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Vaccine-Induced CD8$^+$ Central Memory T Cells in Protection from Simian AIDS

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Critical to the development of an effective HIV vaccine is the identification of adaptive immune responses that prevent infection or disease. In this study we demonstrate in a relevant nonhuman primate model of AIDS that the magnitude of vaccine-induced virus-specific CD8$^+$ central memory T cells (T_{CM}), but not that of CD8$^+$ effector memory T cells, inversely correlates with the level of SIVmac251 replication, suggesting their pivotal role in the control of viral replication. We propose that effective preventive or therapeutic T cell vaccines for HIV-1 should induce long-term protective central memory T cells. ***The Journal of Immunology,*** **2005, 175: 3502–3507.**

A major goal of immunization against cell-associated pathogens, such as HIV, is to generate long-lived protective memory T cells. After the initial exposure to Ags, the expansion of specific CD8$^+$ T cells is followed by a contraction phase by which Ag-specific CD8$^+$ T cells are distinguished into two subsets, central memory T cells (T_{CM})[2] and effector memory T cells (T_{EM}), on the basis of phenotypical and functional features (1). T_{CM} express lymph node homing receptors (CD62L and CCR7), whereas T_{EM} are mainly located at effector sites (2), express β_1 and β_2 integrins, chemokines such as CCR1, CCR3, and CCR5, and homing receptors such as CD103 and CLA (3). Although both the T_{CM} and T_{EM} subsets acquire effector functions such as cytokine production and lytic activity, T_{CM} functions are characterized by a greater capacity for in vivo expansion after re-exposure to Ag (4). In mice, T_{CM} are more efficient in conferring protection in the control of either viral replication or disease (4, 5), suggesting that effective T cell-based vaccines should be programmed to elicit a higher frequency of T_{CM} rather than T_{EM}. We hypothesized that the same may be true for primates and performed a retrospective analysis of cryopreserved samples obtained from macaques vaccinated before or after SIVmac251 infection, because the virological outcome in those studies was already known. In one of these studies, we have shown that a regimen of DNA priming followed by a boost with the highly attenuated NYVAC-based SIV vaccine candidate (6) induced high virus-specific helper responses, increased the frequency and durability of CD8$^+$ T cell responses, and, importantly, was more effective in protecting macaques from disease than NYVAC-SIV alone (7, 8). In a second study we have demonstrated that vaccination with NYVAC-SIV alone of SIVmac251-infected macaques treated with antiretroviral therapy (ART) also resulted in better containment of viral replication (9, 10), i.e., reduced virus level at set point. Viral levels expressed as RNA copies per milliliter after either primary viremia or viral rebound after ART suspension are predictive of disease development (5, 10–12). Interestingly, in both studies an inverse correlation was found between CD4$^+$ Th responses, virus-specific CD8$^+$ T cell responses to a dominant Gag peptide, and plasma virus level at set point (7–10, 13). However, which of the two CD8$^+$ memory response subpopulations, T_{CM} or T_{EM}, boosted/elicited by vaccination contributed to containment of viral replication was not established.

In this study we demonstrate that virus-specific T_{CM} correlated inversely with containment of viral replication in both the preventive and therapeutic vaccine studies, suggesting that the goal of an effective vaccine for HIV should primarily be to elicit central memory cells rather than CTLs.

Materials and Methods

Detection of Gag tetramer-specific CD8β^+ T lymphocytes by flow cytometry in cryopreserved PBMCs of immunized macaques

The animals used in this study were housed and maintained in accordance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care. The macaques were chronically infected with SIVmac251, as previously described (7, 8). The Abs used were anti-human CD8β Ab (clone 2ST8.5H7) from Beckman Coulter; anti-human CD28 Ab (clone CD28.2), anti-human CD95 Ab (clone DX2), anti-human CD45RA (5H9 clone), anti-human IFN-γ (clone 4SB3), anti-human TNF-α (clone MAB11) from BD Pharmingen; and anti-human CD11a (clone G-25.2) from BD Biosciences. The anti-human CCR7 was obtained from R&D Systems. Cryopreserved PBMCs (14) were thawed and maintained in medium for overnight recovery and stained with anti-human CD8β Ab (PE labeled, clone 2ST8.5H7; Beckman Coulter), anti-human CD28 Ab (FITC labeled, clone CD28.2), and anti-human CD95 Ab (PE-Cy5 labeled, clone DX2; BD Pharmingen). Staining was also performed simultaneously with the Mamu-A*01 tetrameric complex, refolded in the presence of the $Gag_{181-189}$ CM9 (p11C) peptide and the Mamu-A*01 molecule (13), and conjugated to allophycocyanin-labeled streptavidin (Molecular Probes). The $Gag_{181-189}$ CM9 (p11C; CTPYDINQM)-specific tetramer was reacted with cells at room temperature in the dark for 30 min. One hundred thousand events were collected in the lymphocyte region (R1) and analyzed with CellQuest software and PAINT-A-GATE (BD Biosciences). For the analysis, the memory T cell population was identified based on forward and side scatter for the lymphocyte population (R1) and on CD8β expression (R2). The gated R2 events were identified as CD28$^+$/CD95$^+$ (R3; T_{CM}) and CD28$^-$/CD95$^+$ (R4; T_{EM}), respectively. Data are presented as percentages of T_{CM} and T_{EM} positive for the tetramer or as percentages of

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[2] Abbreviations used in this paper: T_{CM}, central memory T cell; ART, antiretroviral therapy; *gpe*, *gag-pol-env*; ICS, intracellular cytokine staining; T_{EM}, effector memory T cell; T_N, naive T cell.

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tetramer-positive cells in the $CD28^+/CD95^+$ (T_{CM}) and $CD28^-/CD95^+$ populations (T_{EM}).

Intracellular cytokine staining (ICS)

For ICS, a total of 1×10^6 PBMCs were incubated in RPMI 1640 medium (containing 10% FCS and antibiotics) for 6 h in the presence of specific peptide pool at 2 μg/ml or in the presence of the superantigen staphylococcal enterotoxin B at a 1 mg/ml final concentration as a positive control or with no Ag as a negative control. The costimulatory mAb CD49d (0.5 μg/ml; BD Pharmingen) was added to all the samples to maximize the detection of T cells with higher activation thresholds (15). CD28 was not used as a costimulator molecule as one of the mix of Abs used for the staining for the detection of different subsets. Brefeldin A (Sigma-Aldrich) at a final concentration of 10 mg/ml was added after 1 h. The cells were washed, stained for the surface Abs, permeabilized by incubation in FACS-Perm solution (BD Pharmingen), and stained with anti-TNF-α and anti-IFN-γ.

Statistical analysis

Statistical analysis involved two-group comparisons of log-transformed frequencies performed using repeated measures ANOVA and corrected for multiple tests. Correlations were assessed using the Spearman rank-correlation method.

Results

T_{CM}, but not T_{EM}, elicited by vaccination before SIVmac251 challenge exposure inversely correlate with viremia containment

We performed a retrospective analysis to establish the ability of the two vaccination regimens to generate T_{CM} on cryopreserved PBMC samples (7, 8). At first T_{CM} and T_{EM} were characterized (R3 and R4, respectively; Fig. 1*a*) using Abs to CD28 and CD95, as previously described, in rhesus macaques (16). In parallel, we used other markers that define T_{CM} and T_{EM} in humans and mice, such as CD45RA, CD11a, and CCR7 (Fig. 1*b*). We observed that the T_{CM} population defined as $CD28^+/CD95^+$ in macaques was also $CCR7^+$, as in humans (1). Also, the T_{EM} population defined as $CD28^-/CD95^+$ was $CCR7^{dim}$ or $CCR7^{low}$.

We focused at first on a dominant Gag response induced by vaccination or subsequent viral exposure in Mamu-A*01-positive animals that recognize the immune-dominant Gag peptide $Gag_{181-189}$ CM9 (p11C), because this T cell response can be accurately measured using the Mamu-A*01 $Gag_{181-189}$ CM9 (p11C) tetramer (Fig. 1*a*). This Gag response has been correlated to viremia containment in several studies (17–20).

Four Mamu-A*01-positive macaques previously immunized with four inoculations of 10^8 PFU of NYVAC-SIV, and five with three intradermal and i.m. inoculations of DNA-SIV and boosted with NYVAC-SIV-*gag-pol-env* (*gpe*; Fig. 2*a*) were studied. Two Mamu-A*01-positive macaques were mock-vaccinated with the parental NYVAC. Mucosal challenge exposure to SIVmac251 was performed 6 mo from the last immunization (Fig. 2*a*).

The frequency of tetramer-positive $CD8\beta^+$ T cells was evaluated within the $CD28^+/CD95^+$ and $CD28^-/CD95^+$ PBMCs, because the coordinated assessment of these two markers is sufficient for discrimination, in this macaque species, of naive T cells (T_N; $CD28^+/CD95^-$), T_{CM} ($CD28^+/CD95^+$), and T_{EM} ($CD28^-/CD95^+$) (16). One DNA immunization did not elicit detectable $Gag_{181-189}$ CM9 (p11C) responses (7, 8). In contrast, $Gag_{181-189}$ CM9 (p11C) tetramer-positive $CD8^+$ T cells were induced by a single administration of NYVAC-SIV (Fig. 2*b*; wk 2). Two immunizations with NYVAC-SIV or three immunizations with DNA-SIV induced comparable frequencies of $Gag_{181-189}$ CM9 (p11C) tetramer-positive $CD8^+$ T cells (see the continuous line in Fig. 2*b*). The frequencies of T_{EM} and T_{CM} were comparable within the wk 0–24 interval, but after the last immunization with NYVAC-SIV (wk 52–60 interval), macaques primed with DNA-SIV (group C) developed significantly more T_{CM} ($p = 0.020$) and significantly fewer T_{EM} ($p = 0.047$) specific for $Gag_{181-189}$ CM9 (p11C) than macaques primed with NYVAC-SIV (group B). An example of raw data, which demonstrates a preponderance (~2-fold) of tetramer-positive T_{CM} in animals of group C after the last immunization of animals in groups B and C with NYVAC-SIV, is presented in Fig. 2*c*.

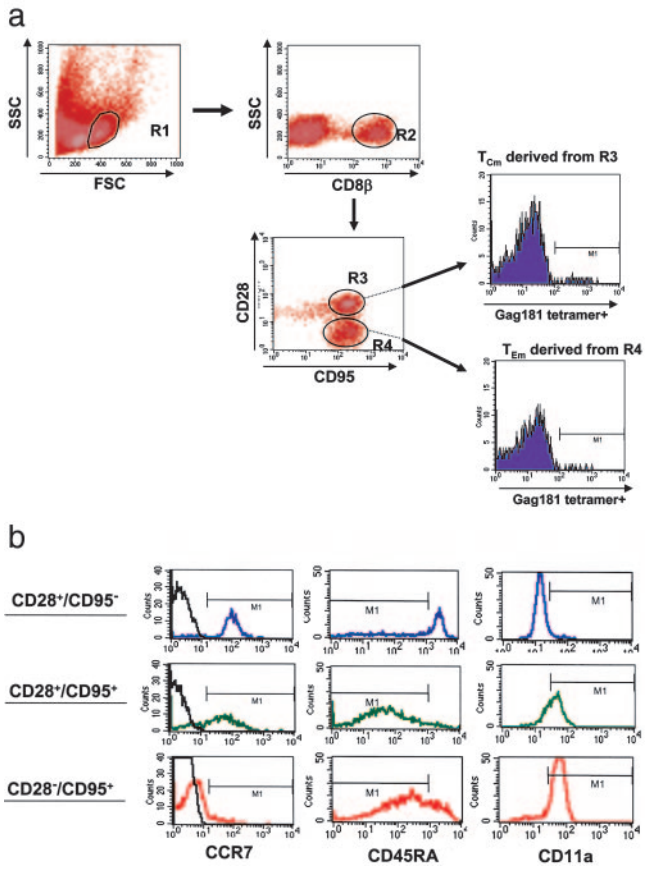

FIGURE 1. Definition of $Gag_{181-189}$ CM9 (p11C) tetramer-positive $CD8^+$ T_{CM} and T_{EM} and study design. *a*, After gating on lymphocyte regions, $CD8\beta^+$ T cells were identified as R3, $CD28^+/CD95^+$ (T_{CM}), and R4, $CD28^-/CD95^+$ (T_{EM}). *b*, T_N (*top panels*), T_{CM} (*middle panels*), and T_{EM} (*lower panels*) were characterized by the expression of CCR7, CD45RA, and CD11a. *c*, Measurement of $Gag_{181-189}$ CM9 (p11C) tetramer-positive $CD8\beta^+$ T cells with T_{CM} (*upper right panel*) or T_{EM} (*lower right panel*) were measured.

After challenge exposure, all macaques experienced an acute phase of viremia with virus level ranging from 10^6 to 10^7 viral RNA copies/ml that subsided to lower levels at the set point (7, 8), particularly in macaques immunized with the DNA-SIV, NYVAC-SIV vaccine combination (8). Comparison of the medians of the log-transformed tetramer-positive T_{CM} percentages over wk 53–60 (memory phase) before viral challenge to the plasma virus level after the set point (wk 6–24) demonstrated a significant negative correlation ($p = 0.045$; $r = -0.77$; Fig. 3), suggesting that T_{CM} play a role in the containment of viral replication. Conversely, we did not find a significant correlation between the levels of T_{EM} and the level of viremia (not shown).

To test whether the response to the single Gag-dominant epitope measured was representative of the quality of the collective virus-specific immune responses elicited by vaccination, we also assessed the quality of the $CD8^+$ T cell response to the Env protein using the ICS assay after in vitro stimulation with overlapping peptides encompassing the C-terminal half of the SIV Env protein.

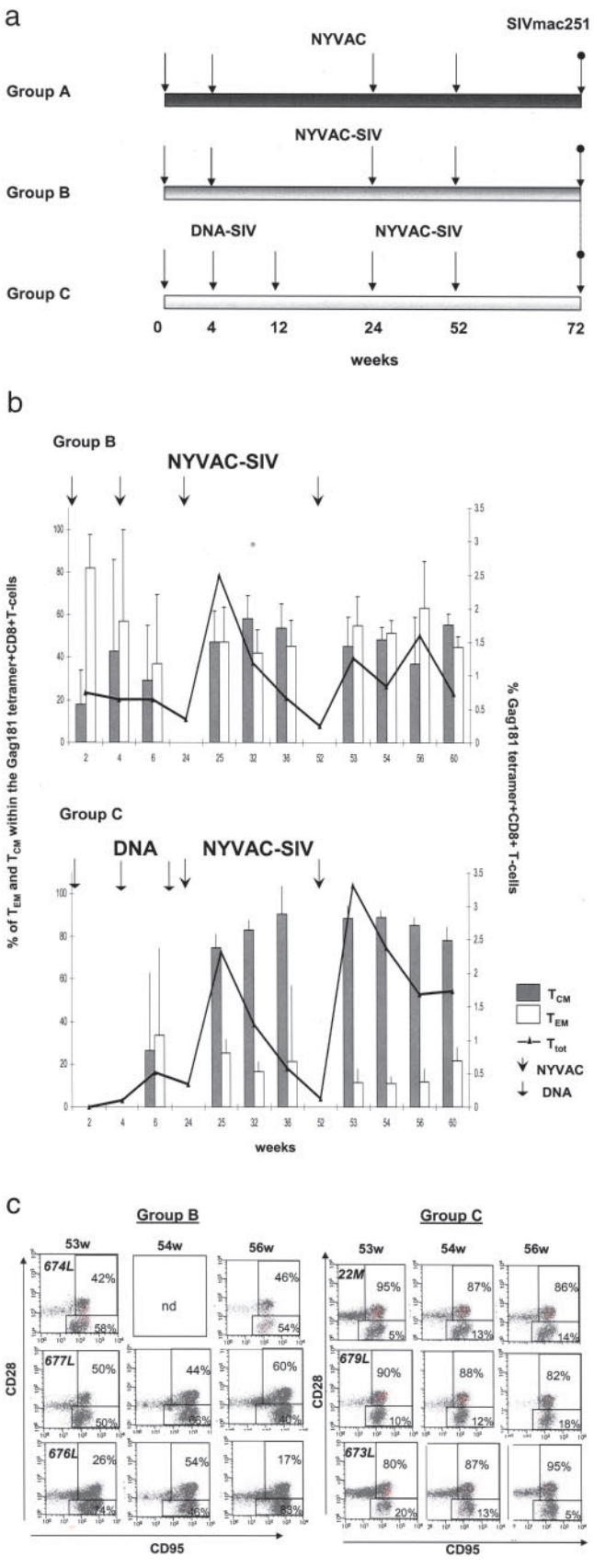

FIGURE 2. $Gag_{181-189}$ CM9 (p11C) tetramer-positive T_{EM} and T_{CM} induced by vaccination. *a*, Study design, as described previously (7, 8). *b*, Mean relative percentages (±SE) of T_{CM} and T_{EM} within the $Gag_{181-189}$ CM9 (p11C) tetramer-positive $CD8^+$ T cells (histogram; see *left axis*) and total percentages of $Gag_{181-189}$ CM9 (p11C) tetramer-positive $CD8^+$ T cells (continuous line; see *right axis*). *c*, An example of raw data obtained after the last immunization with NYVAC-SIV in animals of groups B and C.

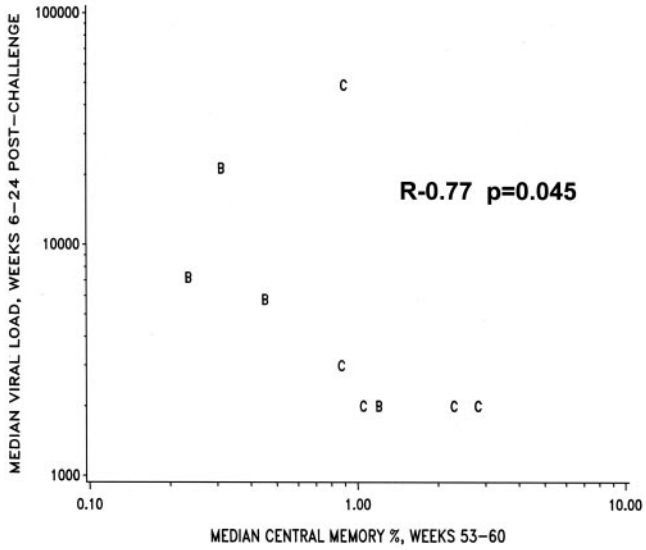

FIGURE 3. Inverse correlation between virus load and T_{CM}. The mean virus level at the set point (wk 6–24) and the frequency of tetramer-positive T_{CM} were correlated. The letters refer to the animals studied in groups B (four macaques) and C (five macaques).

Both vaccination and SIVmac251 infection of rhesus macaques elicit sizeable responses to these Ags, enabling quantitation of the relative frequencies of T_{CM} and T_{EM}. After the last immunization with NYVAC-SIV, the Env-specific responses were highest in the animals in group C, which had received the DNA prime, NYVAC-SIV boost (Fig. 4*a*). Importantly, when CD28 and CD95 expressions were used to dissect the relative contributions of T_{CM} and T_{EM} to the $CD8^+$ SIV Env response, we found that macaques immunized with NYVAC-SIV only developed equivalent frequencies of Env-specific T_{CM} and T_{EM} (Fig. 4*b*). In contrast, macaques immunized with the DNA prime, NYVAC-SIV boost had a higher frequency of Env-specific T_{CM} than T_{EM} (Fig. 4*c*), although the difference did not reach statistical significance. Because in the ICS assay we did not use the Ab to CD28 to costimulate cells, it is possible that we understated the ICS response. Nevertheless, it appears that the ability of the DNA prime, NYVAC-SIV boost vaccine regimen to increase virus-specific T_{CM} may not be restricted to the dominant $Gag_{181-189}$ CM9 (p11C), supporting the hypothesis that DNA priming influences the overall quality of $CD8^+$ T cells in favor of T_{CM} expansion.

SIV-specific T_{CM} inversely correlate with the level of plasma viremia in macaques infected with SIVmac251

In a previous study we demonstrated that ART, alone or in combination with therapeutic vaccination with NYVAC-SIV, of SIVmac251-infected macaques results in better containment of viral replication (9). This previous study had been designed to investigate whether intervention with ART and therapeutic vaccination during primary infection could confer a virological benefit and included eight macaques per group; three animals in each group were Mamu-A*01-positive (Fig. 5*a*). Macaques in group A were treated with ART and mock-vaccinated with NYVAC, whereas the remaining macaques were vaccinated with the NYVAC-SIV construct in the presence (group B) or the absence (group C) of ART

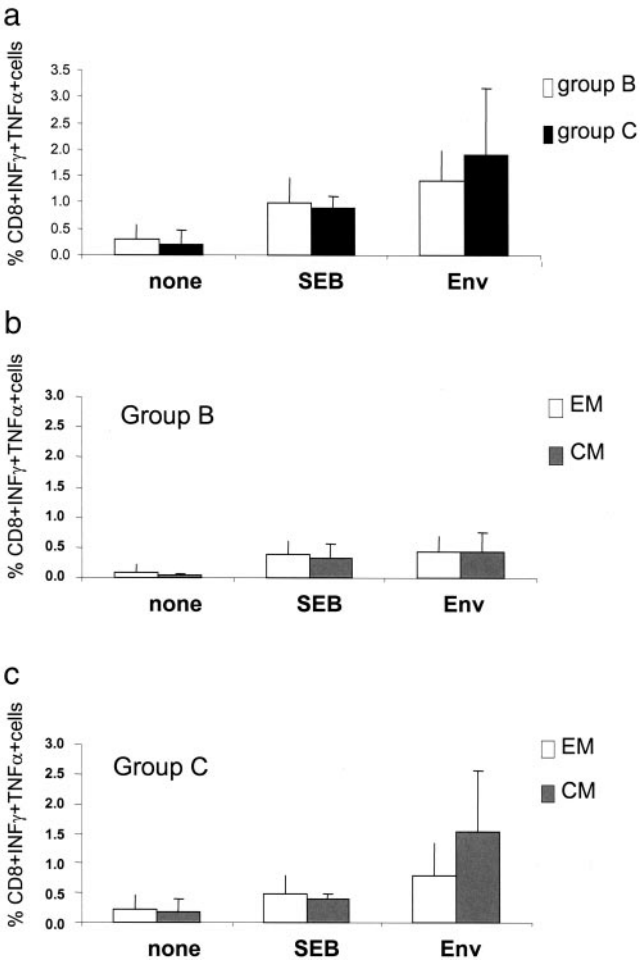

FIGURE 4. Env-specific T_{CM}. *a*, Mean percentages (±SE) of $CD8^+$ T cells producing IFN-γ and TNF-α after stimulation with an Env peptide pool derived from the SIVk6w DNA sequence (32). The data were obtained from cryopreserved PBMCs collected at wk 53, 1 wk after the last immunization (see Fig. 1*a*) and represent the mean frequency values obtained from animals in groups B and C. Mean percentages (with error bar) of cytokine-producing $CD8^+/CD95^+/CD28^-$ (T_{EM}) and $CD8^+/CD95^+/CD28^+$ (T_{CM}) cells from macaques in groups B (*b*) and C (*c*).

(Fig. 5*a*). To investigate the role of T_{CM} in this setting, we retrieved cryopreserved PBMCs from Mamu-A*01-positive macaques and analyzed the relative frequency of $Gag_{181-189}$ CM9 (p11C) tetramer-positive T_{CM} and T_{EM} before, during, and after all treatments. The last immunization with NYVAC-SIV of ART-treated macaques expanded ~8-fold the mean frequency of the $Gag_{181-189}$ CM9 (p11C) tetramer-positive cells in macaques of group B (wk 25; Fig. 5*b*, *middle panel*) compared with unvaccinated controls (group A). In contrast, in the absence of ART, NYVAC-SIV was unable to expand these responses. Collectively, these results were in agreement with previous data obtained from ex vivo PBMCs (9). Qualitative analysis of $Gag_{181-189}$ CM9 (p11C) tetramer-positive T_{CM} and T_{EM} demonstrated a significant increase in the frequency of T_{CM} in macaques vaccinated while on ART (data not shown). Correlative analysis between the level of Gag-specific T_{CM} at wk 25 (2 wk after the last vaccination and before ART suspension) and the level of viremia over subsequent weeks when virus rebounded after ART cessation revealed a Spearman rank correlation of either −0.85 or −0.87 with the same *p* value of 0.029 (data not shown), again suggesting a role for T_{CM} in the containment of viremia. This significant negative correlation persisted after ART cessation when the median viral load over wk 28–35 for each of the nine macaques studied was related to the median log-transformed percentage of central memory cells over the same interval ($r = -0.80$; $p = 0.015$; Fig. 5*c*).

Discussion

Immunological memory is an essential aspect of adaptive immune responses after natural infection or vaccination (21). Although B and T cell dynamics (22) and the role of memory Ab responses in protection against infection are well understood, less is known about the quality of memory T cells required for protection against disease. Evidence in murine systems of viral infection (4, 5) has recently suggested that $CD8^+$ T_{CM} may constitute the correlate of protection from disease. Along the same lines, it was recently reported that $CD4^+$ T_{CM} mediate long-term immunity against the parasite *Leishmania major* in the mouse (23).

In this study, using a model of SIV infection in the rhesus macaque, we show that vaccine-induced protection correlates with the expansion of $CD8^+$ T_{CM} cells in the settings of both preventive and therapeutic vaccinations. We have demonstrated that DNA priming induced higher levels of $CD8^+$ T_{CM} responses than priming with the NYVAC-SIV vaccine. These findings may relate to the ability of DNA to expand both $CD8^+$ and $CD4^+$ T cells, limiting activation-induced cell death (24) and exhaustion (25), which occur when T cells respond to a high Ag dose and/or the response occurs in inflammatory conditions. Indeed, we have previously reported that DNA-SIV prime, NYVAC-SIV boost also induces higher $CD4^+$ T cell responses (7), which inversely correlate with virus levels in SIVmac251-challenged macaques (8). This is consistent with the idea that $CD4^+$ T cell help is required to enhance the survival, maintenance, and further expansion of $CD8^+$ T cells after Ag re-encounter (26–28). Possibly, the low level of Ags expressed by DNA-SIV favor the synchronous activation and expansion of $CD8^+$ and $CD4^+$ T cells. In contrast, the expression of higher levels of Ags by live vector-based vaccination (e.g., NYVAC-SIV) that also induces local inflammatory conditions is less dependent on cognate CD4 help and hinders the expansion of $CD8^+$ T_{CM} cells. Interestingly, NYVAC-SIV boost fully expands $CD8^+$ T_{CM} induced by and maintained through DNA priming. Because the dose of Ag at the time of priming does not dictate the rate of expansion of $CD8^+$ memory T cells (5, 29), one could argue that the contribution of the NYVAC-SIV boost could be either to provide a different or richer cytokine milieu or to recruit different cells to the site of immune expansion. Consistent with this overall interpretation of the data are recent findings in the mouse, where complete protection from lethal virus challenge was obtained by immunization with a low dose of Ag (5).

The data also imply that therapeutic vaccination combined with maneuvers that reduce Ag load in vivo may reach the same objective. We speculate that ART resets the Ag load and concomitantly reduces the inflammatory conditions sustained by high levels of virus replication, hence mimicking the starting conditions of DNA priming in naive macaques. This provides evidence for the plasticity of the immune response and is consistent with the idea that although high doses of Ag may induce unresponsiveness, low doses of Ag are immunogenic (30, 31).

The findings presented in this study suggest that $CD8^+$ T_{CM} can be regarded as an important correlate of protection against disease in the SIV model of infection and provide a rational explanation for why the DNA prime, live vector boost immunization confers greater protection than immunization with live vector alone. They also point to new rules for the induction of protective T cell responses by vaccination, which, based on the present observations, should be focused on programming and maintaining $CD8^+$ T_{CM}

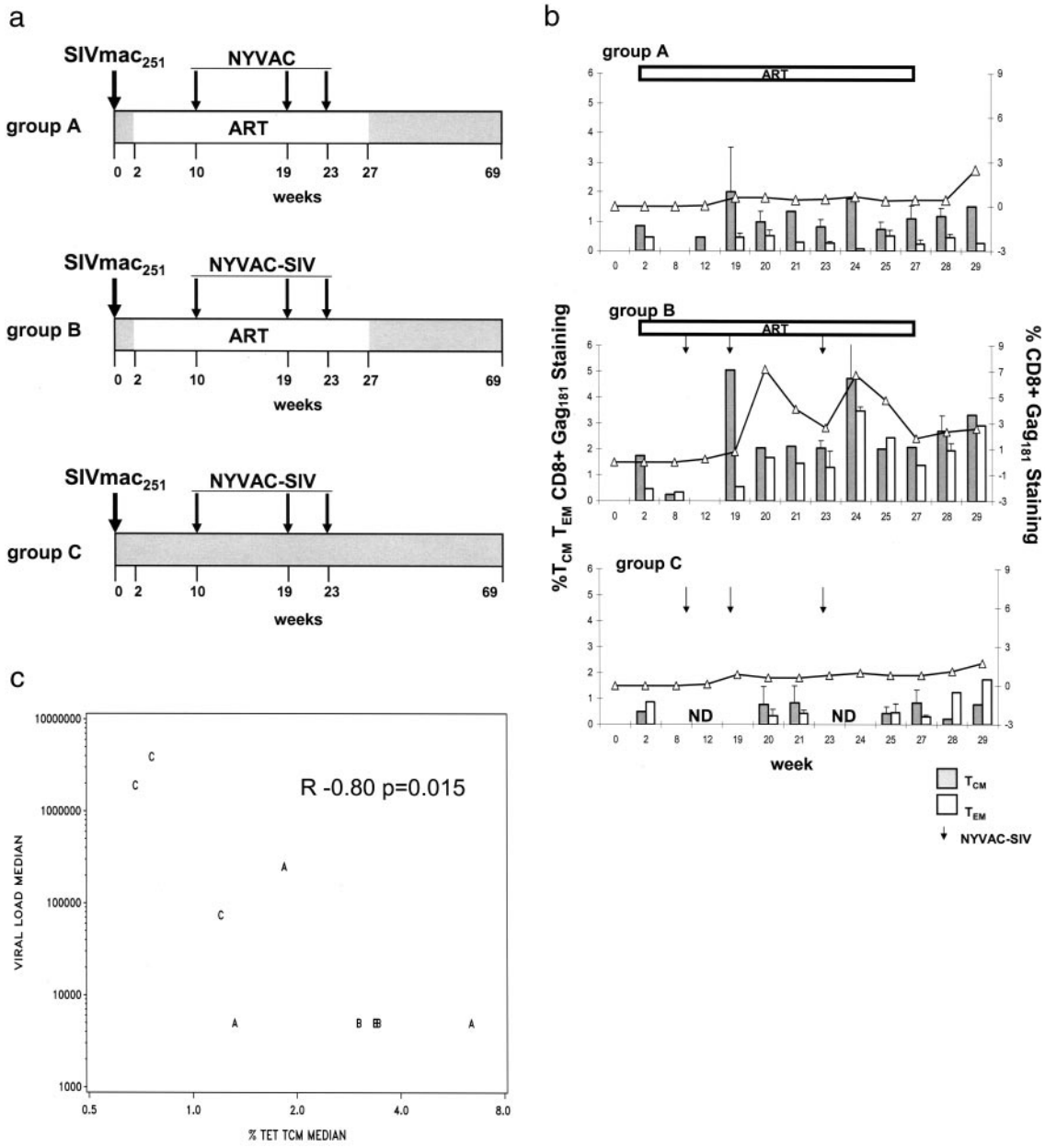

FIGURE 5. Relative frequencies of Gag-specific T_{CM} and T_{EM} in ART-treated and/or vaccinated SIVmac251-infected macaques. *a*, Study design. The overall study results can be found in Ref. 9. *b*, Mean frequencies of total $Gag_{181-189}$ CM9 (p11C) tetramer-specific $CD8^+$ T cells (solid line) or relative mean percentages (with error bar) of tetramer-positive T_{CM} and T_{EM} in macaques after immunization with NYVAC-SIV in the presence or the absence of ART (groups B and C, respectively) or mock immunization (NYVAC parental) in the presence of ART (group A). *c*, Inverse correlation of T_{CM} at wk 25 (2 wk after the last immunization) and viremia levels at wk 28–35 (after ART suspension) in all nine macaques studied.

by appropriate vaccination regimens. Thus, efforts to develop effective therapeutic and preventive vaccines for HIV should promote the generation of long-lived Ag-specific $CD8^+$ T_{CM} in conjunction with helper responses able to sustain the ability of $CD8^+$ T_{CM} to expand adequately upon virus encounter.

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Disclosures

The authors have no financial conflict of interest.

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