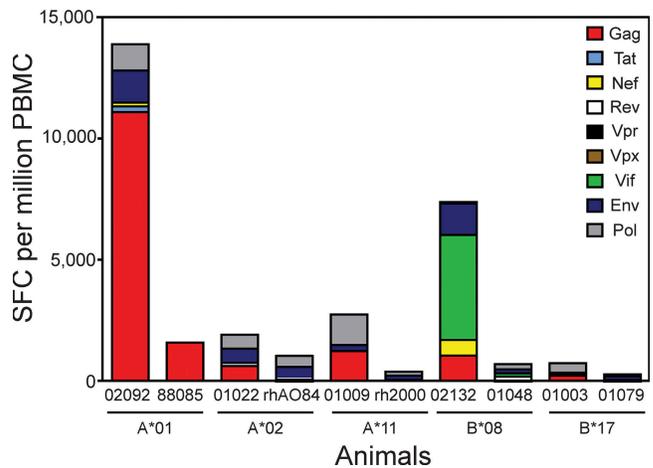


Figure 3. Expansion of virus-specific cells in SIVmac239 Δ nef-vaccinated macaques at 2 wk p.c. with SIVsmE660. Expansion in vaccinated macaques of the number of SFC above prechallenge levels in IFN- γ ELISPOT assays at 2 wk p.c. with SIVsmE660. To determine the expansion of SFC p.c., we subtracted the number of SFC per pool before challenge from the number of SFC detected p.c. The sum of the expanded cells per protein is displayed in the graph.

Table II. Comparison of amino acid sequences of known CD8⁺ T cell epitopes between SIVmac239Δnef, SIVsmE660, and circulating virus during the chronic phase of infection in the vaccinated animals

Sequence origin	Epitope sequence							
Mamu-A*01	Gag ₁₄₉₋₁₅₇ LW9	Gag ₁₈₁₋₁₈₉ CM9	Gag ₂₅₄₋₂₆₂ QI9	Gag ₃₇₂₋₃₇₉ LF8	Pol ₁₄₇₋₁₅₆ LV10	Pol ₃₆₃₋₃₇₂ GM10	Pol ₅₉₂₋₆₀₀ QV9	Pol ₆₂₅₋₆₃₃ SV9
SIVmac239Δnef	LSPRTLNAW	CTPYDINQM	QNPPIVGNL	LAPVPPIPF	LGPHTPKIV	GSPAIFQYTM	QVPKFHLPV	STPPLVRLV
SIVsmE660R.DQL..	...N...V.	E.....
02092A.....R.DQL..	...N...V.	E.....V..
88085
Mamu-A*01	Env ₂₃₃₋₂₄₁ CL9	Env ₆₂₂₋₆₃₀ TL9	Env ₇₂₉₋₇₃₈ ST10	Tat ₂₈₋₃₅ SL8	Vif ₁₄₄₋₁₅₂ QA9	Vpx ₈₋₁₈ II11		
SIVmac239Δnef	CAPPGYALL	TVPWPNASL	SSPPSYFQQT	STPESANL	QVPSLQYLA	IPPGNSGEETI		
SIVsmE660ET.A.V..I	P.....		
02092GT.A.V..I	P.....		
88085ET.A.V..IL..		
Mamu-A*02	Gag ₇₁₋₇₉ GY9	Pol ₃₂₄₋₃₃₂ FF9	Pol ₅₁₈₋₅₂₆ LY9	Env ₂₉₆₋₃₀₄ RY9	Env ₃₁₇₋₃₂₅ KM9	Env ₃₅₉₋₃₆₇ QY9	Env ₅₁₉₋₅₂₈ GF10	Env ₇₆₀₋₇₆₈ SY9
SIVmac239Δnef	GSENLKSLY	FSIPLDEEF	LSQEQEGCY	RTIISLNKY	KTVLVPTIM	QTIVKHPRY	GTSRNRKRGVF	SSWPWQIEY
SIVsmE660	E.L.....	.A.....	R.....
01022
rhA084f....	E.L.....	.A.....	R.....
Mamu-A*02	Env ₇₈₈₋₇₉₅ RY8	Nef ₂₀₋₂₈ LY9	Vif ₈₉₋₉₇ IW9	Vif ₉₇₋₁₀₄ WY8	Vpr ₆₃₋₇₁ RM9			
SIVmac239Δnef	RTLLSRVY	LLRARGETY	ITWYSKNFW	WTDVTPNY	RILQRALFM			
SIVsmE660	.DW.L.T.	..Q.....R...D.I			
01022			
rhA084	.DW.L.T.	..Q.....R...D.	K.....I			
Mamu-A*11	Gag ₁₇₈₋₁₈₆ SI9	Pol ₉₂₋₁₀₀ AL9	Pol ₄₅₇₋₄₆₅ RI9	Pol ₅₀₇₋₅₁₇ AI11	Env ₄₉₅₋₅₀₂ GI8	Vpr ₁₃₋₂₁ RV9		
SIVmac239Δnef	SEGCTPYDI	AERKQREAL	RETWTVNDI	AEAEEENKII	GDYKLVEI	REPWDEWVV		
SIVsmE660	G.-...T.		
01009	RK-...T.		
rh2000	G.-...T.		
Mamu-B*08	Gag ₂₆₃₋₂₇₂ YL9	Env ₅₂₄₋₅₃₂ KF9	Env ₅₇₃₋₅₈₁ KL9	Env ₇₁₇₋₇₂₅ LF9	Env ₈₆₈₋₈₇₆ RL9	Rev ₁₂₋₂₀ KL9	Rev ₄₄₋₅₁ RL8	Nef ₈₋₁₆ RL9
SIVmac239Δnef	YRRWIQLGL	KRGVFLVGF	KRQQELLRL	LRQGYRPVF	RRIRQGLEL	KRLRLIHLL	RRRWQQLL	RRSRPSGDL
SIVsmE660H.....F.	.Q....I.	KQC.RG.N.
02132G...R..	...H.....V.....F.	.Q....I.	KQCRRR.N.
01048S.....
Mamu-B*08	Vif ₁₂₃₋₁₃₁ RL9	Vif ₁₇₂₋₁₇₉ RL8						
SIVmac239Δnef	RRAIRGEQL	RRDNRRL						
SIVsmE660K.	G.N...s.						
02132						
01048G..						
Mamu-B*17	Pol ₃₇₂₋₃₇₉ MF8	Pol ₄₃₅₋₄₄₃ FW9	Pol ₆₀₄₋₆₁₃ VW10	Env ₂₄₁₋₂₅₁ LF11	Env ₈₁₆₋₈₂₅ LY10	Env ₈₃₀₋₈₃₈ FW9	Vif ₄₄₋₅₂ HW9	Vif ₆₆₋₇₃ HW8
SIVmac239Δnef	MRHVLEPF	FQWGMGYELW	VWEQWWTDYW	LRCNDTNSYSGF	LRELTLYLQY	FHEAVQAVW	HFKVGVAWW	HLEVQGYW
SIVsmE660	.N.....	I.....S.....	I..GIA....	.Q....W.	.H.....
01003S.....Y.....	.H.....	Q.....
01079	.N.....	I.....S.....Y.....	.H.....
Mamu-B*17	Vif ₁₃₅₋₁₄₃ CY9	cryptic RW9						
SIVmac239Δnef	CRFPRAHKY	RHLAFKCLW						
SIVsmE660	.K..K...N	.A..S..FR						
01003	.K..K...N	.A..S..FR						
01079	.K..K...N	.A..S..FR						

Periods represent conserved amino acids in comparison to SIVmac239Δnef. The aa sequence for each epitope corresponds to the vaccine strain SIVmac237Δnef. Any aa substitutions in these epitopes present in our stock of SIVsmE660 or in the plasma virus of our vaccinated animals during the chronic phase of infection are identified with capital letters or lowercase letters for mixed populations. Periods represent conserved aa in comparison to SIVmac239Δnef.

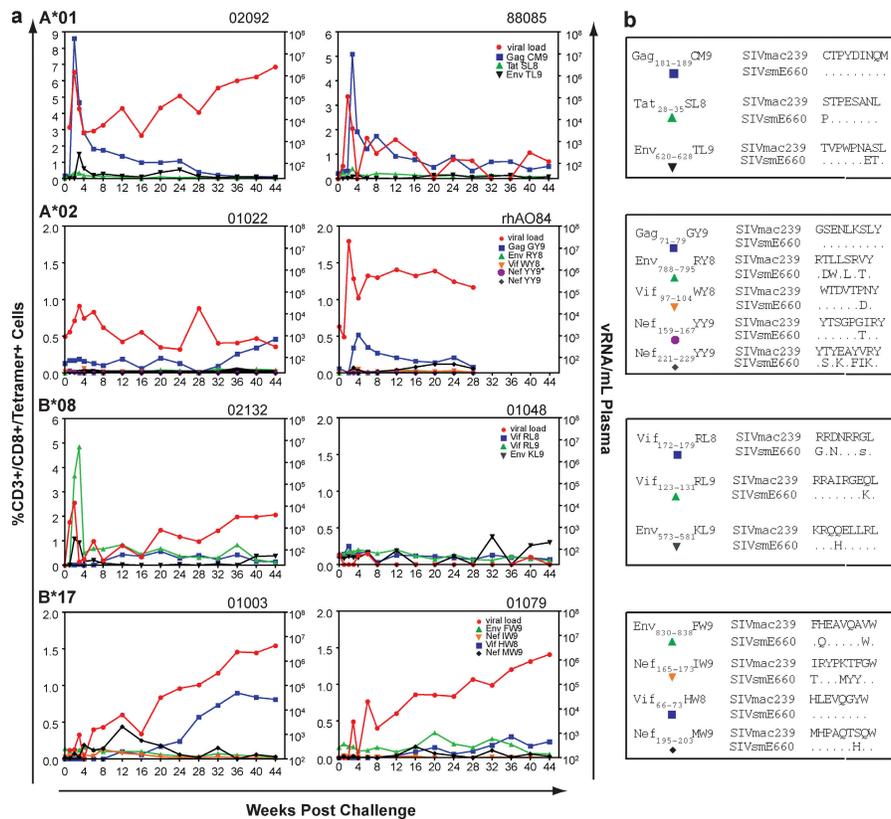


Figure 4. Frequency of tetramer binding cells in the PBMC of SIVmac239 Δ nef-vaccinated macaques p.c. with SIVsmE660. (a) The frequency of MHC-I tetramer binding cells in the PBMC after challenge with SIVsmE660. Frequencies of tetramer positive cells are reported as the percentage of CD3⁺/CD8⁺/tetramer⁺ lymphocytes. The plasma virus concentrations for each animal are displayed with the red line and symbols. * distinguishes Nef₂₂₁₋₂₂₉YY9 from Nef₁₅₉₋₁₆₇YY9. (b) Comparison of amino acid sequences between SIVmac239-derived peptides and SIVsmE660. The SIVmac239 sequence is shown above SIVsmE660 sequence for comparison of the two viruses. Periods represent conserved amino acids, capital letters indicate amino acid substitutions, and lowercase letters represent mixed populations.

Transient depletion of circulating CD8⁺ cells

Because neutralizing antibodies did not appear to be responsible for control of viral replication, we next investigated whether CD8⁺ cells were important in vaccine-mediated control of replication. Therefore, we depleted CD8⁺ cells in vivo at 11 mo p.c. in the four SIVmac239 Δ nef-vaccinated animals controlling virus replication below 5,000 vRNA copy Eq/ml plasma. We administered a monoclonal antibody specific for CD8 α to transiently deplete CD8⁺ cells, which includes both CD8⁺ T cells and NK cells, from the periphery. 1 d after administering the antibody, CD8⁺ T cells were almost completely absent from the PBMC in three of the animals and reduced by >50% in the fourth. During the period of CD8⁺ cell depletion, plasma virus concentrations increased by 1–3 logs (Fig. 5 a). Plasma virus replication peaked 7–10 d after in vivo depletion of CD8⁺ cells and ranged between 2.3×10^4 and 5.5×10^5 vRNA copy Eq/ml. CD8⁺ cells reemerged in the periphery 17–28 d after depletion, concomitant with a decrease in plasma virus concentrations (Fig. 5, b and c). Only animal 01048, however, controlled virus replication at or below predepletion levels by 5 mo after CD8⁺ cell depletion. Conversely, animal 02132 never regained control of virus replication below 5,000 vRNA Eq/ml.

Because these animals controlled virus replication to extremely low levels before CD8⁺ cell depletion, we wanted to determine the predominant species of virus replicating after depletion. Using a sequence-specific quantitative RT-PCR (QRT-PCR) assay, we were surprised to detect only SIVmac239 Δ nef replicating in two of the animals (01022 and 01048), whereas SIVsmE660 was the primary virus replicating in the remaining two animals. These results prompted us to reexamine samples spanning the entire study for all of the vaccinated animals. After this retrospective analysis, we only detected the vaccine strain and not the challenge strain at any point p.c. in 01022 and 01048. In the remaining vaccinated animals, SIVsmE660 was the principal virus replicating at all time points measured after challenge.

For the animals transiently depleted of their CD8⁺ cells, we examined the cellular immune responses by IFN- γ ELISPOT after the CD8⁺ cells returned to the periphery, reasoning that these cells might be involved in reestablishing control of viral replication. We assessed both CD8⁺ and CD4⁺ T cell responses using whole PBMC and PBMC depleted of CD8⁺ cells. We detected broad responses in two of the four animals using freshly isolated PBMC. Animals 01022 and 01048 made responses against 27 and 20 peptide pools, respectively.

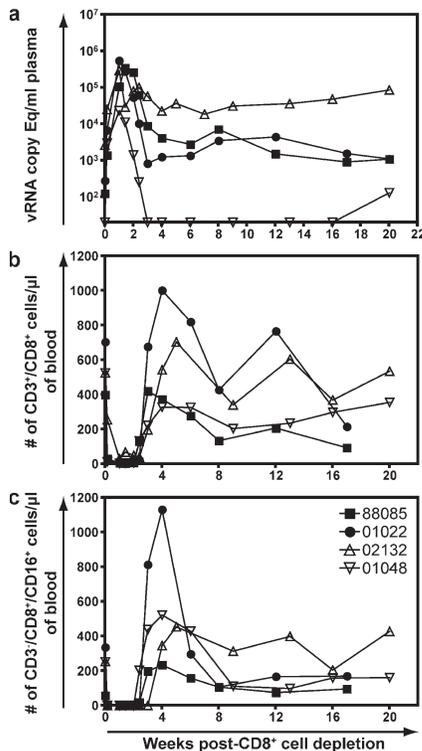


Figure 5. Plasma virus concentrations and CD8⁺ T cell and NK cell counts after in vivo depletion of CD8⁺ cells. (a) Plasma virus concentrations of the four SIVmac239Δnef-vaccinated macaques were transiently depleted of their peripheral CD8⁺ cells. (b and c) The number of CD8⁺ T cells (CD3⁺/CD8⁺ lymphocytes; b) and the number of NK cells (CD3⁻/CD8⁺/CD16⁺; c) in the PBMC after in vivo CD8 depletion.

These responses were directed against multiple viral proteins (Fig. S5, available at <http://www.jem.org/cgi/content/full/jem.20081524/DC1>). Conversely, animals 02132 (11 pools) and 88085 (4 pools) had more focused responses. Of note, 88085 consistently had high background levels of IFN- γ in the PBMC, which resulted in an increased threshold for positive responses and probably accounted for the generally low number of responses detected in this animal.

We also examined CD4⁺ T cell responses in the CD8⁺ cell-depleted animals using a combination of fresh and frozen cells. Because of limitations on cell availability, we restricted our initial analysis of CD4⁺ T cell responses with fresh PBMC depleted of CD8⁺ cells to pools of peptides spanning Gag, Tat, Rev, and Vif at 4 or 5 wk after CD8⁺ cell depletion. We used frozen cells to determine how many peptide pool-specific responses outside of Gag, Tat, Rev, and Vif that were identified using whole PBMC were associated, at least in part, with CD4⁺ cells. Remarkably, animals 88085 (10 pools), 01048 (17 pools), and 01022 (19 pools) made broad CD4⁺ T cell responses against multiple proteins (Fig. S5). The largest magnitude response at 4 wk after depletion in animal 01022 was a CD4⁺ T cell response directed against a peptide pool spanning the first 51 aa of Rev (1,965 SFC per million PBMC and 2,083 SFC per million CD8⁺ cell-depleted PBMC). This region of Rev is known to be the target of potent CD4⁺ T cell

responses (33–35). From animal 02132, however, we only detected three CD4⁺ T cell responses. Although it is likely that some peptide pools stimulated both CD4⁺ and CD8⁺ T cell responses, we were able to associate over half of the detectable peptide pool-specific responses in three of the four animals with CD4⁺ T cells. It is difficult to determine whether these CD4⁺ T cell responses directly contributed to controlling virus replication or their presence is merely the result of low virus replication. These results do underscore, however, the potential importance of SIV-specific CD4⁺ T cell responses in generating successful antiimmunodeficiency virus immunity. Results also indicate the likelihood that SIV-specific CD4⁺ T cell responses are not necessarily deleterious, though some have speculated they would be (36).

Escape in SIVsmE660?

Most of the vaccinated macaques in our study controlled virus replication below 10,000 vRNA copy Eq/ml for at least 4 mo p.c. However, the majority of them experienced gradually increasing virus replication as the study progressed. We therefore set out to determine whether increased plasma virus replication was caused by escape in known CD8⁺ T cell epitopes. We sequenced the virus from the plasma of six of the animals during the chronic phase of infection. Because of the low number of virions in the plasma of the animals controlling virus replication during the chronic phase of infection, we used post-CD8⁺ cell depletion samples for the CD8⁺ T cell escape analysis. We found evidence for amino acid substitutions in known CD8⁺ T cell epitopes in the circulating viruses of these animals. Animal rhAO84, the only vaccinee not to control virus replication at any point p.c. and the fastest to progress to simian AIDS, had circulating virus at its time of death with changes in only one of the 13 Mamu-A*02-restricted epitopes, with respect to the SIVsmE660 sequence, and a mixed population in a second (Table II). This corresponds with the limited expansion of epitope-specific responses detected in this animal p.c. (Fig. 4 and Fig. S3 e). Conversely, animal 02092 made vigorous responses against several Mamu-A*01-restricted epitopes during acute infection, particularly the immunodominant epitope Gag_{181–189}CM9 (Gag CM9; Fig. 4 and Fig. S3). During the chronic phase of infection, however, we detected mutations in several of the Mamu-A*01-restricted epitopes recognized during acute infection, including Gag CM9. Escape from Gag CM9-specific CD8⁺ T cells has previously been associated with a loss of viral control (37, 38).

We also investigated viral evolution in the two vaccinated Mamu-B*17⁺ animals. These animals controlled virus replication at two weeks p.c. below 250 vRNA copy Eq/ml plasma but subsequently experienced near-identical increases in plasma virus replication with respect to time and magnitude (Fig. 6). We detected the same amino acid changes in the two Mamu-B*17-restricted Env epitopes in both animals, Env_{816–825}LY10 (Env LY10) and Env_{830–838}FW9 (Env FW9; Table II). Curiously, the mutations observed in these two epitopes were nearly identical to the sequence of SIVmac239Δnef, with only a histidine-to-tyrosine substitution

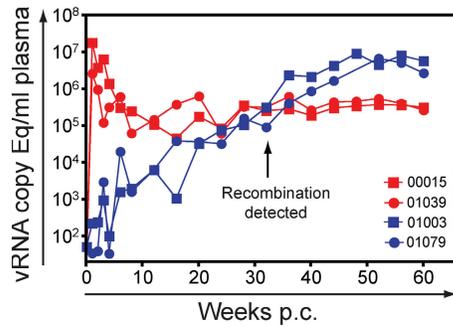


Figure 6. Virus replication in Mamu-B*17⁺ macaques. Plasma virus concentrations in SIVmac239Δnef vaccinated (blue) and unvaccinated (red) Mamu-B*17⁺ macaques for 60 wk p.c.

in Env FW9 differing from the SIVmac239Δnef sequence in these animals.

Recombination between SIVmac239Δnef and SIVsmE660

One possible explanation for the emergence of SIVmac239-like sequences in these two epitopes was that recombination had occurred between the vaccine and challenge viruses. Indeed, bulk sequencing of the entire viral genome at 8 mo p.c. showed that multiple recombination events had occurred in the two Mamu-B*17⁺ animals. Further analysis of all of the vaccinated animals revealed recombination occurring in several other animals (Fig. 7). Additionally, sequence analysis of animals 01022 and 01048 confirmed that only SIVmac239Δnef was replicating in these animals. Interestingly, the virus in the two vaccinated Mamu-B*17⁺ macaques had similar patterns of recombination and replicated to a level ~1 log higher at 1 yr p.c. than the unvaccinated Mamu-B*17⁺ animals infected with SIVsmE660 alone (Figs. 6 and 7). This suggests that recombination provided a fitness advantage for replication in Mamu-B*17⁺ animals.

A recent study found that bulk sequencing of plasma vRNA might result in recombination because of PCR artifacts (39). Therefore, to confirm recombination between the vaccine and challenge strains, we used limiting dilution PCR to achieve single-genome amplification (SGA). We focused our analysis on a putative break point at the end of *env* and the beginning of *nef* in the vaccinated Mamu-B*17⁺ animals. SGA analysis corroborated the bulk sequencing results indicating that recombination had indeed occurred in the two Mamu-B*17⁺ animals, as all 10 clones from 01003 and all 11 clones from 01079 showed evidence of recombination (Fig. S6, available at <http://www.jem.org/cgi/content/full/jem.20081524/DC1>). The presence of recombination in the vaccinees, particularly the Mamu-B*17⁺ animals, indicates another mechanism by which the virus can evade protective immunity in live-attenuated SIV-vaccinated macaques.

DISCUSSION

In this study, we examined the ability of live-attenuated SIV to protect macaques against heterologous virus challenge. The

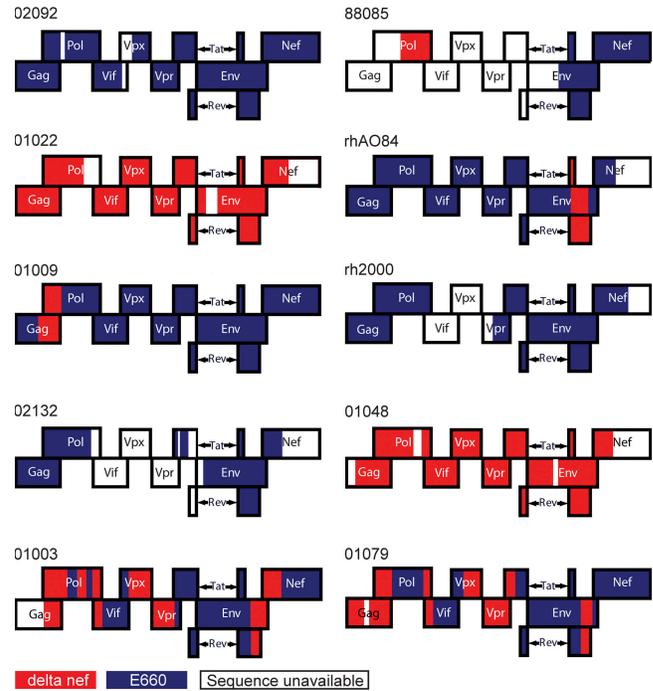


Figure 7. Recombination events between SIVmac239Δnef and SIVsmE660. During the chronic phase of infection or after in vivo CD8 depletion we used bulk sequencing to detect recombination between SIVmac239Δnef and SIVsmE660. Represented are the regions that most closely align to either SIVmac239Δnef (red) or SIVsmE660 (blue). Positioning of putative break points and size of either SIVmac239Δnef or SIVsmE660 regions are approximations based on sequencing data. Open boxes represent regions of the virus where sequences could not be obtained.

vaccinated animals significantly reduced plasma virus replication at most time points between 2 and 32 wk p.c. ($P \leq 0.049$) in comparison to the unvaccinated controls. Surprisingly, we found no evidence of SIVsmE660 replication in two of the vaccinated animals. We also explored the nature of the antiviral immunity that provided protection in the vaccinated animals. Several lines of evidence emerged strongly implicating vaccine-induced MHC-I-restricted CD8⁺ cell responses as the primary force involved in protecting SIVmac239Δnef-vaccinated macaques from heterologous challenge. We discovered, however, evidence of recombination between the vaccine and challenge strains, which likely contributed to a loss of immune-mediated control of virus replication.

We have known that live-attenuated SIV vaccines can protect macaques against pathogenic SIV challenges for almost 20 yr. However, the underlying mechanism of this protective immunity has remained elusive. CD4⁺ and CD8⁺ T cells (10, 12–14, 16), antibodies (9, 10), NK cells (11, 15), and viral interference (16–18) have been implicated in vaccine-induced control. In this study, we provide several lines of evidence showing that MHC-I-restricted CD8⁺ responses are playing the key role in reducing virus replication after heterologous virus challenge in SIVmac239Δnef-vaccinated macaques. First, there is no evidence that vaccine-induced neutralizing antibodies

contributed to controlling virus replication. Second, we found an association between two MHC-I alleles and near complete control of acute virus replication after heterologous challenge. Finally, transient depletion of CD8⁺ cells from the periphery of macaques controlling SIV replication quickly led to a 3–4 log increase in plasma virus concentrations. Our results eliminate viral interference, CD4⁺ T cells, and antibodies as sole providers of protective immunity in live-attenuated SIV vaccinated macaques because these responses are not influenced by protective MHC-I alleles and virus replication in CD8⁺ cell-depleted animals in which SIV-specific antibodies were still present. Our results do not eliminate the possibility that helper CD4⁺ T cells or binding antibodies, either through the development of immune complexes or removal of infected cells via antibody-dependent cell-mediated cytotoxicity, play an important supportive role in controlling virus replication. We also cannot rule out the possibility that NK cells contributed to the control of virus replication. However, a recent study demonstrated that NK cells have a limited role in controlling primary SIV replication (40). But the chronic low level of replication by live-attenuated SIV may provide persistent stimulation and activation to NK cells to allow them to help eliminate pathogenic SIV replication. Additionally, depletion of peripheral CD8⁺ cells during the chronic phase of infection indicates that these cells are responsible for controlling virus replication during this phase of infection but leaves open the possibility that additional factors contribute to the reduction in acute virus replication, particularly in the *Mamu-B*08*⁺ and *-B*17*⁺ animals. To more accurately assess the role of CD8⁺ T cells in controlling acute virus replication in *Mamu-B*08*⁺ and *-B*17*⁺ macaques, additional animals will need to be vaccinated with SIVmac239Δnef and depleted of CD8⁺ cells before SIVsmE660 challenge.

Throughout the course of the study, we used peptides corresponding to the SIVmac239 proteome to detect virus-specific cellular immune responses. We feel that these peptides effectively detected cross-reactive CD8⁺ and CD4⁺ T cell responses between the vaccine and challenge strains because these responses are likely responsible for controlling virus replication in the vaccinated animals. Nevertheless, there may be additional CD4⁺ and CD8⁺ T cell responses that contribute to controlling SIV replication but cannot be detected using SIVmac239-specific peptides. The number of these unaccounted for responses may vary from animal to animal.

Our inability to detect SIVsmE660 replication by QRT-PCR or bulk sequencing in two of the vaccinated animals suggested that they achieved sterilizing immunity against a heterologous virus. These results give hope that CD8⁺ T cell-based HIV vaccines can realize a similar level of efficacy. However, it is unclear why these two animals completely suppressed SIVsmE660 replication, whereas the remaining eight vaccinees did not. Also, we cannot rule out the possibility that these two animals harbor a small number of cells latently infected with SIVsmE660.

The protective capacity of vaccine-induced CD8⁺ T cells elicited by live-attenuated SIV vaccines differs from current prime-boost regimens, which provide only limited reduction

in viral replication after homologous virus challenge (41–44). There may be several reasons for this discrepancy in protective efficacy. CD8⁺ T cell responses induced by SIVmac239Δnef during the vaccine phase of the study were roughly equivalent in frequency to those produced by prime-boost vaccine regimens (41, 45–47). The magnitude of vaccine-induced CD8⁺ T cell responses, therefore, is likely not the reason why live-attenuated SIV vaccines succeed at controlling virus replication and recombinant virus-based vaccines largely fail. Current HIV vaccine modalities focus the immune responses on only a few viral proteins. This may not generate the breadth of responses needed to control replication after a heterologous virus challenge. SIVmac239Δnef vaccination generated broad CD8⁺ T cell responses against several viral proteins, even those which are often considered too variable to be included in HIV vaccines. Cellular immune responses directed against these relatively variable proteins might have contributed to protective immunity in our study. Animal 02132, for example, made a very strong CD8⁺ T cell response to an immunodominant Mamu-B*08-restricted epitope in Vif, likely contributing to its control of virus replication after heterologous virus challenge. SIVmac239Δnef vaccination also provided consistent low-level antigen stimulation throughout the vaccine phase of the study. This contrasts with prime-boost strategies and single cycle SIV viruses where only limited replication of the virus/vector occurs (48). Under these circumstances anamnestic responses induced by prime-boost or single-cycle SIV may be incapable of responding fast enough to limit acute viral replication after exposure to pathogenic viruses. The constant antigen exposure provided by SIVmac239Δnef, alternatively, may induce effector responses that rapidly react after pathogenic immunodeficiency virus infection and limit virus replication. Finally, SIVmac239Δnef may have additional advantages over most current HIV/SIV vaccines in that it primes immune responses in the same tissues where the pathogenic viruses are likely to replicate. The discovery of recombinant viruses indicates that the two virus strains indeed replicated in the same cells, let alone the same tissues. Although we did not specifically sample mucosal tissues in our study, live-attenuated SIV vaccines are known to generate CD8⁺ T cell responses in these tissues (49, 50). Trafficking of antiviral immune responses to the mucosa maximizes the chances that these responses will encounter virally infected cells during the earliest stages of infection, even after i.v. inoculation. Therefore, to mimic live-attenuated SIV vaccines as closely as possible, improved HIV vaccines may need to use replicating vectors to provide constant antigenic stimulation to antiviral immune responses. Ideally these vectors will target responses to mucosal tissues and include as many viral proteins as possible to maximize the number of cross-reactive immune responses with infecting HIV viruses.

In spite of early control of virus replication after SIVsmE660 challenge, particularly those animals expressing *Mamu-B*17*, we observed a progressive increase in plasma virus replication in most vaccinees. This differs from the durable control observed in animals vaccinated with live-attenuated SIV and challenged with homologous viruses. We found evidence of

viral escape from CD8⁺ T cell responses that likely contributed to the loss of viral containment. Although this finding further supports CD8⁺ T cells as playing an important role in controlling virus replication after heterologous virus challenge, it also demonstrates the fragility of this control. The already reduced number of epitopes recognized in the heterologous challenge virus may focus selective pressure on a few regions of the virus and provide an avenue by which only a small number of key mutations can result in a loss of viral control. These results, therefore, also emphasize the need to limit virus replication to as low a level as possible.

We discovered that recombination had occurred between the vaccine and the challenge strains. This may have potentially produced a more pathogenic recombinant strain. In vaccinated *Mamu-B*17*⁺ animals in particular we detected numerous recombination events. Surprisingly, most of these events were shared between the two animals. These recombination events appear to contribute to the loss of containment of virus replication even after control of acute virus replication was achieved. Startlingly, the recombinant viruses eventually replicated to around 5×10^6 copy Eq/ml plasma, approximately one log higher than the unvaccinated *Mamu-B*17*⁺ controls infected with SIVsmE660 alone. Although the number of *Mamu-B*17*⁺ animals in our study is relatively small, our results suggest that the recombinant virus had a fitness advantage in *Mamu-B*17*⁺ animals.

In retrospect, this recombination was hardly surprising. Recombination between different viral strains is well documented in HIV and has been noted before in the setting of live-attenuated SIV vaccines (3, 4, 51–54). However, the prospect of incoming HIV virions recombining with a live-attenuated strain to produce a more pathogenic strain should eliminate any remaining possibility of using live-attenuated HIV vaccines in humans.

We challenged our cohort of animals i.v. because previous studies had shown effective control of homologous virus by live-attenuated SIV-vaccinated macaques challenged via this route. This challenge is comparable to transmission of HIV-1 through contaminated needles or blood products. It is also the most difficult route of transmission for vaccines to protect against because all physical barriers are bypassed and viruses can enter freely into the body. Worldwide, however, sexual contact, and not direct blood-to-blood contact, is the primary route of HIV-1 transmission. Studies in macaques and recent studies in humans suggest that only a small population of virus crosses the mucosal barriers to establish infection after heterosexual transmission (39, 55, 56). Subsequent localized virus replication during the earliest stages of infection may allow vaccine-induced immune responses to prevent systemic infection. Thus, it is possible that the reduction in plasma virus replication observed in our study after i.v. challenge may be even greater in a model of sexual transmission.

In conclusion, our study demonstrates that SIVmac239Δnef-induced CD8⁺ T cell responses can significantly reduce heterologous AIDS virus replication. Most of the vaccinated animals controlled virus replication below 10,000 vRNA

copy Eq/ml through 4 mo p.c. However, only four of them maintained low levels of virus replication out to 11 mo p.c. These four animals have various MHC-I backgrounds, suggesting that a properly designed HIV vaccine that induces CD8⁺ T cell responses can be effective in diverse populations. The combination of the association of the *Mamu-B*08*⁺ and *-B*17*⁺ MHC-I alleles with near complete control of acute virus replication, the rapid increase in virus replication after in vivo depletion of CD8⁺ cells in the periphery, and the inability of neutralizing antibodies to affect ongoing virus replication strongly implicate MHC-I-restricted CD8⁺ T cells as the major contributors to live-attenuated SIV-induced protective immunity. The discovery of recombination between the vaccine and challenge strains, however, militates strongly against the development of live-attenuated HIV vaccines.

MATERIALS AND METHODS

Animals and viruses. Indian rhesus macaques (*Macaca mulatta*) from the Wisconsin National Primate Research Center were chosen based on expression of particular MHC-I alleles as determined by sequence-specific PCR (26, 57). All animals were housed and cared for according to the regulations set forth in the *Guide for the Care and Use of Laboratory Animals* of the National Research Council (National Research Council, 1996. Guide for the care and use of laboratory animals. National Academy Press, Washington DC), as approved by the University of Wisconsin Institutional Animal Care and Use Committee. 10 macaques were inoculated i.v. with 10 ng p27 of SIVmac239Δnef, provided by R. Desrosiers (New England Primate Research Center, Southborough, MA). Vaccinees and control animals were challenged i.v. with 100 50% tissue culture infective doses of SIVsmE660.

In vivo CD8 depletion. We transiently depleted peripheral CD8⁺ cells by administering monoclonal antibody cM-T807 (produced by Centocor and provided via Keith Reimann [Harvard Medical School, Boston, MA] and the National Institutes of Health Nonhuman Primate Reagent Resource) i.v. in a single dose at 50 mg/kg of body weight.

Viral load determination. Levels of circulating plasma virus were determined using a previously described QRT-PCR assay (58). The challenge strain SIVsmE660 was differentiated from SIVmac239Δnef via a second QRT-PCR assay run on the same isolation of vRNA used for quantitating virus concentrations. The E660 primers Nefdel.1 5'-CRCTCTCTTGT-GAGCCTCAGAA-3' and Nefdel.r2 5'-TCTTGGGTGCACTGAGACAC-3' produce a small amplicon in Nef. The Nefdel.r2 primer and the probe 5'-(6-Fam)CAAGTCATCATCTTCATCATCCACATCATCCAT (BHQ1)-3' bind entirely within the deleted portion of *nef* and detect full-length virus exclusively. These primers and probe are used under the same cycling conditions that were described previously (58). The concentration of full-length virus was determined by interpolation onto a standard curve of in vitro-transcribed *nef* RNA standards in serial 10-fold dilutions using the LightCycler 480 software (version 1.2.0; Roche).

IFN-γ ELISPOT assays. Fresh PBMC isolated from EDTA anticoagulated blood were used in ELISPOT assays for the detection of IFN-γ-secreting cells as previously described (59). Peptides used in these assays were obtained through the AIDS Research and Reference reagent program, Division of AIDS, National Institute of Allergy and Infectious Disease, and National Institutes of Health. Additionally, we examined responses by CD8⁺ cells by depleting PBMC of CD8⁺ cells using a CD8 Microbead kit for nonhuman primates (Miltenyi Biotec) according to the manufacturer's instructions.

Tetramer staining. SIV-specific cells were enumerated by staining PBMC with MHC-I (*Mamu-A*01*, *-A*02*, or *-B*17*) tetrameric complexes loaded

with synthetic peptides corresponding to previously identified epitopes (26, 28, 29, 60, 61). The Mamu-B*08 tetramers were refolded with either the synthetic peptides corresponding to Env₅₇₃₋₅₈₁KL9 (62) or the recently identified Vif₁₂₃₋₁₃₁RL9 or Vif₁₇₂₋₁₇₉RL8 epitopes (30). All Mamu-B*17 tetramers and some Mamu-B*08 tetramers were refolded as previously outlined (63). The assay was performed using one million PBMC as previously described (41).

Neutralization assays. Neutralization assays were performed as previously described (64) with the following exceptions. Rhesus PBMC was stimulated for 3 d with PHA and IL-2 before the addition of virus and plasma. After addition of virus and plasma, plates were incubated at 37°C overnight, and the cells were then washed four times with fresh culture medium. Half of the media was replaced with fresh media 3 d later. Culture supernatants were harvested for p27 analysis 7 d after infection.

Intracellular cytokine staining. Intracellular cytokine staining was performed using thawed PBMC as previously described (42). In brief, 5×10^5 – 10^6 PBMC were incubated in the presence of Brefeldin A (Sigma-Aldrich) and pools of 10 peptides, 15 aa in length, for 5 h. The cells were then stained with anti-CD8 α PerCP (Becton Dickinson) and anti-CD4 APC (Becton Dickinson) for 30 min. After the incubation, the cells were washed twice with PBS, fixed with 2% paraformaldehyde, and stored overnight at 4°C. The next day, the cells were washed twice with permeabilization buffer (PBS + 10% FBS + 0.1% saponin) and stained with anti-IL-2 PE (BD Biosciences) and anti-IFN- γ FITC (BD Biosciences) for 50 min. After washing the cells twice with permeabilization buffer, the cells were fixed with paraformaldehyde and stored at 4°C until being acquired on a FACSCalibur Flow Cytometer (Becton Dickinson). The data were analyzed using FlowJo software (Tree Star, Inc.).

CD8⁺ T cell and NK cell quantification. We monitored the CD8⁺ cell depletion kinetics by staining PBMC with fluorescently labeled antibodies specific for CD8-PE (Dako), CD16–Pacific Blue (BD Biosciences), and CD3–Alexa 700 (BD Biosciences). In brief, 500,000 PBMC were incubated with these antibodies for 30 min at room temperature. The samples were then washed twice, fixed with paraformaldehyde, and run on a BD-LSR-II flow cytometer (Becton Dickinson) using FACSDiva software (BD Biosciences). Data were analyzed using FlowJo software. Absolute counts were calculated by multiplying the frequency of CD8⁺ T cells or CD3⁺/CD8⁺/CD16⁺ NK cells within the lymphocyte gate by the lymphocyte counts per microliter of blood obtained from matching complete blood counts.

Sequencing of plasma vRNA. vRNA was extracted from plasma samples containing >1,000 vRNA copy Eq/ml as previously described (58). Samples were sequenced from 8 mo p.c., the time of death, for animals rhAO84 and rh2000, from 10 mo p.c. for animals 02092, 01009, 01003, and 01079, and 2 wk after depletion for animals 88085, 01022, 02132, and 01048. For each vRNA sample, several overlapping amplicons spanning the open reading frames of SIVmac239 or SIVsmE660 were amplified using the One-Step RT-PCR kit (QIAGEN) and pairs of sequence specific primers. The RT-PCR conditions for all amplicons were as follows: 50°C for 30 min, 95°C for 15 min, 45 cycles of 94°C for 30 s, 53°C for 1 min, 72°C for 150 s, and 68°C for 20 min. The amplified cDNAs were purified using the QIAquick 8 PCR Purification kit (QIAGEN). Sequencing reactions for each amplicon were set up using the DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare). The sequencing cycling conditions for all amplicons were as follows: 30 cycles of 95°C for 20 s, 50°C for 15 s, and 60°C for 1 min. Both strands of each amplicon were sequenced on a 3730 DNA Analyzer (Applied Biosystems). Sequences were assembled using Aligner version 2.0.6 (Codon-Corporation). DNA sequences were conceptually translated and aligned to SIVsmE660 in MacVector 9.0 trial version (Accelrys).

SGA PCR. cDNA was created from vRNA extracted from plasma using the MinElute kit (QIAGEN) per the manufacturer's instructions. cDNAs were created using the Superscript III cDNA synthesis kit (Invitrogen) using oligo dT primers per the manufacturer's instructions.

To determine the optimal cDNA concentration resulting in <30% positive reactions in PCR we used Phusion high-fidelity polymerase (Finnzymes) per the manufacturer's instructions. PCR reactions were set up using first round amplifications as template as follows: each reaction contained 4 μ l of 5 \times HF buffer, 0.4 μ l of 10 mM dNTP, 0.5 μ l of 20 μ M sense oligo, 0.5 μ l of 20 μ M antisense oligo, 2 μ l of first-round PCR product, 0.2 μ l Phusion high-fidelity polymerase, and DEPC water to 20 μ l. Thermocycling was performed with the following parameters: initial denaturation at 98°C for 30 s, and then denature at 98°C for 5 s, anneal at 65°C for 10 s, and extend at 72°C for 10 s for 45 cycles. A final extension was done at 72°C for 5 min. The oligos used in this reaction were E543-8858F (ACCAAAGAAGGAGAAGAAG-GAGA) and E453-9449R (TATTGCCAATTTGTATGTCATGG). When nested PCRs are complete, all reaction products were electrophoresed on a 1% agarose gel stained with SYBR safe DNA gel stain (Invitrogen). Positive reactions contained a band at \sim 592 bp which was cut out and from which DNA was isolated using the QIAquick Gel Extraction kit (QIAGEN) as per the manufacturer's instructions.

Isolated DNA was sequenced as described in Sequencing of plasma vRNAs using the following oligos: E543-8858F (ACCAAAGAAGGAGA-AGAAGGAGA), E453-9449R (TATTGCCAATTTGTATGTCATGG), E543_Frag14_F1 (GGCCTTGGCAGATAGAATATTC), and E543_Frag14_R (CTGAKACMCCTACYAAGTCATCATC).

Statistical analysis. Statistical analyses consisted of two sample Student's *t* tests of the variables studied, namely number of virions per milliliter of blood and viral load at different time points p.c. We performed these tests using the TTEST procedure in SAS (version 9.1) to do the following: compare the plasma virus concentrations of the SIVmac239 Δ nef-vaccinated macaques ($n = 10$) with their MHC-I-matched naive controls ($n = 10$); compare Mamu-B*08⁺/-B*17⁺ macaques ($n = 4$) with their MHC-I-matched naive controls ($n = 4$); and compare Mamu-A*01⁺/-A*02⁺/-A*11⁺ macaques ($n = 6$) with their MHC-I-matched controls ($n = 6$).

Before performing inferential testing, we checked key underlying assumptions of the Student's *t* tests (normality of residuals and homoscedasticity). The failure of the data to support these assumptions led us to transform the data via the natural logarithm, which improved the conformity to the assumptions. Student's *t* tests were performed under equal and unequal group variance assumptions via the Welch correction, in which we used the folded form of F test (*F'*) to decide the test statistic (pooled or Satterthwaite). This form of F test gives the probability of a greater F value under the null hypothesis that group variances are equal. We also used one way nonparametric analysis using an exact Wilcoxon Rank-Sum test with the continuity correction, which does not assume normality of residuals, to further verify our results. Conclusions about statistical significance and direction of effects were the same across these three analytic approaches, so herein we report only the Student's *t* test results.

Online supplemental material. Fig. S1 shows that most SIVmac239 Δ nef replication was controlled below 500 copy Eq/ml plasma at the time of the SIVsmE660 challenge. Fig. S2 shows the prechallenge IFN- γ ELISPOT responses for each animal using whole PBMC and CD8⁺-depleted PBMC. Fig. S3 shows the epitope-specific expansion of CD8⁺ T cell responses before challenge and p.c. for all of the animals. Fig. S4 shows prechallenge and p.c. peptide pool-specific responses from animal 02132 in IFN- γ ELISPOT assays. It also shows, through intracellular cytokine staining assays, that we can associate two of these responses with CD4⁺ T cells. Fig. S5 shows peptide-specific responses in whole PBMC and PBMC depleted of CD8⁺ cells after the return of CD8⁺ cells to the periphery. Fig. S6 shows recombination by single-genome analysis of virus circulating in the vaccinated Mamu-B*17⁺ macaques during the chronic phase of infection. Table S1 displays the frequency of tetramer binding cells in the PBMC during the vaccine phase of the study. Table S2 displays the peptide pools to which SIVmac239 Δ nef-vaccinated animals made responses in IFN- γ ELISPOT assays using whole PBMC or PBMC depleted of CD8⁺ cells. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20081524/DC1>.

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