Natural Suppression of HIV-1 Replication Is Mediated By
Transitional Memory CD8⁺ T Cells

(Running Head: HIV-Suppressing CD8⁺ Cells)

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HIV replication is suppressed in vitro by a CD8+ cell non-cytotoxic antiviral response (CNAR). This activity directly correlates with an asymptomatic clinical state. The objective of this study was to identify the phenotype of CD8+ cell subsets having strong CNAR activity. CD8+ cell subset frequencies and CNAR levels were measured for HIV-uninfected individuals and three groups of HIV-1-infected individuals: asymptomatic individuals with low-level viremia (vHIV), antiretroviral drug-treated subjects with undetectable virus levels (TxHIV), and therapy-naïve aviremic elite controllers (EC). CD8+ cells from the vHIV individuals exhibited the highest HIV-suppressing activity and had elevated frequencies of CD45RA-CD27+ and PD-1+ (CD279+) cells. Functional assessments of CD8+ cells sorted into distinct subsets established that maximal CNAR activity was mediated by CD45RA-CCR7-CD27+ and PD-1+CD8+ cells. T cell receptor (TCR) repertoire profiles of CD8+ cell subsets having strong CNAR activity exhibited increased perturbations in comparison to those of inactive subsets. Together, these studies suggest that CNAR is driven by HIV replication and that this antiviral activity is associated with oligoclonally expanded activated CD8+ cells having a transitional memory cell phenotype. The findings better describe the identity of CD8+ cells showing CNAR and should facilitate the evaluation of this important immune response in studies of HIV pathogenesis, resistance to infection, and vaccine development.
INTRODUCTION

CD8+ cells from HIV-infected individuals potently suppress the in vitro replication of HIV in primary CD4+ cells without eliminating the infected cells (24, 32, 50, 51, 54). This CD8+ cell noncytotoxic anti-HIV response (CNAR) becomes evident during the acute stage of infection (22, 38), varies in magnitude among HIV infected persons (21, 35, 52), and directly correlates with a healthy clinical state (3, 6, 7, 14, 25, 35). Strong CNAR activity is a feature of long-term survivors (LTS) of HIV infection (3, 14). CNAR activity is also associated with resistance to HIV infection among exposed seronegative individuals (27, 45). CD8+ cells from uninfected persons, individuals with AIDS, and HIV-infected subjects receiving long-term antiretroviral therapy typically exhibit little or no CNAR activity (21, 22, 44).

CNAR is associated with the production of a soluble CD8+ cell antiviral factor (CAF) (26) that suppresses HIV replication by blocking transcription from the virus promoter (9, 32). CAF is not present in cytolytic granules (37) and CNAR does not involve apoptosis (36). CNAR activity is effective against all HIV and SIV isolates and is not virus type specific (5, 53). And, CD8+ cells are able to suppress HIV replication in MHC mismatched CD4+ cells (28, 34, 51).

CNAR has been found to be associated with an activated CD8+ cell phenotype (25) and with VCAM-1 expressing CD8+ cells (11). To further characterize the CD8+ cells that mediate CNAR, we evaluated this activity in phenotypically distinct CD8+ cell subsets obtained directly from peripheral blood without in vitro stimulation. Here we report that the natural suppression of HIV-1 replication is mediated by memory CD8+ T cells, particularly those that express PD-1 and exhibit a transitional memory cell phenotype.
MATERIALS & METHODS

Human subjects. HIV-1 infected (n = 100) and uninfected (HIV-, n = 19) subjects were selected from participants in ongoing studies at the University of California San Francisco (UCSF).

Among the HIV-1 infected subjects, all infected for more than 5 years, three groups were studied: 1) individuals on antiretroviral therapy with very low viral loads (TxHIV+, n = 44), 2) elite controllers (EC, n = 15) who had been infected with HIV-1 for at least 10 years without exhibiting AIDS-defining symptoms, and had undetectable plasma viral loads (<50 copies HIV RNA / ml) and normal CD4+ T cell counts (>400 CD4+ T cells / µl) in the absence of antiretroviral therapy, and 3) viremic individuals (vHIV+, n = 41) who were asymptomatic, had viral loads ranging from 3.6 – 4.8 logs RNA copies per ml, and were not receiving antiretroviral therapy. Each subject signed informed consent forms, and the study received approval from the Committee for Human Research at UCSF. Salient features of the study population are provided in Table 1.

Clinical measures. Complete differential blood cell counts (CBCs) for erythrocyte number, hemoglobin, total leukocytes, granulocytes, lymphocytes, monocytes, platelets, and T cell subsets were performed by the UCSF clinical laboratories. Measurements of plasma HIV RNA levels were performed using a branched DNA (bDNA) assay (Seimens Diagnostics, Emeryville, CA) or were self-reported.

Cell specimens. Whole blood was collected in evacuated tubes (BD) containing EDTA and sodium heparin for immunophenotyping and functional studies respectively. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood collected in evacuated tubes containing EDTA (BD Biosciences) by density gradient separation over ficoll (Sigma). From the PBMC of each study subject, CD4+ and CD8+ cells were serially isolated by immunomagnetic
bead separation (Miltenyi) prior to cell sorting. Purities of the isolated cells were >95% as measured by flow cytometry.

**CNAR assays.** To determine the respective levels of CNAR activity, incremental numbers of CD8⁺ cells (without in vitro stimulation) were cocultured with HIV acutely infected autologous or heterologous CD4⁺ cells and the ensuing level of HIV replication was measured. Briefly, CD4⁺ cells were resuspended (3x10⁶ cells/ml) in growth medium [RPMI 1640 medium supplemented with fetal calf serum (heat inactivated at 56°C for 30 min, 10% v/v), penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2mM), and recombinant IL-2 (100 U/ml, Invitrogen)] and stimulated for 3 days in the presence of phytohemagglutinin-leucoagglutinin (PHA-L, 3 µg/ml, Sigma) in a 37°C humidified incubator. Subsequently, 10x10⁶ cells were treated with polybrene (2 µg/ml, Sigma) for 20 minutes at 37°C and pelleted. The pelleted cells were resuspended in 1 ml of HIV-1 SF33 (10,000 TCID₅₀/ml in PBMC) for 1hr at 37°C with periodic mixing. This syncytium inducing (SI), CXCR4-tropic (X4), HIV-1 SF33 has been maintained in primary cells since its isolation, exhibits rapid replication kinetics with a high degree of cytopathicity in cell culture, and is not sensitive to β-chemokine mediated antiviral effects (31). The acutely infected CD4⁺ cells were then washed, resuspended at 10⁶ cells/ml of growth medium, and 200 µl aliquots were placed into a flat bottom 96-well tissue culture plate (Falcon 3072, BD) in triplicate. Co-cultures were established by adding CD8⁺ cells to wells containing acutely infected CD4⁺ cells at 1:1, 0.5:1, and 0.25:1 CD8⁺ cell to CD4⁺ cell input ratios.

**Measurement of CNAR activity.** To measure HIV replication levels in the cultures, 100 µl aliquots of culture supernatant from each well were collected on days 3 and 6 of culture, centrifuged at 12,000 x g for 1 hour at 4°C, and the resulting virus pellets were assayed for reverse transcriptase (RT) activity as described (17). In ongoing cultures, the supernatant
removed for measurement of HIV levels was replaced with an equal volume of fresh growth medium. In the assays for RT activity, 2.5 units (U) of purified Avian Myeloblastosis Virus (AMV) reverse transcriptase (Roche) were used as a positive control. The extent of CNAR activity in each culture, assessed as percent suppression, was calculated based on the relative magnitude of HIV replication in the co-cultures in comparison to replication levels in CD4+ cell cultured alone as follows: Percent Suppression = \[
\left(1 - \frac{\text{HIV level in coculture}}{\text{HIV level in CD4+ cells}}\right) \times 100 \%
\] Differential CNAR activity was defined to be present when the CD8+ cell subsets compared were discordant (e.g. >60% vs. <40% suppression) at various CD8+ cell to CD4+ cell input ratios.

**Conditioned medium and transwell assays.** All experiments were performed without the in vitro stimulation of CD8+ cells, except those involving conditioned medium from CD8+ cells or their use in transwell inserts. For such experiments, the CD8+ cells were stimulated with anti-CD3 beads following their separation into distinct subsets. Conditioned medium was generated by culturing the stimulated CD8+ cells of HIV-infected individuals in serum-free F12 medium and its anti-HIV activity was measured as previously described (33). For the transwell assays, the CD4+ cells and CD8+ cells were placed into a 24-well plate where they were physically separated by a semipermeable insert (0.45µ, BD). These cultures were established with the upper chamber containing 1x10^6 HIV-infected CD4+ cells and the lower reservoir having 4x10^5 CD8+ cells.

**Immunophenotyping and cell sorting.** To enumerate the frequencies of distinct CD8+ cell subsets, fresh whole blood was stained with various combinations of fluorescein isothiocyanate (FITC), phycoerythrin (PE), and allophycocyanin (APC) conjugated monoclonal antibodies. Anti-CD3 (clone UCHT1), CD7 (M-T701), CD11b (D12), CD25 (2A3), CD27 (L128), CD28 (L293), CD38 (HB7), CD45RA (L48), CD57 (HNK-1), CD62L (SK11), CD95 (DX2), PD-1
(MIH4), HLA-DR (L243), CD122 (TU27), CD127 (M21), and CCR7 (3D12) were obtained from BD whereas anti-CD8β (IM217U), and 2B4 (C1.7.1) were purchased from Beckman Coulter. Negative cell populations and cursor settings for each specimen were established using the appropriate isotype control reagents (BD). 4-color flow cytometric analyses were performed on a FACSort (BD) using CellQuest (BD). 7-color analyses were performed using a LSRII cytometer (BD) and FlowJo (Treestar) software. To isolate subsets within the CD8+ cell compartment, CD8+ cells were first separated from PBMC using immunomagnetic beads (see above). The CD8+ cells were stained with various antibodies (described above) and then sorted into distinct subsets using a FACSDiva (BD) or FACSAria (BD) instrument (UCSF core facilities). Post-sort cell populations were analyzed and confirmed to exceed 90% purity. Where presented in figures, dot plots have been gated on CD8+ cells that fall within a lymphocyte region of characteristic size and complexity.

CD107 degranulation assay. To evaluate cytotoxic potential (i.e. the release of lytic granules), CD107 degranulation assays were performed similarly to those described (4). Briefly, CD8+ cells alone or mixed with HIV-infected heterologous CD4+ cells were cultured in complete medium (10^6 cells/ml) in the presence of anti-CD107a and anti-CD107b FITC-conjugated monoclonal antibodies (BD, 50μl/ml each) for 1 hour at 37°C. After 1 hour, monensin (BD, 1μl/ml) was added and the cells were cultured for another 4-5 hours at 37°C. Then, the cells were collected, stained with anti-CD8-APC (BD), and analyzed for CD107 surface expression. For use as positive controls in the CD107 degranulation assay, primary CD8+ cells from HLA-A0201+ HIV-infected individuals were stimulated for 3 days in complete medium containing the HIV-1 gag consensus peptide SLYNTVATL (1μg/ml) or a cocktail of CMV/EBV/Flu (CEF, 10μg/ml) peptides (NIH AIDS Research and Reference Reagent Program) (23). The peptide-stimulated CD8+ cells were then cultured for an additional 11 days to generate appreciable
frequencies of the antigen-specific cells. These HIV-specific and CMV-specific CD8+ cells were then used in CD107 degranulation assays in the absence or presence of SLYNTVATL and CEF peptides. Flow cytometric analyses were performed following staining of the cells with CMV- and HIV-specific tetramer reagents (Beckman Coulter).

*T cell receptor repertoire analysis.* In evaluating the clonal diversity of the T cells mediating strong CNAR activity, T cell receptor (TCR) repertoire analysis was performed as previously described (19, 20, 23). Historical nomenclature for the TCRβ families is used in this text in order to maintain consistency with these prior reports. Briefly, RNA was extracted from cell lysates using RNAeasy columns (Qiagen) and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (www.invitrogen.com) and a TCRβ chain constant region primer (CTCAGCTCCAGTG). Specific combinations of one, two, or three TCRβ-specific forward primers (0.1 µM each) were then used for each multiplex PCR on the resulting cDNA. Each reaction also contained a 6-Fam fluorescently labeled reverse primer (0.1 µM) specific for the TCRβ constant region. The nucleotide lengths and fluorescent intensities of the resulting amplicons were measured using an ABI PRISM 3730xl DNA analyzer (Applied Biosystems). Fluorescence data were collected and processed using Genescan and Genotyper software packages (ABI).

*Statistical Analyses.* All data were compiled in an Access database (Microsoft). Group comparisons were performed using the non-parametric Mann-Whitney test with Splus 6.1 (Insightful). Graphs were prepared using SigmaPlot 11.2 (Systat).
RESULTS

CNAR activity is associated with persistent low-level HIV replication in vivo. In previous studies, we have consistently observed that mitogen-stimulated CD8+ cells from HIV uninfected individuals, HIV-infected individuals with advanced disease, and those who are receiving antiretroviral therapy exhibit reduced or no CNAR activity in comparison to those from asymptomatic HIV-infected individuals (see Introduction). In the present study, we evaluated unstimulated (ex vivo) CD8+ cells from healthy uninfected blood donors (HIV-), elite controllers (EC), viremic HIV-infected individuals (vHIV), and HIV-infected subjects receiving antiretroviral therapy (TxHIV) (Figure 1 and Table 1). We observed the following trend of CNAR activity among CD8+ cells from individuals in these groups: vHIV >> EC = TxHIV > HIV- (Fig. 1A).

To identify potential subsets of cells that mediate strong CNAR activity, we measured the whole blood frequencies of CD8+ cell subsets in the aforementioned groups (Table 2). In comparison to the HIV- and EC subjects, vHIV individuals exhibited elevated frequencies (p<0.05) of CD45RA-, CD11b-, HLA-DR+, and CD27+ CD8+ cells. vHIV subjects also exhibited the highest frequencies of CD45RA CD27+, CD57+CD28+, and PD-1+ CD8+ cells (Fig. 1B, 1C, 1D respectively). A direct correlation (r² = 0.62) was observed between CNAR activity and the frequency of CD45RA CD27+ cells within the CD8+ cell compartment (Fig. 1D). These cross-sectional comparisons of CD8+ cells from aviremic and viremic individuals demonstrate an association between low level HIV replication, frequencies of distinct CD8+ cell subsets, and CNAR activity.

Maximal CNAR activity is mediated by activated CD8+ T cells. Having identified correlations between the heightened CNAR activity of bulk CD8+ cells and the frequencies of selected CD8+ cell subsets (e.g., CD45RA+CD27+ cells) measured by FACS, we next evaluated the functional...
ability of those subsets (without in vitro stimulation procedures) to suppress HIV-1SF3 (X4) replication in primary CD4+ cell cultures (Figure 2). Toward this objective, more than 50 independent cell sorting experiments were performed. First, to determine whether or not CNAR activity is a characteristic of all CD8+ lymphocytes or restricted to CD8+ T cells, CD3+ and CD3- CD8+ cells were evaluated for their CNAR activity (Fig. 2A). As shown, most CD8+ cells express CD3. Note that this and all other flow cytometry plots presented in Figures 2 and 3 are gated on CD8+ lymphocytes. The CD8+CD3+ (T) cells exhibited substantially greater CNAR activity than did the CD8+CD3- (NK) cells (95% vs. 35% suppression of HIV replication at a 0.5:1 input ratio). Thus, with respect to CD8+ cells having distinct hematopoietic lineages, CNAR is mediated by CD8+ T cells.

CD8+ T cells, as they encounter environmental stimuli, become “activated” and newly express a variety of surface antigens. To investigate their association with CNAR, cells differing in their expression of several activation markers (e.g., CD8 beta chain (CD8β), HLA-DR and CD38, IL-25, IL-122, C1.7, CD95, CD11b and PD-1) were evaluated. Among the CD8+ T cells, two distinct populations were apparent: CD8βdim and CD8βbright cells (Fig. 2B). In cocultures with HIV-1-infected cells, the CD8βdim subset consistently exhibited greater suppression of virus replication than did the CD8βbright subset (90% vs. 20% at a 0.5:1 input ratio). Noteworthy is that CD8+ NK cells do not express CD8β (39). Upon evaluation of CD8+ cells differing in their expression of HLA-DR and CD38, HLA-DR+ cells had the strongest CNAR activity, regardless of CD38 expression levels (Fig. 2C). In addition to CD8βdim and HLA-DR+ cells, we observed that maximal CNAR activity was mediated by C1.7+, CD95+, and CD11b PD-1+ CD8+ cells (Figs. 2D-F). Notably, CD8βdim cells and C1.7+ cells were primarily CD95+ cells and CD28- cells respectively. These results for unstimulated CD8+ cells, with respect to HLA-DR, CD38, and
CD11b, confirm previous studies with mitogen-stimulated CD8^+ cells (25), and further establish that CNAR is mediated by activated CD8^+ cells.

We also investigated CD8^+ cells differing in their expression of the IL-2 receptors CD122 and CD25. Differential expression of the intermediate-affinity IL-2 receptor, CD122, was not observed to be associated with CNAR activity (data not shown). Moreover, depletion of the minor population of CD8^+ cells that express the low-affinity IL-2 receptor, CD25, had no appreciable effect on CNAR levels (data not shown). Thus, IL-2 receptor expression does not distinguish ex vivo CD8^+ cells with high and low CNAR activity.

**CD8^+ cells with a memory phenotype exhibit maximal CNAR activity.** To investigate the differentiation state of CNAR-mediating cells, CD8^+ cells differing in their co-expression of CD45RA/CD62L, CD45RA/CCR7, CD11b/CD57, CD11b/CD28, CD57/CD28, and CD45RA/CD27/CD28 were assessed (**Figure 3**). First, we compared the CNAR activities of CD8^+ cells that differed in their co-expression of CD45RA and CD62L or CCR7 (**Figs. 3A, B**). In comparison to naïve (CD45RA^+CD62L^+ or CD45RA^+CCR7^+) cells, the more immunologically mature CD8^+ cells (CD45RA^−CD62L^− or CD45RA^−CCR7^−) exhibited superior CNAR activity (e.g. > 90% suppression vs. < 50% suppression when comparing the 0.5:1 cell input values).

Next, we evaluated the CNAR activity of CD8^+ cells that differed in their co-expression of CD11b and CD57 or CD28 (**Figs. 3C, D**). CD11b^+ cells were observed to be mostly CD57 negative, whereas these cells were heterogeneous for expression of CD28. Maximal suppression was associated with a CD57^− phenotype, while both CD11b^+CD28^− and CD11b^+CD28^+ cells exhibited strong CNAR activity. Then, we evaluated the CNAR activity of CD8^+ cells that varied in their co-expression of CD28 and CD57 or CD27 (**Figs. 3E, F**). Maximal suppression was exhibited by CD57^CD28^− and CD27^CD28^− cells. Finally, among six CD8^+ cell subsets that differentially express CD45RA, CD27, and CD28, the two CD45RA^−CD27^+ subsets were found to most
potently suppress HIV replication (Fig. 3G). Therefore, the CD8+ cells that exhibited maximal suppression of HIV replication were CD45RA-CD27+CD28- cells, although appreciable anti-HIV activity was also mediated by CD45RA-CD27-CD28+ cells. Comparisons of bulk CD8+ cells, that were unstained or stained with antibodies, revealed no substantial differences in CNAR activity (data not shown). Thus, activation of the cells and/or blocking of the surface antigens due to antibody binding was not involved. These results show that CNAR is associated with memory CD8+ cells, particularly those with a transitional memory phenotype.

CNAR is associated with oligoclonal CD8+ cell populations. To investigate the diversity of T cell receptor usage among CD8+ cell subsets having strong CNAR activity, T cell receptor (TCR) profiling was performed (Figure 4). Specifically, bulk CD8+ cells (Fig. 4C), CD45RA-CD27-CD28-, CD45RA-CD27+CD28+, CD45RA-CD27+CD28+ (Fig. 4B), CD45RA-CD27+CD28+ (Fig. 4A), CD45RA-CD27-CD28-, and CD45RA-CD27+CD28- CD8+ cells were analyzed from 3 HIV-1-infected (vHIV+) individuals. Among the subsets evaluated, the CD45RA-CD27+CD28- CD8+ cells exhibited Gaussian-like distributions of CDR3 lengths in each TCRV family, indicating a lack of clonal dominance within this subset. As described above (Fig. 3G), this population exhibited poor CNAR activity. In contrast, TCRV families within the CD45RA-CD27+CD28- CD8+ cell subset, a population with robust CNAR activity (Fig. 3G), exhibited a striking degree of perturbation (Fig. 4B). These data provide evidence that CNAR is associated with CD8+ cell subsets having a biased (i.e., a non-Gaussian-like distribution) T cell receptor usage.

CD8+ cells with strong CNAR activity do not exhibit classical CTL activity. Past studies have shown that CNAR does not involve cell killing (28). To confirm those findings in the present studies, the CD8+ cells were removed after 3 days of cocultivation with the HIV-infected CD4+ cells (Figure 5). As shown previously (50, 51), HIV-infected cells persist in the presence of
CNAR activity. HIV levels rapidly increased in the cell culture supernatants upon removal of the CD8\(^+\) cells (Fig. 5A).

In separate experiments, CD8\(^+\) cells were evaluated for degranulation upon their exposure to HIV peptides or HIV-infected heterologous CD4\(^+\) cells in CD107 mobilization assays. The mobilization of lysosomal associated membrane glycoproteins (LAMPs), including CD107a (LAMP-1) and CD107b (LAMP-2) to the cell surface of CD8\(^+\) cells is directly associated with CTL activity (4). CD8\(^+\) cells that were expanded \textit{in vitro} with HIV-specific (Fig. 5B, left) or CMV-specific (data not shown) antigens and then incubated in the presence of their cognate peptides underwent marked degranulation. In comparison, CD8\(^+\) cells did not degranulate when placed into culture with heterologous CD4\(^+\) cells that had been infected for 3 days and were producing substantial levels of HIV (Fig. 5B, right). Also, the levels of CD107 expression on CD8\(^+\) cells were not found to differ between CD8\(^+\) cells cultured alone and those cocultured with acutely infected CD4\(^+\) cells (data not shown). Notably, the CD8\(^+\) cells that were stimulated with HIV-peptides were able to suppress HIV replication in acutely infected heterologous CD4\(^+\) cells post-stimulation.

In another series of experiments, we assessed the ability of various subsets of CD8\(^+\) cells to produce soluble factors having anti-HIV activity. In two independent experiments (Fig. 5C), conditioned medium from the CD57\(^-\)PD-1\(^+\) CD8\(^+\) cells exhibited increased anti-HIV activity in comparison to conditioned medium from bulk CD8\(^+\) cells or those lacking a CD57\(^-\)PD-1\(^+\) cells phenotype. Similarly, CD57\(^-\)PD-1\(^+\) cells suppressed HIV replication by >50% when assessed in transwell assays (data not shown). These results demonstrate that HIV-suppressing CD8\(^+\) cells do not eliminate HIV-infected cells, do not exhibit detectable degranulation in the presence of HIV-infected CD4\(^+\) cells, and that CD8\(^+\) cell subsets with strong CNAR activity do suppress HIV
replication via the secretion of a soluble factor(s). Thus they are unlike classical CTL in function.
DISCUSSION

In previous studies, we observed that CD8+ cells from HIV-infected individuals vary in their ability to suppress HIV replication in primary CD4+ cells; CD8+ cells from asymptomatic persons have the highest CD8+ cell noncytotoxic antiviral response (CNAR) (3, 7, 14, 21, 25, 35, 52). To further characterize this anti-HIV activity, we systematically compared the whole blood frequencies (Table 1) and HIV-suppressing activity of various CD8+ cell subsets (Fig. 2, Fig. 3) among HIV-infected and uninfected individuals.

In comparison to CD8+ cells from aviremic (EC and TxHIV) HIV-infected individuals, those from asymptomatic subjects with low-level viremia (vHIV) were found to exhibit the strongest CNAR activity (Fig. 1). These findings support past results showing that when viral loads are below detectable levels, as characteristic of elite controllers and subjects treated with antiretroviral therapy, CNAR is generally low or not detectable (44). This observation indicates that CNAR responds to HIV replication. Those elite controllers who exhibited some CNAR activity (Fig. 1) most likely had blips of virus replication that were sufficient to sustain this response (16).

Importantly, the viremic individuals in this study were healthy long-term survivors of HIV infection and exhibited low viral loads (median 3.9 logs, Table 1). Indeed, our findings are consistent with previous studies establishing this anti-HIV response as a characteristic of CD8+ cells from asymptomatic long-term survivors with low level viremia (3, 7, 14, 25, 35).

Cross-sectional analyses of whole blood revealed increased frequencies of CD45RA-CD27+, and CD57-CD28- CD8+ cells in the vHIV group (Fig. 1). In addition, we observed a direct correlation between CNAR activity and the frequency of CD45RA-CD27+ CD8+ cells. These observations link CNAR activity with transitional memory cells (see below). The finding of decreased frequencies of CD45RA-CD27+ CD8+ cells in patients receiving antiretroviral therapy
(Fig. 1A, B) provides an explanation for the previously observed loss of CNAR activity in CD8+ cells from these subjects (22, 44). Still, differences in CD8+ cell subset frequencies, as measured by flow cytometry, do not necessarily account for a loss or gain of antiviral function. Therefore, cell sorting experiments were performed to evaluate directly the antiviral activities of CD8+ cell subsets that change in frequency with HIV infection.

In assessing cell function, our experiments demonstrate that CD8+ cell subsets (without prior in vitro stimulation) exhibit differential abilities to suppress HIV replication. With respect to CD8+ cells of distinct hematopoietic lineages, CD3+ (T) cells expressing CD8E have strong CNAR activity whereas CD3+ (NK) cells do not (Fig. 2). Of note, CD8E is not expressed by circulating CD8+ γδ T cells (30), thus excluding γδ T cells from being part of CNAR.

In previous investigations with mitogen-stimulated CD8+ cells, we observed that noncytotoxic anti-HIV activity was highest among HLA-DR+, CD11b−, and VCAM+ cells (11, 25). Similarly, in this study of peripheral blood CD8+ cells that were not mitogen-stimulated, CNAR activity was found to be mediated by CD8+ cells having activated phenotypes (Fig. 2). Specifically, CD8βdim, HLA-DR+, CD95+, C1.7+, and PD-1+ CD8+ cells exhibited maximal CNAR activity. CD8β is downmodulated upon activation and the frequency of this CD8βdim cells is increased in HIV-infected individuals (43). CD8βdim cells also suppress virus replication in FIV-infected cats (13).

Moreover, as noted in other studies of CD8+ cells that were activated in vitro, CNAR was chiefly mediated by CD57−CD8+ cells (2). In the present studies of CD8+ cells not stimulated in vitro, both CD57−CD28+ and CD57−CD28− subsets were able to suppress HIV replication, although the CD28− cells showed superior CNAR activity. At the time of the earlier study (2), CD28 and CD57 were believed to be mutually exclusive antigens on CD8+ cells and the depletion of CD57−
cells was thought to yield relatively pure populations of CD28+ cells. However, as described in this study (Fig. 1C), HIV-infected persons can harbor appreciable numbers of CD57−CD28+ CD8+ cells. Furthermore, CD8+ cells have now been shown to downmodulate CD28 expression during the process of immunologic maturation (46).

Further phenotypic analyses of CD8+ cells that potently suppress HIV replication provided insight into the differentiation state of cells mediating CNAR. Circulating CD8+ cells can be classified into the following subsets: naïve cells (CD45RA+CD27+CD28+), central memory cells (CD45RA−CCR7+CD62L+), transitional memory cells (CD45RA−CD27+CCR7−), and effector cells (CD45RA−CD27+CCR7−) (15, 42). Memory cells express high surface levels of CD95 and exhibit little cytolytic activity in the absence of in vitro prestimulation. Effector cells express high levels of CD11b (10) and have high cytolytic activity without in vitro prestimulation. As noted above, we found that CNAR activity is mediated by CD8+ memory cells (Fig. 3), chiefly of the transitional memory (CD45RA−CD27+CCR7−) phenotype. Supportive evidence that CNAR activity is mediated by memory CD8+ cells is our observation that the CD8+ cell population having strong CNAR activity exhibits skewed T cell receptor usage (Fig. 4). Additional studies are needed establish the overall contribution of clonally expanded CD8+ cells to CNAR. In this regard, CD8+ cell clones isolated from HIV-infected individuals can exhibit CNAR activity without HIV-specific CTL activity (18, 47). Also, our findings are consistent with the very recent report that memory CD8+ cells, particularly CD45RA−CD27+ cells, effectively suppress HIV replication (12). However, our studies used a primary HIV-1 isolate and included biologic assessments of the antiviral function of CD8+ cells from HIV-1-infected individuals. Thus we were able to distinguish CNAR from classical CTL activity (see below).

HIV-specific (tetramer positive) CD8+ cells have been described to predominantly exhibit a CD45RA−CD27+CD57−CCR7− phenotype characteristic of transitional memory cells (8).
contrast, strong CTL responses have traditionally been associated with a CD45RA-CD27-CD28- effector cell phenotype (46). In comparison to bulk CD8+ cells or those that are specific for other viruses (e.g. CMV), HIV-specific CD8+ cells contain substantially lower levels of perforin, a protein required for granule-mediated cytolysis (1). These observations have led to speculation that HIV-specific CD8+ cells are defective killers (29, 49). Indeed, CD8+CD45RA+CD27+ cells exhibit very little lytic activity in CD3 mAb–mediated redirected cytotoxicity assays (15). This finding is consistent with our past and present observations that HIV-suppressing CD8+ cells do not eliminate HIV-infected cells and their antiviral effect is rapidly reversible (Fig. 5A) (50, 51). Moreover, CD8+ cells exhibiting strong CNAR activity do not degranulate in the presence of HIV-infected CD4+ cells (Fig. 5B), yet do secrete a soluble antiviral factor (Fig. 5C). Therefore, we propose that CD8+ cells having a CD45RA-CD27+ phenotype, likely including some that are HIV-specific, are noncytotoxic HIV-suppressing cells (8).

Considerable attention has been given to the role of PD-1 expressing CD8+ cells in HIV infection. PD-1 is a member of the CD28 family that has immunoregulatory functions (40) and this antigen is frequently expressed on HIV-specific CD8+ cells having a transitional memory phenotype (41). In agreement with other studies (48), we observed that PD-1 expression on CD8+ cells is increased in the context of HIV infection (Table 2). However, in those studies the HIV-specific CD8+ cells exhibiting high levels of PD-1 expression were found functionally defective (48). In contrast, our studies demonstrate that PD-1+ CD8+ cells have a previously unappreciated anti-HIV activity (Fig. 2F).

In summary, maximal CNAR activity is associated with CD8+ T cells having a CD3+, CD8βdim, CD11b-, CD57-, CD95+, C1.7+, and PD-1+ cell phenotype. These markers, along with HLA-DR (25) indicate that CNAR is mediated by activated CD8+ cells. Furthermore, strong suppression of HIV replication is associated with CD8+ cells having a CD45RA+CD27+CD28-CCR7-
phenotype that is characteristic of transitional memory cells. Notably as well, PD-1+CD8+ cells, previously considered dysfunctional, exhibit strong CNAR activity. Overall, our immunophenotyping and functional studies indicate that fewer than 50% of CD8+ cells mediate greater than 90% of CNAR activity. These studies better distinguish the ex vivo phenotypes of CD8+ T cells that mediate CNAR, and provide insight for why HIV-infected subjects can differ in this important antiviral activity. This information can be helpful in developing novel immunotherapeutic strategies and directing vaccine design.
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REFERENCES


**Table 1.** Demographic and immunologic characteristics of the study subjects.

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<th>HIV-</th>
<th>TxHIV+</th>
<th>EC</th>
<th>vHIV+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (N, ♂/♀)</td>
<td>16/3</td>
<td>44/0</td>
<td>15/0</td>
<td>39/2</td>
</tr>
<tr>
<td>Age, years *</td>
<td>43 (33-56)</td>
<td>50 (40-56)</td>
<td>51 (48-53)</td>
<td>48 (39-56)</td>
</tr>
<tr>
<td>Lymphocytes / ul (x 1000)</td>
<td>1.7 (1.4-2.0)</td>
<td>2.1 (1.8-2.4)</td>
<td>2.1 (1.7-2.4)</td>
<td>2.3 (1.6-2.8)</td>
</tr>
<tr>
<td>CD4⁺ T cells / ul</td>
<td>835 (650-1209)</td>
<td>497 (312-662)</td>
<td>690 (638-830)</td>
<td>447 (357-633)</td>
</tr>
<tr>
<td>CD4⁺ T cell %</td>
<td>40 (37-48)</td>
<td>29 (20-35)</td>
<td>43 (35-49)</td>
<td>27 (20-33)</td>
</tr>
<tr>
<td>CD8⁺ T cells / ul</td>
<td>580 (513-647)</td>
<td>802 (588-1160)</td>
<td>693 (554-890)</td>
<td>901 (687-1350)</td>
</tr>
<tr>
<td>CD8⁺ T cell %</td>
<td>26 (23-30)</td>
<td>47 (39-55)</td>
<td>37 (32-42)</td>
<td>52 (44-60)</td>
</tr>
<tr>
<td>CD4:CD8 ratio</td>
<td>1.6 (1.2-1.8)</td>
<td>0.6 (0.4-0.9)</td>
<td>1.1 (0.9-1.5)</td>
<td>0.5 (0.4-0.8)</td>
</tr>
<tr>
<td>HIV-1 RNA log copies / ml</td>
<td>-</td>
<td>&lt; 1.7</td>
<td>&lt; 1.7</td>
<td>3.9 (3.6-4.8)</td>
</tr>
</tbody>
</table>

* Numbers provided are median values with interquartile ranges in parentheses.
Table 2. *CD8* cell subset frequencies in HIV-infected and uninfected individuals.

<table>
<thead>
<tr>
<th>Cell subset</th>
<th>HIV- (n = 19)</th>
<th>TxHIV+ (n = 12)</th>
<th>EC (n = 15)</th>
<th>vHIV+ (n = 31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of CD8+ cells *:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD38*</td>
<td>64 (50-69)</td>
<td>62 (49-69)</td>
<td>60 (55-70)</td>
<td>70 (59-76)</td>
</tr>
<tr>
<td>HLA-DR*</td>
<td>15 (10-32)</td>
<td>31 (22-46)</td>
<td>33 (25-46)</td>
<td>49 d (36-62)</td>
</tr>
<tr>
<td>CD27*</td>
<td>22 (19-26)</td>
<td>26 (20-31)</td>
<td>21 (17-23)</td>
<td>31 d (25-37)</td>
</tr>
<tr>
<td>CD28*</td>
<td>57 (49-71)</td>
<td>47 (38-53)</td>
<td>40 (33-56)</td>
<td>35 a,c (27-46)</td>
</tr>
<tr>
<td>CD25*</td>
<td>4 (3-6)</td>
<td>5 (3-6)</td>
<td>3 (2-4)</td>
<td>2 a,c (1-4)</td>
</tr>
<tr>
<td>CD122*</td>
<td>31 (19-44)</td>
<td>16 (11-25)</td>
<td>21 (18-29)</td>
<td>17 a (13-26)</td>
</tr>
<tr>
<td>CD45RO+</td>
<td>35 (28-46)</td>
<td>50 (43-54)</td>
<td>42 (34-48)</td>
<td>47 a (38-58)</td>
</tr>
<tr>
<td>CD45RA+</td>
<td>69 (59-83)</td>
<td>67 (57-72)</td>
<td>73 (64-76)</td>
<td>59 a,b (52-71)</td>
</tr>
<tr>
<td>CD57+</td>
<td>19 (12-22)</td>
<td>43 (30-54)</td>
<td>36 (32-41)</td>
<td>41 a (34-55)</td>
</tr>
<tr>
<td>CD62L+</td>
<td>62 (54-71)</td>
<td>50 (45-57)</td>
<td>52 (43-58)</td>
<td>42 d (36-49)</td>
</tr>
<tr>
<td>CD11b+</td>
<td>31 (19-41)</td>
<td>15 (7-27)</td>
<td>17 (11-21)</td>
<td>10 a,b (6-16)</td>
</tr>
<tr>
<td>PD-1+</td>
<td>9 (6-15)</td>
<td>N.D.</td>
<td>18 (15-23)</td>
<td>44 a,b (34-51)</td>
</tr>
</tbody>
</table>

* Median (interquartile range)

a Significantly different from HIV- (p < 0.05).
b Significantly different from EC (p < 0.05).
c Significantly different from Tx (p < 0.05).
d Significantly different from HIV-, EC, and Tx (p < 0.05).

N.D., no data
FIGURES

Figure 1. **CNAR activity and cell subset frequencies of CD8⁺ cells from viremic and aviremic individuals.** A) Shown are the relative abilities of primary CD8⁺ cells from HIV-uninfected (HIV-) individuals, HIV-infected subjects receiving antiretroviral therapy (TxHIV+), elite controllers (EC), and low viremic HIV-infected individuals (vHIV+) to suppress HIV replication in heterologous primary CD4⁺ cells. Results are provided for day 6 cultures containing CD8⁺ cells and acutely HIV-infected CD4⁺ cells that were plated at 0.5:1 and 1:1 input ratios respectively. HIV replication levels consistently peaked 6 days post-infection in the cultures of CD4⁺ cell alone, with RT activity exceeding 10⁶ CPM / 100 μl of cell culture supernatant. Bars show median values. Box plots detail whole blood levels of B) CD45RA⁻CD27⁺CD28⁻, C) CD57⁻CD28⁻, and D) PD-1⁺ CD8⁺ cells among HIV infected and uninfected individuals. The lower, central, and upper lines of the boxes identify quartiles; dotted lines demark mean values; whiskers and dots mark the 5th, 10th, 90th, and 95th percentiles. * denotes significantly different from aviremic groups (p < 0.05) E) Correlation between CNAR activity (y-axis, 1:1 CD8⁺ cell:CD4⁺ cell ratio) and the frequency of CD45RA⁻CD27⁺ cells within the CD8⁺ cell compartment (x-axis).

Figure 2. **CNAR activity is mediated by CD8⁺ T cells that are activated in vivo.** Primary CD8⁺ cells from asymptomatic low viremic HIV-1-infected individuals were sorted into distinctive subsets and then cocultured with heterologous HIV-infected primary CD4⁺ cells at 0.5:1 and 1:1 CD8⁺ cell to CD4⁺ cell input ratios. Shown are representative staining profiles (left) and antiviral activities (right) for CD8⁺ cell subsets differing in their expression of A) CD3, B) CD8β, C) HLA-DR and CD38, D) C1.7, E) CD95, and F) CD11b and PD-1. All FACS plots shown are gated on live CD8⁺ lymphocytes. Results are representative of at least 2 independent experiments with different CD8⁺ cell sources.
Figure 3. Transitional memory CD8+ cells exhibit maximal CNAR activity. Shown are staining profiles (left panel) and anti-HIV activities (right panel) of CD8+ cells that were separated into distinct populations based on the expression of A) CD45RA and CD62L, B) CD45RA and CCR7, C) CD57 and CD11b, D) CD28 and CD11b, E) CD57 and CD28, F) CD27 and CD28, and G) CD45RA, CD27, and CD28. Suppression data are shown for CD8+ cell : infected CD4+ cell coculture ratios of 0.25:1 (G only), 0.5:1, and 1:1 (left to right). Results are representative of at least 2 independent experiments with different CD8+ cell sources.

Figure 4. HIV-suppressing CD8+ cells are increased in frequency among cells that exhibit biased T cell receptor repertoires. T cell receptor diversity profiling was performed on A) CD45RA+CD27+CD28+ (naïve), B) CD45RA-CD27+CD28- (differentiated), and C) bulk CD8+ cells that were freshly isolated from the blood of an HIV-infected individual. Elevated CNAR activity was exhibited by the CD45RA-CD27+CD28- CD8+ cells in parallel assays. Shown are the resulting spectratypes for 16 of the 24 TCRVβ families evaluated. Within each TCRVβ family, peaks are separated by 3 nucleotides (1 amino acid). Similar results were observed among 3 HIV-infected individuals.

Figure 5. Non-cytotoxic features of HIV-suppressing CD8+ cells. A) HIV replication levels were evaluated in cultures from which CD8+ cells were removed following suppression of HIV replication. Shown are reverse transcriptase (RT) levels in the supernatants of HIV-infected cells cultured alone (●), in the presence of HIV-suppressing CD8+ cells (◆), and upon removal of the HIV-suppressing CD8+ cells after 4 days of coculture (●). B) CD107a/b levels were measured in HIV-specific CD8+ cells (left) and bulk CD8+ cells (right) upon exposure to cognate antigen and heterologous HIV-infected CD4+ cells respectively. C) CD8+ cells from a viremic HIV-infected individual were sorted into 2 subsets: CD57-PD1+ cells and those lacking this
phenotype (not CD57^PD1^). Conditioned medium collected from cultures containing these CD8^+ cell subsets was placed onto CD4^+ cells that were acutely infected with HIV. Shown are the RT levels in each culture at the time of peak virus replication in the control. Data in each panel are representative of at least 2 separate experiments.
Figure 1. CNAR activity and frequencies of CD8+ cell subsets from viremic and aviremic individuals.
Figure 2. CNAR activity is mediated by activated CD8+ T cells.
Figure 3. Transitional memory CD8+ cells exhibit maximal CNAR activity.
Figure 4. HIV-suppressing CD8+ cells are increased in frequency among cells that exhibit biased T cell receptor repertoires.
Figure 5. Non-cytotoxic features of HIV-suppressing CD8+ cells.