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Preservation of Functional Virus-Specific Memory CD8⁺ T Lymphocytes in Vaccinated, Simian Human Immunodeficiency Virus-Infected Rhesus Monkeys¹

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Functional impairment of virus-specific memory CD8⁺ T lymphocytes has been associated with clinical disease progression following HIV, SIV, and simian human immunodeficiency virus infection. These lymphocytes have a reduced capacity to produce antiviral cytokines and mediators involved in the lysis of virally infected cells. In the present study, we used polychromatic flow cytometry to assess the frequency and functional capacity of central memory (CD28⁺CD95⁻) and effector memory (CD28⁻CD95⁺) subpopulations of Gag-specific CD8⁺ T cells in SIV/simian human immunodeficiency virus-infected rhesus monkeys. The aim of this study was to determine whether Ag-specific, memory CD8⁺ T cell function could be preserved in infected monkeys that had been immunized before infection with a vaccine regimen consisting of a plasmid DNA prime followed by a recombinant viral vector boost. We observed that vaccination was associated with the preservation of Gag-specific central memory CD8⁺ T cells that were functionally capable of producing IFN-γ, and effector memory CD8⁺ T cells that were capable of producing granzyme B following viral Ag exposure. The Journal of Immunology, 2006, 176: 5338–5345.

Virus-specific CD8⁺ T lymphocytes play a central role in viral containment in HIV-infected individuals (1–4). The antiviral activity of these lymphocytes is likely mediated by direct cytotoxicity as well as the production of chemokines and other small molecules (5). Recent studies have suggested that clinical disease progression in infected individuals may be associated with the functional impairment of HIV-specific CD8⁺ T cells (6–10). This functional impairment can include abnormalities in the ability of effecter cells to produce antiviral cytokines, chemokines, and molecules required for lytic activity.

We have recently shown that virus-specific CD8⁺ T cells in simian human immunodeficiency virus (SHIV)³-infected rhesus monkeys have a reduced capacity to produce cytokines such as IL-2 and TNF-α in response to viral Ag exposure (10). Importantly, Gag-specific CD8⁺ T cells in a cohort of monkeys that were vaccinated and then subsequently infected with SHIV were protected against these cytokine production abnormalities. However, only a limited spectrum of the functional capabilities of these cells was evaluated in this study.

Recent advances in flow cytometry have facilitated the elucidation of the maturation status and functional capacities of T lymphocyte subpopulations (11–15). This technology has provided a valuable tool for evaluating the expression of cell surface molecules, cytokine production, and the lytic potential of lymphocyte populations (13, 16–20). In fact, the use of polychromatic flow cytometry has broadened our understanding of the generation and function of memory T lymphocyte populations. Memory T lymphocytes have been divided into two distinct subsets, central memory (CM) and effector memory (EM) T cells, based on their migratory capacity, effector functions, and phenotypic profile (21, 22). CM T lymphocytes have the capacity to home to lymphoid organs, have limited effector functions, and, upon secondary exposure to Ag, will rapidly proliferate to become effector T cells. EM T lymphocytes have the capacity to home to peripheral tissues, have limited proliferative capacity, and have the ability to rapidly produce effector cytokines such as IFN-γ following antigenic stimulation.

In the present study, we have used polychromatic flow cytometry in conjunction with MHC class I/peptide tetramer and intra-cellular cytokine staining to characterize the frequency and functional capacity of virus-specific memory CD8⁺ T cells in simian immunodeficiency virus-infected rhesus monkeys. We have also used these technologies to determine the degree to which vaccination protects against the evolution of these functional T cell abnormalities.

Materials and Methods

Animals and viruses

Blood samples were obtained from rhesus monkeys (Macaca mulatta). All animals were maintained in accordance with the guidelines of the Committee on Animals for the Harvard Medical School and the “Guide for the Care and Use of Laboratory Animals” (23). Twenty milliliters of whole blood in sodium heparin collection tubes (Vacutainer; BD Biosciences) were processed either within several hours following collection or shipped overnight at ambient temperature and processed the next day. In extensive

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³Abbreviations used in this paper: SHIV, simian human immunodeficiency virus; rAd, recombinant adenovirus; rPox, recombinant poxvirus; CM, central memory; EM, effector memory.
studies of flow cytometry-based functional assays, we have determined that when sodium heparin is used as an anticoagulant, functional cytokine responses by PBMC derived from freshly collected and day-old blood are comparable (data not shown). Monkeys used in the present study were infected with either pathogenic SIVmac251 or SHIV-89.6P.

Selection of monkeys

Monkeys selected for study were genotypically Mamu-A*01′, determined using a PCR-based technique, as previously described (24). Moreover, to assure accurate assessment of small subpopulations of T cells, only monkeys with a p11C tetramer population >0.5% of CD3 CD8 lymphocytes were evaluated.

CD4 counts and viral loads

Peripheral blood CD4 T lymphocyte counts were calculated by multiplying the total lymphocyte count by the percentage of CD3 CD4 T cells determined by mAb staining and flow cytometric analysis. Plasma viral RNA levels were measured by an ultrasensitive branched DNA amplification assay with a detection limit of 125 copies/ml (Bayer Diagnostics).

Vaccination protocols

Two groups of vaccinated, SHIV-89.6P-infected Mamu-A*01′ rhesus monkeys were included in this study (Table I). Each monkey received one of the following immunizations and viral challenges:

DNA recombinant adeno virus (rAd) vaccine. Monkeys WFB, VWT, 414, KPA, WHJ, 420, VFA, and 419 received three DNA immunizations by i.m. injections at weeks 0, 4, and 8 with 4.5 mg of plasmid DNA vectors expressing the SIVmac239 Gag-Pol-Nef fusion protein and one of the following: 4.5 mg of clade B HIV-1 Env protein, 1.5 mg of clade B HIV-1 Env protein, 4.5 mg of clade C HIV-1 Env protein, or a combination of 1.5 mg each of the clade A-, B-, and C HIV-1 Env proteins (clade A B + C). At week 26, these animals were boosted i.m. with rAd serotype 5 (Ad5) vectors (2.0 × 10^11 total particles) expressing SIVmac Gag-Pol and HIV-1 Env genes matched to those administered during priming. At week 42, all animals were challenged i.v. with 50 MID50 of pathogenic SHIV-89.6P (25).

DNA recombinant pox virus (rPox) vaccine. Monkeys 151M, 89M, 114M, 116M, 2M, 85M, 76M, 103M, 100M, and 120M received three DNA priming immunizations i.m. at weeks 0, 4, and 8 with 5 mg of HIV-1 89.6P Env gp140 (KB9) and 5 mg of SIVmac239 Gag DNA, both expressed by pVIR plasmids and adjuvanted by the coadministration of 5 mg of pVIR plasmid expressing IL-2/Δg. At week 42, the DNA-primed monkeys were boosted i.m. and intradermally with 10^9 PFU of either recombinant fowlpox (rFVP), recombinant modified vaccinia Ankara (rMVA) or recombinant vaccinia virus (rVac) expressing HIV-1 89.6P Env and SIVmac239 Gag, or boosted again with 5 mg of HIV-1 89.6P Env gp140 (KB9) and 5 mg of SIVmac239 Gag DNA vaccines. At week 60, all animals were challenged i.v. with 50 MID50 of pathogenic SHIV-89.6P (26).

Antibodies

The Abs used in this study were directly coupled to the following fluorochromes: FITC, PE-Texas Red (ECD), PerCP-cyanine 5.5 (PerCP-Cy5.5), PE-cyanine 7 (PE-Cy7), allophycocyanin, and Alexa Fluor 700. Mamu-A*01′ p11C tetramer complexes were prepared and labeled with PE-labeled streptavidin, as previously described (27, 28). The following mAbs were used: anti-CD28-FITC (CD28.2; Beckman Coulter), anti-CD8-PE-Cy7 (B27; BD Pharmingen), anti-CD95-allophycocyanin (DX2; BD Pharmingen), and anti-granzyme B-Alexa 700 (GB11; BD Pharmingen). All mAbs were titrated for optimal staining in preliminary experiments before their use in a seven-color panel.

PBMC stimulation and polychromatic intracellular staining

PBMC were separated from 10 to 20 ml of heparinized whole blood by Ficoll density gradient centrifugation (Ficoll-Paque Plus; Amersham Biosciences) at 3000 rpm for 20 min. Cells were washed twice with PBS/2% FBS. Any residual RBC were lysed using a 3-min incubation at room temperature in PBS/2% FBS. Any residual RBC were lysed using a 3-min incubation at room temperature in PBMC (2.0 × 10^7) in RPMI 1640/10% FBS medium plus 2 µg/ml anti-CD49d (9F10; BD Pharmingen) were incubated at 37°C in a 5% CO2 environment for 1 h in either medium alone (unstimulated), in medium containing 5 µg/ml p11C (CTPYDINQM) peptide (peptide-stimulated), or in medium containing 10 ng/ml PMA + 1 µg/ml ionomycin as a positive control. After a 1-h incubation, the Golgi transport inhibitor monensin (GolgiStop; BD Pharmingen) was added to all cultures to allow for the accumulation of cytokine within the cells. The cultures were then incubated for an additional 5 h at 37°C in a 5% CO2 environment. After incubation, the cells were stained with p11C tetramer and cell surface mAbs, washed twice with PBS/2% FBS, and then fixed and permeabilized with Cytotox/Cytoperm solution (BD Pharmingen), according to the manufacturer’s protocol. Cells were washed twice with 1× PermWash buffer and stained with mAbs specific for intracellular cytokine and granzyme. Cells were washed twice using 1× PermWash buffer and

Table I. Clinical data on rhesus monkeys infected with pathogenic immunodeficiency viruses

<table>
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<tr>
<th>Monkey Groups</th>
<th>Infecting Virus</th>
<th>Weeks Since Infection</th>
<th>Plasma Virus RNA (copies/ml)</th>
<th>CD4⁺ T Cells (counts/µl)</th>
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given a final wash with PBS before fixation in 1.5% formaldehyde/PBS. Samples were collected using the BD LSRII flow cytometer which is supported by FACSDiva acquisition software (BD Immunocytometry Systems). Whenever possible, 50,000 lymphocyte events or more were collected.

To obtain an accurate assessment of tetramer+ CD8+ T cells, a sample of unstimulated, nonpermeabilized whole blood from each monkey was stained with anti-CD3, anti-CD8, and p11C tetramer. We then compared this percentage of tetramer-binding CD8+ T cells in whole blood with the percentage of these cells in epitope-stimulated PBMC to determine the magnitude of TCR down-regulation during Ag stimulation. Data were collected on the peptide-stimulated cells only if the observed TCR expression on these cells was 80% or more than that on the unstimulated CD3+ CD8+ tetramer-binding cells.

To determine the reproducibility of this assay, we assessed the phenotype and the function of CD8+ T cell responses at two time points in the cohort of monkeys that had received the DNA/rAd immunization. We found in these repeated studies that the median percentage of tetramer+ CM CD8+ T cells in these PBMC was 39.8 and 37.5%, and the median percentage of tetramer+ IFN-γ+ CD8+ T cells in the PBMC of these monkeys was 62.6 and 60%.

Data analysis and gating strategy

Data analyses were performed using FlowJo 6.0 (Tree Star). Lymphocytes were gated based on cell size and granularity on a forward scatter (FSC) vs side scatter (SSC) plot. CD8+ T lymphocytes were identified based on their positive surface expression of CD3 and CD8. In Fig. 1A, CD3+ CD8+ T cells were gated based on CD28 and CD95 expression to define memory CD8+ T cell subpopulations: naive (CD28+CD95-), CM (CD28+CD95+), and EM (CD28-CD95+). Using the analysis software, we were then able to evaluate the functional capacity of these T cell subpopulations. As shown in Fig. 1B, the same data analyses were performed, however, CD8+ T lymphocytes were first gated based on their binding of p11C tetramer and then by expression of CD28/CD95. In some cases, a contour plot was used rather than a dot plot to separate cell subpopulations. Samples were considered positive if the percentage of tetramer+ cytokine-staining cells was at least twice that of background.

Data reporting and statistical analyses

Data presented in this study concerning the frequency of lymphocyte populations are reported as percentages of populations and as absolute cell numbers in counts per microliter or milliliter. However, in the experiments that deal with Ag-specific memory CD8+ T cell function, we chose to report the data as percentages of populations because these cell subsets are rare, and computation of their absolute numbers would represent values with very high error coefficients.

Comparisons of median percentages between the three cohorts of monkeys were first performed using the Kruskal-Wallis test. If the results of these tests were significant (p < 0.05), the Mann-Whitney U test was performed to compare data pairs. The Wilcoxon rank-sum test was performed to compare the absolute numbers of memory CD8+ T cell subsets of unvaccinated and vaccinated animals. The Spearman correlation test was performed to compare the percentage of CD4+ T cell counts and the percent of p11C tetramer+ CM CD8+ T cells in vaccinated, infected animals. The paired t test was performed to compare the percentages of intracellular granzyme B levels of p11C tetramer+ memory CD8+ T cells before and after epitope peptide stimulation.

Results

Loss of Gag-specific CM CD8+ T cells following SIV/SHIV infection was prevented by vaccination

Because the rhesus monkey MHC class I molecule Mamu-A*01 presents the 9-aa p11C peptide fragment of the SIV and SHIV Gag protein as a dominant epitope to CD8+ T cells, p11C tetramer staining can be used to define a viral Ag-specific CD8+ T cell population in immunodeficiency virus-infected monkeys. Cell staining and polychromatic flow cytometry were therefore used to identify subpopulations of memory CD8+ T cells in SIV/SHIV-infected Mamu-A*01+ rhesus monkeys based on p11C tetramer binding and cell surface expression of CD28 and CD95. Tetramer+ CD8+ T cells with a CD28+CD95- phenotype were designated as CM T cells, while those with a CD28+CD95+ phenotype were designated as EM T cells (29, 30). The distribution of these virus-specific CD8+ T cell memory subsets was evaluated in PBMC of three cohorts of monkeys: 1) SIV-infected with progressive disease (SIV+), 2) animals that were vaccinated with a DNA/rAd regimen and then subsequently infected with SHIV-89.6P (DNA/rAd), and 3) animals that were vaccinated with a DNA/rPox regimen and then subsequently infected with SHIV-89.6P (DNA/rPox).

Monkeys infected with SHIV-89.6P without prior vaccination were not evaluated because we have previously shown that these animals do not have sufficiently preserved immune function to generate detectable CD8+ T cell responses (data not shown). Vaccinated, SIVmac251-infected monkeys were not available to evaluate because Mamu-A*01+ rhesus monkeys are not routinely used in vaccine/challenge studies because the substantial contribution of the Mamu-A*01 allele to protection against progression of SIV-induced disease confounds the contribution of protection afforded by vaccination (31). Therefore, because we were interested in evaluating the ramifications of vaccine protection on CD8+ T cell function following primate immunodeficiency virus infection, we

![FIGURE 1. Representative intracellular staining profile of functional Ag-specific memory CD8+ T lymphocytes from an SIVmac251- or SHIV-89.6P-infected, Mamu-A*01+ rhesus monkey following a 6-h epitope peptide (p11C) stimulation. Total CD8+ T cells (A) and p11C tetramer+ CD8+ T cells (B) were gated and analyzed for their expression of CD28 and CD95, with CD28+CD95+ cells designated as CM and CD28+CD95+ cells designated as EM. A, IFN-γ production and granzyme B expression in CM and EM CD8+ T cells. Left column panels, Dot plots showing background levels of IFN-γ in both p11C tetramer+ CM and EM CD8+ T cells cultured in medium alone. Center column panels, Dot plots showing IFN-γ production by these same Ag-specific memory CD8+ T cells following p11C peptide stimulation. Right column panels, Intracellular granzyme B staining by tetramer+ memory CD8+ T cells following peptide stimulation. Numbers indicate percent of gated CD3+CD8+ T cells in each quadrant. All dot plots show a minimum of 50,000 CD3+CD8+ events acquired for each sample and condition. B, Representative p11C tetramer staining in combination with memory phenotype and intracellular staining. P11C tetramer+ CD8+ T cells were gated based on CD28/CD95 expression. The total tetramer+ T cells (left), tetramer+ CM cells (center), and tetramer+ EM cells (right) were analyzed for IFN-γ production and expression of granzyme B. Numbers indicate the percent of gated p11C tetramer+ CD3+CD8+ T cells in each quadrant.](http://www.jimmunol.org/)

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compared CD8+ T cells in vaccinated, SHIV-89.6P-infected monkeys with those in unvaccinated, SIVmac251-infected monkeys.

In the total memory CD8+ T lymphocyte population, the representation of CM T cells in the two groups of vaccinated, infected monkeys (medians 23 and 25%) was comparable to that in unvaccinated, infected monkeys (median 19%) (Fig. 2A). Accordingly, the percentages of EM CD8+ T cells in vaccinated and unvaccinated, infected animals were also comparable (medians 77, 75, and 81%) (data not shown). The absolute numbers of total memory CD8+ T lymphocytes with a CM phenotype were also comparable in SIV-infected progressor animals and vaccinated, SHIV-infected animals (medians 674 and 583 counts/μl, respectively, p = 0.12, Wilcoxon rank-sum test) (Fig. 2B).

Importantly, the percentage of SIV Gag p11C-specific CD8+ T cells that were CM was higher in the infected animals that were immunized with either the DNA/rAd or DNA/rPox regimen (medians 40 and 24%, respectively) than in the unvaccinated, SIV-infected group of animals (median 9%) (Fig. 2C). Consistent with this finding, the percentages of p11C-specific EM CD8+ T cells in both vaccinated groups (medians 60 and 76%) were lower than in the unvaccinated, infected group (median 91%) (data not shown). The absolute numbers of tetramer+ CD8+ T cells that were CM were higher in the total vaccinated, infected monkeys (median 4104 counts/ml) than in the unvaccinated group of monkeys (median 2968 counts/ml) (Fig. 2D), with the difference in absolute T cell counts between these groups almost achieving statistical significance (p = 0.06, Wilcoxon rank-sum test). When the vaccinated, infected monkeys were evaluated in groups based on vaccine regimen, the absolute numbers of their tetramer+ CM CD8+ T cells were comparable to those of unvaccinated, SIV-infected monkeys (data not shown, p = 0.16, Kruskal-Wallis test). Although the absolute numbers of these T cell populations were not significantly different between the three cohorts of monkeys, a trend was apparent showing an association between vaccination

**FIGURE 2.** The distribution of memory CD8+ and tetramer+ memory CD8+ T cells of different cohorts of SIV/SHIV-infected *Mamu-A*/*B* rhesus monkeys. The monkeys were divided into three groups: 1) SIV+, monkeys (n = 5) infected with pathogenic SIVmac251 with progressive disease; 2) DNA/rAd, monkeys (n = 8) immunized with a DNA/rAd regimen before SHIV-89.6P infection with no signs of clinical progression; and 3) DNA/rPox, monkeys (n = 10) immunized with a DNA/rPox regimen before SHIV-89.6P infection with no signs of clinical progression. A group comparison of the median percentages of memory CD8+ T cells with a CM phenotype is represented in A and of p11C tetramer+ CD8+ T cells that are CM in C. A group comparison of the absolute numbers of memory CD8+ T cells with a CM phenotype is represented in B and of p11C tetramer+ CD8+ T cells that are central memory in D. Statistical differences shown in A and C were determined by the Kruskal-Wallis test (in bold, upper left corner). Statistical differences between individual groups in C were calculated using the Mann-Whitney U test, and are represented where significant by brackets. Statistical differences shown in B and D were determined by the Wilcoxon rank-sum test (in bold, upper left corner).

**FIGURE 3.** IFN-γ-producing tetramer+ memory CD8+ T cells in the three cohorts of infected monkeys. The p11C tetramer+ IFN-γ+ CD8+ T cell population in each group of the animals was evaluated for expression of CD28 and CD95. The median percentages of these cells that were CM (CD28+CD95+) in each cohort of monkeys are shown. Comparisons between groups were determined using the Kruskal-Wallis test (in bold, upper left corner). Statistical differences between individual groups were calculated using the Mann-Whitney U test, and are represented where significant by brackets.

**FIGURE 4.** Intracellular granzyme B expression in unstimulated p11C tetramer+ CD8+ T cells in the three cohorts of infected monkeys. Median percentages of tetramer+ granzyme B+ CD8+ T cells for each group of animals are shown. Statistical comparison between groups was determined using the Kruskal-Wallis test (in bold, upper left corner). Statistical differences between individual groups were calculated using the Mann-Whitney U test, and are represented where significant by brackets.
and higher numbers of Ag-specific CD8\(^+\) T cells with a CM phenotype. The results of these analyses suggest that vaccination before infection was associated with a preservation of Ag-specific CM CD8\(^+\) T cells.

**Preservation of IFN-\(\gamma\) production in Gag-specific CM CD8\(^+\) T cells of infected, vaccinated monkeys**

Staining to assess intracellular IFN-\(\gamma\) production by p11C tetramer\(^+\) memory CD8\(^+\) T cells was performed in association with cell surface staining on p11C epitope peptide-stimulated PBMC from vaccinated and unvaccinated rhesus monkeys that were infected with SIV/SHIV. The production of IFN-\(\gamma\) by tetramer\(^+\) memory CD8\(^+\) T cells following p11C peptide exposure was comparable in all three cohorts of monkeys (data not shown, \(p = 0.70\), Kruskal-Wallis test). However, further analysis of tetramer\(^+\) IFN-\(\gamma\)^+ CD8\(^+\) T cell populations of each group of monkeys demonstrated that the ability to produce IFN-\(\gamma\) was better preserved in the tetramer\(^+\) CM CD8\(^+\) T cell population of infected animals that had been previously vaccinated with either DNA/rAd (\(p = 0.02\), Mann-Whitney U test) or DNA/rPox (\(p = 0.03\), Mann-Whitney U test) than in the unvaccinated, infected monkeys (Fig. 3). Although this comparison was done using percentage values, a similar analysis done using absolute numbers of tetramer\(^+\) IFN-\(\gamma\)^+ CD8\(^+\) T cell subpopulations demonstrated a similar difference that approached statistical significance (\(p = 0.06\), Kruskal-Wallis test).

**SIV Gag-specific CD8\(^+\) T cells of unvaccinated, infected monkeys with progressive disease had higher levels of intracellular granzyme B than vaccinated, infected monkeys**

The intracellular production of the serine protease granzyme B was used as an indicator of cytotoxic effector function in the CD8\(^+\) T cells of these monkeys (32, 33). Granzyme B is stored along with other lytic enzymes in granules in CD8\(^+\) T lymphocytes. Following cytotoxic CD8\(^+\) T cell activation, these granules are exocytosed in a process referred to as degranulation, and granzyme B is released to act synergistically with the membrane pore-forming protein perforin to induce target cell death (34). Intracellular staining for granzyme B was performed on p11C tetramer\(^+\) CD8\(^+\) T cells in association with surface staining and intracellular staining for IFN-\(\gamma\) production. A comparison of intracellular granzyme B levels in unstimulated tetramer\(^+\) CD8\(^+\) T cells of the study animals is shown in Fig. 4. Rhesus monkeys that were not vaccinated and had detectable plasma virus RNA had a higher level of intracellular granzyme B (median 74%) than both groups of vaccinated, infected monkeys (medians 52 and 53%) (\(p = 0.01\), unvaccinated vs monkeys vaccinated with either DNA/rAd or DNA/rPox, Mann-Whitney U test; however, in this particular case, when tetramer\(^+\) granzyme B CD8\(^+\) T cells were evaluated as absolute numbers rather than as percentages, these differences did not achieve statistical significance (\(p = 0.29\), Kruskal-Wallis test)). Statistically significant differences were also observed for tetramer\(^+\) memory CD8\(^+\) T cell subpopulations in comparing unvaccinated and vaccinated cohorts of monkeys (data not shown). Levels of intracellular granzyme B following p11C peptide stimulation were also evaluated in tetramer\(^+\) IFN-\(\gamma\)^+ memory CD8\(^+\) T cells from each cohort of monkeys. Once again, granzyme B levels were higher in both the CM and EM subsets of tetramer\(^+\) IFN-\(\gamma\)^+ CD8\(^+\) T cells of the SIV\(^+\) progressor animals than in the vaccinated, SHIV-infected animals (data not shown). These results suggest that the elevated levels of granzyme B in viremic animals with advanced disease may reflect the fact that functional Ag-specific CD8\(^+\) T lymphocytes are being called upon to control high levels of immunodeficiency virus replication.

**Tetramer\(^+\) EM CD8\(^+\) T cells degranulate upon exposure to epitope peptide**

The ability of p11C-specific CD8\(^+\) T cells to degranulate upon epitope peptide exposure and subsequent activation was assessed in all infected monkeys. For this particular analysis, the two groups of vaccinated, infected animals were combined into one cohort of monkeys (SHIV\(^+\) vaccinated). In all study cohorts, a reduction from background granzyme B levels was seen in epitope peptide-stimulated CD8\(^+\) T cells (data not shown). When these p11C-specific granzyme B CD8\(^+\) T cells were analyzed by subdividing them according to their memory phenotypes, cells in both animal groups that were CM did not degranulate upon stimulation with p11C peptide (Fig. 5, A and B). However, tetramer\(^+\) EM CD8\(^+\) T cells degranulated on activation with p11C epitope peptide (Fig. 5, C and D).
cells in both groups consistently degranulated following a brief p11C peptide stimulation (Fig. 5, C and D). For example, p11C-specific CM CD8+ T cells of SHIV+ vaccinated monkeys showed no reduction in intracellular granzyme B levels following epitope peptide stimulation (Fig. 5B, unstimulated, median 24% vs peptide-stimulated, median 24%, p = 0.89, paired t test). The same analyses applied to absolute numbers of p11C-specific CM CD8+ T cell subpopulations also did not demonstrate a statistically significant reduction in granzyme B expression in this particular subset of p11C-specific CD8+ T cells from vaccinated, SHIV-infected monkeys (unstimulated, median 871 cells/ml vs peptide-stimulated, median 829 cells/ml, p = 0.09, paired t test). However, a significant fall (12%, p = 0.0003, paired t test) in intracellular granzyme B levels in tetramer+ EM CD8+ T cells of these animals was observed following stimulation with the epitope peptide (Fig. 5D, unstimulated, median 64% vs peptide-stimulated, median 52%, p = 0.0003, paired t test). Correspondingly, a reduction in intracellular granzyme B levels was also demonstrated in assessing absolute numbers of tetramer+ EM CD8+ T cells of these animals following stimulation with epitope peptide (unstimulated, median 9411 cells/ml vs peptide-stimulated, median 5005 cells/ml, p = 0.002, paired t test). This reduction in intracellular granzyme B production can likely be attributed to its release during granule exocytosis following p11C-specific EM CD8+ T cell activation.

Intracellular granzyme B is best preserved in Gag-specific EM CD8+ T cells of vaccinated, infected monkeys

Tetramer+ granzyme B+ CD8+ T cells following epitope-peptide stimulation from each group of infected animals were further analyzed for their expression of CD28/CD95. In all three groups of animals, a greater percentage of the p11C tetramer+ granzyme B+ CD8+ T cells were EM than CM T cells. Moreover, both groups of vaccinated, SHIV-infected animals had a significantly higher percentage of p11C-specific granzyme B+ EM CD8+ T cells (medians 68 and 69%) than unvaccinated, infected animals with progressive disease (median 55%) (Fig. 6, p = 0.002, DNA/rAd vs SIV+ monkeys, Mann-Whitney U test; p = 0.003, DNA/rPox vs SIV+ monkeys, Mann-Whitney U test). These data suggest that prior vaccination was associated with the preservation of cytotoxic potential in virus-specific EM CD8+ T cells.

Discussion

We used polychromatic flow cytometry to assess the frequency of virus-specific CD8+ memory T cell subpopulations in SIV- and SHIV-infected monkeys. These data showed the preservation of virus-specific CM CD8+ T cells following SHIV infection in previously immunized monkeys. In contrast to this, infected, viremic monkeys that were not previously immunized demonstrated a significant loss of SIV Gag-specific CM CD8+ T cells, with ~90% of their Gag-specific CD8+ T cells having an EM phenotype.

The mechanism by which prior vaccination protected these cells following SHIV infection remains unclear. It has been recently reported that the rate and extent of memory CD4+ T cell loss may serve as a good prognosticator of disease progression in rhesus monkeys (35). Moreover, we have recently shown that memory CD4+ T cell immune function is maintained in animals that were immunized before viral challenge (36). Therefore, we expected to find a correlation between the extent of preservation of memory CD4+ T cells and virus-specific CM CD8+ T cells in vaccinated, infected animals. However, no relationship was found between the extent of preservation of tetramer+ CM CD8+ T cells and either the absolute total CD4+ T cell counts, the memory CD4+ T cell counts, or the absolute and relative declines of memory CD4+ T cells (data not shown). It is possible that the preservation of certain CD4+ T cell functional capacities was associated with this preservation of tetramer+ CM CD8+ T cells (36).

Because vaccination has been shown to preserve the frequency of virus-specific CM CD8+ T cells following viral challenge, we asked whether vaccination might preserve the ability of these memory T cells to produce antiviral cytokines and lytic molecules involved in the killing of virally infected cells. Although clinical disease progression in infected individuals may be a consequence of functional CD8+ T cell impairment, the mechanism by which chronic immunodeficiency virus infection inhibits the ability of CTL to control viral replication and mediate killing of virally infected cells is not understood. Suggested explanations for this functional CD8+ T cell impairment include the absence of CD4+ T cell help and CD8+ T cell exhaustion due to persistent exposure to viral Ag (37, 38). In the present study, we evaluated SIV Gag-specific memory CD8+ T cell functional capacity in these three cohorts of monkeys by assessing the ability of these cells to produce the antiviral cytokine IFN-γ and the lytic enzyme granzyme B following exposure to p11C peptide Ag.

Ag-stimulated IFN-γ production by virus-specific CD8+ T lymphocytes has been used as an indicator of CD8+ T cell functional capacity in cellular assays. Recently, however, it has been suggested that the production of other cytokines such as IL-2 and TNF-α may be more useful indicators of CD8+ T cell dysfunction because the loss of these cytokines has been shown to precede the loss of IFN-γ production by virus-specific CD8+ T cells (7, 10). In fact, IL-2 responses are 3-fold lower than IFN-γ responses by epitope-specific CD8+ T lymphocytes of SIV/SHIV-infected monkeys following epitope peptide stimulation in vitro (data not shown). In the present study, we focused on the evaluation of IFN-γ because its production following antigenic stimulation is more robust and persistent than that of the other cytokines.

Intracellular cytokine staining assays used in the present study demonstrated that IFN-γ production by Ag-specific memory CD8+ T cells following viral peptide stimulation was comparable in all infected animal groups in this study. However, evaluation of the tetramer+ IFN-γ-producing memory CD8+ T cell populations of the monkeys demonstrated that a substantially larger fraction of

FIGURE 6. Granzyme B-producing, tetramer+ memory CD8+ T cells in the three cohorts of infected monkeys. The total p11C tetramer+ granzyme B+ CD8+ T cell population was evaluated for expression of CD28 and CD95 for each animal. The median percentage of cells that were EM (CD28+CD95+) in each cohort of infected monkeys is shown. Statistical differences shown are based on the Kruskal-Wallis test (in bold, upper left corner). Statistical differences between individual groups were calculated using the Mann-Whitney U test, and are represented where significant by brackets.
these cells were CM in the vaccinated than in the unvaccinated, infected monkeys. Therefore, the clinical outcome in vaccinated animals appeared to be associated with the ability of virus-specific CM CD8⁺ T cells to produce IFN-γ.

The impact of persistent viral replication on granzyme B expression in Gag-specific CD8⁺ T-cells of these monkeys was complex. The present analysis of granzyme B expression in SIV/SHIV-infected rhesus monkeys showed that tetramer⁺ CD8⁺ T-cells of monkeys with high levels of viremia expressed more granzyme B than those of monkeys that controlled viral replication. Nevertheless, viral burden had no discernable effect on granzyme B exocytosis by virus-specific EM CD8⁺ T cells. Moreover, chronic viral infection did not interfere with the ability of virus-specific CD8⁺ T-cells to undergo granzyme B exocytosis following antigenic stimulation. It remains unclear, however, whether vaccination had any influence on the frequency of virus-specific granzyme B⁺ CD8⁺ T-cells and their cytolytic function. Nevertheless, we found that vaccinated, infected monkeys had a significantly greater percentage of tetramer⁺ granzyme B⁺ CD8⁺ T-cells that were EM than were seen in unvaccinated, infected monkeys. This finding suggests that prior vaccination was associated with the preservation of virus-specific CD8⁺ EM T-cells that can function to control viral infection via granzyme-mediated cytocidal activity of infected cells. Although Ag-specific CD8⁺ T-cells in unvaccinated, viremic monkeys had significantly elevated levels of granzyme B, nearly half of these cells were found to have a CM phenotype, cells that are incapable of degranulation upon activation. Therefore, Ag-specific CD8⁺ T lymphocytes in monkeys with progressive disease may be poorly suited for controlling high levels of immunodeficiency virus replication because few of these cells are capable of granzyme-mediated cytotoxicity of virally infected cells.

The present studies therefore show that clinical protection from SHIV-89.6P infection in previously immunized rhesus monkeys is associated with the preservation of functional memory CD8⁺ T cell subsets. Moreover, the protection conferred by the DNA/Ad vaccination regimen may be more durable than that conferred by DNA/AdPox immunization. Nevertheless, vaccination with either DNA prime/recombinant viral vector boost regimen was effective in preserving SIV Gag-specific CM CD8⁺ T-cells that are functionally capable of producing IFN-γ and EM CD8⁺ cells that are able to release granzyme B.

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Disclosures

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15. The authors have no financial conflict of interest.


