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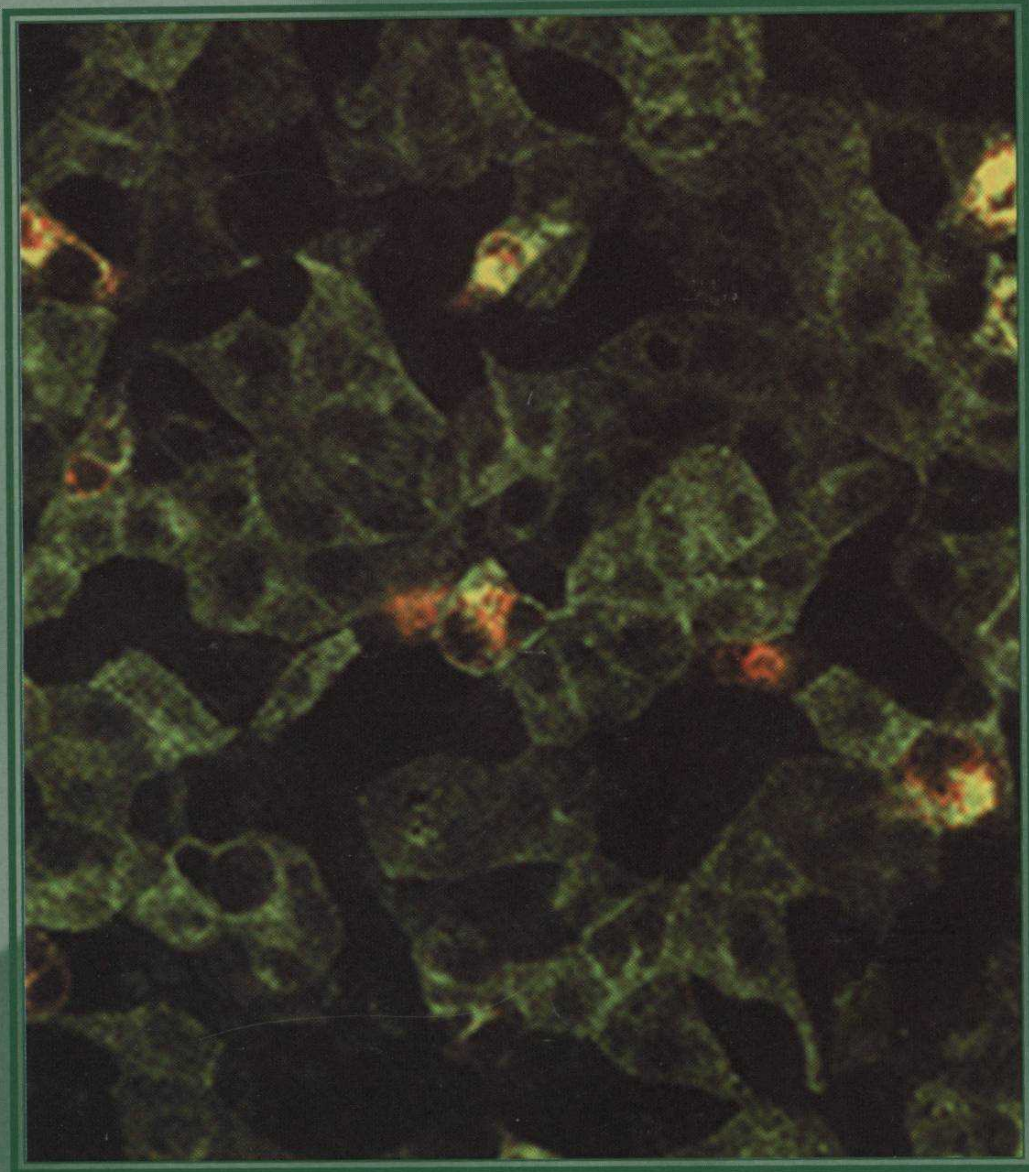
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Dysfunction of Simian Immunodeficiency Virus/Simian Human Immunodeficiency Virus-Induced IL-2 Expression by Central Memory CD4⁺ T Lymphocytes¹

Yue Sun,* Jörn E. Schmitz,* Paula M. Acierno,* Sampa Santra,* Ramu A. Subbramanian,* Dan H. Barouch,* Darci A. Gorgone,* Michelle A. Lifton,* Kristin R. Beaudry,* Kelledy Manson,[†] Valerie Philippon,[†] Ling Xu,[‡] Holden T. Maecker,[§] John R. Mascola,[‡] Dennis Panicali,[†] Gary J. Nabel,[‡] and Norman L. Letvin^{2,*}

Production of IL-2 and IFN- γ by CD4⁺ T lymphocytes is important for the maintenance of a functional immune system in infected individuals. In the present study, we assessed the cytokine production profiles of functionally distinct subsets of CD4⁺ T lymphocytes in rhesus monkeys infected with pathogenic or attenuated SIV/simian human immunodeficiency virus (SHIV) isolates, and these responses were compared with those in vaccinated monkeys that were protected from immunodeficiency following pathogenic SHIV challenge. We observed that preserved central memory CD4⁺ T lymphocyte production of SIV/SHIV-induced IL-2 was associated with disease protection following primate lentivirus infection. Persisting clinical protection in vaccinated and challenged monkeys is thus correlated with a preserved capacity of the peripheral blood central memory CD4⁺ T cells to express this important immunomodulatory cytokine. *The Journal of Immunology*, 2005, 174: 4753–4760.

Virus-specific CD4⁺ T lymphocytes play a central role in the immune containment of HIV. They contribute to HIV clearance both by providing help for B cell responses and by maintaining effective CTL (1, 2). Recent studies suggest that functional CD4⁺ T lymphocytes are also required at the time of immune priming for the development of memory CD8⁺ T lymphocytes (3–5).

CD4⁺ T lymphocytes of HIV-infected individuals display functional defects, including reduced proliferative responses to both Ags and mitogens (6). These functional CD4⁺ T lymphocyte defects are associated with reduced production of IL-2, which can be partially corrected in vitro by the addition of exogenous IL-2 (7, 8). The integrity of CD4⁺ T lymphocytes that secrete IL-2, or a subset of CD4⁺ T lymphocytes that secrete both IFN- γ and IL-2, is associated with good clinical outcome in HIV-infected individuals (9–11). Thus, production of IL-2 by CD4⁺ T lymphocytes is important for the maintenance of a functional immune system in infected individuals.

CD4⁺ T lymphocytes have been divided into central memory and effector memory cell subsets based on their homing capacity and effector function (12, 13). Central memory T lymphocytes

home to lymphoid organs, have little or no effector function, produce predominantly IL-2, and have a high capacity to proliferate. In contrast, effector memory T lymphocytes migrate to peripheral tissues, display effector function, produce primarily IFN- γ , and lack significant proliferative capacity. Precisely how the defective cellular production of cytokines is associated with the development of memory CD4⁺ T lymphocytes in HIV-infected individuals remains to be elucidated.

In the present study, the cytokine production of CD4⁺ T lymphocyte subsets was analyzed in rhesus monkeys infected with pathogenic viruses and attenuated viruses, and in vaccinated monkeys that were protected from developing disease following pathogenic simian human immunodeficiency virus (SHIV)³ challenge. The results of this study suggest that the ability of SIV/SHIV-stimulated central memory CD4⁺ T lymphocytes to synthesize IL-2 is an immune correlate of disease protection following primate immunodeficiency virus infection.

Materials and Methods

Animals and viruses

Heparinized 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 (14). Viruses used in this study were the nonpathogenic SHIV-89.6, the pathogenic SHIV-89.6P, the pathogenic SIVmac251, the attenuated J5 strain of SIVmac251, and the pathogenic SIVsmE660.

CD4⁺ T lymphocyte counts and plasma viral RNA levels

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 per ml (Bayer Diagnostics).

³ Abbreviations used in this paper: SHIV, simian human immunodeficiency virus; rAd5, recombinant adenovirus serotype 5; MVA, modified vaccinia virus Ankara; SEB, staphylococcal enterotoxin B.

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Table I. Vaccination/challenge history of study animals

	Immunogens	Immunizations	Challenge
Vaccinated only (Vaccinated) 90-98, 95-98, 135-97, 128-97, 132-97, 196-97	Plasmid DNAs expressing genes encoding <i>gag</i> , <i>pol</i> , <i>nef</i> , and <i>env</i> CTL epitopes Pox virus or rAd5 expressing SIVmac239 Gag/Pol, HIV-1 89.6P Env	Plasmid DNA i.m. MVA, vaccinia, or rAd5 i.m.	No
(Vaccinated) AW2P, AW13, AW28, AV83	Plasmid DNAs and/or rAd5 expressing SIVmac239 Gag/Pol/Nef, HIV-1 89.6P Env	Plasmid DNA i.m.—wk 0, 4, 8 rAd5 i.m.—wk 26, 159	No
Vaccinated/SHIV-89.6P challenged (Gag-Pol-Nef/Env) PKB, AV8T, AK67, AK85, AW2V, PWE, AV2E, AV67, AX7H, AE6B	Plasmid DNAs and rAd5 expressing SIVmac239 Gag/Pol/Nef, HIV-1 89.6P Env	Plasmid DNA i.m.—wk 0, 4, 8 rAd5 i.m.—wk 26	SHIV-89.6P i.v.—wk 38
(Gag-Pol-Nef/Mock Env) JBA, TKF, AV65, TCJ, AK6X, AV5K	Plasmid DNAs and rAd5 expressing SIVmac239 Gag/Pol/Nef	Plasmid DNA i.m.—wk 0, 4, 8 rAd5 i.m.—wk 26	SHIV-89.6P i.v.—wk 38

Vaccination protocol

Three groups of vaccinated monkeys were included in this study (Table I). The first group of monkeys (vaccinated/SHIV-89.6P challenged) was immunized three times by the i.m. route with 10 mg of plasmid DNA containing SIVmac239 Gag/Pol/Nef, with or without HIV-1 89.6P Env. Following an 18-wk rest, each of the DNA-primed monkeys was boosted with 2×10^{12} PFU of recombinant adenovirus serotype 5 (rAd5) expressing the same SIV/HIV-1 genes by the i.m. route. Animals were challenged at wk 38 with 100 50% monkey infectious doses (MID₅₀) of SHIV-89.6P by the i.v. route. The second group of monkeys (vaccinated only) was immunized two to four times by the i.m. route with plasmid DNA containing *gag*, *pol*, *nef*, and *env* genes encoding for CTL epitopes (15), and boosted by i.m. injection

with recombinant modified vaccinia virus Ankara (MVA), vaccinia virus, or rAd5 constructs. The third group of monkeys (vaccinated only) either was immunized three times by i.m. route with 10 mg of plasmid DNA containing SIVmac239 Gag/Pol/Nef and HIV-1 89.6P Env, or received no plasmid DNA immunization. Following an 18-wk rest, all DNA-primed monkeys, as well as a group of naïve monkeys, received 2×10^{12} PFU of rAd5 expressing the same SIV/HIV-1 genes. Monkeys received a second inoculation of rAd5 constructs 133 wk later.

Antibodies

The Abs used in this study were directly coupled to FITC, PE, PE-Texas Red (ECD), allophycocyanin, PerCP-Cy5.5, Alexa Fluor 700, or PE-Cy7.

Table II. Clinical data on rhesus monkeys infected with pathogenic or attenuated viruses

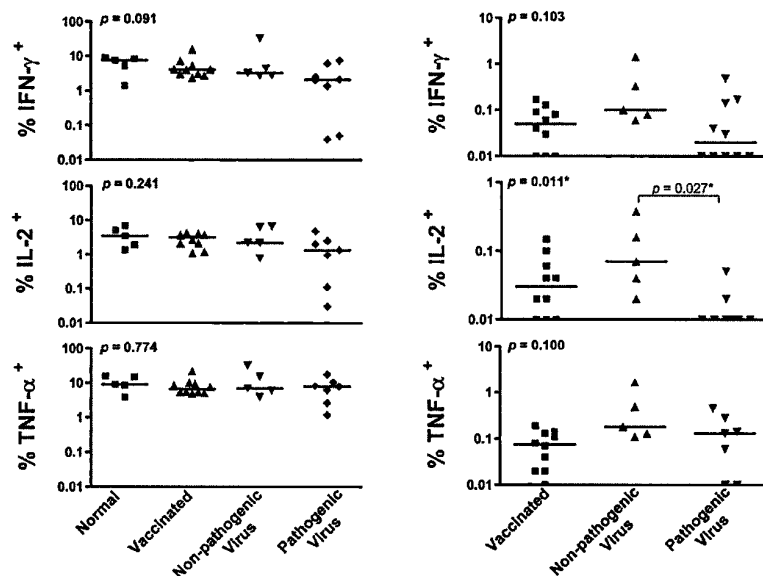
Group	Infecting Virus	Years of Infection	Plasma Viral RNA (copies/ml)	CD4 ⁺ T Cells (count/ μ l)
Monkeys with no detected plasma viral RNA ^a				
403-91	SIVmac251 (J5)	12	<125	773
191-96	SIVmac251	6	<125	374
556-92	SHIV-89.6	6	<125	689
287-94	SHIV-89.6	6	<125	756
206-93	SHIV-89.6	6	<125	368
267-95	SHIV-89.6	4	<125	1,117
196-88	SHIV-89.6P	1	135	961
Median		6	<125	756
Monkeys with detected plasma viral RNA ^b				
AV52	SIVmac251	1	14,122	167
PH0465	SIVmac251	1	4,118,200	91
JHB	SIVmac251	1	4,925,000	236
PH0685	SIVmac251	1	95,510	249
PH0305	SIVmac251	1	3,313,400	190
AV8P	SIVmac251	1	29,430	365
136-99	SIVsmE660	1	2,389,900	232
238-95	SIVsmE660	1	1,015,665	150
223-95	SIVsmE660	1	369,611	437
261-95	SIVsmE660	1	2,389,900	180
BE86	SIVmac251	0.6	126,049	659
BR32	SIVmac251	0.6	9,444	536
BH25	SIVmac251	0.6	9,276	726
Median		1	369,611	236

^a Referred to in the figures as nonpathogenic virus.

^b Referred to in the figures as pathogenic virus.

A SEB-stimulated CD4⁺ T lymphocytes B Gag-specific CD4⁺ T lymphocytes

FIGURE 1. Cytokine expression profiles of CD4⁺ T lymphocytes of normal, vaccinated, and SIV/SHIV-infected rhesus monkeys. The evaluated animals included healthy, uninfected monkeys; monkeys primed with plasmid DNA and boosted with a recombinant poxvirus vaccine or recombinant adenovirus vaccine; monkeys infected with attenuated SIV/SHIV; and monkeys infected with pathogenic SIV isolates. CD4⁺ T lymphocyte responses to SEB (A) or pooled Gag peptides (B) were measured. IFN- γ , IL-2, and TNF- α expression are shown as percent positive CD4⁺ T lymphocytes. Values for each monkey are depicted as separate points, and the bars represent the median value for each group. Nonresponders (zero values) are shown on the base of each log plot at the frequency of 0.01. The Kruskal-Wallis test was used to evaluate significant differences between groups (bolded *p* values), and the Mann-Whitney *U* test was used to investigate the statistical significance of the individual data pairs. To account for multiple comparisons, the *p* values were adjusted by the Holm's method. Statistically significant differences using this test are highlighted in the figure by an asterisk (*).



The following mAbs were used: anti-CD28-FITC (28.2; BD Biosciences), anti-Ki67-FITC (B56; BD Biosciences), anti-CD28-PE (28.2; BD Biosciences), anti-CD95-PE (DX2; BD Biosciences), anti-CCR5-PE (3A9; BD Biosciences), anti-CCR7-PE (150503; R&D System), anti-CD11a-PE (HI111; BD Biosciences), anti-CD45RA-PE (2H4; Beckman Coulter), anti-CD62L-PE (SK11; BD Biosciences), anti-CD25-PE (M-A215; BD Biosciences), anti-Bcl-2-PE (Bcl-2/100; BD Biosciences), anti-Granzyme B-PE (CLB-GB11; PeliCluster), anti-CD4-ECD (19thy5d7; Beckman Coulter), anti-CD8 α -ECD (7PT; Beckman Coulter), anti-CD95-allophycocyanin (DX2; BD Biosciences), anti-IFN- γ -allophycocyanin (B27; BD Biosciences), anti-TNF- α -allophycocyanin (MAB11; BD Biosciences), anti-IL-2-allophycocyanin (MQ1-17H12; BD Biosciences), anti-CD4-PerCP-Cy5.5 (L200; BD Biosciences), anti-CD3-Alexa Fluor 700 (SP34-2; BD Biosciences), and anti-IFN- γ -PE-Cy7 (B27; BD Biosciences).

PBMC stimulation and intracellular cytokine staining

PBMC were separated from whole blood by Ficoll density gradient centrifugation. PBMC (10^6) were incubated at 37°C in a 5% CO₂ environment for 6 h in the presence of RPMI 1640/10% FCS medium alone (unstimulated), a pool of 15-mer Gag peptides (5 μ g/ml each peptide), or staphylococcal enterotoxin B (SEB) (5 μ g/ml, Sigma-Aldrich) as a positive control. All cultures contained Monensin (GolgiStop; BD Biosciences) as well as 1 μ g/ml anti-CD49d (BD Biosciences). The cultured cells were stained with mAbs specific for cell surface molecules before fixation. The PBMC were washed twice with PBS-2% FCS and then fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) in accordance with the manufacturer's protocol. Cells were washed twice with 1 $\times$ Perm/Wash buffer (BD Biosciences) and then stained with anti-cytokine mAb. Anti-cytokine mAb titers for optimal staining were determined in preliminary experiments. Cells were washed twice with 1 $\times$ Perm/Wash buffer and then fixed in 1.5% formaldehyde-PBS. The following mAb combinations were used: CD28-FITC/CD95-PE/CD4-ECD/cytokine-allophycocyanin (IFN- γ , IL-2, and TNF- α) and CD28-FITC/CD95-PE/CD8 α -ECD/IL-2-allophycocyanin/CD4-PerCP-Cy5.5/CD3-Alexa700/IFN- γ -PE-Cy7. Samples were collected either on a FACSCalibur instrument (BD Biosciences) and analyzed with CellQuest software (BD Biosciences) or on a LSR II instrument (BD Biosciences) and analyzed using FlowJo software (Tree Star). Approximately 100,000 to 300,000 events in the lymphocyte gate were acquired. The background level of cytokine staining varied from sample to sample, but was typically <0.04% of the CD4⁺ T lymphocytes. The only samples considered positive were those in which the percentage of cytokine-staining cells was at least twice that of the background or in which there was a distinct population of cytokine brightly positive cells.

Statistical analyses

A comparison of values was first performed using the Kruskal-Wallis test. If any results were statistically significant (*p* < 0.05), the data pairs were

compared by the Mann-Whitney *U* test. The Holm's method was used to account for multiple comparisons, and the *p* values were adjusted accordingly; only significant values (*p* < 0.05) after applying the Holm's method were indicated in the figures. A Spearman correlation test was performed to analyze the association between the cytokine responses and plasma viral RNA level. A value of *p* < 0.05 was considered significant and was highlighted in the figures with an asterisk (*), and *p* values that approached 0.05 were also shown.

Results

IL-2, IFN- γ , and TNF- α production by CD4⁺ T lymphocytes in naive, vaccinated, and SIV/SHIV-infected rhesus monkeys

To determine the functional capacity of CD4⁺ T lymphocytes from naive, vaccinated, and SIV/SHIV-infected rhesus monkeys, the expression of selected cytokines by SEB-stimulated lymphocytes was first characterized. One of the cohorts of vaccinated animals that was evaluated received plasmid DNA prime and recombinant poxvirus boost immunizations 6 mo before evaluation

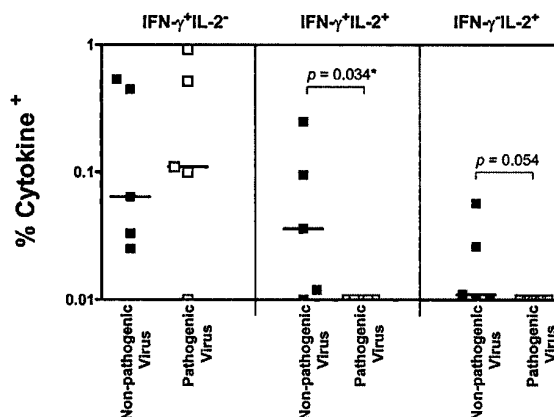
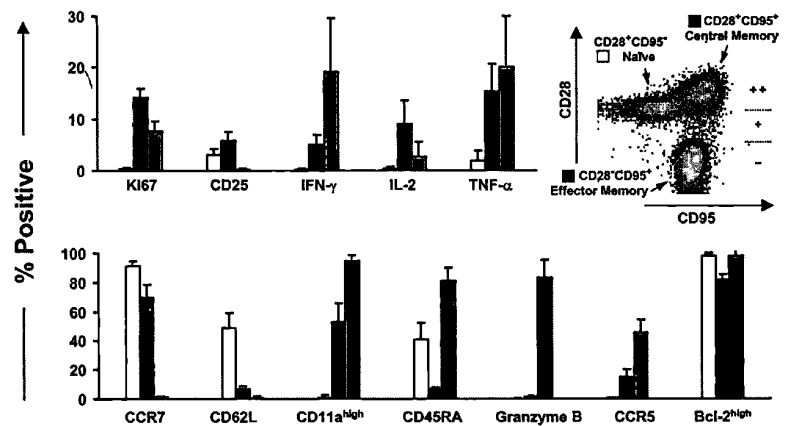


FIGURE 2. Comparison of CD4⁺ T cell frequencies of IFN- γ only, IL-2⁺ only, and IFN- γ +IL-2⁺ double-positive cells following Gag stimulation in PBMC of monkeys infected with attenuated SIV/SHIV and monkeys infected with pathogenic SIV isolates. Nonresponders (zero values) are shown on the base of each log plot at the frequency of 0.01. Significant differences are shown as determined by the Mann-Whitney *U* test.

FIGURE 3. The expression of molecules associated with maturation and function on CD4⁺ T lymphocyte subsets defined by CD28 and CD95. Cell surface expression of molecules associated with maturation and function on CD28⁺CD95⁺ (naive), CD28⁺CD95⁺ (central memory), and CD28⁺CD95⁺ (effector memory) CD4⁺ T cell subsets was evaluated. Lymphocytes from five healthy, uninfected monkeys were studied. The symbols ++, +, and - reflect the relative staining intensities of the anti-CD28 mAb.



(Table I). The other cohort of vaccinated monkeys that was studied was given plasmid DNA prime and recombinant adenovirus boost immunizations 5 wk before analysis (Table I). Because the monkeys vaccinated by either of the regimens developed comparable immune responses, the data from both cohorts were combined for purpose of analysis. Monkeys infected with nonpathogenic SHIV-89.6, attenuated SIVmac251(J5), or highly pathogenic SIVmac251 and SIVsmE660 isolates were characterized to determine plasma viral RNA levels and CD4⁺ T lymphocyte counts (Table II).

No statistically significant differences were noted in the frequencies of IFN- γ , IL-2-, and TNF- α -producing CD4⁺ T lymphocytes in response to SEB from normal, vaccinated, and infected monkeys (Kruskal-Wallis test) (Fig. 1A). However, a trend toward lower frequencies of IFN- γ - and IL-2-producing CD4⁺ lymphocytes was seen in monkeys infected with the pathogenic SIV isolates. We also noticed markedly decreased *in vitro* viability of PBMC from monkeys with advanced disease following stimulation with SEB, with the remaining CD4⁺ T cells having no cytokine-producing capacity after stimulation (data not shown). Of note, monkeys infected with pathogenic SIV isolates had significantly lower frequencies of Gag-specific IL-2-producing CD4⁺ T cells than those infected with nonpathogenic virus ($p = 0.027$, Holm's test) (Fig. 1B). Thus, the capacity of Gag-specific CD4⁺ T lymphocytes to express IL-2 was associated with the clinical status of the infected monkeys.

Gag-specific IFN- γ alone, IL-2⁺ alone, and IFN- γ IL-2⁺ CD4⁺ T cell frequencies in monkeys infected with attenuated SIV/SHIV or pathogenic SIV isolates

Recent studies have shown that the maintenance of a subset of HIV-specific CD4⁺ T cells that produce both IFN- γ and IL-2 is associated with good clinical outcome in HIV-infected individuals (11). We therefore sought to determine whether the loss of IL-2-producing cells in the monkeys infected with pathogenic virus reflected a loss of cells producing IL-2 alone or cells producing IL-2 and IFN- γ . To this end, we quantitated Gag-specific CD4⁺ T cells that were IFN- γ ⁺ alone, IL-2⁺ alone, and IFN- γ ⁺IL-2⁺ in PBMC of monkeys infected with attenuated SIV/SHIV and of monkeys infected with pathogenic SIV isolates (Fig. 2). PBMC were stained simultaneously for both IFN- γ and IL-2, and the frequency of CD4⁺ T cells that produced IFN- γ or IL-2, as well as cells that produced both IFN- γ and IL-2 was measured in a seven-color flow cytometric assay. The percentage of Gag-specific CD4⁺ T cells producing IFN- γ alone was similar in PBMC of both groups of monkeys. However, the median frequency of Gag-specific IFN- γ ⁺IL-2⁺CD4⁺ T cell responses was significantly higher in monkeys infected with the attenuated SIV/SHIV than in monkeys in-

fectured with pathogenic SIV isolates ($p = 0.034$). Moreover, although very few Gag-specific CD4⁺ T cells produced IL-2 alone in the evaluated monkeys, a trend toward lower frequencies of these cells was seen in monkeys infected with pathogenic SIV

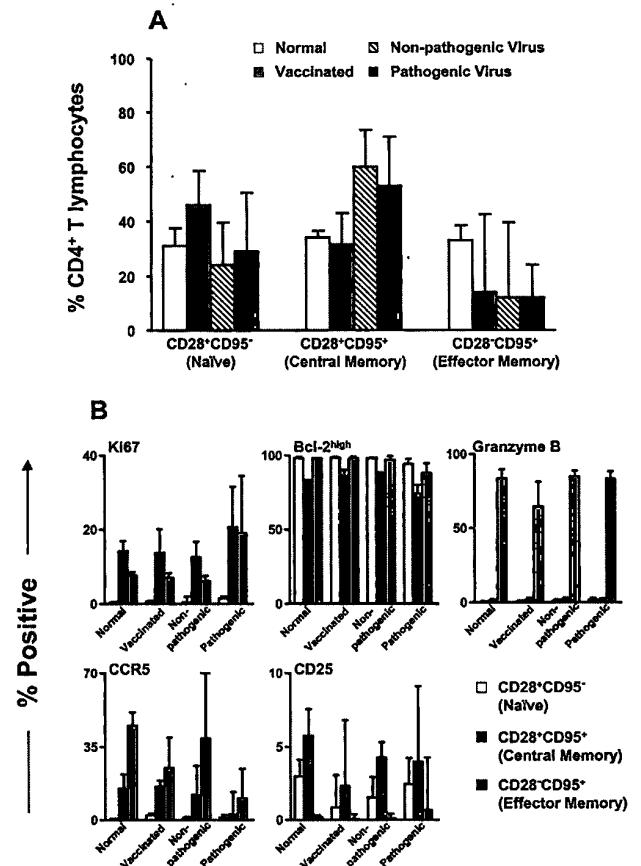
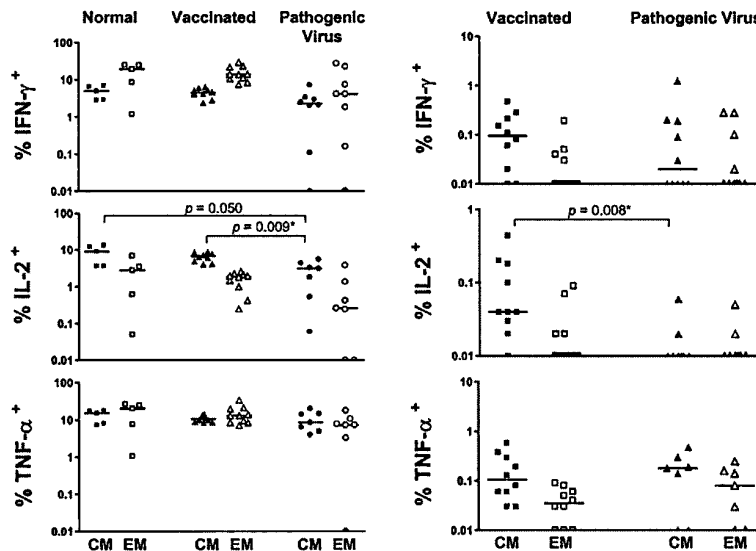


FIGURE 4. Maturation and function-associated molecules on naive and memory subsets of CD4⁺ T lymphocytes from normal, vaccinated, and SIV/SHIV-infected rhesus monkeys. A, The expression of CD28 and CD95 on peripheral blood CD4⁺ T lymphocytes of healthy uninfected monkeys ($n = 5$), monkeys primed with plasmid DNA and boosted with a recombinant poxvirus vaccine ($n = 6$), monkeys infected with attenuated SIV/SHIV ($n = 5$), and monkeys infected with pathogenic SIV isolates ($n = 10$). B, Comparison of the expression of molecules associated with maturation and function on CD4⁺ T lymphocyte subsets defined by CD28 and CD95 expression in each of these four groups of monkeys. The interquartile range is indicated for each bar.

A SEB-stimulated CD4⁺ T lymphocytes B Gag-specific CD4⁺ T lymphocytes

FIGURE 5. Cytokine expression profiles of rhesus monkey CD4⁺ T lymphocyte subsets. Intracellular cytokine expression was measured in SEB-stimulated (A) or Gag pooled peptide-stimulated (B) PBMC from healthy uninfected monkeys, monkeys primed with plasmid DNA and boosted with a recombinant poxvirus vaccine or a recombinant adenovirus vaccine, and monkeys infected with pathogenic SIV isolates. IFN- γ , IL-2, and TNF- α expression are shown as percent positive CD28⁺CD95⁺ central memory (CM) and CD28⁻CD95⁺ effector memory (EM) CD4⁺ T lymphocytes. Values for each monkey are depicted as separate points, and the bars represent the median value for each group. Nonresponders (zero values) are shown on the base of each log plot at the frequency of 0.01. Significant differences are shown as determined by the Mann-Whitney *U* test. To account for multiple comparisons, the *p* values were adjusted by the Holm's method.



isolates than in monkeys infected with attenuated virus ($p = 0.054$). These results suggest that the presence of Ag-specific CD4⁺ T cells able to produce IL-2 is associated with effective immune control of virus.

Cell surface characterization of CD4⁺ T lymphocytes: maturation and functional markers on lymphocyte subsets defined on the basis of CD28 and CD95 expression

The expression of CD28 and CD95 has been used to define distinct subsets of CD4⁺ T lymphocytes (16). Naive CD4⁺ T cells can be identified by their intermediate expression of CD28 and lack of

CD95 expression. Memory CD4⁺ T cells acquire CD95 expression and can be divided into central memory and effector memory subsets based on CD28 expression.

To examine the expression of other putative naive and memory cell-associated surface molecules on rhesus monkey CD4⁺ T lymphocytes, we performed phenotypic and functional analyses of PBMC defined by CD28 and CD95 expression in five normal animals. Naive CD4⁺ T cells (CD28⁺CD95⁻) were homogenous small lymphocytes that expressed moderate-to-high levels of lymphoid tissue-homing molecules (CCR7⁺, CD62L⁺), low levels of adhesion molecules (CD11a^{low}), and lacked expression of CCR5, a molecule associated with homing to effector sites (Fig. 3). These cells showed minimal proliferation (Ki67⁻, Bcl-2^{high}) and no effector function (no cytokine production capacity, Granzyme B⁻). In contrast, effector memory CD4⁺ T cells (CD28⁻CD95⁺) expressed cell surface molecules that facilitate homing to effector sites (CCR7⁻, CD62L⁻, CD11a^{high}, CCR5⁺). They had phenotypic characteristics of effector cells (Granzyme B⁺, and were capable of producing IFN- γ and TNF- α). Central memory CD4⁺ T cells (CD28⁺CD95⁺) had a different phenotypic profile. Two-thirds of the central memory CD4⁺ T cells were CCR7⁺. The central memory CD4⁺ T cells had the capacity to produce IL-2 and proliferate, but lacked cytotoxic effector function (Granzyme B⁻). Some naive and central memory CD4⁺ T cells were CD25⁺ and may therefore have regulatory function. These observations confirmed that the rhesus monkey CD4⁺ T lymphocyte subsets defined by CD28 and CD95 expression exhibited physiologic characteristics of naive, central memory, and effector memory CD4⁺ T cells as previously described (12, 16).

Maturation and function of CD4⁺ T lymphocytes in naive, vaccinated, and SIV/SHIV-infected rhesus monkeys

Having demonstrated a loss of IL-2 production by Gag-specific CD4⁺ T cells in monkeys with progressive disease (Fig. 1B), we sought to determine whether this reflected changes in the relative representation of naive and memory CD4⁺ T cells in the peripheral blood of these animals or resulted from the loss of cytokine production capacity by a particular subset of CD4⁺ T cells. We therefore evaluated naive and memory CD4⁺ T cells defined by CD28 and CD95 expression in the naive, vaccinated, and infected cohorts

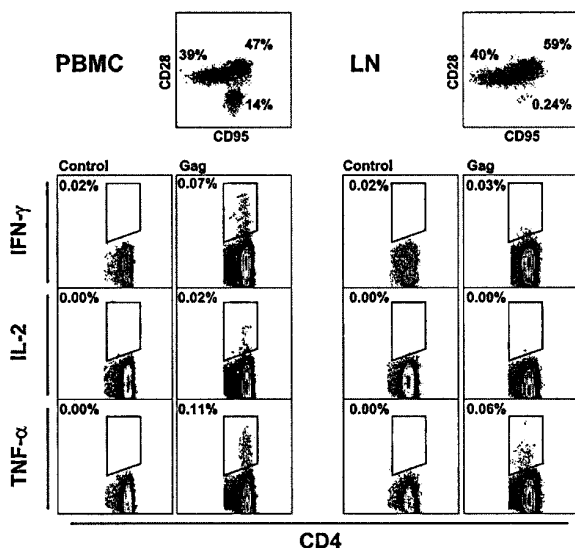


FIGURE 6. Naive and memory CD4⁺ T lymphocytes are present in blood and peripheral lymph node of a SHIV-89.6P-infected rhesus monkey. CD4⁺ T cells from PBMC and from a peripheral lymph node (LN) were examined for their expression of CD28 and CD95 (percent positive noted in each dot plot for the designated cell subsets). Cytokine expression profiles of rhesus monkey CD4⁺ T lymphocyte from PBMC and LN were compared. A study of cells from the representative monkey 196-88 infected with pathogenic SHIV-89.6P is shown.

Table III. Clinical data on vaccinated/SHIV-89.6P-infected rhesus monkeys^a

Group	Plasma Viral RNA (copies/ml)	CD4 ⁺ T Cells (count/ μ l)
Gag-Pol-Nef/Env		
PKB	5,639	519
AV8T	1,425	633
AK67	335	712
AK85	662	636
AW2V	223	1,211
PWE	<125	3,041
AV2E	684	821
AV67	494	919
AX7H	1,178	1,708
AE6B	227	744
Median	578	783
Gag-Pol-Nef/Mock Env		
JBA	1,075,346	54
TKF	<125	893
AV65	230,826	139
TCJ	171	514
AK6X	<125	869
AV5K	21,954	15
Median	11,603	327

^a All monkeys were infected for 15 mo.

of monkeys (Fig. 4A). Although the numbers of CD4⁺ T cells decreased in the monkeys infected with pathogenic viruses, no significant difference in the percentage of total CD4⁺ T cells with a naive (CD28⁺CD95⁻) ($p = 0.264$), central memory (CD28⁺CD95⁺) ($p = 0.296$), or effector memory (CD28⁻CD95⁺) ($p = 0.117$) phenotype was noted among the various groups of monkeys. A more detailed phenotypic analysis showed that monkeys infected with pathogenic virus had a relatively higher turnover of naive and memory CD4⁺ T cells (increased expression of Ki67, $p = 0.06$; decreased expression of Bcl-2, $p = 0.06$), and a significantly lower percentage of CCR5⁺CD4⁺ T cells compared with the other groups ($p = 0.005$) (Fig. 4B). Taken together, these data suggest that the loss of IL-2 production by Gag-specific CD4⁺ T cells in monkeys with progressive disease is not associated with depletion of naive or memory T cell subsets. However, as expected, there was a decrease in cells that might serve as targets for SIV infection (CD4⁺CCR5⁺).

Cytokine expression by CD4⁺ T cell subsets in naive, vaccinated, and pathogenic SIV-infected monkeys

To evaluate further the functional activity of CD4⁺ T cell subsets in normal, vaccinated, and SIV-infected monkeys, cytokine production by central memory and effector memory cells was analyzed (Fig. 5). In normal monkeys, SEB-induced IFN- γ production by CD4⁺ T lymphocytes was found predominantly in the effector memory cell population (median, 19.2 vs 4.97%). Conversely, IL-2 production by SEB-stimulated CD4⁺ T lymphocytes originated predominantly from central memory cells (median, 8.98 vs 2.77%). TNF- α expression was comparable in SEB-stimulated central memory and effector memory CD4⁺ T lymphocytes (median, 15.34 vs 20.47%). No significant differences in SEB-induced cytokine expression patterns were observed between the CD4⁺ T lymphocyte populations of vaccinated and normal monkeys. However, there was a significant decrease of IL-2 expression by central memory CD4⁺ T cells of monkeys infected with pathogenic virus (Fig. 5A). A trend toward a statistical difference between the normal monkeys and the monkeys infected with pathogenic virus in the SEB-stimulated IL-2-producing CD4⁺ T cells was also seen.

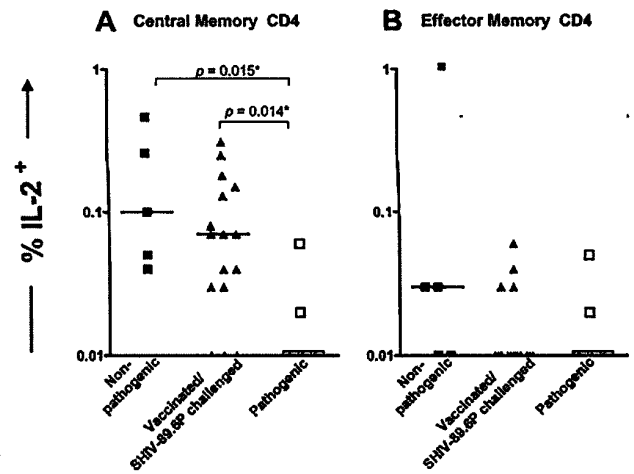


FIGURE 7. Vaccine protection against SHIV-89.6P-induced clinical disease is associated with preserved central memory CD4⁺ T lymphocyte functional capacity. Rhesus monkeys were vaccinated with immunogens expressing Gag/Pol/Nef with or without Env. Gag-specific IL-2 expression was evaluated in CD4⁺ T lymphocytes of previously vaccinated rhesus monkeys 15 mo after SHIV-89.6P challenge. A comparison of IL-2-producing CD28⁺CD95⁺ central memory CD4⁺ T cells (A) and IL-2-producing CD28⁻CD95⁺ effector memory CD4⁺ T cells (B) from vaccinated/challenged monkeys and unvaccinated monkeys infected with pathogenic virus demonstrated that, similar to monkeys infected with attenuated SIV/SHIV, central memory CD4⁺ T cells of the vaccinated/SHIV-89.6P-challenged monkeys have a preserved capacity to produce IL-2. Significant differences are shown as determined by the Mann-Whitney U test. The p values were adjusted by the Holm's method.

In contrast, Ag stimulation by a Gag peptide pool induced very different cytokine expression patterns in subpopulations of CD4⁺ T lymphocytes of vaccinated or SIV-infected monkeys (Fig. 5B). There were low but measurable levels of IFN- γ , IL-2, or TNF- α expression by Gag-specific CD28⁻CD95⁺ effector memory cells. Cytokine production by Gag-specific CD4⁺ T lymphocytes was found predominantly in cells with a central memory phenotype (CD28⁺CD95⁺). A selective decrease in IL-2 expression was seen in central memory CD4⁺ T cells of monkeys with progressive clinical disease, sometimes in the absence of changes in the expression of IFN- γ or TNF- α . These data suggest that loss of IL-2 production by Gag-specific CD4⁺ T cells in monkeys with progressive disease does not reflect changes in the relative representation of naive and memory CD4⁺ T cells in the peripheral blood of these animals. Rather, it results from the loss of IL-2 production capacity by central memory CD4⁺ T cells.

Naive and memory CD4⁺ T lymphocytes are present in blood and peripheral lymph nodes of infected rhesus monkeys

Because it has been shown that central memory CD4⁺ T cells can preferentially be found in lymphoid tissues, we sought to determine whether the frequency of central memory CD4⁺ T cells was high enough in the peripheral blood to reflect the biology of this cell subpopulation. We therefore compared the relative frequencies of naive and memory phenotype CD4⁺ T cells in the peripheral blood and the secondary lymphoid tissues in infected monkeys. Data from a representative monkey with a normal CD4⁺ T cell count and undetectable viral load are shown in Fig. 6. Central memory CD4⁺ T cell frequencies in peripheral blood tended to be a little lower than frequencies in the lymph node, and as expected, effector memory CD4⁺ T cells were absent from the lymph node. More importantly, Gag-specific cytokine-producing CD4⁺ T cells

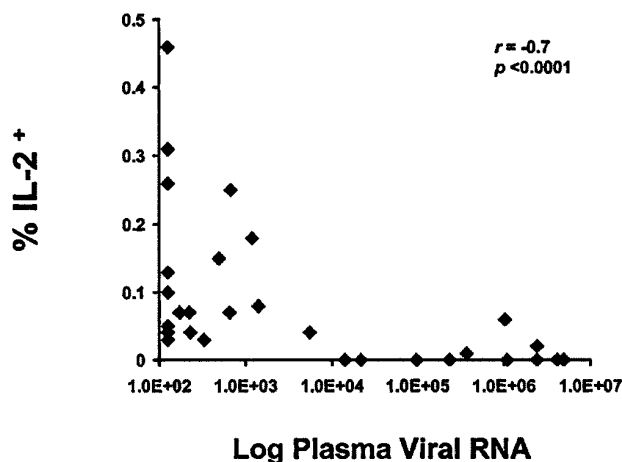


FIGURE 8. Inverse correlation between the frequencies of IL-2-producing CD28⁺CD95⁺ central memory CD4⁺ T cells and plasma viral RNA levels. Data on all evaluated animals (monkeys infected with attenuated SIV/SHIV, monkeys infected with pathogenic virus, and monkeys vaccinated and challenged with SHIV-89.6P) are indicated by individual filled diamonds (◆). The relationship between the percentage of IL-2-producing central memory CD4⁺ T cells and plasma viral RNA levels was evaluated using the Spearman correlation test ($r = -0.7$; $R^2 = 0.49$; $p < 0.0001$).

were present at twice the frequency in the peripheral blood than in the lymph node. Thus, central memory CD4⁺ T lymphocytes in the peripheral blood should reflect those seen in lymph nodes.

Vaccine protection against SHIV-induced clinical disease is associated with preserved central memory CD4⁺ T lymphocyte function

We next assessed cytokine expression profiles of Gag-specific CD4⁺ T cells from a cohort of monkeys that was vaccinated and subsequently challenged with SHIV-89.6P (Tables I and III). In this cohort, monkeys were vaccinated first with plasmid DNA and then with recombinant adenovirus constructs expressing SIV Gag/Pol/Nef with or without HIV-1 Env. Following challenge with SHIV-89.6P, all vaccinated monkeys maintained low plasma viral RNA levels and normal peripheral blood CD4⁺ T lymphocyte counts. Three monkeys that received Gag/Pol/Nef but no Env-containing vaccines had relatively high plasma viral RNA levels and reduced CD4⁺ T lymphocyte counts. Gag peptide-stimulated CD4⁺ T lymphocyte cytokine expression profiles were assessed in PBMC of these monkeys 15 mo postchallenge.

Similar to monkeys infected with attenuated SIV/SHIV, IL-2 production by central memory CD4⁺ T cells of the vaccinated/challenged monkeys was better preserved than IL-2 production by central memory CD4⁺ T cells in monkeys infected with pathogenic virus in the absence of prior vaccination ($p = 0.014$, Holm's test) (Fig. 7A). The only vaccinated and then challenged monkeys that did not demonstrate preserved SIV Gag-stimulated IL-2 production by central memory CD4⁺ T lymphocytes were those with high plasma viral RNA levels. These results demonstrate that vaccine protection against SHIV-induced clinical disease is associated with preserved central memory CD4⁺ T lymphocyte function.

Correlation between the frequency of IL-2-producing central memory CD4⁺ T cells and plasma viral RNA levels

The observed association between high viral loads and loss of Gag-specific IL-2-producing central memory CD4⁺ T cells in this group of vaccinated/challenged monkeys suggested that such an association be evaluated in a larger cohort of animals. Therefore,

the frequencies of Gag-specific IL-2-producing central memory CD4⁺ T cells were assessed for an association with plasma viral RNA levels for all evaluated monkeys, including monkeys infected with attenuated SIV/SHIV, monkeys infected with pathogenic virus, and monkeys vaccinated and challenged with SHIV-89.6P. A significant inverse correlation was observed between these parameters ($r = -0.70$, $p < 0.0001$) (Fig. 8). However, no significant correlation was noted between IFN- γ or TNF- α production by central memory CD4⁺ T cells and the plasma viral RNA levels in these animals (data not shown). Therefore, the maintenance of IL-2-producing central memory CD4⁺ T cells is ultimately associated with virologic control in the monkeys.

Discussion

Although IFN- γ production assays are commonly used to measure T lymphocyte immune responses to HIV Ags, these analyses do not reflect the complete profile of the functional capacity of HIV-specific T cells. In fact, considerable recent work suggests that the production of other cytokines by CD4⁺ T lymphocytes plays an important functional role in disease pathogenesis. IL-2 is essential to the expansion and survival of T lymphocytes, and accruing data suggest that IL-2-secreting Ag-specific CD4⁺ T lymphocytes represent a key component of an effective immune response (7, 11, 17). In the present study, CD4⁺ T lymphocytes of infected monkeys with uncontrolled viremia and reduced CD4⁺ T lymphocyte counts preserved their capacity to produce IFN- γ and TNF- α , whereas their ability to synthesize IL-2 was impaired. Therefore, the preservation of Gag-specific IL-2-producing CD4⁺ T lymphocytes is associated with effective immune control of virus. IL-2 production by CD4⁺ T lymphocytes may be lost during an early stage of disease in these monkeys, with a loss of IFN- γ and TNF- α production as disease progresses. This possibility is consistent with the recent finding that lymphocytic choriomeningitis virus-specific T cells lose their ability to produce IL-2 before their ability to produce TNF- α and IFN- γ as lymphocytic choriomeningitis virus-induced disease progresses in mice (18).

A number of studies have shown that Ag-specific CD8⁺ T cells can be separated into three functional subsets: early-, intermediate-, and late-differentiated phenotypes. HIV-specific CD8⁺ T lymphocytes can exist in an intermediate differentiated state in the peripheral blood of infected individuals (19, 20). A similar maturation phenotype has also been observed for HIV-specific CD4⁺ T lymphocytes in the peripheral blood of these individuals (21, 22). The phenotypic profiles of the SIV Gag-specific CD4⁺ T cells of the infected monkeys evaluated in the present study are consistent with this intermediate differentiated state. Cytokine production by Gag-specific CD4⁺ T lymphocytes was found predominantly in cells with a central memory phenotype (CD28⁺CD95⁺). The reason for this atypical state of T cell maturation is likely multifactorial. First, because compartmentalization of T lymphocyte subpopulations may occur, the population of cells identified in the peripheral blood of infected individuals might reflect changes in T cell trafficking and are not necessarily representative of the entire virus-specific T cell population. Second, intermediate T cell differentiation may reflect the normal immune response to an immunodeficiency virus during chronic infection. HIV-specific CD4⁺ T cells that have a central memory/effector memory phenotype may be targeted to lymph nodes where HIV replication is ongoing. A third possible explanation for this atypical T cell maturation is that replicating HIV may inhibit or redirect T cell differentiation and, in so doing, affect the clinical course of disease progression. Conflicting data have been reported bearing on this last possibility. Some investigators have reported that, although HIV-specific CD4⁺ T cells are less mature than CMV-specific CD4⁺ T cells,

there is no correlation between HIV-specific T cell maturation and disease progression (22). Other investigators have reported that HIV-1 replication skews Gag-specific CD4⁺ T cells away from an IL-2-producing central memory phenotype and toward a poorly proliferating effector memory phenotype, and this change may limit the effectiveness of the HIV-specific immune response (23).

These conflicting results may reflect the different approaches used by these investigators to define naive, central memory, and effector memory T lymphocyte subsets. CD4⁺ T lymphocytes have been divided into three major functional subsets on the basis of their expression of pairs of surface molecules (12, 20, 24). The pairing molecules used for such studies include CCR7 or CD62L with CD45RA, CD28 with CD27, and CD28 or CD27 with CD45RA. However, it is very likely that a more precise definition of functional subsets of cells can be accomplished using mAbs that bind to additional surface molecules. For example, CD28⁺CD45RA⁺ central memory T cells might be further divided into CD28⁺CD45RA⁺CCR7⁺ and CD28⁺CD45RA⁺CCR7⁻ T cell subpopulations. Therefore, a more precise definition of functional T lymphocyte subpopulations may facilitate an elucidation of the role of these cells in disease pathogenesis.

In the present study, surface expression of CD28 and CD95 has been used to define CD4⁺ T lymphocyte subsets. We showed that cell subpopulations defined in this way have physiologic characteristics of naive, central memory, and effector memory CD4⁺ T cells as has been previously described (16, 25). Most of the cytokine production by Gag-specific CD4⁺ T lymphocytes was contributed by cells with a CD28⁺CD95⁺ central memory phenotype. This was true in healthy vaccinated monkeys, and in monkeys infected with pathogenic virus. The consistency of these findings suggests that the function of SIV-specific CD4⁺ T cells with a central memory phenotype is correlated with disease progression. Finally, it is also possible that late effector cells that acquire CCR5 expression become targets for HIV infection, and are therefore depleted. This would result in cells of intermediate-differentiated phenotype being detected predominantly in infected individuals with progressive disease.

We and others have previously shown that monkeys vaccinated and challenged with a pathogenic immunodeficiency virus have low viral loads, preserved CD4⁺ counts, and no evidence of clinical disease (26–28). However, the mechanism accounting for this persistent clinical protection is poorly understood. In the present study, we show that the central memory CD4⁺ T lymphocytes from such vaccinated and then infected monkeys had a preserved capacity to produce IL-2, comparable with that seen in animals that were infected with nonpathogenic virus. This observation suggests that persistent clinical protection in vaccinated and challenged monkeys is correlated with a preserved capacity of the peripheral blood central memory CD4⁺ T cells to express virus-induced immunomodulatory cytokine IL-2.

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Disclosures

The authors have no financial conflict of interest.

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