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Effect of CD8$^+$ Lymphocyte Depletion on Virus Containment after Simian Immunodeficiency Virus SIVmac251 Challenge of Live Attenuated SIVmac239Δ3-Vaccinated Rhesus Macaques

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Although live attenuated vaccines can provide potent protection against simian immunodeficiency virus (SIV) and simian-human immunodeficiency virus challenges, the specific immune responses that confer this protection have not been determined. To test whether cellular immune responses mediated by CD8$^+$ lymphocytes contribute to this vaccine-induced protection, we depleted rhesus macaques vaccinated with the live attenuated virus SIVmac239Δ3 of CD8$^+$ lymphocytes and then challenged them with SIVmac251 by the intravenous route. While vaccination did not prevent infection with the pathogenic challenge virus, the postchallenge levels of virus in the plasmas of vaccinated control animals were significantly lower than those for unvaccinated animals. The depletion of CD8$^+$ lymphocytes at the time of challenge resulted in virus levels in the plasma that were intermediate between those of the vaccinated and unvaccinated controls, suggesting that CD8$^+$ cell-mediated immune responses contributed to protection. Interestingly, at the time of challenge, animals expressing the Mamu-A*01 major histocompatibility complex class I allele showed significantly higher frequencies of SIV-specific CD8$^+$ T-cell responses and lower neutralizing antibody titers than those in *Mamu-A*01*$^-$ animals. Consistent with these findings, the depletion of CD8$^+$ lymphocytes abrogated vaccine-induced protection, as judged by the peak postchallenge viremia, to a greater extent in *Mamu-A*01*$^+$ than in *Mamu-A*01*$^-$ animals. The partial control of postchallenge viremia after CD8$^+$ lymphocyte depletion suggests that both humoral and cellular immune responses induced by live attenuated SIV vaccines can contribute to protection against a pathogenic challenge and that the relative contribution of each of these responses to protection may be genetically determined.

Several unique biological features render human immunodeficiency virus (HIV) infection an especially challenging infection to prevent by vaccination (7, 12, 34). The development of an AIDS vaccine is further hindered by a lack of information on the types of immune responses that a vaccine should elicit in order to block or limit HIV infection (25). Both clinical observations of HIV-infected humans and experimental data from nonhuman primate models have indicated that antibodies induced by currently available immunogens are unlikely to elicit broadly protective immunity (6, 7). The temporal concordance of emerging virus-specific responses by CD8$^+$ T cells with declining viremia during primary HIV-1 infection (26), together with observations in nonhuman primates demonstrating that the experimental depletion of CD8$^+$ lymphocytes results in striking increases in virus levels (21, 45), suggests that the induction of virus-specific cytotoxic T lymphocyte (CTL) responses should be a key objective of vaccine development strategies. However, vaccine approaches that elicit high-frequency virus-specific CTL responses have proven incapable of

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TABLE 1. Animals and experimental groups used for this study

Treatment group	Characteristics of experimental animals[b]			Characteristics of attenuated virus vaccination[c]			Challenge virus	Antibody treatment
	Animal no.	Sex	MHC class I status (Mamu-A*01)	Virus	Inoculation route	Mos of infection		
Vaccinated with attenuated virus, treated with control MAb	N374	F	Neg	SIVmac239Δ3	i.v.	63	None	Control MAb
	N029	F	Neg	SIVmac239Δ3	i.v.	63	None	Control MAb
Vaccinated with attenuated virus, treated with anti-CD8 MAb	JW3	M	Neg	SIVmac239Δ3	CNS	57	None	Anti-CD8
	BB4	M	Pos	SIVmac239Δ3	CNS	57	None	Anti-CD8
Vaccinated with attenuated virus, treated with control MAb challenged with pathogenic virus	17141	M	Neg	SIVmac239Δ3	i.v.	66	SIVmac251	Control MAb
	17152	M	Neg	SIVmac239Δ3	i.v	66	SIVmac251	Control MAb
	KY3	F	Neg	SIVmac239Δ3	i.v. and p.o.	55	SIVmac251	Control MAb
	SR3	F	Pos	SIVmac239Δ3	i.v. and p.o.	55	SIVmac251	Control MAb
	N437[a]	F	Neg	SIVmac239Δ3	i.v.	63	SIVmac251	Control MAb
	6543[a]	F	Neg	SIVmac239Δ3	i.v.	63	SIVmac251	Control MAb
Vaccinated with attenuated virus, treated with anti-CD8 MAb challenged with pathogenic virus	17175	M	Neg	SIVmac239Δ3	i.v.	66	SIVmac251	Anti-CD8
	17178	M	Pos	SIVmac239Δ3	i.v.	66	SIVmac251	Anti-CD8
	63–88	F	Neg	SIVmac239Δ3	i.v.	55	SIVmac251	Anti-CD8
	LY3	M	Pos	SIVmac239Δ3	CNS	57	SIVmac251	Anti-CD8
	QS3	M	Neg	SIVmac239Δ3	i.v. and p.o.	55	SIVmac251	Anti-CD8
	RN913[a]	F	Pos	SIVmac239Δ3	p.o.	62	SIVmac251	Anti-CD8
	96H[a]	F		SIVmac239Δ3	i.v.	64	SIVmac251	Anti-CD8
Challenged with pathogenic virus only	17181	M	Neg	None	NA	NA	SIVmac251	None
	17174	M	Neg	None	NA	NA	SIVmac251	None
	RSZ-7	M	Neg	None	NA	NA	SIVmac251	None
	RUA-7	M	Neg	None	NA	NA	SIVmac251	None

[a] These animals received the first antibody administration 6 h prior to SIV challenge. All other antibody-treated and SIV-challenged animals received the first antibody administration 3 days prior to SIV challenge.
[b] M, male; F, female; Neg, negative; Pos, positive.
[c] i.v., intravenous; CNS, central nervous system; p.o., per os; NA, not applicable.

preventing AIDS in rigorous challenge models (5, 20). Thus, a clearer definition of the immune mechanisms that are able to mediate a protective immune response against HIV is critically needed.

The immunization of macaques with live attenuated simian immunodeficiency virus (SIV) strains has proven to be one of the most effective vaccine strategies for inducing protection against challenges with pathogenic SIV strains. Attenuated SIV strains have been effective at mediating protection against both intravenous and mucosal challenges, resulting in either sterile protection or a solid, long-term suppression of replication of the pathogenic challenge virus strain with prevention of disease progression in most animals (11, 24, 53, 54). Infections with live attenuated SIVs elicit neutralizing antibody (NAb) responses (8, 10, 27, 31, 37, 54), which have been shown to correlate with protection from a pathogenic challenge (54). However, the passive transfer of serum or immunoglobulin from vaccinated animals has largely been unsuccessful at preventing infection (2, 22). A variety of SIV-specific cellular immune responses have also been documented for monkeys vaccinated with live attenuated SIVs (15, 16, 23, 36, 48, 49), and major histocompatibility complex (MHC) class I-restricted CTL responses have been correlated with protection against pathogenic virus challenges (23, 40). However, the immune mechanisms responsible for this potent vaccine-induced protection remain largely inferred and have never been experimentally evaluated.

For the present study, we tested the hypothesis that the protective immunity induced by vaccination with the live attenuated virus SIVmac239Δ3 is mediated by the $CD8^+$ lymphocyte population by challenging vaccinated monkeys after $CD8^+$ lymphocyte depletion. The depletion of $CD8^+$ lymphocytes from vaccinated animals resulted in a partial abrogation of protection against a challenge with SIVmac251. However, the relative magnitude of this effect was larger for animals expressing the Mamu-A*01 MHC class I allele than for those that did not express this allele. Our results provide evidence of the involvement of both cellular and humoral immune responses in the containment of wild-type SIV and highlight the potential effects of host MHC genes on immune responses to lentiviral vaccination.

MATERIALS AND METHODS

Viruses. SIVmac239Δ3, a virus attenuated by deletion mutations in *nef*, *vpr*, and upstream sequences of U3 (54), was prepared by transfecting cloned DNA into CEMx174 cells as described previously (17). SIVmac251, a pathogenic biological isolate, was expanded and titrated in rhesus monkeys as described previously (30). This stock was negative for foamy and type D viruses. Challenged animals received 1 ml of the SIVmac251 culture supernatant diluted with phosphate-buffered saline to yield 0.3 ng/ml SIV p27 Gag antigen.

Animal inoculations and in vivo $CD8^+$ lymphocyte depletion. Seventeen rhesus monkeys were vaccinated with SIVmac239Δ3 by the intravenous, oral, or central nervous system route 55 to 66 months prior to the experiment, and they are described in Table 1. Infection was confirmed for all animals by serology and virus isolation. At the time of this study, all had >400 $CD4^+$ T cells/μl and viral levels in plasma below the limit of detection, except for two monkeys used in a pilot study (illustrated in Fig. 1).

SIVmac239Δ3-vaccinated monkeys were transiently depleted of $CD8^+$ lymphocytes by the administration of a chimeric anti-human CD8 monoclonal antibody (MAb), cM-T807, at a dose of 10 mg/kg of body weight subcutaneously on day 0 followed by 5 mg/kg intravenously on days 3 and 7. A chimeric MAb against respiratory syncytial virus (chimeric 1129) was administered at the same doses to a control group of monkeys.

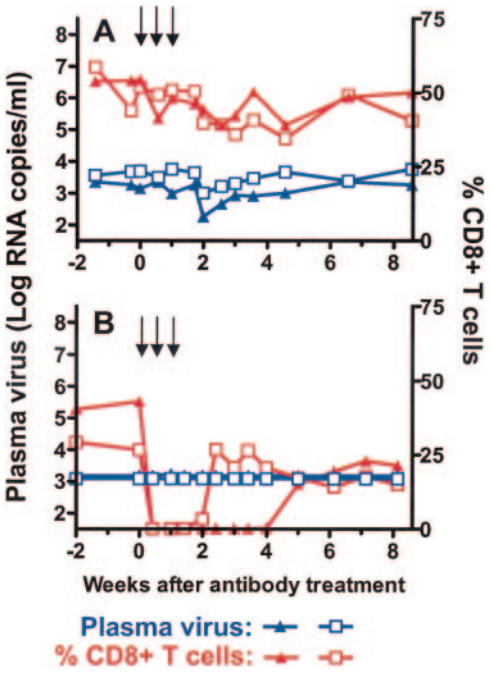


FIG. 1. Effect of MAb administration on SIVmac239Δ3 replication in vaccinated rhesus monkeys. Either an isotype-matched irrelevant MAb (A) or a depleting anti-CD8 MAb (B) was administered to two groups of two vaccinated monkeys each. The time points for MAb administration are indicated with arrows. The viral load assay limits of detection were 100 RNA copies/ml for panel A and 1,500 RNA copies/ml for panel B.

To first determine the effect that the MAb treatment would have on the replication of the vaccine virus, we administered either the anti-CD8 or control MAb to groups of two previously vaccinated rhesus monkeys.

To assess whether $CD8^+$ lymphocytes contribute to vaccine-induced protection, we administered the depleting anti-CD8 MAb to seven previously vaccinated rhesus monkeys either 3 days or 6 h before the intravenous challenge with SIVmac251. Six vaccinated rhesus monkeys received the control MAb before the intravenous challenge. One animal assigned to the anti-CD8 MAb group developed progressive anemia 2 weeks after the SIV challenge and died from a small intestinal perforation and peritonitis 5 weeks later. Since this occurrence was deemed unrelated to MAb administration or SIV infection, the data from this animal were removed from the analysis. Four naïve, unvaccinated rhesus monkeys were inoculated with SIVmac251 and served as controls for the challenge virus infection. This experiment was performed in two parts. Each part of the experiment included approximately one-half of the animals from each treatment group.

Five monkeys expressing the MHC class I gene *Mamu-A*01* were included in the study (Table 1), with the *Mamu-A*01* status being determined by PCR as described previously (28).

For all procedures, animals were anesthetized with ketamine HCl and maintained in accordance with the guidelines of the Institutional Animal Care and Use Committees for Harvard Medical School, Dana-Farber Cancer Institute, and Emory University and the *Guide for the Care and Use of Laboratory Animals* (39A).

Lymphocyte immunophenotyping and Mamu-A*01/SIV Gag p11C tetramer staining. EDTA-anticoagulated whole-blood specimens or lymph node lymphocytes obtained from peripheral lymph node biopsies were immunophenotyped by use of the following antibodies: anti-CD3(FN18)-allophycocyanin, anti-CD4-fluorescein isothiocyanate (19Thy5D7), anti-CD8-phycoerythrin (DK25; Dako, Carpenteria, Calif.), and anti-CD8αβ-ECD (2ST8-5H7; Beckman Coulter, San Diego, Calif.). Specimens from *Mamu-A*01*$^+$ monkeys were further analyzed for the binding of Mamu-A*01/SIV Gag p11C tetrameric complexes as described previously (28). Erythrocytes were lysed from blood specimens by use of the ImmunoPrep reagent system and a TQ-Prep workstation (Beckman Coulter), washed, fixed in phosphate-buffered saline–1% formalin, and analyzed by four-color flow cytometry. Absolute lymphocyte counts on blood specimens were obtained with an automated hematology analyzer.

In situ hybridization. SIVmac replication in lymph node cryostat sections was detected with an ^{35}S-labeled antisense probe (Lofstrand Labs Ltd., Gaithersburg, Md.) as described previously (52).

Determination of virus levels in plasma and of viral species. SIV levels in plasma were determined by a branched DNA amplification assay (Bayer Diagnostics, Emeryville, Calif.; limit of detection, 1,500 RNA copies/ml) or a real-time reverse transcription-PCR assay (Quantitative Molecular Diagnostic Section, AIDS Vaccine Program, NCI-Frederick, Frederick, Md.; limit of detection, 100 RNA copies/ml). The concordance between the assay methods was confirmed for >50 specimens. The vaccine strain SIVmac239Δ3 was discriminated from the challenge virus, SIVmac251, in plasma by a nested PCR using PCR primers that spanned the *nef* deletion, as described previously (54).

Immune assays. Peripheral blood mononuclear cell (PBMC) specimens were cryopreserved from nine animals (five anti-CD8 MAb-treated animals and four control MAb-treated animals). Gamma interferon (IFN-γ)-specific enzyme-linked immunospot (ELISPOT) assays were performed on PBMC depleted of $CD4^+$ T cells by StemSep negative cell separation (Stem Cell Technologies, Vancouver, British Columbia, Canada), resulting in populations of cells with <5% residual $CD4^+$ T cells. Serial dilutions of the PBMC (3×10^5 and 1×10^5 cells/well) in RPMI 1640–10% fetal bovine serum were plated in duplicate in 96-well Multiscreen ELISPOT IP plates (Millipore, Billerica, Mass.) coated with an anti-IFN-γ antibody (diaPharma, West Chester, Ohio) and then incubated with pools of SIV peptides or medium alone containing an equivalent concentration of dimethyl sulfoxide. SIV Gag, Env, Rev, Vif, Tat, and Nef peptide pools consisted of 15-mers that overlapped by 11 residues, corresponding to either the SIVmac239 or SIVmac251 sequence, with the final concentration of each individual peptide being 2 μg/ml. All peptides except the Nef pool were synthesized by Ashok Khatri of the Massachusetts General Hospital Peptide Core Facility (Boston, Mass.), using fluorenylmethoxycarbonyl chemistry; the Nef pool was obtained from the NIH AIDS Research and Reference Reagent Program. PBMC stimulated with concanavalin A (5 μg/ml, 10^5 cells/well) served as a positive control. Plates were incubated for 12 to 18 h. After the plates were washed, IFN-γ spots were detected with a biotin-conjugated anti-IFN-γ Ab (diaPharma) and alkaline phosphatase-streptavidin and were developed by use of an alkaline phosphatase substrate kit (Bio-Rad, Hercules, Calif.). Spots were counted with a KS ELISPOT automated reader system (Carl Zeiss Inc., Thornwood, N.Y.) using KS ELISPOT 4.2 software (performed by Zellnet, New York, N.Y.). The results were calculated as frequencies of SIV-specific spot-forming cells (SFC) per 10^6 PBMC minus the frequency of SFC per 10^6 PBMC obtained with medium alone. The results were considered positive if there were >10 SFC per well and threefold more than the background level.

CTL activity was measured as previously described (23). Briefly, PBMC were stimulated with autologous herpesvirus papio-transformed B-cell lines (B-LCL) infected with a recombinant vaccinia virus (vAbt388; provided by D. Panicali, Therion Biologics, Cambridge, Mass.) expressing the SIVmac251 *gag* and *pol* genes and the SIVmac239 *env* gene in RPMI 1640–10% fetal calf serum, with 20 U/ml recombinant human interleukin-2 added after 3 days. CTL assays were performed 10 to 14 days after stimulation. ^{51}Cr-loaded target cells consisted of autologous B-LCL infected with recombinant vaccinia viruses expressing either Gag derived from SIVmac251 or Env derived from SIVmac239. Cold targets consisted of unlabeled autologous B-LCL infected with the control vaccinia virus strain NYCBH and were used at a cold-to-hot target ratio of 15:1.

Measurements of neutralizing (NAb) and whole virus antibody responses. The antibody-mediated neutralization of SIVmac251 was assessed in a CEMx174 cell killing assay as previously described (38). Cell-free stocks of T-cell-line-adapted SIVmac251 (TCLA SIV251) prepared in H9 cells or of the primary SIVmac251 isolate (PI SIV251) prepared in human peripheral blood mononuclear cells containing 500 50% tissue culture infective doses/50 μl were added in triplicate to multiple dilutions of test serum in 100 ml of RPMI 1640–12% fetal bovine serum containing 50 μg gentamicin in 96-well culture plates. After incubation for 1 h at 37°C, CEMx174 cells (5×10^4 cells in 100 μl) were added to each well. Infection led to extensive syncytium formation and virus-induced cell killing in approximately 4 to 6 days in the absence of antibodies. Neutralization was measured by staining viable cells with Finter's neutral red in poly-L-lysine-coated plates. The percent protection was determined by calculating the difference in absorption (A_{540}) between test wells (cells plus serum sample plus virus) and virus control wells (cells plus virus), dividing this result by the difference in absorption between cell control wells (cells only) and virus control wells, and multiplying the result by 100. Neutralization was measured at a time when virus-induced cell killing in virus control wells was >70% but <100%. Neutral-

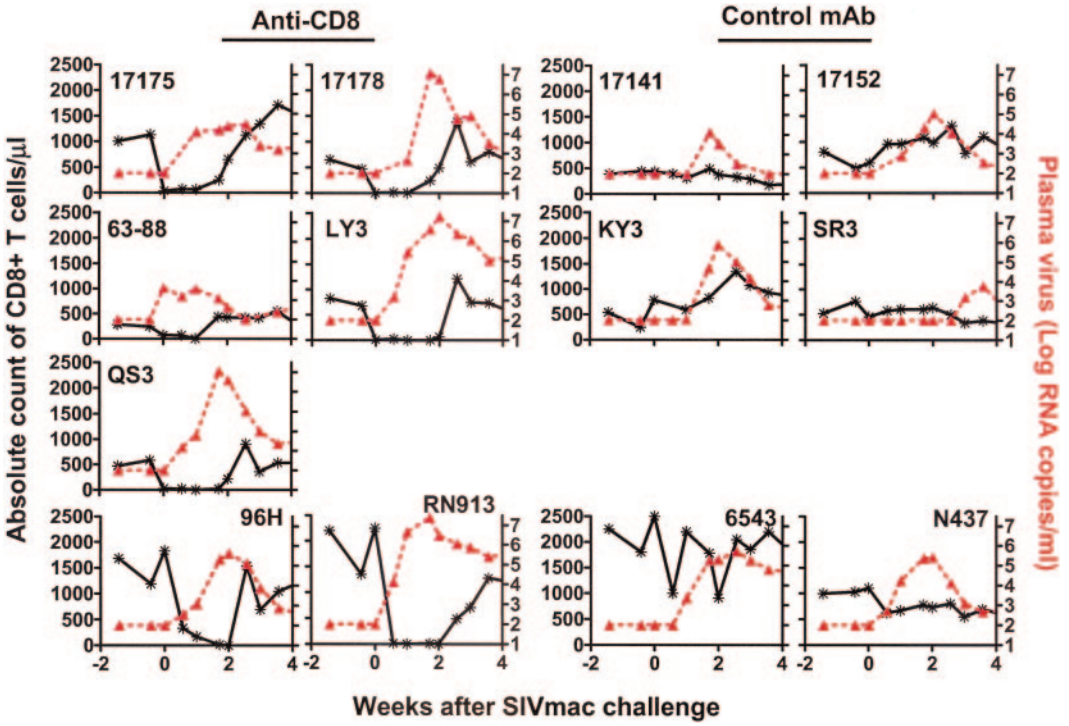


FIG. 2. Effectiveness of anti-CD8 MAb treatment. The graphs show $CD8^+$ T-cell numbers and levels of plasma virus for individual vaccinated monkeys during anti-CD8 or control antibody treatment and challenge with pathogenic SIVmac251. Antibody administration was begun either 3 days (rows 1 to 3) or 6 h (row 4) before monkeys were challenged with SIVmac251.

izing titers are given as the reciprocal dilutions required to protect 50% of cells from virus-induced killing.

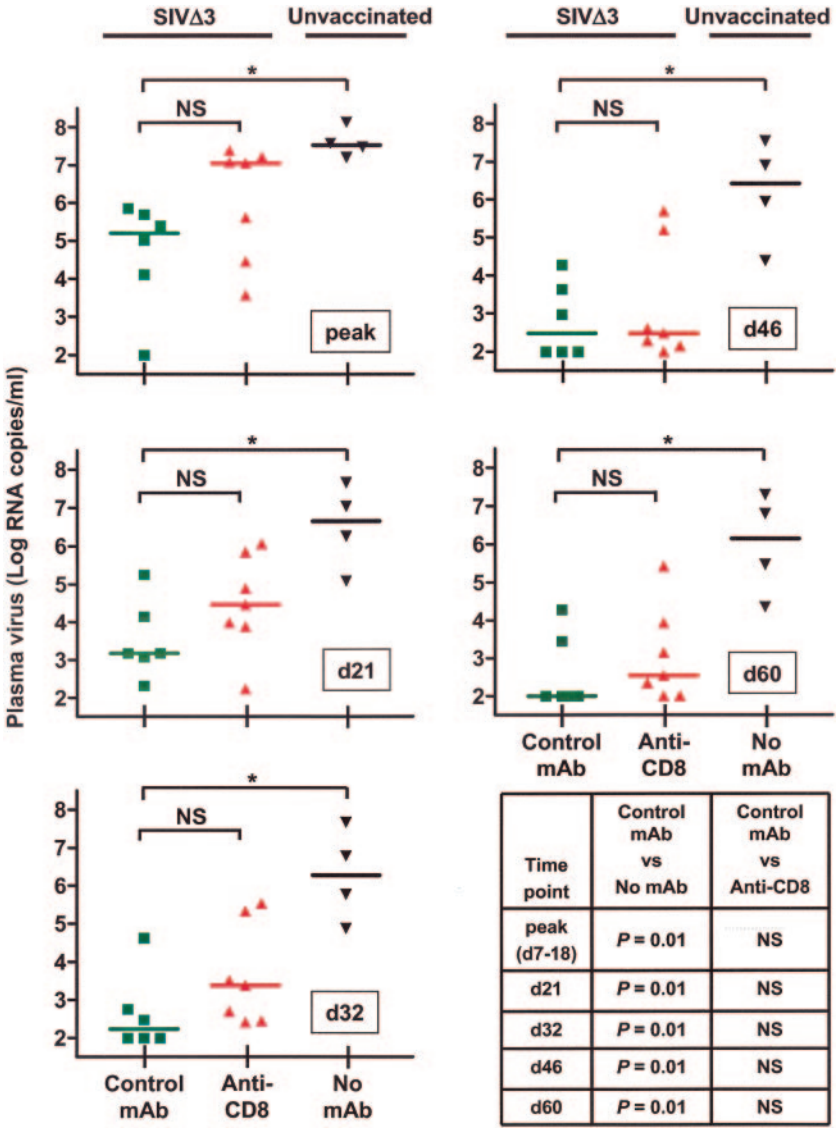
Antibody responses against whole SIV were measured at dilutions of 1:20 and 1:100 or 1:200 in an enzyme-linked immunosorbent assay (ELISA) using purified whole SIVmac251 as described previously (54).

Data analysis. The $\log_{10}$ virus levels in plasma or the area under the curve (AUC) for $\log_{10}$ virus levels in plasma over time (calculated by using the trapezoidal rule) and the $\log_{10}$ neutralizing antibody titers were compared between groups of animals by use of the exact Mann-Whitney U test. We chose a priori to compare (i) vaccinated, control MAb-treated monkeys with unvaccinated monkeys with no MAb treatment and (ii) vaccinated, CD8-depleted monkeys with vaccinated, control antibody-treated monkeys without CD8 depletion. NAb titers from the TCLA or PI assays were compared by use of the Wilcoxon sign rank test. The correlation of NAb titers and SIV RNA levels in plasma and the correlation of spot-forming $CD8^+$ cells and NAb titers were estimated and tested by use of the Spearman rank correlation. All significance levels were two-sided. To adjust for the two treatment comparisons, we used Holm's method, wherein the smaller of the two significance levels was compared to a cutoff of 0.025. If that comparison was significant, then the larger of the two significance levels was compared to a cutoff of 0.05. The AUC calculations and plots were done with GraphPad Prism, version 3.02 (GraphPad Software, San Diego, Calif.). For AUC calculations, the background (set at 100 copies of viral RNA/ml) was subtracted. All statistical calculations were performed with SPSS, version 11.5 (SPSS Inc., Plover, Wis.).

RESULTS

Effect of $CD8^+$ lymphocyte depletion and MAb administration on replication of live attenuated SIVmac239Δ3. Experiments to determine the contribution made by $CD8^+$ lymphocytes to the protection afforded by live attenuated vaccines required the administration of a MAb and the elimination of $CD8^+$ lymphocytes which might result in increased replication of the vaccine virus, which would confound the interpretation of results following a SIVmac251 challenge. In a previous study of monkeys vaccinated with the less attenuated virus SIVmac 239Δnef, $CD8^+$ lymphocyte depletion did result in substantial replication of the attenuated virus (36). Therefore, a pilot study was performed with four rhesus monkeys that were vaccinated with SIVmac239Δ3 and treated with either anti-CD8 or a control MAb. Both monkeys receiving the control MAb had stable levels of plasma virus and $CD8^+$ T cells (Fig. 1A). More importantly, in two additional vaccinated monkeys, the virus levels in plasma remained below the limit of detection (1,500 RNA copies/ml) throughout the entire period of $CD8^+$ lymphocyte depletion (Fig. 1B), confirming that neither anti-CD8 nor the control MAb resulted in substantial changes in the replication of live attenuated SIVmac239Δ3.

Effect of $CD8^+$ lymphocyte depletion on pathogenic challenge of monkeys vaccinated with SIVmac239Δ3. We next wished to determine whether the protection against a pathogenic SIV challenge afforded by attenuated SIV vaccines is mediated by cellular immune responses contained in the $CD8^+$ lymphocyte subset. To evaluate the contribution of these cells to vaccine protection, we assigned 13 monkeys previously vaccinated with SIVmac239Δ3 and with undetectable levels of vaccine virus (<100 copies of RNA/ml) to receive either a depleting anti-CD8 or control MAb (Table 1). Antibody administration was begun either 3 days or 6 h before the monkeys were challenged with SIVmac251 (Fig. 2). In previous studies, this vaccine provided significant control against SIV challenge compared to the case for unvaccinated animals (54). Monkeys that received the anti-CD8 MAb showed a total depletion of $CD8^+$ lymphocytes in the peripheral blood and a nearly total depletion of lymph node $CD8^+$ lymphocytes for those five



Time point	Control mAb vs No mAb	Control mAb vs Anti-CD8
peak (d7-18)	$P = 0.01$	NS
d21	$P = 0.01$	NS
d32	$P = 0.01$	NS
d46	$P = 0.01$	NS
d60	$P = 0.01$	NS

FIG. 3. Virus levels in plasma after challenge with SIVmac251. Rhesus monkeys vaccinated with live attenuated SIVmac239Δ3 were challenged with pathogenic SIVmac251 after the depletion of $CD8^+$ lymphocytes by an anti-CD8 MAb (anti-CD8) or after receiving an irrelevant MAb (control MAb). As a virus challenge control, naïve unvaccinated monkeys that received no MAb treatment also received the challenge virus (no MAb). Data are illustrated for each group, showing the peak level of primary viremia (highest level observed on days 7 through 18 postchallenge) and the levels on days 21, 32, 46, and 60 postchallenge. The bars indicate the median values for the groups. NS, no significant difference.

animals that were biopsied (data not shown), and the depletion persisted for 10 to 18 days (median, 14 days). $CD8^+$ lymphocyte numbers were stable in the control MAb-treated group.

In four unvaccinated and untreated rhesus monkeys that served as controls for SIVmac251 infection, the levels of plasma virus peaked at 10^7 to 10^8 RNA copies/ml and were maintained at $>10^4$ copies/ml for >8 weeks postchallenge (Fig. 2 to 4). In seven monkeys vaccinated with SIVmac239Δ3 and treated with the control MAb, the peak levels of postchallenge viremia (days 7 to 18) were significantly lower (approximately 1,000-fold) than those seen in naïve control monkeys. By day 60 postchallenge, viremia was undetectable in four of six vaccinated, control MAb-treated monkeys. In seven vaccinated monkeys depleted of $CD8^+$ lymphocytes at the time of challenge, the level of peak viremia was intermediate between those in the control MAb-treated and naïve control groups and the median level was approximately 100-fold higher than that for the control MAb-treated group. This trend toward higher levels of plasma virus in vaccinated monkeys depleted of $CD8^+$ lymphocytes during virus challenge than in control MAb-treated monkeys was maintained through day 46 (Fig. 4), which was approximately 4 1/2 weeks after $CD8^+$ lymphocytes began to reappear.

To determine whether postchallenge plasma contained the vaccine or challenge virus strain, we performed nested PCR to distinguish the two virus species. When virus levels in the plasma were <5,000 copies/ml, only the vaccine strain was detected. A mixture of bands indicating the presence of both viruses was seen when viral RNA levels in the plasma ranged from 5,000 to 100,000 copies/ml. However, when the virus level

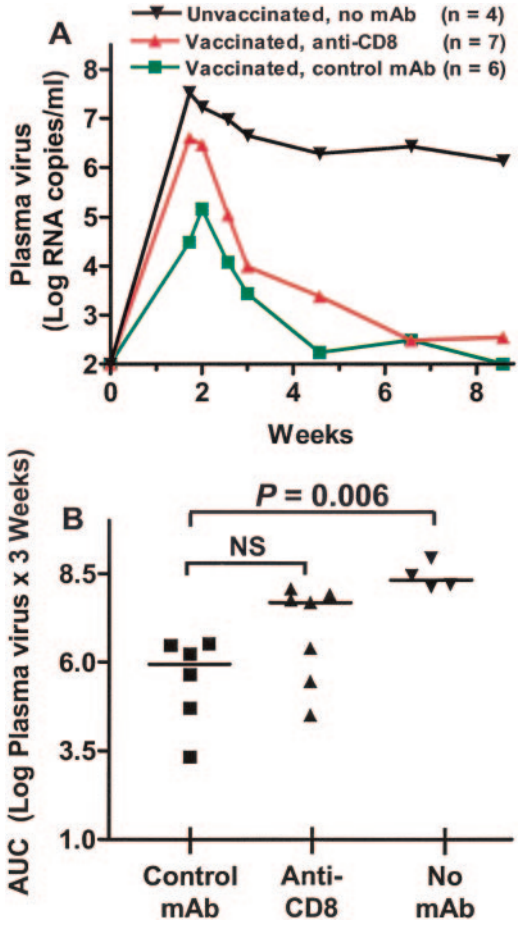


FIG. 4. Summary of virus levels in plasma over time. The group designations are the same as those shown in Fig. 2. (A) Median virus levels in plasma. (B) Areas under the curve minus the background level for days 0 to 21 postchallenge. Bars indicate median values. NS, no significant difference.

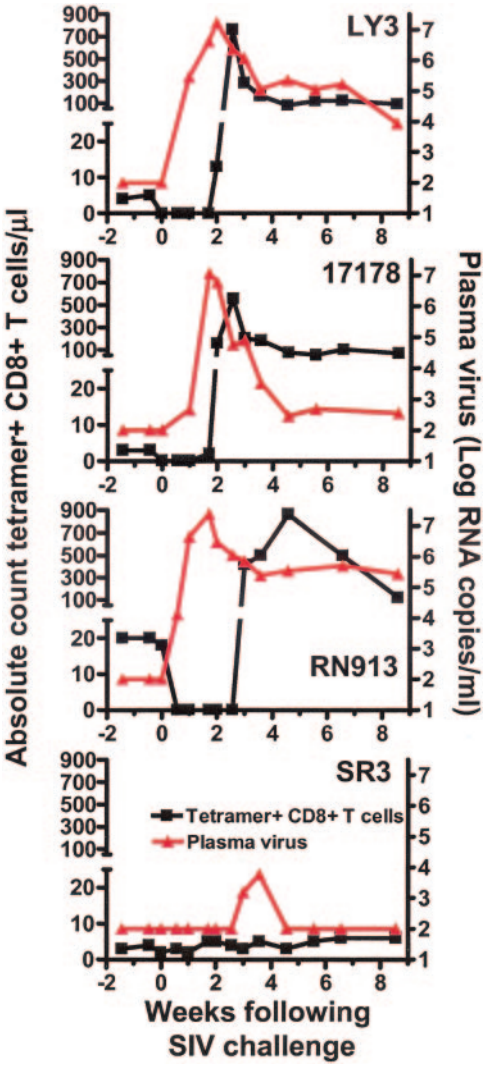


FIG. 5. Absolute numbers of tetramer-positive $CD8^+$ T cells and virus levels in plasma for vaccinated monkeys during anti-CD8 antibody (LY3, 17178, and RN913) and control antibody (SR3) treatment after SIVmac251 challenge.

in plasma was >100,000 copies/ml, only the challenge virus was detected (data not shown).

The level of virus replication detected in peripheral blood correlated well with the detection of viral RNA by in situ hybridization in lymph nodes. Prior to $CD8^+$ lymphocyte depletion and SIV challenge, replicating viruses were only rarely detected in the lymph nodes of vaccinated animals (data not shown). However, following the SIVmac251 challenge, a large increase in productively infected cells was observed, which again decreased when virus replication was controlled.

CTL assays confirmed the depletion of SIV-specific $CD8^+$ T cells from the blood after anti-CD8 MAb administration. SIV-specific CTL activity was present in vaccinated monkeys prior to $CD8^+$ lymphocyte depletion and following the reappearance of $CD8^+$ lymphocytes and in all control MAb-treated animals (data not shown). In addition, binding of the Mamu-A*01/SIV Gag p11C tetramers to $CD8^+$ T cells was detected in animals expressing the *Mamu-A*01* allele prior to $CD8^+$ lymphocyte depletion (baseline frequencies, 0.1 to 0.5% of $CD8^+$ T cells) and following the reappearance of $CD8^+$ lymphocytes (Fig. 5). However, during the depletion of $CD8^+$ lymphocytes, no binding of this tetramer to $CD3^+$ T cells was observed. Tetramer-positive cells were among the first $CD8^+$ lymphocytes to reappear in vaccinated animals challenged with the pathogenic virus. As $CD8^+$ lymphocytes returned, tetramer-positive cells expanded rapidly (prior to challenge and $CD8^+$ lymphocyte depletion, there were <20 cells/μl; following the reappearance of $CD8^+$ lymphocytes, there were 550 to 863 cells/μl) and eventually declined over several weeks, concomitant with a decline in the level of the pathogenic virus.

These analyses of virus replication in the presence and absence of $CD8^+$ lymphocytes may suggest that cellular immune responses contribute to the control of viremia afforded by infection with a live attenuated SIV. However, the elimination of $CD8^+$ lymphocytes did not completely abrogate this vaccine-induced protection.

Correlation between NAb titer and level of viremia achieved postchallenge. To assess the contribution that NAbs may have made to the control of virus replication, we tested whether a correlation existed between the NAb titer against the challenge

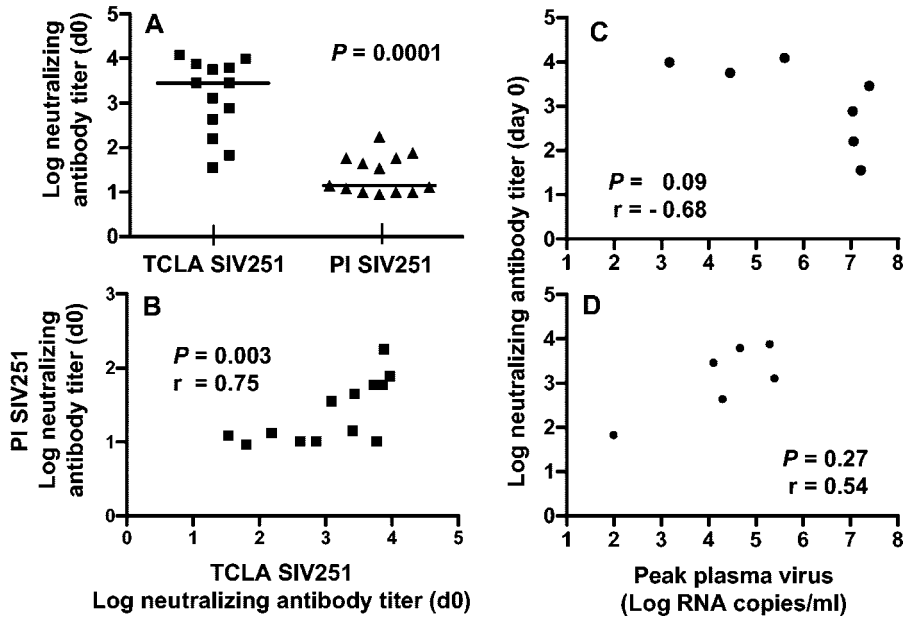


FIG. 6. Comparison of NAb assays and correlation of prechallenge NAb titers to peak plasma viremia. NAb titers in a TCLA SIV251 or PI SIV251 assay were compared for all SIVmac239Δ3-vaccinated monkeys prior to challenge and antibody treatment. (A) Comparison of NAb titers by using TCLA SIV251 and PI SIV251 assays. Bars indicate medians. (B) Correlation of NAb titers in TCLA SIV251 or PI SIV251 assay. (C and D) Comparison of NAb titers on the day of SIVmac251 challenge (TCLA SIV251 assay) to peak levels of plasma virus achieved after the challenge of anti-CD8 antibody-treated (C) or control MAb-treated (D) monkeys.

virus on the day of challenge and the peak level of viremia achieved postchallenge. In addition, this experiment provided a unique opportunity to assess the effect of NAbs at a time when cellular immune responses had been eliminated from the $CD8^+$ lymphocyte-depleted group. We measured the neutralization of both T-cell-line-adapted (TCLA) and primary isolate (PI) SIVmac251. As predicted from previous studies (29, 35), NAb titers against the PI virus were substantially lower than those against the TCLA virus (Fig. 6A). The neutralizing titers in the TCLA assay had a range of about 1,000-fold, whereas titers against the PI SIV varied only 20-fold. However, titers from both the TCLA and PI assays were significantly correlated ($r = 0.75$; $P = 0.003$), indicating that the more robust titers from the TCLA assay may serve as a surrogate marker for neutralization of the PI SIV (Fig. 6B). Using either the TCLA (Fig. 6D) or PI (data not shown) SIV assay, we were unable to show a significant correlation between the prechallenge NAb titer and the peak level of viremia in control MAb-treated monkeys. However, for animals that underwent $CD8^+$ lymphocyte depletion, a trend toward a negative correlation between NAb titers and peak levels of viremia was observed (Fig. 6C) (Spearman rank $= -0.68$; $P = 0.09$). In addition, we made a similar observation by using an ELISA to measure plasma antibody binding to purified whole SIV wherein the ELISA titers prior to inoculation correlated negatively with the peak level of viremia postchallenge (data not shown). These findings suggest a contributory role for NAbs in the control of challenge viremia that becomes apparent only after the $CD8^+$ lymphocyte-mediated effects are removed.

Effect of MHC class I gene expression on the magnitude of SIV-specific cellular and humoral immune responses in SIVmac239Δ3-vaccinated monkeys. Recent reports have demonstrated that expression of the *Mamu-A*01* allele is associated with more efficient control of virus replication, an effect that may in part be mediated by *Manu-A*01*-restricted $CD8^+$ T-cell responses (39, 41, 42). Therefore, we compared the magnitudes of humoral and cellular immune responses in rhesus monkeys that expressed the MHC class I allele *Mamu-A*01* with those in monkeys that did not express this allele. First, we compared NAb titers prior to challenge in vaccinated animals that expressed *Mamu-A*01* with those in vaccinated animals that did not express this allele. Interestingly, the NAb titers were significantly lower in *Mamu-A*01*$^+$ than in *Mamu-A*01*$^-$ animals in the TCLA assay (Fig. 7A), and a similar trend was seen with the PI assay (data not shown).

To assess the effect that MHC class I allele expression would have on the magnitude of $CD8^+$ cellular immune responses, we compared SIV-specific IFN-γ ELISPOT responses in $CD8^+$ lymphocyte-enriched PBMC from *Mamu-A*01*$^+$ animals with those in PBMC from *Mamu-A*01*$^-$ animals prior to challenge by using overlapping peptides from six different SIV genes (Fig. 7B). For the subset of animals with which these assays were performed, animals expressing *Mamu-A*01* had significantly stronger $CD8^+$ T-cell-mediated, SIV-specific immune responses than did *Mamu-A*01*$^-$ animals ($P < 0.02$). Furthermore, the magnitude of spot-forming $CD8^+$ T-cell responses showed a trend toward a negative correlation with NAb titers immediately prior to pathogenic challenge ($P = 0.08$) (Fig. 7C). This suggests that within an individual, vaccinated animal, either cellular or humoral immune responses may predominate and that this difference may be genetically determined.

We then compared the postchallenge levels of plasma viremia detected in anti-CD8 antibody- and control antibody-

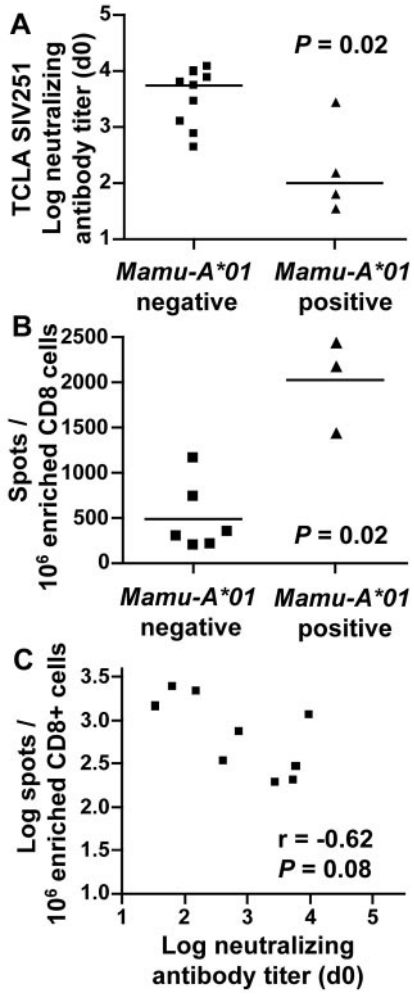


FIG. 7. Influence of MHC class I allele on NAb titers and SIV-specific $CD8^+$ T-cell responses in monkeys vaccinated with live attenuated SIVmac239Δ3. (A) Comparison of median NAb titers in *Mamu-A*01*$^-$ and *Mamu-A*01*$^+$ monkeys. Bars indicate medians. (B) Comparison of SIV-specific $CD8^+$ cellular immune responses in *Mamu-A*01*$^+$ and *Mamu-A*01*$^-$ animals by IFN-γ ELISPOT assay. CD8-enriched lymphocytes were assessed by an IFN-γ ELISPOT assay using Gag, Env, Rev, Vif, Tat, and Nef peptide pools. The sums of the total responses against all peptide pools used are shown. Bars indicate medians. (C) Correlation of SIV-specific $CD8^+$ spot-forming cells and NAb titers (TCLA SIV251 assay) in vaccinated monkeys prior to challenge.

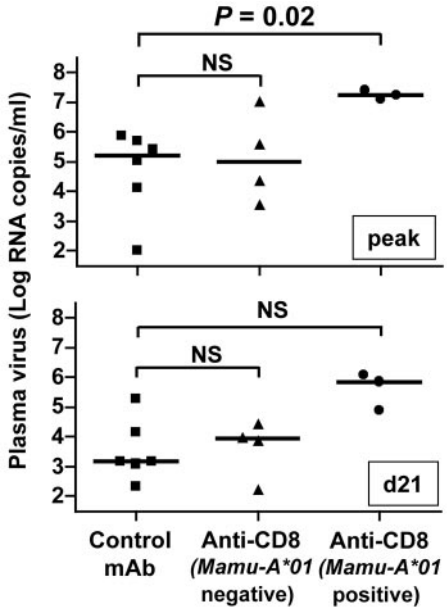


FIG. 8. Comparison of postchallenge viremia levels in vaccinated monkeys treated with control or anti-CD8 antibody and challenged with SIVmac251.

treated animals. $CD8^+$ lymphocyte-depleted *Mamu-A*01*$^+$ animals showed significantly higher levels of viremia at their peak ($P = 0.02$) than their *Mamu-A*01*$^-$ counterparts (Fig. 8). In contrast, the peak viremia in $CD8^+$ lymphocyte-depleted *Mamu-A*01*$^-$ animals was indistinguishable from that in control antibody-treated animals. Such differences were not significant on day 21.

Taken together, the data from the virus neutralization and cellular immune assays suggest that animals vaccinated with SIVmac239Δ3 can utilize both the $CD8^+$ cellular and the humoral adaptive immune responses to control the replication of a pathogenic challenge virus. However, the relative magnitudes of the cellular and humoral immune responses are affected by the presence of the *Mamu-A*01* allele.

DISCUSSION

Although safety concerns have dampened enthusiasm for the use of live attenuated vaccines to prevent HIV infection (3, 18), live attenuated SIVs provide a unique opportunity to define correlates of protective immunity against AIDS virus infection. In the present study, we directly assessed the role of cellular immune responses mediated by $CD8^+$ lymphocytes in the protection afforded in rhesus monkeys by vaccination with a live attenuated SIV vaccine. By depleting $CD8^+$ lymphocytes at the time of challenge with a pathogenic SIV, we showed that immune responses mediated by $CD8^+$ lymphocytes can contribute to vaccine-induced protection, as assessed by the levels of plasma viremia postchallenge. However, the depletion of $CD8^+$ lymphocytes did not completely abolish the ability of vaccinated animals to control viremia after challenge.

A previous study (36) showed that the depletion of $CD8^+$ lymphocytes in animals vaccinated with the less attenuated SIVmac239Δnef strain resulted in a substantial increase in vaccine virus replication. We reasoned that a more highly attenuated vaccine virus such as SIVmac239Δ3 would be less susceptible to reemergence during anti-CD8 treatment and would therefore be more suitable for our studies. Indeed, our pilot study confirmed that the vaccine virus remained undetectable in the plasma during $CD8^+$ lymphocyte depletion, thus eliminating this factor as a confounding effect in our study.

Compared to animals vaccinated with the less attenuated SIVmac239Δnef strain (9, 11), most animals vaccinated with SIVmac239Δ3 exhibited a substantial replication of pathogenic SIVmac251 after challenge. Nevertheless, all vaccinees had levels of plasma virus that were significantly lower than those in nonvaccinated animals from 32 days onward following challenge. Eight weeks after challenge, the mean levels of plasma

virus in vaccinated animals were >1,000-fold lower than those in naïve controls. This sustained control of viral replication after challenges with highly pathogenic SIVmac strains, such as SIVmac251, is uncommon with vaccine strategies other than live attenuated vaccines (22). The depletion of $CD8^+$ lymphocytes in vaccinated animals resulted in peak and postpeak levels of viremia that fell between those for the vaccinated and unvaccinated controls. This observation suggested that $CD8^+$ lymphocytes contribute to vaccine-induced protection, but depletion was not able to totally ablate this protection, implicating potential contributions from other mechanisms as well (51).

The failure of $CD8^+$ lymphocyte depletion to completely abolish vaccine-induced protection may have been due in part to the transient nature of cell depletion that occurs in this model or to an incomplete depletion of $CD8^+$ lymphocytes. The total depletion of $CD8^+$ lymphocytes in peripheral blood in our study did not persist for more than 18 days in any animal. A similar study in which only a partial depletion of $CD8^+$ lymphocytes was achieved failed to block live attenuated vaccine-induced protection as well (50). An incomplete depletion of $CD8^+$ lymphocytes in lymph nodes or other extravascular sites may also have contributed to the only partial reversal of the protective effect of vaccination. Thus, it is conceivable that more complete cell depletion and/or a longer duration of cell depletion may have more efficiently eliminated the vaccine-induced protection.

Alternatively, we considered that immune responses mediated by other mechanisms may have contributed to the protection induced by SIVmac239Δ3. To evaluate the contribution of humoral immunity in this study, we measured NAb titers in sera of vaccinated animals on the day of challenge. As predicted, we found low NAb titers against a primary SIV isolate but detected much higher titers against a tissue culture lab-adapted SIV strain. However, the NAb titers against primary isolate and lab-adapted SIVs were strongly correlated. The anti-SIV NAb titer needed to impact virus replication has yet to be definitely determined. However, it is conceivable that even low-level titers measured in an in vitro NAb assay using a primary isolate SIV may provide some antiviral activity in vivo. For this study, we used the more robust NAb titers against a lab-adapted SIV, which correlated with the NAb titers against a primary SIV isolate, as a surrogate for NAb titers against the challenge SIV. While the data did not reach statistical significance, we did observe a trend toward a negative correlation between the NAb titer on the day of challenge and the postchallenge peak level of viremia ($P = 0.09$). While this does not constitute direct proof that the protection against the challenge virus was mediated in part by NAbs, our data suggest that humoral immunity may contribute to vaccine-induced protection. This interpretation is also supported by a previous study that showed significant correlations between both SIV-specific $CD8^+$ T-cell responses and antibody responses and protection against a vaginal challenge with SIVmac251 in animals vaccinated with attenuated SIV strains (24). Similarly, other vaccine modalities have shown that both cellular and humoral immune responses provide protection against other pathogenic challenge viruses (14). A detailed analysis of the mechanisms of protection induced by an attenuated murine retrovirus provided compelling evidence that the combined contributions of immune $CD4^+$ and $CD8^+$ T cells, along with those of B cells, are necessary to provide complete protection against a challenge with the pathogenic Friend virus (13). Similarly, studies of the immune protection induced by postexposure chemoprophylaxis against Rauscher murine leukemia virus demonstrated that the adoptive transfer of both $CD4^+$ and $CD8^+$ T cells was required for the effective protection of naïve recipients (19, 44). Taken together, these studies strongly suggest that an effective AIDS vaccine will likely need to induce $CD4^+$ and $CD8^+$ T-cell responses, as well as NAb responses.

We also considered the possibility that genetic factors might have affected the responses of some animals to the live attenuated vaccine. Recent observations have indicated that rhesus monkeys expressing certain MHC class I alleles, such as *Mamu-A*01*, can control SIV replication more effectively than animals not expressing this allele (39, 41, 42), possibly due to the generation of stronger CTL responses. Consistent with a genetically determined difference in response to SIV, we found different vaccine-induced immune responses in *Mamu-A*01*$^+$ animals than those in animals that did not express this allele. In response to vaccination, *Mamu-A*01*$^+$ animals developed strong $CD8^+$ CTL responses and relatively weak NAb responses, possibly because the control of the initial replication of the vaccine virus by cellular responses limited the antigen exposure necessary for the induction of humoral responses. In these animals, the depletion of $CD8^+$ lymphocytes was quite effective at abrogating vaccine-induced protection during the initial postchallenge period. However, these animals subsequently showed a strong induction of NAb responses following the increase in plasma viremia associated with $CD8^+$ lymphocyte depletion, which brought viral replication under substantial control by 6 to 8 weeks postchallenge. In contrast, following vaccination with the attenuated SIVmac239Δ3 strain, *Mamu-A*01*$^-$ animals developed stronger humoral immune responses. In the presence of these more robust prechallenge NAb responses, the depletion of $CD8^+$ lymphocytes at the time of pathogenic virus challenge had a much less dramatic effect on the control of postchallenge viral replication.

Thus, both cellular and humoral SIV-specific immune responses may contribute to the control of a pathogenic challenge virus induced by vaccination with live attenuated vaccine strains. The relative contributions of SIV-specific cellular and humoral immune responses to the protection induced by attenuated SIV vaccines are likely consequences of differential responses to the attenuated vaccine strain, influenced in part by MHC haplotypes and other genetic factors. The difficulty with identifying correlates of protection in nonhuman primate AIDS vaccine experiments thus far might be explained by the facts that multiple immune mechanisms can mediate protection and that the relative strengths of these responses vary between individuals.

It is also interesting that while the *Mamu-A*01* allele had a distinct effect on the relative strength of the cellular or humoral immune response, the presence or absence of this allele had no noticeable effect on the replication of the live attenuated vaccine virus. All vaccinated animals were able to limit the replication of the vaccine virus to below the limit of detection prior to the pathogenic virus challenge.

Studies using T-cell-depleting antibodies have shown that $CD8^+$ lymphocyte responses are important for the early con-

trol of SIV replication and contribute substantially to the containment of SIV replication during the chronic phase of infection (21, 45). However, vaccine modalities capable of eliciting high frequencies of simian-human immunodeficiency virus (SHIV)-specific CTLs have been unable to provide sterilizing immunity after challenge (1). Mounting evidence suggests that humoral immune responses can also play a role in controlling SIV replication. Correlative studies have shown that macaques exhibiting a rapidly progressive disease course after SIV infection develop weak humoral immune responses (46) and that antibodies may serve as a second line of defense in some vaccine approaches (43). Interestingly, the administration of virus-specific MAbs provided sterile protection of rhesus monkeys from SHIV infection (4, 33). However, the passive transfer of immunoglobulin from live attenuated vaccinated monkeys to naïve monkeys has not conferred protection thus far (2; unpublished observations). The failure to achieve adequate levels of SIV-specific NAbs in recipient animals may in part explain these negative results, since the level of SIV-specific antibodies needed to confer in vivo protection has not been definitively determined. Those studies that did achieve sterilizing protection from SHIV challenge via the passive transfer of either polyclonal immunoglobulin or MAbs required very high antibody levels (4, 32, 47). Alternatively, these failures of passive antibody transfer to protect animals may reflect the need for NAbs to work in concert with cellular immune responses to mediate protection.

These results have implications for AIDS vaccine development by providing evidence for the involvement of both $CD8^+$ T-cell responses and NAbs in controlling SIV viremia and attenuating disease. They also highlight the potential impact that the host genotype can have on immune responses and reinforce the importance of incorporating information on host MHC genes into future efforts to examine mechanisms of protective immunity in the SIV/macaque model.

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