

Induction of a virus-specific effector–memory CD4⁺ T cell response by attenuated SIV infection

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We investigated simian immunodeficiency virus (SIV)-specific CD4⁺ T cell responses in rhesus macaques chronically infected with attenuated or pathogenic SIV strains. Analysis of SIV Δ nef-infected animals revealed a relatively high frequency of SIV-specific CD4⁺ T cells representing 4–10% of all CD4⁺ T lymphocytes directed against multiple SIV proteins. Gag-specific CD4⁺ T cells in wild-type SIV-infected animals were 5–10-fold lower in frequency and inversely correlated with the level of plasma viremia. SIV-specific CD4⁺ cells from SIV Δ nef animals were predominantly CD27⁻CD28⁻CD45RA^{low}CCR7⁻CCR5⁻, consistent with an effector–memory subset, and included a fully differentiated CD45RA⁺CCR7⁻ subpopulation. In contrast, SIV-specific CD4⁺ T cells from SIV-infected animals were mostly CD27⁺CD28⁺CD45RA⁻CCR7⁺CCR5⁺, consistent with an early central memory phenotype. The CD45RA⁺CCR7⁻CD4⁺ subset from SIV Δ nef animals was highly enriched for effector CD4⁺ T cells, as indicated by the perforin expression and up-regulation of the lysosomal membrane protein CD107a after SIV Gag stimulation. SIV-specific CD4⁺ T cells in attenuated SIV-infected animals were increased in frequency in bronchioalveolar lavage and decreased in lymph nodes, consistent with an effector–memory T cell population. The ability of SIV Δ nef to induce a high frequency virus-specific CD4⁺ T cell response with direct effector function may play a key role in protective immunity produced by vaccination with attenuated SIV strains.

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Abbreviations used: BAL, bronchoalveolar lavage; ICS, intracellular cytokine staining; SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; SIV, simian immunodeficiency virus.

In recent years, increasing attention has focused on HIV-1-specific CD4⁺ T lymphocytes, which are thought to play a central role in initiating and maintaining antiviral immunity and may be critical for control of HIV-1 replication (1). Several reports have suggested that control of HIV viremia is associated with robust HIV-specific CD4⁺ T cell proliferative responses. Long-term nonprogressors with low virus loads have higher levels of HIV-specific CD4⁺ T cell proliferative responses compared with those of HIV-1-infected subjects with progressive disease (2, 3), and similar results

have been reported in untreated patients with chronic HIV-1 infection (4). Studies in HIV-exposed but seronegative individuals also suggest that HIV-specific CD4⁺ T cell responses may be associated with a reduced risk of HIV infection (5). However, other reports have identified HIV-specific CD4⁺ T cell responses in subjects with progressive disease but have not observed a significant relationship between the magnitude of these responses and control of viremia (6, 7). Therefore, the significance of virus-specific CD4⁺ T cell responses in HIV-1 infection and the factors contributing to progression to AIDS remain unclear. Moreover, HIV-specific CD4⁺ T cells are preferentially infected by HIV and, in the context of structured treatment interruptions, may serve as a reservoir for ongoing HIV replication (8).

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Although emerging data indicate a significant role for both CD4⁺ and CD8⁺ T lymphocytes in determining viral set point in HIV-1 infection (9), it is difficult to draw definitive conclusions from these studies.

The dramatic ability of live attenuated mutants of simian immunodeficiency virus (SIV) to induce protection against subsequent challenge with pathogenic strains of SIV (10–13) has provided a valuable model to examine potential mechanisms responsible for protective immunity (14). Several studies have demonstrated that animals vaccinated with live attenuated SIV develop neutralizing antibodies (10, 12), a relatively strong CTL response (15) associated with the production of soluble antiviral factors (16) and the induction of a vigorous and sustained virus-specific T helper response (17). However, the relative roles of each of these responses in protective immunity induced by live attenuated SIV remain poorly understood.

The objective of this study was to characterize in detail the phenotype and function of SIV-specific CD4⁺ T cell responses in rhesus macaques chronically infected with live attenuated or pathogenic SIV strains. Using optimized conditions described previously for the detection of SIV-specific CD4⁺ T lymphocytes (18), we examined the phenotype, cytokine expression, and functional characteristics of SIV-specific CD4⁺ T cells from animals infected with attenuated SIV compared with wild-type SIV-infected animals. Our data demonstrate clear differences in the maturation of SIV-specific CD4⁺ T cell responses between macaques infected with attenuated or pathogenic SIV strains. Macaques chronically infected with attenuated SIV had a relatively high frequency virus-specific CD4⁺ T cell response with an effector–memory phenotype, whereas monkeys chronically infected with wild-type SIV had a low frequency response with a less mature phenotype. Moreover, a subset of SIV-specific CD4⁺ T cells in attenuated virus-infected animals possessed the potential for direct effector function, as indicated by the up-regulation of the lysosomal membrane protein CD107a and the presence of perforin.

RESULTS

Detection of high frequency SIV Gag-specific CD4⁺ T lymphocyte responses in rhesus macaques with chronic attenuated SIV infection

Our initial studies focused on rhesus macaques that had been chronically infected for 13–15 yr with attenuated SIV Δ nef (Table S1, available at <http://www.jem.org/cgi/content/full/jem.20060134/DC1>) and had previously been shown to have strong SIV-specific CD4⁺ T cell proliferative responses (17). SIV-specific CD4⁺ T cell responses were quantitated by intracellular cytokine staining (ICS) using a protocol optimized for macaques (18). Representative responses of CD4⁺ T cells to stimulation with SIV Gag protein are shown in Fig. 1 A. Using this assay, we found high frequencies of SIV p55 Gag-specific CD4⁺ T cells secreting TNF- α , IFN- γ or IL-2 (mean = 6.7, 4.6, and 1.2%, respectively); however, no significant secretion of IL-4 or IL-10 was detected in any

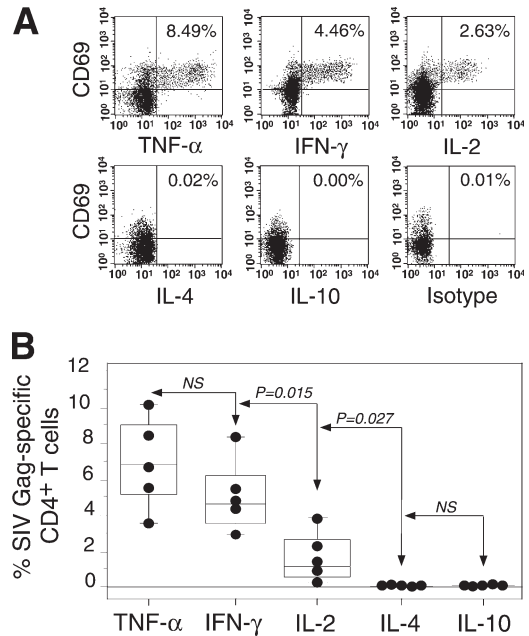


Figure 1. Detection of SIV Gag-specific CD4⁺ T cells in rhesus macaques immunized with SIV Δ nef. (A) Representative ICS data for the analysis of cytokine production by CD3⁺CD4⁺ T cells from an SIV Δ nef-immunized animal (71.88) after SIV p55 stimulation. The percentage of CD4⁺ T lymphocytes coexpressing CD69 and the appropriate intracellular cytokine is shown in the top right quadrant of each plot. These data are representative of results generated from five SIV Δ nef-infected macaques, each tested in at least 10 independent experiments. (B) The frequency of responding CD4⁺ T cells from SIV Δ nef-infected animals after SIV p55 stimulation. p-values were determined by the non-parametric Mann-Whitney U test. NS, not significant.

SIV Δ nef-infected macaque (mean = 0.024%; Fig. 1 A). As expected, <0.03% of CD4⁺ T cells from SIV-seronegative uninfected macaques responded to SIV p55 with any of the cytokines tested (18 and not depicted). In all SIV Δ nef-immunized animals, the frequency of SIV Gag-specific CD4⁺ T cells secreting TNF- α or IFN- γ was four- to sixfold higher than the frequency of SIV Gag-specific CD4⁺ T cells secreting IL-2 ($P \leq 0.015$; Fig. 1 B).

Correlation between the magnitude of SIV Gag-specific CD4⁺ T cell responses and the degree of attenuation of the SIV strain

We next examined the relationship between the frequency of SIV Gag-specific CD4⁺ T cell responses and the degree of attenuation of the SIV strains. Several different groups of macaques were studied according to their SIV infection status, including animals chronically infected with live attenuated SIV Δ nef ($n = 5$), SIV Δ 3 ($n = 6$), or SIV Δ 4 ($n = 3$) (listed in increasing order of the degree of attenuation; Table S1), and uninfected control animals ($n = 6$).

The frequency of CD4⁺ T lymphocytes expressing either IFN- γ or TNF- α in response to Gag was highest in animals immunized with SIV Δ nef, with frequencies up to

8.2 and 10.1%, respectively (Fig. 2 A). Although the frequency of TNF- α -secreting SIV-specific CD4⁺ T lymphocytes exceeded the frequency of SIV-specific IFN- γ -secreting CD4⁺ T lymphocytes in most animals, the production of both cytokines was highly correlated ($R^2 = 0.97$; Fig. 2 B, right), and >65% of SIV-specific TNF- α -secreting CD4⁺ T lymphocytes also coexpressed IFN- γ (Fig. 2 B, left, and not depicted). In addition, when we compared the frequencies of SIV-specific CD4⁺ T lymphocytes expressing IFN- γ and TNF- α induced by different strains of attenuated SIV, we found an inverse relationship between the degree of attenuation of the SIV strains and the magnitude of SIV-specific CD4⁺ T lymphocyte responses (Mann-Whitney U test; $P \leq 0.025$ for all comparisons between vaccinated groups; Fig. 2 A).

We also examined the frequency of SIV Gag-specific CD4⁺ T cell responses in four SIV Δ nef-immunized macaques by the TNF- α ICS assay over a period of 5 yr. During this time, Gag-specific CD4⁺ T cell responses were analyzed using cells stimulated with either recombinant p55 or overlapping peptide pools corresponding to the Gag protein. Although the observed frequency of Gag-specific CD4⁺ T cells was slightly lower after stimulation with Gag peptides than with p55, the results of both were highly correlated (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20060134/DC1>). Regardless of the mode of stimulation, we observed that the levels of Gag-specific CD4⁺ T cells in all four Δ nef-infected animals slowly decayed over time ($T_{1/2} = 3.78$ yr), despite consistently undetectable plasma viremia (SIV RNA < 120 copies/ml; Fig. 2 C, left) and normal CD4⁺ T cell counts (mean = 950/mm³; Fig. 2 C, right).

Breadth of SIV-specific CD4⁺ T cell responses in macaques infected with SIV Δ nef

To determine recognition of multiple SIV proteins by CD4⁺ T cells, PBMCs isolated from macaques infected with SIV Δ nef were stimulated with overlapping peptide pools corresponding to the full sequence of SIVmac239 proteins (18). Significant TNF- α production in response to multiple SIV peptide pools was observed in peripheral blood CD4⁺ T lymphocytes from SIV Δ nef-immunized animals (Fig. 3 A). High frequencies of CD4⁺ T cell responses to Gag were observed in all SIV Δ nef-immunized animals (Fig. 3 B) and ranged between 0.85 and 3.82% (mean \pm S.D., 1.72 \pm 1.1%) of all CD4⁺ T lymphocytes. The dominant target proteins for SIV-specific CD4⁺ T lymphocytes from SIV Δ nef-vaccinated animals were Gag and Env (Fig. 3 B). Similar results were also found using PBMCs isolated from five animals infected with SIVmac Δ 3 (not depicted). However, in each Δ nef-vaccinated animal two to four additional SIV-specific peptide pools were found to elicit CD4⁺ T cell responses (generally Rev, Vif, Vpx, and Pol). Occasional low frequency CD4⁺ T cell responses to the Nef peptide pool (ranging from 0.09 to 0.15%) were observed in some SIV Δ nef-immunized animals (Fig. 3 B). Epitope mapping of these CD4⁺ T cell responses demonstrated recognition of

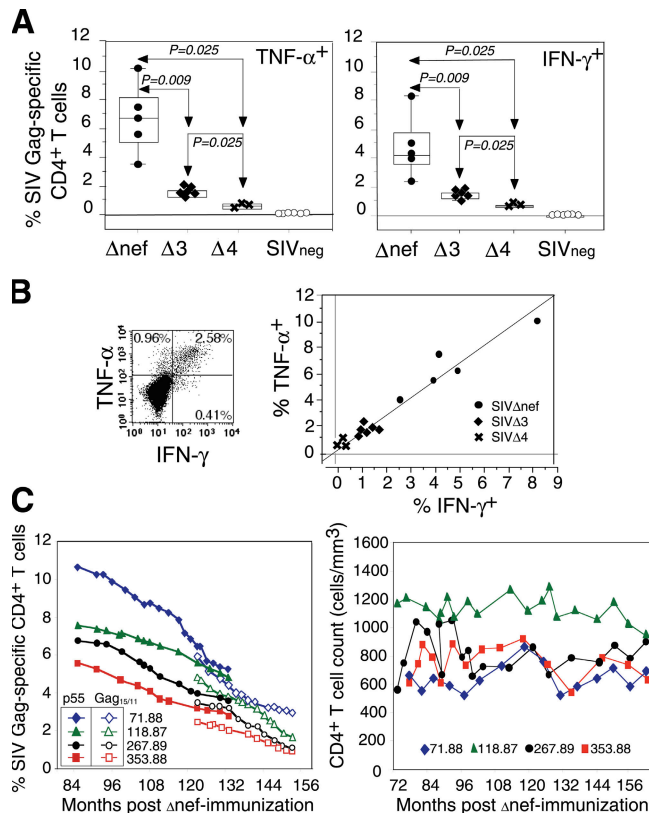


Figure 2. The frequency of SIV Gag-specific CD4⁺ T lymphocytes varies with the degree of attenuating of the vaccine strain and the time since immunization. (A) Percentage of Gag-specific CD4⁺ T lymphocytes coexpressing CD69 and TNF- α (top left) or IFN- γ (top right). p -values were determined by the nonparametric Mann-Whitney U test. (B) Positive correlation between the percentage of SIV Gag-specific CD4⁺ T lymphocytes secreting TNF- α or IFN- γ among individual animals. A representative dot plot gated on CD4⁺ T lymphocytes is shown (left; 397.88). All percentages shown have had background staining of unstimulated PBMCs activity subtracted. (C) Longitudinal analysis of SIV Gag-specific CD4⁺ T cell responses and absolute CD4⁺ T cell counts in four SIV Δ nef-vaccinated macaques. SIV-specific CD4⁺ T cell responses were evaluated after stimulation with SIV Gag p55 protein (filled symbols) or SIV Gag_{15/11} peptide pool (open symbols).

epitopes contained in the NH₂-terminal 57 amino acids of Nef proximal to the deletion in SIV Δ nef (unpublished data). The sum of all SIV-specific CD4⁺ T cell responses in Δ nef-vaccinated animals ranged from 2.8 to 6.4% and thus constituted a significant portion of all CD4⁺ T cells. Based on the immunodominance of the SIV Gag protein, as has been observed for HIV-specific CD4⁺ T cell responses in humans (19), our subsequent studies of SIV-specific CD4⁺ T cell responses focused on Gag.

Detection of SIV Gag-specific CD4⁺ T cell responses in rhesus macaques with wild-type SIV infection: association with plasma SIV RNA

To provide a point of reference for interpretation of our data on SIV-specific CD4⁺ T cells in macaques chronically infected

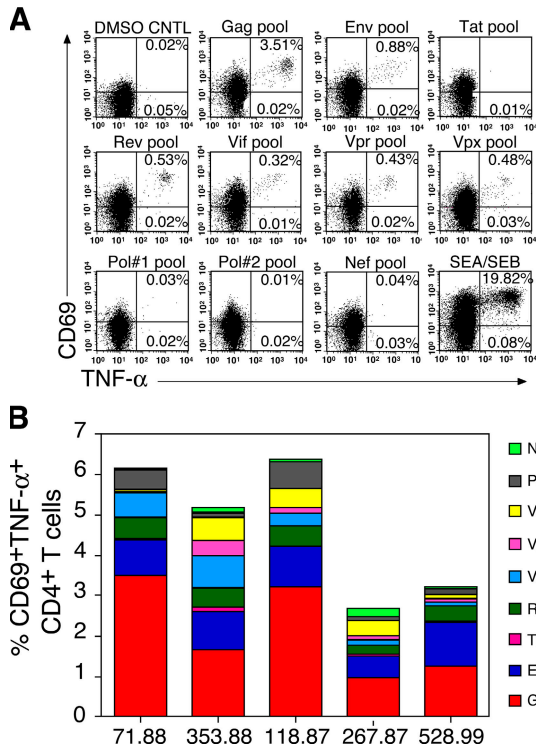


Figure 3. Breadth of SIV-specific CD4⁺ T cell responses in SIVΔnef-infected macaques. (A) Representative dot plots of SIV-specific CD4⁺ T cell responses (macaque 71.88) to multiple SIV overlapping 15-mer peptide pools corresponding to the full predicted sequence of the SIVmac239 proteome. DMSO alone and the superantigen SEB served as negative and positive controls, respectively. All plots were gated on CD4⁺ T lymphocytes. (B) Responses to the indicated peptide pools are shown for each SIVΔnef-infected animal. These data are representative of results of two independent experiments for each of the five SIVΔnef-infected macaques studied.

with attenuated SIV strains, we also investigated responses in animals infected with wild-type SIVmac239 for 9–12 mo. Using our optimized ICS assay, we identified SIV Gag-specific CD4⁺ T lymphocytes after stimulation with Gag peptides in most wild-type SIV-infected animals (Fig. 4 A, top) at frequencies ranging from 0.09 to 0.91% (mean = 0.50 ± 0.41%; Fig. 4 B, left). However, when stratified according to viral load, the frequency of Gag-specific TNF-α-expressing CD4⁺ T cells in animals with VL < 5 × 10⁵ copies/ml (mean = 0.58 ± 0.33%; n = 10) was significantly higher than in animals with VL > 5 × 10⁵ copies/ml (mean = 0.27 ± 0.16%; n = 12; P < 0.023). In comparison, the magnitude of SIV-specific CD4⁺ T cells in SIVmacΔnef-immunized animals was significantly higher than in wild-type SIV-infected macaques with VL < 5 × 10⁵ or VL > 5 × 10⁵ copies/ml in response to SIV Gag peptide pool stimulation (P < 0.0007 and P < 0.0001, respectively; Fig. 4 B, left). Similar results were found after stimulation with superantigen (Fig. 4 B, right), a finding that may reflect the depletion of memory CD4⁺ T cells (and resulting enrichment of naive CD4⁺ T cells) in SIV-infected macaques (20) and the fact that naive

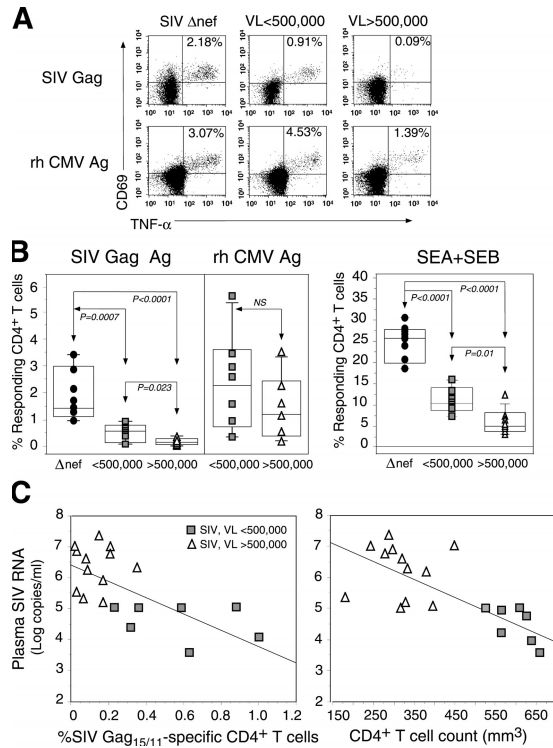


Figure 4. Cross-sectional analysis of the frequency of SIV Gag-specific and CMV-specific CD4⁺ T lymphocytes in wild-type SIV-infected compared with SIVΔnef-infected macaques. (A) Representative dot plots gated on CD4⁺ T lymphocytes from wild-type SIV-infected macaques with plasma viral RNA < 5 × 10⁵ copies/ml (macaques 244.99 and 327.98) or > 5 × 10⁵ copies/ml (345.99 and 222.93) and compared with PBMCs isolated from SIVΔnef-infected (71.88 and 267.87) macaques. (B) Cross-sectional analysis of the frequency of SIV-specific CD4⁺ T lymphocytes in SIVΔnef-infected and wild-type SIV-infected animals after SIV Gag_{15/11} peptide pool, rhesus CMV, or SEA plus SEB stimulation. p-values were calculated by the nonparametric Mann-Whitney U test. NS, not significant. (C) Inverse correlation between the magnitude of SIV Gag-specific CD4⁺ T lymphocyte responses and plasma viremia in wild-type SIV-infected animals (left). Correlation of total CD4⁺ T cell counts and plasma viral RNA in wild-type SIV-infected macaques with plasma SIV RNA < 5 × 10⁵ copies/ml (n = 7) and > 5 × 10⁵ copies/ml (n = 12; right). Spearman rank correlation coefficients (Rho) and p-values were calculated for all SIV-infected macaques for plasma SIV RNA versus SIV Gag-specific CD4⁺ T cell frequencies (Rho = -0.815; P = 0.0008), and for plasma SIV RNA versus absolute CD4⁺ T cell counts (Rho = -0.646; P = 0.0074).

CD4⁺ T cells produce substantially less TNF-α and IFN-γ after superantigen stimulation (21). However, depletion of memory CD4⁺ T cells in wild-type-infected macaques did not account for the progressive decrease in SIV-specific CD4⁺ T cells in wild-type-infected animals shown in Fig. 4 B (left). A similar relationship was observed when the frequency of SIV-specific CD4⁺ T cells was expressed as a percentage of memory CD4⁺ T cells (Fig. S2 A, available at <http://www.jem.org/cgi/content/full/jem.20060134/DC1>).

We next investigated if the decreased frequency of SIV Gag-specific CD4⁺ T cells observed in wild-type SIV-infected

macaques with $VL > 5 \times 10^5$ copies/ml was limited to SIV or reflected a generalized depletion of antigen-specific $CD4^+$ T lymphocytes. We therefore performed a cross-sectional analysis of CMV-specific $CD4^+$ T lymphocytes in a subset of SIV-infected animals that were CMV seropositive with plasma viral RNA $> 5 \times 10^5$ copies/ml or $< 5 \times 10^5$ copies/ml (Fig. 4 A, bottom). CMV-specific $CD4^+$ T lymphocytes were detected in all wild-type SIV-infected animals at frequencies ranging from 0.51 to 5.53% ($1.87 \pm 1.43\%$; $n = 12$; Fig. 4 B, right). However, no significant difference in the frequency of CMV-specific $CD4^+$ T cell responses was observed between wild-type-infected animals with high and low viral loads (Fig. 4 B). Therefore, SIV-specific but not CMV-specific $CD4^+$ T cell responses appear to be preferentially lost during chronic SIV infection in rhesus macaques that have not yet progressed to clinical simian AIDS.

We next examined the relationship between the total SIV Gag-specific $CD4^+$ T cell frequency and plasma SIV RNA loads in wild-type SIV-infected macaques. Previous studies with HIV-infected patients suggested that there is an inverse correlation between HIV Gag-specific $CD4^+$ T cell responses and HIV viral load (2, 22). However, other studies were unable to detect a correlation between the frequency of responding HIV Gag-specific $CD4^+$ T cells and viral load (6, 7). We found a significant inverse relationship between the frequency of SIV-specific $CD4^+$ T cell responses and plasma viral RNA load for all SIV-infected animals ($Rho = -0.81$; $P = 0.0008$; Fig. 4 C, left). A similar relationship was observed between the frequency of memory-corrected SIV-specific $CD4^+$ T cells and plasma SIV RNA (Fig. S2 B). As has been observed in HIV-infected subjects (23), there was an inverse relationship between plasma viral RNA and

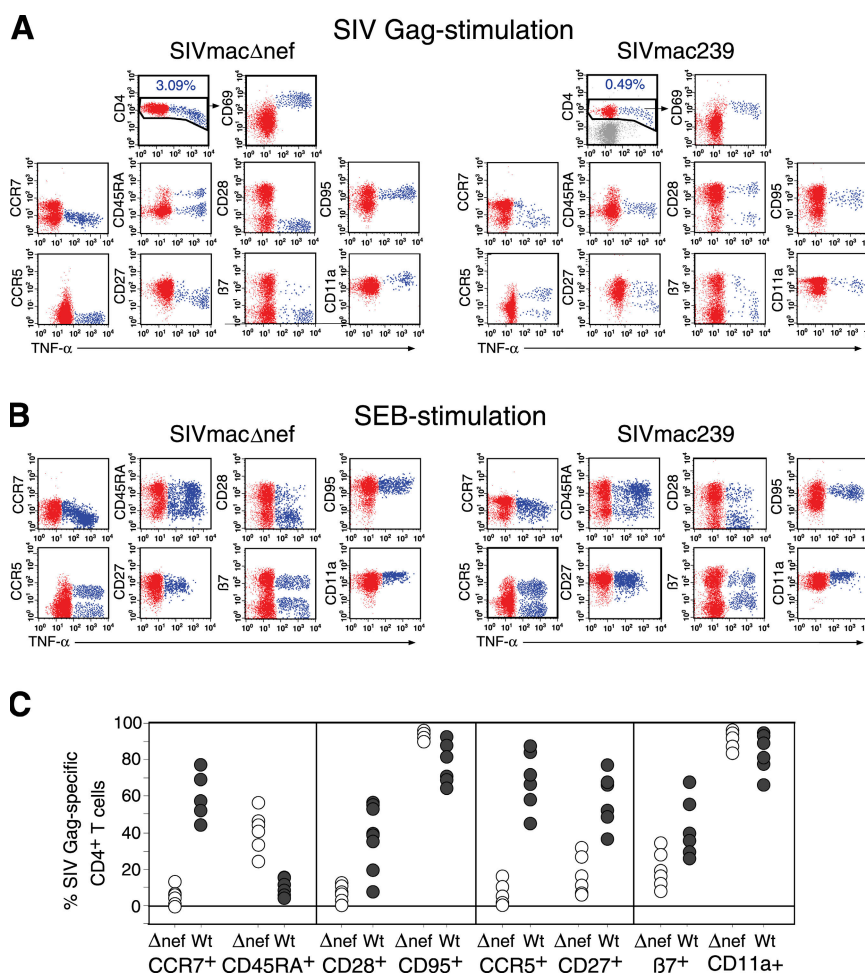


Figure 5. Phenotypic analysis of SIV-specific $CD4^+$ T lymphocytes in SIV Δ nef- and wild-type SIV-infected macaques. Representative dot plots of TNF- α -secreting $CD4^+$ T cells from one SIV Δ nef-immunized macaque (118.87) and one wild-type SIV-infected animal (104.98; $VL < 5 \times 10^5$ copies/ml) are shown. All plots are gated on $CD4^+$ cells after SIV Gag_{15/11} peptide pool stimulation (A) or SEB stimulation (B). The blue-colored population designates $CD4^+$ CD69 $^+$ TNF- α $^+$ cells, and the

percentages are shown in the top dot plot. (C) Comparative analysis of SIV-specific TNF- α -secreting $CD4^+$ lymphocytes expressing the indicated phenotypic marker, including SIV Δ nef-infected macaques (\circ ; $n = 6$) and wild-type SIV-infected animals with plasma viral RNA $< 5 \times 10^5$ copies/ml (\bullet ; $n = 6$). These results reflect data generated from three independent experiments performed on all 12 animals.

absolute CD4⁺ T cell counts in wild-type SIV-infected macaques (Fig. 4 C, right).

Phenotypic differences in SIV-specific CD4⁺ T cells in macaques infected with attenuated or pathogenic SIV strains

Differentiation of antigen-specific CD4⁺ T cell responses can be tracked by analysis of cell surface molecules such as CD28, CD45RA, and CCR7 (24–26). We analyzed the expression of a panel of phenotypic markers on SIV-specific CD4⁺ T cells defined by ICS in rhesus macaques infected with SIV Δ nef or SIVmac239. To ensure a sufficient population of SIV-specific CD4⁺ T cells for comparative analysis, we focused our analysis on wild-type SIV-infected animals with plasma viral RNA < 5 × 10⁵ copies/ml, CD4 counts > 500/mm³, and no sign of disease progression for a period of 3 mo before and after our study (Table S2, available at <http://www.jem.org/cgi/content/full/jem.20060134/DC1>).

As illustrated in Fig. 5 (A, left, and C), SIV-specific CD4⁺ T lymphocytes from Δ nef-vaccinated animals were predominantly CD27^{-/low}CD28⁻CCR5⁻CCR7⁻ and largely β 7 integrin^{-/low} but CD95⁺ and CD11a⁺ with variable CD45RA expression, consistent with a late effector–memory CD4⁺ T cell subset (Fig. 5 A, left). Repeated analysis of SIV Δ nef-infected animals for up to 2 yr revealed no significant change in the phenotype of these cells (not depicted). SIV-specific CD4⁺ T lymphocytes from wild-type SIV-infected macaques, however, exhibited an intermediate memory phenotype that maintained expression of CD28 and CD27, were typically β 7 integrin^{-/low} and mostly CCR5⁺, but were CD45RA^{-/low} with variable expression of CCR7 (Fig. 5, A, right, and C). Little to no difference was seen for the expression of CD95 and CD11a because SIV-specific CD4⁺ T lymphocytes in both animal groups were CD95⁺ and CD11a⁺ (Fig. 5, A and C). In contrast, staphylococcal enterotoxin B (SEB) stimulation elicited high frequency TNF- α production by CD4⁺ T lymphocytes in SIV Δ nef-vaccinated animals that were exclusively CD95⁺ but variable with respect to CD28, CD45RA, CCR5, and β 7 integrin expression (Fig. 5 B).

A summary of the phenotype of SIV-specific CD4⁺ T cells in Δ nef and wild-type SIV-infected macaques is presented in Fig. 5 C. These results clearly demonstrate that SIV-specific CD4⁺ T cells exhibit a more fully differentiated effector–memory phenotype during chronic infection with attenuated SIV Δ nef compared with the early intermediate memory phenotype observed in chronic wild-type SIVmac239 infection.

Identification of SIV-specific CD4⁺ T lymphocytes that degranulate in response to antigenic stimulation

Several recent reports have identified subsets of virus-specific CD4⁺ T cells with cytolytic function and provided evidence that this population of effector CD4⁺ T cells may play a role in the control of viral infections, including HIV (27, 28). We therefore examined if SIV-specific CD4⁺ T

cells could degranulate in response to SIV-specific stimulation. By measuring the mobilization of cytolytic granule membrane proteins (i.e., CD107a) to the cell surface (29), we were able to detect SIV-specific CD4⁺ T cell degranulation by flow cytometry.

We analyzed SIV-specific CD4⁺ T lymphocyte responses in SIV Δ nef-infected animals by examining cell surface expression of CD107a in conjunction with intracellular TNF- α production after Gag peptide pool stimulation. Three distinct populations of SIV-specific CD4⁺ T cells in SIV Δ nef-infected animals could be identified based on expression of CD107a and TNF- α . The majority of CD107a⁺ cells also expressed TNF- α , although a significant minority expressed CD107a alone (Fig. 6, A and B). Thus, a significant subpopulation of SIV-specific CD4⁺ T cells in SIV Δ nef-infected animals is able to degranulate in response to antigen-specific stimulation.

Perforin⁺ CD4⁺ T lymphocytes are a distinct subset of SIV-specific cells during chronic infection with attenuated or pathogenic SIV strains

To further characterize the subset of SIV-specific CD4⁺ T cells with direct effector function, we also analyzed expression of perforin in CD4⁺ T lymphocytes. Analysis of unstimulated CD4⁺ T lymphocytes from SIV Δ nef-infected animals revealed a significant increase in the frequency of perforin-expressing cells (14.6 ± 2.3%; *n* = 4), compared with normal (6.1 ± 2.0%) and SIV-infected animals (1.9 ± 1.1%; Fig. 7 A). Notably, after stimulation of lymphocytes from SIV Δ nef or wild-type SIV-infected animals with SIV Gag peptides, the frequency of perforin-expressing CD4⁺ T cells decreased to negligible levels, accompanied by the expected increases in TNF- α or CD107a expression (Fig. 7 A), suggesting that the majority of perforin-expressing CD4⁺ T cells in SIV-infected animals was SIV specific.

To more precisely characterize the subset of effector CD4⁺ T cells, we analyzed perforin expression in conjunction with CD45RA and CCR7. Analysis of expression of single phenotypic markers on SIV-specific CD4⁺ T cells

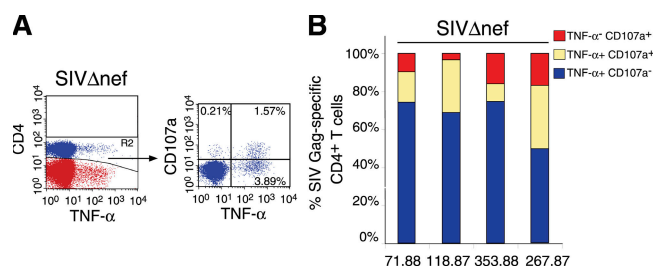


Figure 6. Degranulation of SIV-specific CD4⁺ T cells as assessed by up-regulation of the lysosomal membrane protein CD107a.

(A) Representative dot plots of CD107a and TNF- α expression on CD4⁺ T lymphocytes after SIV Gag peptide pool stimulation in freshly isolated PBMCs from an SIV Δ nef-infected macaque (118.87). The right panel is gated on CD4⁺ T cells. (B) Distribution of CD107a and TNF- α expression on SIV-specific CD4⁺ T cells from SIV Δ nef-infected animals.

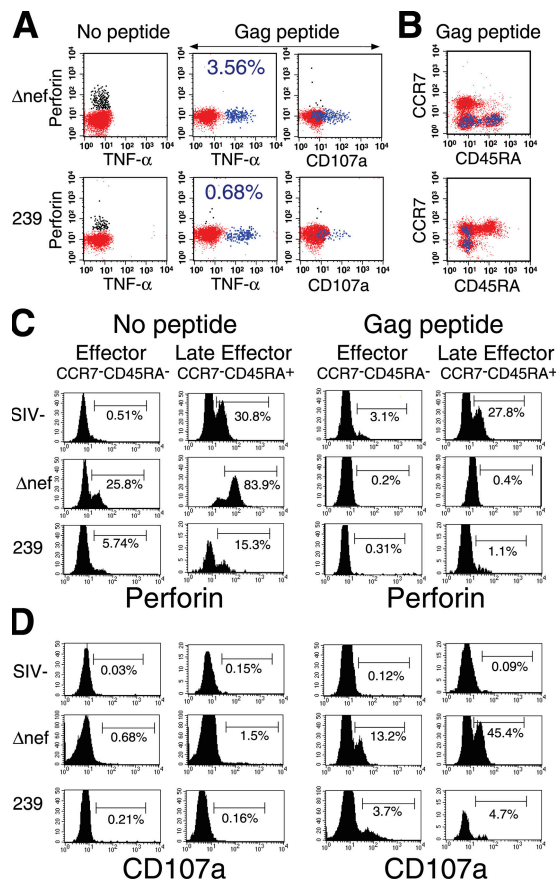


Figure 7. Expression of perforin in subsets of CD4⁺ T lymphocytes from macaques infected with SIV Δ nef or wild-type SIVmac239. (A) Expression of perforin (shown in black) in unstimulated and SIV Gag-stimulated CD4⁺ lymphocytes (118.87 and 397.98). SIV-specific CD4⁺ T cells identified on the basis of TNF- α secretion are displayed in blue. The total percentage of TNF- α -secreting CD4⁺ lymphocytes is shown. (B) Expression of CD45RA and CCR7 on SIV-specific CD4⁺ lymphocytes. PBMCs from SIV Δ nef- (118.87) or SIVmac239- (327.98) infected animals were stimulated with SIV Gag peptides, and antigen-specific cells (shown in blue) were identified based on TNF- α secretion. The remaining CD4⁺ lymphocytes are displayed in red. (C) Loss of perforin expression in subpopulations of CD4⁺ lymphocytes after SIV stimulation. (D) Up-regulation of CD107a in subpopulations of CD4⁺ lymphocytes after SIV Gag peptide stimulation. Data are representative of four uninfected (SIV-), four SIV Δ nef, and four wild-type SIVmac239-infected animals.

described in Fig. 5 suggested that the majority of SIV-specific CD4⁺ T cells in SIV Δ nef-infected animals was either CCR7⁻CD45RA⁻ or CCR7⁻CD45RA⁺, a conclusion confirmed by ICS assays conducted with simultaneous analysis of both markers (Fig. 7 B). We therefore analyzed perforin expression in subsets of CD4⁺ T cells defined by CCR7 and CD45RA expression before and after SIV stimulation. The relatively high levels of perforin expression in CCR7⁻CD45RA⁻ and CCR7⁻CD45RA⁺ CD4⁺ T cells (26 and 84%, respectively) declined precipitously after SIV-specific stimulation (compare the left with the right panels in Fig. 7 C). The decrease in perforin expression in these subsets

was accompanied by an increase in CD107a expression, although to a lesser extent (Fig. 7 D), again confirming the significant enrichment of CD4⁺ T cells with effector function in the CCR7⁻CD45RA⁻ and CCR7⁻CD45RA⁺ subsets in SIV Δ nef-infected animals.

Preferential trafficking of SIV-specific CD4⁺ T cells in attenuated SIV-infected animals to extralymphoid sites

Central memory T cells preferentially circulate to secondary lymphoid tissues, whereas effector–memory T cells traffic through extralymphoid tissues (24). To determine if the effector–memory phenotype of SIV-specific CD4⁺ T cells in attenuated SIV-infected animals predicted preferential localization of these cells to extralymphoid sites, we compared the frequency of SIV-specific CD4⁺ T cells in peripheral blood, peripheral LNs, and bronchoalveolar lavage (BAL). The SIV Gag-specific response was assessed by examination of CD107a and TNF- α expression in CD4⁺ T lymphocytes after Gag peptide pool stimulation (Fig. 8 A). The total SIV Gag-specific response was calculated by combining the percentage of CD107⁺TNF- α ⁻, CD107⁺TNF- α ⁺, and CD107⁻TNF- α ⁺ CD4⁺ T lymphocytes. As compared with peripheral blood, the average frequency of SIV-specific CD4⁺ T lymphocytes was decreased eightfold in LNs and increased twofold in BAL from attenuated SIV-infected macaques (Fig. 8 B). Correction of the frequency of SIV-specific CD4⁺ T cells for the varying percentage of memory T cells in each of these compartments revealed a similar relationship (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20060134/DC1>). Analysis of the range of effector function of SIV-specific CD4⁺ T cells in each of these sites revealed a significant increase in the percentage of TNF- α ⁺ CD107a⁺ cells in peripheral blood, whereas LN and BAL exhibited an increased percentage of TNF- α ⁻ CD107a⁺ virus-specific CD4⁺ T cells (Fig. 8 C). Thus, analysis of extralymphoid tissues revealed an enrichment of SIV-specific CD4⁺ T cells as compared with LN or peripheral blood and a shift in the range of effector functions.

DISCUSSION

CD4⁺ T cells are central to the development, interaction, and regulation of both antibodies and CD8⁺ T cell responses to viral infections (1); however, the relationship of virus-specific CD4⁺ T cell responses to control of HIV/SIV infection remains poorly understood. Efforts to understand the relationship between HIV-specific CD4⁺ T cell responses and control of viremia in infected patients have led to discrepant results (2, 6, 7, 22), in part due to the different techniques used by these groups to quantitate virus-specific T cell responses. Several factors inherent in the study of HIV-infected subjects may confound the ability to determine the relationship between virus-specific CD4⁺ T cell responses and viral suppression, including the heterogeneity of infecting strains (leading to underrepresentation of type-specific responses and uncertainty as to the replicative fitness of the HIV-1 strain infecting a given patient), as well as the effects

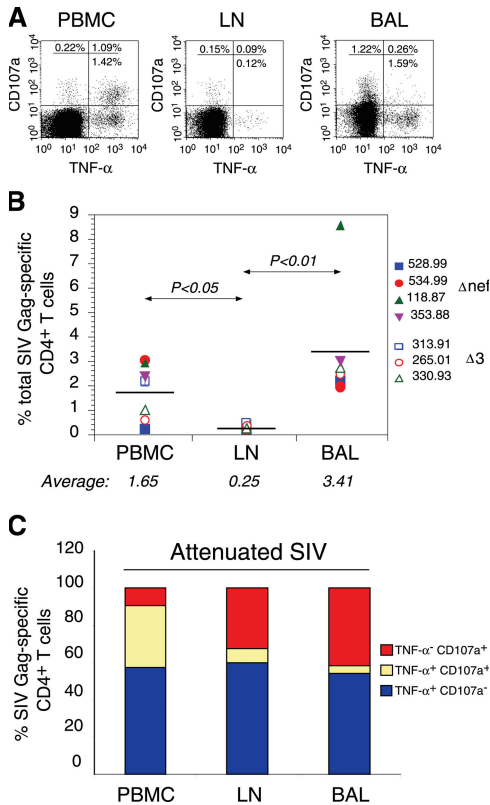


Figure 8. Frequency and effector function of SIV-specific CD4⁺ T lymphocytes in PBMCs, LNs, and lung lavage. (A) Representative dot plots of CD107a versus TNF- α expression on CD4⁺ T lymphocytes after SIV Gag peptide pool stimulation of lymphocytes from an SIV Δ nef-infected animal (118.87). (B) Total SIV-specific CD4⁺ T cells isolated from peripheral blood, LNs, and BALs from SIV Δ nef- and SIV Δ 3-infected macaques. The total Gag-specific response was calculated by combining the percentage of CD107⁺TNF- α ⁻, CD107⁺TNF- α ⁺, and CD107⁻TNF- α ⁺ CD4⁺ T lymphocytes. (C) Distribution of CD107a and TNF- α expression on SIV-specific CD4⁺ T cells isolated from various lymphoid and nonlymphoid compartments in attenuated SIV Δ nef- ($n = 4$) and SIV Δ 3- ($n = 3$) infected macaques. The decrease in the frequency of TNF- α ⁺CD107a⁺ cells between PBMCs and LNs or BAL is significant ($P < 0.05$; Kruskal-Wallis with Dunn's multiple comparisons test).

of antiretroviral therapy. Studies in macaques infected with SIV can address many of these shortcomings, permitting analysis of immune responses using homologous sequences in animals infected with viruses of known and variable pathogenicity without the confounding effects of antiretroviral therapy.

Our present findings confirm and extend our previous findings of relatively strong SIV-specific CD4⁺ T cell responses in macaques vaccinated with live attenuated SIV strains (17). However, by the use of an optimized ICS technique (18) and examination of responses to all SIV proteins, we now show that this response is much higher than previously appreciated. The total magnitude of SIV-specific CD4⁺ T cell responses can represent up to 4–10% of all CD4⁺ T cells. This high frequency response stands in contrast to that

observed for many viruses such as mumps, influenza, and vaccinia that are characterized by clearance of the infection (30–32). The magnitude of the virus-specific CD4⁺ T cell response in SIV Δ nef-infected animals is comparable to that observed in CMV-infected humans (32) and macaques (33), an observation that likely reflects ongoing low level antigenic stimulation in animals chronically infected with SIV Δ nef despite undetectable levels of plasma viremia. Although several of the attenuated SIV-infected macaques used in this study had been challenged with wild-type SIV, no difference in the frequency or phenotype of SIV-specific CD4⁺ T cells was observed between challenged and unchallenged animals (not depicted) and no evidence for wild-type SIV replication was evident at the time of study. The strength of the SIV-specific CD4⁺ T cell response in Δ nef-infected animals is also reflected by the breadth of the response, with an average of four different proteins recognized by each animal. The presence of a high frequency of virus-specific CD4⁺ T cells in SIV Δ nef-infected macaques is analogous to that reported in HIV-infected long-term nonprogressors (2, 34), although this finding has not been confirmed by all groups (6) and is confounded by the effects of viremia on proliferative responses in vitro (35). Several groups have failed to observe any significant correlation between the frequency of HIV-specific CD4⁺ T cell responses and control of viremia (6, 7). In contrast, we observed a clear, statistically significant inverse correlation between the magnitude of SIV Gag-specific CD4⁺ T cell responses and plasma viremia in wild-type SIV-infected animals, although we cannot determine whether SIV-specific CD4⁺ T cell responses represent an independent correlate of control of viremia or reflect an immune response that is lost with increasing viral replication. Finally, SIV-specific CD4⁺ T cell responses in Δ nef-infected animals were also distinguished by their ability to produce IL-2, whereas we observed no significant IL-2 production by SIV-specific CD4⁺ T cells from wild-type SIV-infected macaques (not depicted). These observations are consistent with those in HIV-infected patients that have shown that IL-2 production by HIV-specific CD4⁺ T cells is lost in the presence of ongoing viremia (36, 37).

Our data also provide insight into the maturation of HIV and SIV-specific CD4⁺ T cell responses. As assessed by analysis of multiple specific markers, SIV-specific CD4⁺ T cells in macaques infected with attenuated SIV strains display a late effector–memory phenotype, as demonstrated by the lack of expression of CD27, CD28, and CCR7, and by the presence of a subset of cells that are CCR7⁻ and CD45RA⁺. Consistent with an effector–memory phenotype, SIV-specific CD4⁺ T cells in attenuated SIV-infected animals were increased in frequency in lung lavage and decreased in LNs as compared with peripheral blood. In contrast, SIV-specific CD4⁺ T cell responses in wild-type SIV-infected animals displayed a less differentiated central memory phenotype that maintained expression of CD27, CD28, and CCR7 but lacked expression of CD45RA. The relative immaturity of SIV-specific CD4⁺ T cells appears similar to that previously reported in

an analysis of HIV-specific CD4⁺ T lymphocytes that focused primarily on expression of CD27 and CD28 (25). Interestingly, SIV-specific CD4⁺ T cells in wild-type SIV-infected animals maintained expression of CCR5, suggesting that these cells would be readily susceptible to SIV infection, as has been reported for HIV-specific CD4⁺ T cells (8). In contrast, SIV-specific CD4⁺ T cells in SIV Δ nef-vaccinated animals were CCR5⁻, suggesting that after pathogenic challenge these cells would be relatively resistant to SIV infection. Many factors may contribute to the apparent paradox of the phenotypic immaturity of SIV-specific CD4⁺ T cells in wild-type-infected animals that are chronically stimulated with large amounts of antigen. Direct infection of virus-specific CD4⁺ T cells, as previously reported for HIV (8), may play a dominant role. In this scenario, virus-specific CD4⁺ T cells in wild-type-infected animals would die a premature death, whereas CD4⁺ T cell differentiation would be allowed to proceed along a normal path in SIV Δ nef-infected animals. Alternatively, defects in antigen presentation and costimulation as well as adverse effects of chronic high level antigenic stimulation may also play a role.

Interestingly, longitudinal analysis of Gag-specific CD4⁺ T cell responses in four SIV Δ nef-infected macaques over a five-year period showed a progressive decline. Whether this reflects selective loss of SIV-specific CD4⁺ T cell responses, decreased antigenic stimulation, or a normal process of senescence of antigen-specific CD4⁺ T cell responses over extended periods of time is unknown. Slow declines in the frequency of virus-specific CD4⁺ T cell responses have been reported for measles in humans (38) and lymphocytic choriomeningitis virus in mice (39), even when stable frequencies of virus-specific CD8⁺ T cells have been observed.

Although CD4⁺ T lymphocytes have traditionally been viewed as helper cells that mediate their effect on infectious pathogens through other lymphocyte populations, emerging evidence suggests that under certain conditions CD4⁺ T cells may mediate direct antiviral effects. In murine models, evidence for the ability of virus-specific CD4⁺ T cells to directly suppress viral replication, either by cytokine secretion or direct cytolysis, has been obtained for several viruses, including the γ -herpesvirus MHV-68 and Friend murine leukemia virus (40, 41). Expansion of perforin- or granzyme-expressing CD4⁺ T cells has been described in humans infected with HIV, EBV, or CMV (27, 28). Our data suggest that a significant subset of SIV-specific CD4⁺ T cells in Δ nef-vaccinated animals is likely to possess direct cytolytic activity, as indicated by their antigen-specific up-regulation of the lysosomal membrane protein CD107a and loss of perforin expression. Additional evidence for the cytolytic activity of SIV-specific CD4⁺ T cells comes from the observation that CD4⁺ T cell lines obtained by stimulation with an MHC class II-restricted peptide are able to lyse class II-expressing B cell lines sensitized with the cognate peptide (unpublished data). In light of the ability of HIV and SIV to evade CD8⁺ T cell responses by Nef-mediated down-regulation of MHC class I molecules (42), generation of virus-specific CD4⁺ T cells with cytolytic

function may be particularly advantageous, especially in targeting MHC class II-expressing reservoirs such as activated CD4⁺ T cells, macrophages, and dendritic cells.

The identification of a previously uncharacterized virus-specific immune response in SIV Δ nef-vaccinated animals may have implications for our understanding of protective immunity induced by live attenuated SIV. Previous efforts to define mechanisms of immune protection mediated by live attenuated SIV strains have focused primarily on CD8⁺ T lymphocytes and neutralizing antibodies (11, 13, 43). Several of these studies have provided evidence for involvement of both CD8⁺ T lymphocyte responses and neutralizing antibody responses in protection mediated by attenuated SIV (12, 13, 43), but analysis of these responses alone does not appear to account for the substantial protection induced by SIV Δ nef (11, 44). As noted above, the induction of virus-specific CD4⁺ T cells with direct effector function may preferentially emerge under conditions of chronic antigenic stimulation such as those provided by infection with SIV Δ nef. As such, SIV-specific CD4⁺ T cells with effector function may represent a distinct feature of antiviral immune responses induced by live attenuated SIV infection that may play an important role in mediating immune protection. Additional studies to define the kinetics of induction of this response after infection with SIV Δ nef and to correlate this response with protection will be necessary to better understand the role that effector CD4⁺ T lymphocytes play in protective immunity.

MATERIALS AND METHODS

Animals. Animals were housed in the biocontainment facilities of the New England Primate Research Center (NEPRC) and were maintained in accordance with the guidelines of the local institutional animal use committees and the Department of Health and Human Services (DHHS) Guide for the Care and Use of Laboratory Animals. 59 rhesus macaques were selected for this study, including animals infected with live attenuated SIV ($n = 16$; Table S1), pathogenic SIV strains ($n = 35$; Table S2), and uninfected macaques as negative controls ($n = 8$). Macaques vaccinated with attenuated SIV strains had been infected for 5–15 yr with SIVmac239 Δ nef ($n = 7$) (10), SIVmac239 Δ 3 ($n = 6$) (45), or SIVmac239 Δ 4 ($n = 3$) (45). A subset of these animals had been challenged 7–12 yr before our study with SIVmac239 or SIVmac251 but remained healthy without evidence of wild-type SIV infection or progression to simian AIDS. Selection criteria for wild-type SIV-infected macaques included chronic infection for 9–12 mo and no symptoms of simian AIDS for 3 mo before and after our analysis. When indicated, animals were screened for naturally acquired CMV infection by testing sera for antibodies to rhesus CMV by ELISA, as described previously (33).

mAbs. mAbs were obtained from Becton Dickinson, Caltag Laboratories, DakoCytomation, or Immunotech and were conjugated with FITC, PE, peridinin chlorophyll protein, or allophycocyanin. A detailed list of specific mAbs is provided in Table S3. Anti-CCR7 was used as a purified antibody, which was then bound with a biotin-conjugated rat anti-mouse IgM mAb (clone R6-60.2; BD Biosciences) and detected with streptavidin peridinin chlorophyll protein (BD Biosciences). CD28 and CD49d costimulation for ICS assays was performed as described previously (18).

Antigens and peptides. Antigens used for CD4⁺ T lymphocyte stimulation included recombinant SIV p55 Gag protein derived from SIVmac239 and VSV-nucleocapsid (VSV-N) (17). SIV peptide pools used in our experiments

consisted of 20-mer peptides overlapping by 10 amino acids corresponding to the SIVmac239 p55 Gag sequence and 15-mer peptides overlapping by 11 amino acids corresponding to the SIVmac239 Gag, Env, Tat, Rev, Nef, Vif, Vpr, Vpx, and Pol sequences. The Nef and Pol peptides were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH). All other peptides were synthesized by A. Khatri (Massachusetts General Hospital/Peptide Core Facility, Boston, MA). Peptides were reconstituted at 100 mg/ml in DMSO (Sigma-Aldrich) and combined in a pool such that each individual peptide was equally represented in the pool. For superantigen stimulation, staphylococcal enterotoxin A (SEA) and SEB were used together (100 ng/ml each; Sigma-Aldrich). Rhesus CMV antigen and control antigens were prepared as described previously (33).

Cell processing and phenotypic analysis. Rhesus macaque PBMCs were isolated from heparinized venous blood and resuspended at $1.5\text{--}2.0 \times 10^6$ cells/ml in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS (Sigma-Aldrich), 10 mM Hepes, 2 mM L-glutamine, 50 U of penicillin per ml, and 50 μg of streptomycin per ml (R-10 medium). LN cells were isolated from minced LNs, and BAL was performed as described previously (20). Surface staining was performed as described previously (18). Data analysis was performed with CELLQuest (BD Biosciences) or PAINT-A-GATE Plus (BD Biosciences).

ICS analysis. Detection of antigen-specific CD4⁺ T cells was performed as described previously using techniques optimized for rhesus macaque PBMCs (18, 33). In brief, fresh or thawed PBMCs, resuspended in complete R-10 medium, were stimulated for 6 h at 37°C with the appropriate antigen in the presence of cross-linked costimulatory anti-CD28 and anti-CD49d as well as 10 $\mu\text{g}/\text{ml}$ brefeldin A (Sigma-Aldrich) that was added for the last 4.5 h of stimulation (18). Stimulated cells were surface stained for 30 min at 4°C with anti-CD4 mAb and various combinations of mAbs to CD3, CD27, CD28, CD45RA, CD95, β 7 integrin, CCR5, or CD107a. Cells were fixed and permeabilized with successive incubations in FACS Lysing and FACS Permeabilizing solutions (BD Biosciences) and incubated with anti-CD69 and the appropriate anti-cytokine mAbs for 30 min at 4°C. PBMC aliquots unstimulated or stimulated with SEA and SEB for 6 h served as a negative and positive control, respectively. Positive controls for the detection of CD4⁺ T cell production of IL-4 and IL-10 included a 6–8-h stimulation with PMA/ionomycin and Con A, and ranged between 2 and 6%, respectively (not depicted). 200,000 events were collected on a FACSCalibur (BD Biosciences), and the proportion of CD4⁺ or CD3⁺CD4⁺ T cells coexpressing CD69 and the cytokine of interest were determined using CELLQuest or PAINT-A-GATE Plus. Gates for the determination of cytokine staining cells were established after stimulation with control antigen VSV-N or to yield <0.05% cytokine-expressing unstimulated cells. Where indicated, the memory-corrected frequency of SIV-specific CD4⁺ cells was calculated by dividing the frequency of SIV-specific CD4⁺ T cells by the fraction of memory CD4⁺ T cells determined at the same time point (6). The fraction of memory CD4⁺ T cells was calculated as the sum of the CD45RA⁺CCR7⁺, CD45RA⁺CCR7⁻, and CD45RA⁻CCR7⁻ populations. Intracellular staining of perforin, granzyme A, and/or granzyme B versus cell surface markers was similar to cytokine analysis, except that permeabilization was performed in a 1 \times concentration of fixation/permeabilization solution (Caltag Laboratories).

Plasma SIV RNA viral load measurements. Plasma SIV viral RNA levels were measured using a real-time RT-PCR assay as described previously (46, 47).

Statistical analysis. Statistical analysis was performed using StatView (Abacus Concepts, Inc) and Instat (Graphpad Software, Inc.). Tests used included the Wilcoxon Signed Rank test, Spearman rank test, Mann-Whitney U test, and Kruskal-Wallis test with Dunn's posttest analysis, as appropriate.

Online supplemental material. Table S1 shows the characteristics of attenuated SIV-vaccinated rhesus macaques. Table S2 shows the characteristics of wild-type SIV-infected rhesus macaques, and Table S3 lists the mAbs used in these studies. Fig. S1 is an analysis of SIV Gag-specific CD4⁺ T cell responses in SIV Δ nef-vaccinated macaques using peptide pools. Fig. S2 is a cross-sectional analysis of the frequency of SIV Gag-specific CD4⁺ memory T lymphocytes in SIV Δ nef-infected and wild-type SIV-infected macaques. Fig. S3 shows the frequency of SIV-specific CD4⁺ memory T lymphocytes in PBMCs, LNs, and lung lavages from SIV Δ nef- and SIV Δ 3-infected macaques. The online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20060134/DC1>.

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