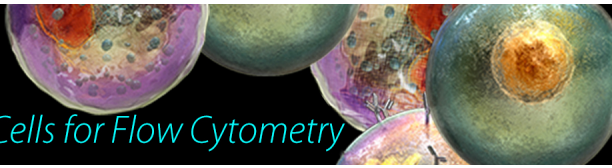


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Functional and Phenotypic Characterization of CD57⁺CD4⁺ T Cells and Their Association with HIV-1-Induced T Cell Dysfunction¹

Brent E. Palmer, Naomi Blyveis, Andrew P. Fontenot, and Cara C. Wilson²

HIV-1 replication is associated with reduced or absent HIV-1-specific CD4⁺ T cell proliferation and skewing of HIV-1-specific CD4⁺ T cells toward an IFN- γ -producing, CCR7⁻ phenotype. The CCR7⁻ T cell population is heterogeneous and can be subdivided based on the expression of CD57. Although CD57 expression on CD8⁺ T cells is associated with proliferation incompetence and replicative senescence, less is known about the function of CD57-expressing CD4⁺ T cells. In this study, the frequency, phenotype, and function of CD57⁺CD4⁺ T cells were evaluated in 25 HIV-1-infected subjects and 10 seronegative controls. CD57⁺CD4⁺ T cells were found to be proliferation incompetent, even after strong mitogen stimulation. Percentages of CD4⁺ T cells that expressed CD57 were significantly higher in untreated HIV-1-infected subjects than in HIV-1-seronegative donors, and CD57 expression did not normalize in subjects receiving at least 6 mo of effective antiretroviral therapy. CD57 was predominately expressed on the CCR7⁻ fraction of the CD4⁺ T cell compartment and accounted for the majority of cells in the CCR7⁻CD45RA⁺ population from untreated HIV-1-infected subjects. HIV-1-specific CD4⁺ T cells producing only IFN- γ had the highest expression of CD57, whereas few cells producing IL-2 alone expressed CD57. These findings further define a novel population of proliferation-incompetent CD4⁺ T cells that are generated in the presence of chronic Ag exposure. A better understanding of the generation and persistence of CD57⁺ T cells in HIV-1 infection could provide important insights into the immunopathogenesis of this disease. *The Journal of Immunology*, 2005, 175: 8415–8423.

Functionally distinct T cell populations can be defined by the expression of specific cell surface Ags. Numerous studies describe associations between T cell surface phenotype and function, and this information is extensively used to track and delineate HIV-1 pathogenesis (1–7). CD57, a surface molecule expressed on T and NK cells, is a marker with functional associations. Recently, it was shown that a subpopulation of CD8⁺ T cells expressing CD57 were proliferation incompetent (8). These cells were terminally differentiated and underwent apoptosis upon T cell activation (7–9). The expression of CD57 on the surface of CD8⁺ T cells is also linked with chronic immune activation, and increased numbers of CD8⁺ T cells expressing CD57 have been associated with HIV-1 and other persistent viral infections (7, 9, 10). Expansions of CD4⁺ T cells expressing CD57 are associated with a number of chronic pathological conditions such as tuberculosis, malaria, rheumatoid arthritis, and HIV-1 infection (11–15). However, the function of these cells is not well characterized, and the association of this cell population with HIV-1 disease remains poorly understood.

HIV-1 replication is associated with profound changes in CD4⁺ T cell function, including reduced proliferative responses to recall Ags and mitogens (16, 17). Changes in CD4⁺ T cell function are

associated with changes in memory T cell maturation state that take place in the presence of viral replication and chronic Ag exposure. We and others (6, 18) have shown that HIV-1 replication skews HIV-1-specific CD4⁺ T cells away from an IL-2-producing, proliferation competent, CCR7⁺ central memory T cell (Tcm)³ phenotype, toward an IFN- γ -producing, CCR7⁻ effector memory T cell (Tem) phenotype. Skewing of the HIV-1-specific CD4⁺ T cell population is directly correlated with increased HIV-1 viral load and decreased HIV-1-specific proliferation (6). CD4⁺ T cell-proliferative responses are partially restored with the suppression of HIV-1 replication with antiretroviral therapy (ART), and the recovery of proliferation in these subjects is in part due to an increase in the frequency of IL-2-producing CCR7⁺CD45RA⁻CD4⁺ T cells (19, 20). Interestingly, the increased proliferation potential of adoptively transferred CCR7⁺CD8⁺ T cells was associated with their ability to control lymphocytic choriomeningitis virus replication in a mouse model of chronic viral infection (21). Studies of the proliferative potential of virus-specific CD4⁺ T cells associated strong proliferative responses with control of viral replication, although this has not been conclusively shown in HIV-1 disease (22, 23).

It was recently proposed that CCR7⁻ effector memory CD4⁺ T cells (Tem) may be divided into at least two subpopulations based on proliferative capacity (18). This model holds that when naive CD4⁺ T cells are initially exposed to Ag, they develop into an early CCR7⁻ effector population (Tem1) with strong proliferative potential, subsequently reverting to CCR7⁺ Tcm cells that persist over time when Ag burden is low or absent. If Ag exposure remains high and constant, as in the case of untreated HIV-1 infection, these “Tem1” CD4⁺ T cells may instead develop into a more

Department of Medicine, University of Colorado Health Sciences Center, Denver, CO 80262

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² Address correspondence and reprint requests to Dr. Cara C. Wilson, Associate Professor of Medicine and Immunology, Campus Box B-164, Division of Clinical Immunology, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Denver, CO 80262. E-mail address: cara.wilson@UCHSC.edu

³ Abbreviations used in this paper: Tcm, central memory T cells; Tem, effector memory T cells; ART, antiretroviral therapy; HAART, highly active ART; HS, HAART suppressed; TN, treatment naïve; TR, Texas Red; SEB, staphylococcal enterotoxin B; Temtd, terminally differentiated effector memory cells.

differentiated “Tem2” effector population, which is able to produce IFN- γ but lacks the capacity to produce IL-2, and thus, the ability to self-renew. HIV-1-specific CCR7⁻CD4⁺ T cells from viremic subjects, in whom Ag levels remain high, are only able to produce IFN- γ , whereas those from aviremic subjects can produce both IFN- γ and IL-2 (6, 18). We hypothesized that the expression of CD57 on CD4⁺ T cells might be used as a marker to differentiate Tem1 from Tem2 and as an indicator to assess the functionality of the T cell compartment in HIV-1-infected subjects.

In this study, we examined the frequency, phenotype, and functional properties of Ag-specific CD57⁺ T cells in untreated, viremic HIV-1-infected subjects as well as in infected subjects whose viral loads were suppressed with ART using multiparameter flow cytometry, intracellular cytokine staining, and CFSE staining. We conclusively demonstrate that CD57⁺CD4⁺ T cells can produce IFN- γ but are unable to proliferate or produce IL-2, even when stimulated with a powerful mitogen. We also show that CD4⁺CD57⁺ T cells are expanded in HIV-1-infected subjects, and that CD57⁺ T cells constitute a high proportion of the CCR7⁻ T cell population, especially in subjects with untreated HIV-1 disease. These data suggest that the expression of CD57 on CD4⁺ T cells may be an important phenotypic marker to assess the functional competence of the CD4⁺ T cell compartment.

Materials and Methods

Study population

HIV-1-infected study subjects were selected from a cohort of HIV-1-infected individuals followed in the Adult Infectious Diseases Group Practice at the University of Colorado Health Sciences Center. Twenty-five HIV-1-infected subjects enrolled into two clinical cohorts based upon their treatment status: highly active ART (HAART)-suppressed (HS) or treatment naive (TN). Efforts were made to match CD4⁺ T cell counts in the HS and TN groups based on having a screening peripheral CD4⁺ T cell count in the range of 350–800 cells/ μ l. Inclusion criteria for the HS cohort ($n = 13$) included receiving a combination of three or more antiretroviral agents with suppression of plasma viral load to <20 copies HIV-1 RNA per milliliter of plasma for ≥ 6 mo (median CD4⁺ T cell count, 594 cells/ μ l; range, 270–1,020). TN subjects ($n = 12$) were TN with a median viral load of 15,650 copies HIV-1 RNA per milliliter of plasma (range, 2,280–644,909), a median CD4⁺ T cell count of 471 cells/ μ l (range, 20–1,218). HIV-1-seronegative subjects ($n = 10$) were normal healthy adult volunteers. All of the study subjects participated voluntarily and gave informed consent, and the study was approved by the University of Colorado Health Sciences Center Institutional Review Board.

Collection and preparation of PBMC

Blood from HIV-1-seropositive and -seronegative adults was collected in Vacutainer tubes containing sodium heparin (BD Vacutainer). Within 2 h of venipuncture, PBMC were isolated from whole blood by density gradient centrifugation on Ficoll. PBMC for intracellular staining were rested overnight in RPMI 1640 with 10% human serum at 3×10^6 cells/ml. CFSE staining was performed immediately.

Cell sorting

Cell sorting was accomplished using a FACS Aria cell sorter (BD Biosciences). PBMC were isolated, and a minimum of 1×10^8 total PBMC were incubated with anti-CD57 FITC, anti-CD45RA PE Cy5, anti-CD4 allophycocyanin Cy7, and anti-CD3 PE-Texas Red (TR) for 30 min at 4°C. Cells were sorted into four different populations, CD3⁺CD4⁺CD57⁺, CD3⁺CD4⁺CD57⁻CD45RA⁻ and CD3⁺CD4⁺CD57⁻CD45RA⁺ as well as CD4⁺-depleted PBMC, and were collected. To obtain the highest purity possible, a doublet discriminating gate was used. The sort logic was set by gating lymphocytes by forward light scatter and side scatter and then gating on CD3⁺CD4⁺ cells. The CD3⁺CD4⁺CD57⁺ T cells were then separated and collected, and the remaining CD57⁻ cells were further segregated by expression of CD45RA into naive and memory populations. CD4-depleted PBMC were collected to be used as APCs from a large forward light scatter vs side scatter gate, which encompassed most live cells. Within the lymphocyte gate, all non-CD3⁺CD4⁺ T cells were also added to the CD4-depleted PBMC fraction. The purity of the sorted CD4⁺ T cell populations ranged from 94 to $>99\%$.

Proliferation assay

Assays were performed on either whole PBMC or with combinations of sorted CD4⁺ T cells and CD4-depleted PBMC. Cells were labeled with CFSE (Molecular Probes) by incubating 6×10^6 cells in a 3- μ M solution of CFSE in HBSS for 20 min at 37°C followed by two washes, before incubation with Ag. Sorted CD4⁺ T cell subsets (CD57⁺, CD57⁻CD45RA⁺, and CD57⁻CD45RA⁻) were mixed at a 1:1 ratio with CD4⁺ T cell-depleted PBMC. Both whole PBMC and the sorted PBMC were plated at 1×10^6 cells/ml, stimulated with 5 μ g/ml PHA (REMEL), and cultured for 4 days in RPMI 1640 plus 10% human serum at 37°C in a humidified 5% CO₂ atmosphere. On day 4, cells were gently removed from the wells, washed with PBS containing 1% BSA, and surfaced stained with anti-CD4 allophycocyanin Cy7 (Caltag Laboratories), anti-CD8 allophycocyanin (BD Pharmingen), anti-CD57 PE (BD Pharmingen), and anti-CD3 PE-TR (Beckman Coulter) for 30 min at 4°C. Cells were washed again and resuspended in 1% formaldehyde. In the whole PBMC cultures, the percentage of CD57⁺ and CD57⁻CD4⁺ T cells that proliferated was determined by first gating on CD3⁺CD4⁺CD57⁺ and CD3⁺CD4⁺CD57⁻ subpopulations, then determining the percentage of CFSE^{low} cells in each subpopulation. CD4⁺ T cells from unstimulated cultures were used to set the gate for the determination of the percentage of CFSE^{low} cells in the PHA-stimulated cultures.

Apoptosis assay

PBMC were stained directly ex vivo and after 24 h in culture with and without PHA stimulation. PBMC were placed at 1×10^6 cell/ml in 24-well plates in RPMI 1640 plus 10% human serum with or without 1 μ g/ml PHA (Sigma-Aldrich) and incubated for 24 h. PBMC were washed and incubated with unlabeled anti-CD57 mAb (BD Pharmingen) for 30 min at 4°C, washed once with PBS containing 1% BSA, and then stained with the secondary anti-mouse IgM label with biotin for 30 min at 4°C. Cells were washed again with PBS-BSA and stained with anti-CD4 (allophycocyanin Cy7; BD Pharmingen), anti-CD3 (PE-TR; Beckman Coulter), and streptavidin-PE. Washed cells were then resuspended in 1 \times annexin V buffer and incubated with annexin V-FITC (BD Pharmingen) for 15 min in the dark at room temperature. Two hundred microliters of annexin V buffer was added, and the samples were analyzed immediately on the FACS ARIA.

Peptides

HIV-1 Gag peptides (122 peptides) consisted of 15 mers overlapping by 11 aas and corresponding to sequences of clade B H \times B2 strain HIV-1 (National Institutes of Health AIDS Research and Reference Reagent Program; catalog no. 5107). HIV-1 Pol peptides (100 peptides) were 20 mers overlapping by 10 aas corresponding to clade B strain H \times B2R (National Institutes of Health AIDS Research and Reference Reagent Program; catalog no. 4358), and HIV-1 Nef clade B peptides (49 peptides; National Institutes of Health AIDS Research and Reference Reagent Program; catalog no. 5189) were 15 mers overlapping by 11 aas. Peptides were individually resuspended at 100 mg/ml in DMSO, then pooled into a group containing all Gag, Pol, and Nef peptides.

T cell stimulations for Ag-specific cytokine production

The frequency of Ag-specific, IFN- γ - and IL-2-producing T cells in PBMC was determined using a previously reported method, with minor modifications (10). Briefly, 2.5×10^6 PBMCs were placed in 12 \times 75 mm culture tubes containing 3 μ g/ml anti-CD28 and -CD49d Abs (BD Biosciences) in RPMI 1640 plus 10% human serum under one of the following stimulation conditions: pooled HIV-1 Gag (122 peptides), Pol (100 peptides), and Nef (49 peptides) peptides (1 μ g/ml final concentration of each peptide; National Institutes of Health AIDS Research and Reference Reagent Program); CMV lysate (1/10 dilution, derived from G-lung cell line infected with CMV strain AD169, virus titer 2×10^7 PFU/ml (provided by A. Weinberg, Denver, CO)) or control lysate (derived from uninfected G-lung cells); staphylococcal enterotoxin B (SEB) (1 μ g/ml; Toxin Technology); or medium alone. The cultures were incubated at a 5-degree slant at 37°C in a humidified 5% CO₂ atmosphere for 6 h with Brefeldin A (BD Biosciences) added after the initial 2 h.

Immunofluorescence staining of stimulated T cells

Stimulated PBMC were washed and then surfaced stained with unlabeled anti-CCR7 mAb (BD Pharmingen) for 30 min at 4°C, washed once with PBS containing 1% BSA, and stained with the secondary anti-mouse IgM label with biotin for 30 min at 4°C. Cells were washed once with PBS-BSA and stained with anti-CD4 (allophycocyanin Cy7; BD Pharmingen), anti-CD3 (PE-TR; Beckman Coulter), anti-CD8 (Alexa Flour 405; Caltag Laboratories), anti-CD57 (FITC; BD Pharmingen), anti-CD45RA (PE Cy5;

BD Biosciences), and streptavidin-PE for 30 min at 4°C. In some subjects, degranulation was measured by staining with CD107a + b (FITC; BD Pharmingen). This was accomplished by adding both CD107a and b (FITC; BD Pharmingen) at the beginning of the cell stimulation. Cells were fixed and held overnight at 4°C. Cells were permeabilized, and stained with anti-IFN- γ (PE Cy7; BD Pharmingen) and anti-IL-2 mAbs (allophycocyanin; Caltag Laboratories) for 30 min at 4°C. Permeabilized cells were washed and resuspended in 1% formaldehyde.

Flow cytometric acquisition and analysis

Ten-parameter flow cytometry was performed using a FACS Aria flow cytometer. FITC or CFSE, PE, TR, PE Cy5, PE Cy7, allophycocyanin, allophycocyanin Cy7, and Alexa Fluor 405 were used as fluorophores. At least 5×10^5 live CD3⁺ lymphocytes were collected for the Ag-specific analysis. The data files were analyzed using Diva software (BD Biosciences). When PBMC were cultured with only medium and costimulatory molecules to determine the background frequency of cytokine-producing CD4⁺ T cells, the median response for IFN- γ only was 0.01% (range, 0.0–0.06%), IFN- γ /IL-2 was 0.0% (range, 0.0–0.03%), and for IL-2 only was 0.04% (range, 0.0–0.15%). Analysis of CD8⁺ T cells was performed by gating on the CD3⁺CD4⁻ T cells because anti-CD8 Ab was not added in all experiments. However, ~50% of the patient samples were stained with anti-CD8, and within a subject the CD3⁺CD4⁻ population was almost all CD8⁺, confirming the validity of our negative gating strategy.

Statistical analysis

The Mann-Whitney *U* test and the Kruskal-Wallis test with pairwise comparison (Dunn multiple comparison test) were used to determine significance of differences between subject groups. A Spearman correlation test was performed to analyze the association between CD57⁺CD4⁺ T cells.

Results

CD57 expression on CD4⁺ T cells is associated with proliferation incompetence

Because it has been shown that CD57⁺CD8⁺ T cells have reduced proliferative capacity, we first sought to determine whether this held true for CD4⁺ T cells expressing CD57. CD57⁺CD4⁺ T cell proliferation was initially examined using bulk PBMC cultures from 10 healthy HIV-1-seronegative subjects. CFSE-labeled PBMC were incubated with PHA for 4 days, and the percentage of CD57⁺CD4⁺ T cells that were CFSE^{low} was calculated as described in *Materials and Methods*. Fig. 1A shows that CD4⁺ T cells that expressed CD57 proliferated significantly less well than did those not expressing CD57. The median percentage of CFSE^{low}, proliferating CD57⁺CD4⁺ T cells was 21.5% (range, 14.3–54.4%), whereas the median percentage of proliferating CD57⁻CD4⁺ T cells was 65.75% (range, 52–88%; $p < 0.0001$). Significantly fewer CD57⁺CD4⁺ T cells also proliferated in the presence of anti-CD3 Ab or SEB than did cells not expressing CD57 ($p = 0.01$ and $p = 0.002$, respectively; data not shown).

The limitation of the bulk PBMC assay was the inability to determine whether CD57 expression on the CD4⁺ T cells was altered after stimulation. To examine whether CD57 expression fluctuated during the culture period following stimulation, CD57⁺CD4⁺ and CD57⁻CD4⁺ T cells were first sorted and then stimulated. As the proliferative potentials of naive and memory CD4⁺ T cells may differ (24), the CD57⁻ population was further sorted into naive and memory CD4⁺ T cells based on CD45RA expression. It is of note that expression of CD45RA alone only provides an approximate maker of naive cells, but for this purpose it was sufficient because both populations of CD57⁻CD4⁺ T cells exhibited similar proliferation. CD57⁺ T cells are almost exclusively of an effector memory (CCR7⁻) phenotype, thus these cells were not further sorted. The three sorted populations of CD4⁺ T cells were cocultured with CD4-depleted PBMC to more closely mimic their normal physiological environment as well as to provide APCs necessary for optimal T cell stimulation. Sorted, CFSE-labeled CD4⁺ T cells were incubated with APC and PHA for 4

days, then stained for CD4 and CD57. A representative example of CD57 vs CFSE staining for each sorted CD4⁺ T cell population is shown in Fig. 1B. As is clearly evident, CD57⁺CD4⁺ T cells did not proliferate well in response to PHA, whereas both naive and memory CD57⁻CD4⁺ T cells vigorously proliferated (Fig. 1B). Fig. 1C shows combined proliferation data from three such sorting experiments. These experiments also demonstrated that there was minimal change in the expression of CD57 on CD4⁺ T cells after stimulation in culture. The median percentage of CD57⁺ cells before stimulation in the CD57-selected culture from the three sorts was 94.6%, and after 5 days it had dropped to 86.4%. There was virtually no change in CD57 expression on the CD57⁻ naive population after stimulation (<1–2.8%), whereas greater up-regulation of CD57 was observed in the CD57⁻ memory population (<1–7.3%). Taken together, these data demonstrate that the ability of CD57⁺CD4⁺ T cells to proliferate is limited, and that there is minimal change in the expression of CD57 after stimulation.

CD57 expression on CD4⁺ T cells is associated with increased apoptosis

To better understand the mechanism responsible for the reduced in vitro proliferation seen in CD4⁺ T cells expressing CD57, we examined apoptosis using annexin V staining. PBMC from five seronegative donors were stained with annexin V directly ex vivo as well as after 24 h in culture, and the percentage of CD4⁺ T cells undergoing spontaneous apoptosis was determined. A representative example of annexin V staining on CD4⁺ T cells expressing CD57 directly ex vivo and after 24 h of culture is shown in Fig. 2A. Fig. 2B shows a significantly higher percentage of CD4⁺ T cells expressing CD57 stained with annexin V than cells not expressing CD57 directly ex vivo ($p = 0.04$). The median percentage of annexin V-positive CD4⁺ T cells expressing CD57 was 15.9% (range, 4.3–27.8%) compared with 3.1% (range, 1.5–5.6%) for CD57⁻ cells. Fig. 2C shows that when PBMC were cultured for 24 h without stimulation, CD4⁺ T cells expressing CD57 also had significantly higher percentages of annexin V staining than did CD57⁻ cells ($p = 0.003$). After 24 h in culture, the median percentage of annexin V-positive CD4⁺ T cells expressing CD57 was 38.2% (range, 16.3–53.4%) compared with 13.6% (range, 4–25.8%) of CD57⁻ cells. To determine the level of activation-induced apoptosis, PBMC were stimulated for 24 h with PHA. Stimulation substantially increased the level of annexin V staining on all CD4⁺ T cells, but again, more CD57⁻ expressing cells stained annexin V-positive than did CD57⁺ cells ($p = 0.006$; data not shown). These data suggest that CD57 expression on CD4⁺ T cells is associated with an increased rate of spontaneous as well as activation-induced apoptosis.

HIV-1 infection is associated with increased percentages of CD57⁺ T cells in the peripheral blood

CD57 expression on T cell subpopulations from 25 HIV-1-infected subjects and 10 seronegative control subjects was measured by flow cytometry. Fig. 3A shows that the median percentage of CD4⁺ T cells that expressed CD57 in HIV-1-infected subjects (12.3%; range, 2.0–63.4%) was significantly higher than in seronegative subjects (4.6%; range, 2.6–15.6%; $p = 0.004$). As shown in Fig. 3B, the HIV-1-infected cohort was subdivided into antiretroviral TN and HS subjects to determine whether suppression of viral replication had an effect on CD57 expression. The median percentage of CD4⁺ T cells expressing CD57 in the TN and HS cohort was 12.4 (range, 2–63.4%) and 11.5% (range, 3.6–26.5%), respectively. Despite the fact that several viremic subjects expressed CD57 on >25% of their CD4⁺ T cells, no significant difference in CD57 expression was seen between these groups in the

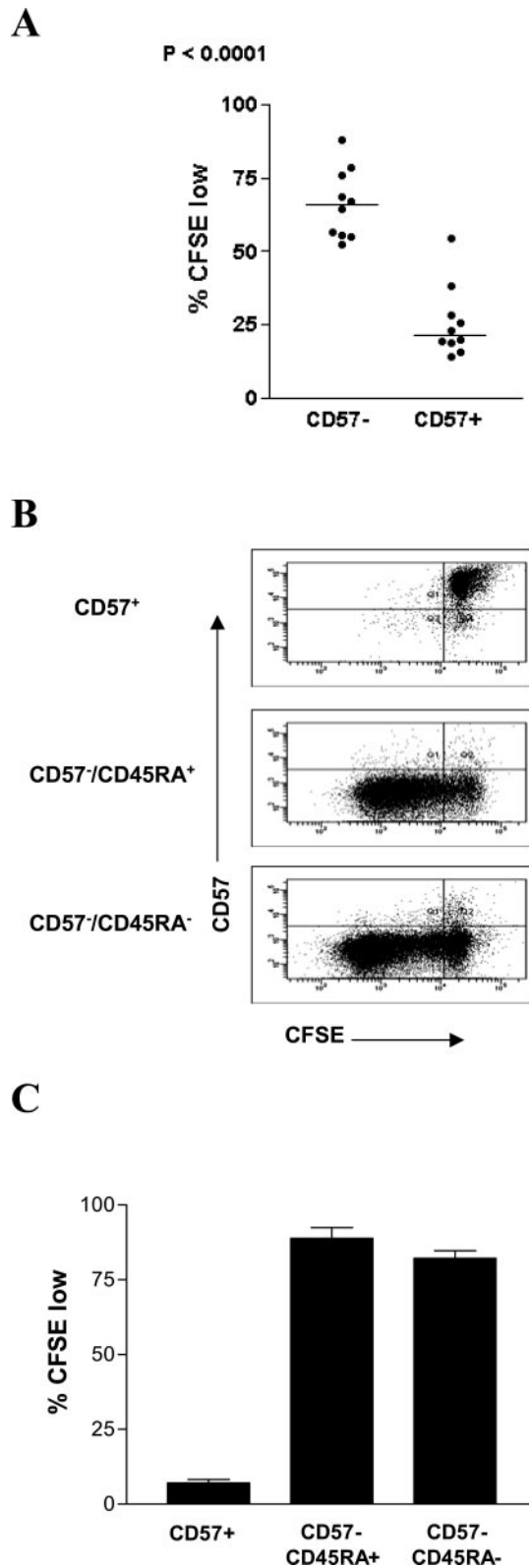


FIGURE 1. Proliferation of CD4⁺ T cell populations defined by expression of CD57. *A*, PBMC from HIV-seronegative individuals ($n = 10$) were labeled with CFSE and cultured with PHA for 5 days. Cultured PBMC were stained with anti-CD57 PE, anti-CD3 Tricolor, and anti-CD4 APC mAbs, and the percentages of CFSE^{low}CD57⁺ and CD57⁻CD4⁺ T cells were determined. *B*, CD4⁺ T cells were sorted into two populations based on CD57 expression. CD45RA expression, which provides an approximate marker of naive T cells, was used to divide the CD57⁻ population into naive and memory cells. The three sorted populations were labeled with CFSE, mixed with CD4-depleted PBMC, and cultured with PHA for 5

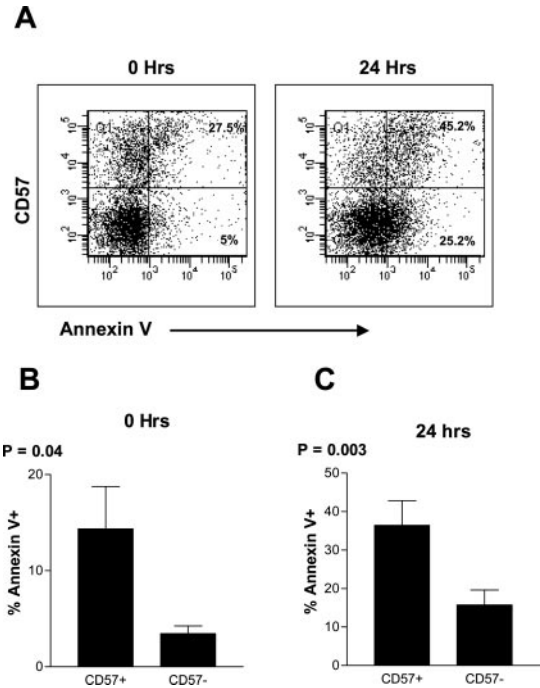


FIGURE 2. Spontaneous apoptosis of CD4⁺ T cells expressing CD57. *A*, Representative example of annexin V staining on CD4⁺ T cells expressing CD57 directly ex vivo and after 24 h in culture without stimulation. Mean and SE ($n = 5$) for percentages of CD4⁺ T cells staining with annexin V-FITC directly ex vivo (*B*) and after 24 h (*C*) are shown. Paired *t* tests were performed to determine differences in the percentage of CD57⁺ or CD57⁻CD4⁺ T cells with annexin V staining.

CD4⁺ T cell subset ($p = 0.34$). Although this finding could indicate that CD57 expression on CD4⁺ T cells does not completely normalize after ART, interpretation of these results is limited by a small sample size. These data show that there is an increased percentage of CD4⁺ T cells expressing CD57 in HIV-1-infected subjects, and that suppression of HIV-1 replication does not result in the complete normalization of CD57 expression in this T cell compartment even after 6 mo of treatment.

CD57 is expressed on more differentiated Tem cell populations

CD57 expression has been associated with terminal differentiation of CD8⁺ T cells, but less has been reported about the expression of this marker on CD4⁺ T cells. CD57 expression was analyzed on memory CD3⁺CD4⁺ T cell populations in TN, HS, and HIV-seronegative subjects using multicolor flow cytometry (Fig. 4). CD45RA and CCR7 expression was used to divide the memory T cell compartment for each subject into distinct populations: Tem (CD45RA⁻CCR7⁺), Tem (CD45RA⁻CCR7⁻), and terminally differentiated effector memory cells (Temtd) (CD45RA⁺CCR7⁻) (25, 26). Representative examples of CD57 staining on the three memory CD4⁺ T cell populations, as defined by the expression of CCR7 and CD45RA, are shown in Fig. 4A. CD57 was infrequently expressed on CCR7⁺CD45RA⁻ central memory CD4⁺ T cells; among all subjects, a median of 4.8% of cells expressed CD57, and there was no statistically significant difference in CD57 expression

days. Cultured cells were stained as described above. A representative example of the CFSE staining is shown in *B*. The percentages of CFSE^{low} CD4⁺ T cells from each sorted populations are depicted in *C*. The mean and SD of three separate sorting experiments are shown. Statistical significance was determined using Wilcoxon matched-pair test.

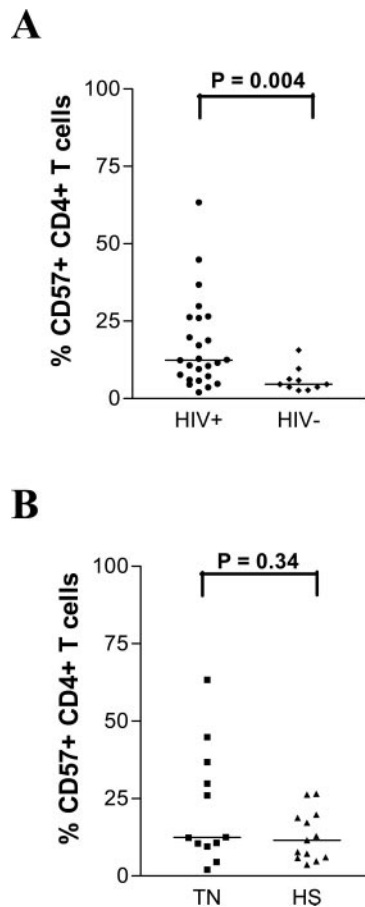


FIGURE 3. Expression of CD57 on CD4⁺ cells from 25 HIV-1-infected and 10 seronegative subjects (*A*). *B* segregates HIV-1-infected subjects into subjects who have never received HAART (TN) and subjects whose viral loads are currently suppressed by HAART (HS). PBMC were stained with anti-CD3, anti-CD4, and anti-CD57 mAbs, and the percentage of T cells expressing CD57 was determined. Values for each subject are depicted as separate points, and the bars represent the median values for each group. Statistical significance was determined using Mann-Whitney *t* test.

on CD4⁺ Tcm cells between subject groups (Fig. 4*B*). CD57 was expressed on a higher percentage of CD4⁺CCR7⁻CD45RA⁻ Tem cells, with a median of 18.5% for all subjects. The median percentage of CD57⁺CD4⁺ Tem cells was 26.2% (range, 3.7–52.7%) in the TN group, 16.0% (range, 5.8–45.0%) in the HS group and 9.6% (range, 0.18–37.5%) in the HIV-1-seronegative group (Fig. 4*B*). Although the median percentage of CD4⁺ Tem cells expressing CD57 in the TN group was almost three times higher than in the HIV-1-seronegative control group, this difference was not statistically significant, perhaps due to the broad range of CD57 expression on CD4⁺ Tem cells within each cohort. CD57 expression on the CCR7⁻CD45RA⁺CD4⁺ Temtd cells was the highest of the memory subpopulations, with a median of 42.7% (range, 0.28–91.5%) for all subjects. The median percentage of CD57⁺CD4⁺ Temtd cells was 68.3% (range, 7.8–91.5%) in the TN group, 35.6% (range, 10.7–80.9%) in the HS group, and 28.1% (range, 0.28–48.7%) in the HIV-1-seronegative group. The difference in percentages of CD57⁺CD4⁺ Temtd cells between the TN and HIV-seronegative groups was found to be statistically significant ($p = 0.02$). Interestingly, a significant direct correlation was found between the percentage of CD4⁺CD57⁺ Temtd cells and plasma viral load ($r = 0.53$; $p = 0.02$; data not shown) within the HIV-1-infected subject cohort. In addition, we found that intracohort variation in

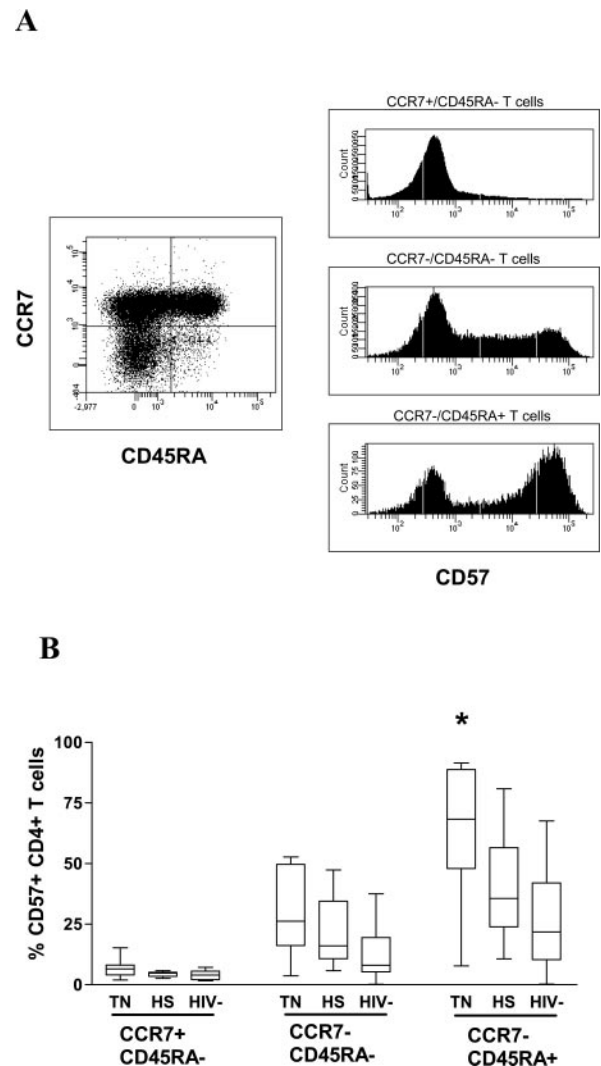


FIGURE 4. The distribution of CD57 on memory CD4⁺ T cell populations. *A*, Representative example of CD57 staining on the three memory populations of CD4⁺ T cells as defined by CCR7 and CD45RA expression. *B*, The percentage of CCR7⁺CD45RA⁻ (Tcm), CCR7⁻CD45RA⁻ (Tem), and CCR7⁻CD45RA⁺ (Temtd) CD4⁺ T cells expressing CD57⁺ T cells was determined for 25 HIV-1-infected subjects comprised of 12 TN and 13 HS as well as 10 HIV seronegative. The box and whiskers graph shows the median and range. ANOVA (Kruskal-Wallis test) was used to determine differences in CD57 expression on a given memory subset between patient groups. Asterisk indicates statistical significance within a memory population.

CD57 expression on the memory CD4⁺ T cell populations was higher for the HIV-1-infected groups than the seronegative controls ($p = 0.0002$ and $p = 0.0003$, respectively, vs $p = 0.01$).

CD57 is predominantly expressed on IFN- γ -producing, Ag-specific T cells

We have previously shown that there is a discordance between HIV-1-specific IFN- γ -producing CD4⁺ T cells and HIV-1-specific CD4⁺ T cell proliferation in untreated HIV-1-infected subjects with progressive disease (19). Additionally, a direct correlation between the percentage of HIV-1-specific IL-2-producing cells and CD4⁺ T cell proliferation has been described (18, 27). We hypothesized that CD57 expression would also vary on distinct cytokine-producing CD4⁺ T cell populations (single IFN- γ , single IL-2, and double IFN- γ /IL-2), and that CD57 expression might reflect their known proliferative potential. We also wanted to determine

whether HIV-1 replication induced changes in CD57 expression on cytokine-producing T cells of other specificities. To address these questions, we analyzed CD57 expression on HIV-1- and CMV-specific as well as SEB-stimulated T cells that produced either IFN- γ only, IFN- γ and IL-2, or IL-2 only from all 25 HIV-1-infected subjects. A representative example of CD57 expression on cytokine-producing CD4⁺ T cells after stimulation with HIV-1 peptides is shown in Fig. 5A. Because the frequency of HIV-1-specific CD4⁺ T cells in peripheral blood tends to be low, we only examined the expression of CD57 on cytokine-producing cells with frequencies $\geq 0.04\%$ to ensure an adequate number of events for a valid analysis. Additionally, due to these low frequency responses and the nature of multicolor parameter flow, which subdivides small populations into even smaller subsets, 5×10^6 PBMC from HIV-1-infected subjects were simultaneously incubated with Gag, Pol, and Nef overlapping peptides to stimulate a maximal number of cytokine-producing cells for detailed multiparametric analysis.

Examination of CD57 expression on HIV-1-specific CD4⁺ T cells from these subjects, as depicted in Fig. 5B, revealed that the median percentage of HIV-1-specific IFN- γ -producing cells (58.7%; range, 20.8–77.5%) expressing CD57 was significantly higher than that of the IFN- γ /IL-2- (42.08%; range, 36.4–47.3%) or IL-2- (19.35%; range, 2.1–51.6%) producing cells ($p = 0.0007$). Interestingly, the distribution of CD57 on the three discrete cytokine-secreting T populations was mirrored in the CMV-specific CD4⁺ T cell response in HIV-1-infected subjects. Evaluation of CD57 expression on CMV-specific CD4⁺ T cells from these subjects revealed that the median percentage of CMV-specific IFN- γ ⁺CD4⁺ T cells expressing CD57 (60.5%; range, 38.3–80.3%) was also much higher than that of the IFN- γ ⁺IL-2⁺ cells (35.6%; range, 20–61.3%) or IL-2⁺ only (22.2%; range, 4.1–47.3%) producing cells ($p < 0.0001$). To examine the expression of CD57 on superantigen-induced, cytokine-producing CD4⁺ T cells, SEB was used as a stimulus. CD57 expression on SEB-stimulated CD4⁺ T cells from these subjects revealed that the median percentage of SEB-stimulated IFN- γ ⁺ cells expressing CD57 (56.4%; range, 16.6–77.8%) was also much higher than that of the IFN- γ ⁺IL-2⁺ (16.6%; range, 5.6–55.5%) or IL-2 only (4.8%; range, 1.1–10.5%) producing cells ($p < 0.0001$). There was no significant difference between TN and HS subjects in the percentage of HIV-1-specific cytokine-producing CD4⁺ T cells that expressed CD57 (TN, ●; HS, ○) (Fig. 5). Comparison between HIV-1-, CMV-, and SEB-stimulated IFN- γ -producing cells also revealed no difference in CD57 expression ($p = 0.57$). However, when CD4⁺ IL-2-producing T cells were examined, a significantly greater percentage of both HIV-1- and CMV-specific CD4⁺ T cells expressed CD57 compared with SEB-stimulated CD4⁺ T cells ($p = 0.002$). Further analysis revealed that SEB-responsive IL-2-producing CD4⁺ T cells were comprised of more CCR7⁺, CD45RA⁺ (naive), and CCR7⁺CD45RA⁻ (Tcm) cells than either CMV- or HIV-specific IL-2-producing CD4⁺ T cells, which were skewed toward a CCR7⁻CD45RA⁻ (Tem) phenotype (data not shown). These data show that CD57 is expressed on a high percentage of CD4⁺ T cells capable of producing only IFN- γ , regardless of Ag specificity.

Although no significant difference between TN and HS subjects was seen in CD57 expression on HIV-1-specific CD4⁺ T cells expressing a particular cytokine profile, we theorized that CD57 expression on total HIV-1-specific T cells from untreated subjects would be greater due to a shift toward IFN- γ production in the presence of viremia. To address this, we first examined the distribution of HIV-1-specific IFN- γ and IL-2-producing CD4⁺ T cells. Fig. 5C shows the distribution of HIV-1-specific CD4⁺ cytokine-producing T cells for TN ($n = 9$) and HS ($n = 13$) subjects. The

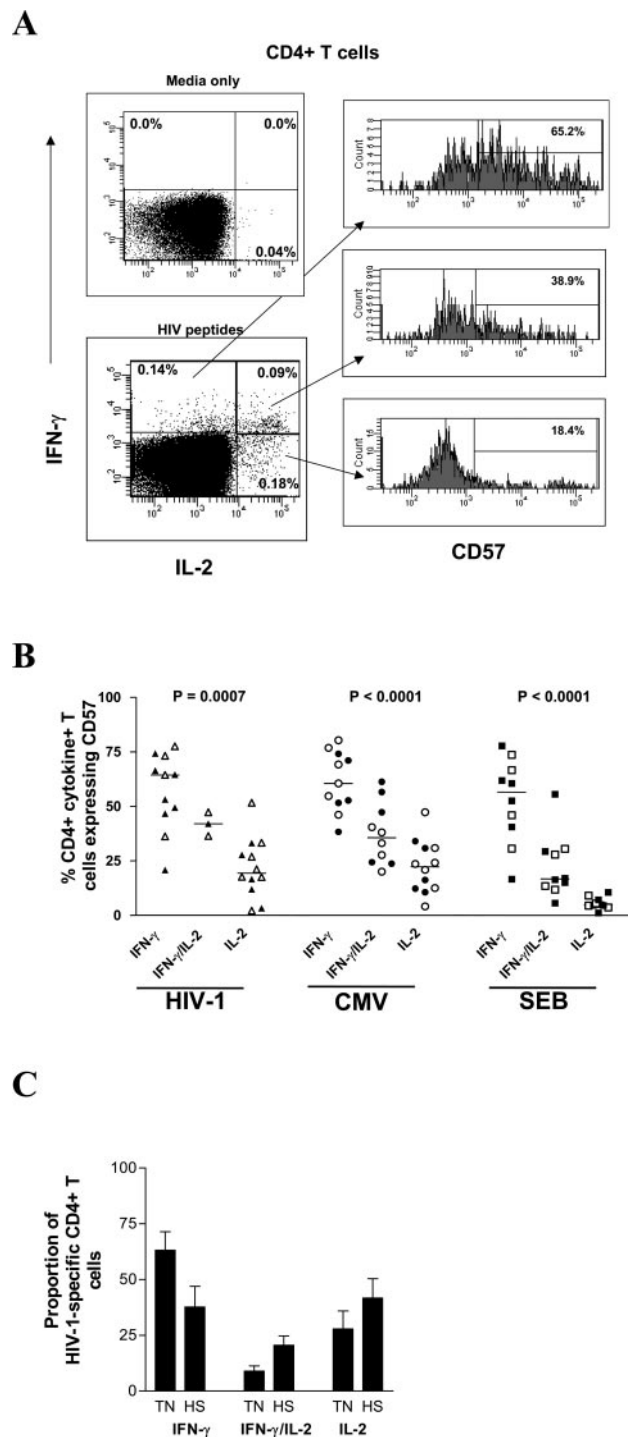


FIGURE 5. The distribution of CD57 on virus-specific cytokine-producing CD4⁺ T cells. **A**, Representative histograms of CD57 expression on HIV-1-specific IFN- γ , IL-2, and dual cytokine-producing CD4⁺ T cell populations. **B**, Shows the percentage of HIV-1, CMV, and SEB-responsive cytokine-producing CD4⁺ T cells that express CD57 in HIV-seropositive subjects. **C**, Mean \pm SE of the proportion of the HIV-1-specific CD4⁺ T cells within the different cytokine-secreting T cell populations in TN ($n = 12$) and HS ($n = 13$) subjects. CD57 expression was only analyzed on HIV-1-infected subjects with particular cytokine response of 0.04% or greater. Values for each subject are depicted as separate points, and the bars represent the median values for each group. Open symbols are HS subject, and closed symbols are TN subject. ANOVA (Kruskal-Wallis test) demonstrated that there was a significant difference in the proportion of cytokine-producing cell in the TN but not the HS cohorts ($p = 0.0007$ and $p = 0.14$, respectively). Kruskal-Wallis test with pair-wise comparison was also performed to determine differences in the percentage of CD57⁺ T cells between distinct cytokine-producing populations.

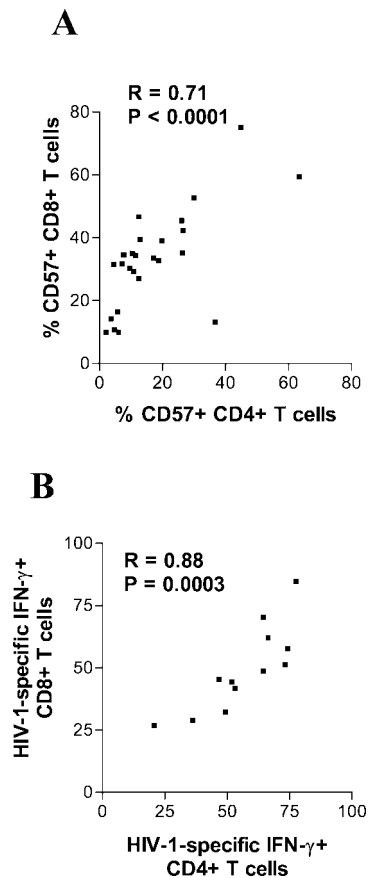


FIGURE 6. Correlation between CD4⁺ and CD8⁺ T cells expressing CD57. Expression of CD57⁺ on total CD4⁺ and CD8⁺ T cells (A) and HIV-1-specific IFN- γ -producing CD4⁺ and CD8⁺ T cells (B) was determined by flow cytometry. Relationship between the percentage of CD4⁺CD57⁺ and CD8⁺CD57⁺ T cells was evaluated using the Spearman correlation test.

mean percentage of HIV-1-specific CD4⁺ T cells from TN subjects producing single IFN- γ or IL-2 and double-positive IFN- γ /IL-2 was 63.1, 8.9, and 27.8%, respectively. In the HS cohort, the mean percentage of HIV-1-specific CD4⁺ T cells producing single IFN- γ or IL-2 and double-positive IFN- γ /IL-2 was 37.7, 20.5, and 41.7%, respectively. ANOVA (Kruskal-Wallis test) determined that there were significant differences in the proportion of HIV-1-specific CD4⁺ T cells with a given cytokine profile in the TN ($p = 0.0007$) but not the HS cohort ($p = 0.14$), with a greater percentage of cells from TN subjects producing IFN- γ than IL-2. These findings are consistent with previous reports of skewing toward IFN- γ production by CD4⁺ T cells in the setting of viremia (4, 6). When we examined CD57 expression on all HIV-1-specific CD4⁺ T cells, combining those that producing IFN- γ , IL-2, or both from a subset of TN and HS, the expression of CD57 on HIV-1-specific CD4⁺ T cells was more than twice as high as on the total CD4⁺ T cell populations (data not shown). The median percentage of HIV-1-specific CD4⁺ T cells that expressed CD57 in TN subjects was 29.7%, whereas CD57 was expressed on a median of 21% in HS subjects. Although CD57 expression was almost one third lower in treated subjects, this difference was not found to be statistically significant (data not shown).

A positive correlation exists between CD57-expressing CD4⁺ and CD8⁺ T cells

Because CD57⁺CD8⁺ and CD57⁺CD4⁺ T cells are both found at higher levels in HIV-1-infected subjects than in uninfected controls, we next sought to determine whether associations existed

between the frequencies of these two cell populations in the peripheral blood and with markers of disease progression. A strong positive correlation was found between CD57-expressing total CD4⁺ and CD8⁺ T cell populations in the peripheral blood of HIV-1-infected patients ($r = 0.71$; $p > 0.0001$) (Fig. 6A). In addition, a strong positive correlation was found between HIV-1-specific IFN- γ ⁺CD57⁺CD4⁺ and IFN- γ ⁺CD57⁺CD8⁺ T cells when all HIV-1-infected subjects were evaluated ($r = 0.88$; $p = 0.0003$; Fig. 6B) as well as when only untreated HIV-1-infected patients were included in the analysis ($r = 0.73$; $p = 0.04$; data not shown). This association may indicate that the same virological factors independently drive CD57 expression on each cell type or may reflect direct interactions between CD57⁺CD4⁺ and CD57⁺CD8⁺ T cells.

To determine whether associations existed between CD57 expression on T cells and markers of HIV-1 disease progression, we next evaluated the relationships between frequencies of CD57⁺ T cells in the peripheral blood and either peripheral CD4⁺ T cell count or plasma HIV-1 viral load. No statistically significant correlations were found between total CD4⁺CD57⁺ T cells or HIV-1-specific CD4⁺CD57⁺ T cells and either viral load or CD4⁺ T count in HIV-1-infected subjects.

Discussion

Diminished virus-specific CD4⁺ T cell-proliferative responses are associated with uncontrolled viral replication and disease progression in both human and animal viral infections (19, 20, 28). Numerous studies (16, 17, 29) have documented that HIV-1-infected subjects have reduced CD4⁺ and CD8⁺ T cell-proliferative responses to recall as well as HIV-1 Ags. In this study, we characterize a population of CD4⁺ T cells, identified based on CD57 expression, that is both unable to proliferate in vitro and is expanded in the peripheral blood of HIV-1-infected subjects. We have previously shown that weak HIV-1-specific CD4⁺ T cell proliferation is associated with increased numbers of IFN- γ -producing, CCR7⁻CD45RA⁻ effector memory CD4⁺ T cells (6). This current study demonstrates that increased expression of CD57 on CCR7⁻CD4⁺ T cells may be responsible for the diminished proliferative responses observed in viremic HIV-1-infected subjects. Furthermore, these data suggest that not only are CD4⁺ T cells skewed toward a CCR7⁻ phenotype, as we have shown previously, but that distinct functional changes take place within this population in untreated subjects with progressive HIV-1 disease. This study expands on our previous findings by defining a novel subpopulation of CCR7⁻CD4⁺ T cells that express CD57, produce IFN- γ , and lack the ability to proliferate.

In concordance with studies of CD8⁺ T cells and NK cells expressing CD57, our results clearly demonstrate that CD4⁺ T cells expressing CD57 also proliferate poorly. In fact, when CD57⁺CD4⁺ T cells were sorted and stimulated with mitogen, little to no proliferation was detected, whereas the majority of both naive and memory CD57⁻CD4⁺ T cell populations vigorously divided. Very little down-regulation of CD57 was seen poststimulation, indicating that these cells do not readily change phenotype or regain the ability to proliferate after in vitro stimulation. These observations are supported by other studies, which demonstrate that CD57⁺CD8⁺ T cells are terminally differentiated, and that proliferation of these cells is not recovered even in the presence of IL-2 or IL-15 (8). Brenchley et al. (8) demonstrated that CD57 but not CCR7, CD28, or CD27 expression on CD8⁺ T cells correlated with diminished HIV-1-specific proliferation. Our results suggest that expression of CD57 on CD4⁺ T cells is a better marker of proliferation incompetence than CCR7 expression. Although studies have shown that the proliferative capacity of CCR7⁻ T cells is

less than that of CCR7⁺ T cells, it is clear that some fraction of CCR7⁻ cells are still capable of dividing. For example, a recent study (30) demonstrated that CMV-specific CCR7⁻ T cells stimulated with their cognate peptide in concert with either CD4 help or IL-2 and IL-15 induced massive clonal expansion of these cells. Because CD57 is mainly expressed on CCR7⁻ cells, our data suggest that the increased proportion of CD4⁺ T cells expressing CD57 may account for the reduced proliferation observed in HIV-1-specific IFN- γ -producing CCR7⁻ CD4⁺ T cells.

The percentage of peripheral blood CD4⁺ T cells expressing CD57 was significantly increased in HIV-1-infected subjects in this study. Increased frequencies of CD57⁺ T cells have been associated with a number of pathological conditions where Ag persists, such as *Mycobacterium tuberculosis*, rheumatoid arthritis, malaria, and HIV-1 (11–15). To determine whether there was an association between CD57 expression on CD4⁺ T cells and a decrease in HIV-1-specific proliferative responses, we studied two well-defined cohorts of HIV-1-infected subjects: untreated subjects with ongoing HIV-1 replication and ART-treated subjects with suppressed viral loads. These cohorts were chosen because it is well documented that HIV-1-specific proliferative responses are low to absent in untreated subjects with progressive disease, whereas improvement in proliferation is seen in a significant fraction of successfully ART-treated subjects who have detectable HIV-1-specific cytokine responses (6, 19, 20, 31). In this study, when HIV-1-specific CD4⁺ T cell responses were dissected into discrete cytokine-producing populations, we found that HIV-1-specific IFN- γ -producing T cells contained the highest percentage of CD57-expressing cells. This is in accordance with the finding that subjects whose HIV-1-specific response is skewed toward increased frequencies of CD4⁺ IFN- γ -producing T cells generally have the weakest proliferative responses (6). Interestingly, when CMV and SEB responses were analyzed, the percentage of IFN- γ -producing T cells expressing CD57 was similar to the fraction of HIV-1-specific, IFN- γ -producing CD4⁺ T cells that expressed CD57. Conversely, CD4⁺ T cells that produced IL-2 generally did not express CD57, again correlating with the known proliferative competence of these cells. These findings suggest that CD4⁺ T cells, which produce only IFN- γ , regardless of specificity, have increased CD57 expression, and that CD57 up-regulation may be a physiological response to persistent Ag exposure.

Chronic Ag exposure has been shown to drive T cells to a more differentiated effector memory state (7, 32). The expression of CD57 on CD8⁺ T cells has been associated with replicative senescence, shortened telomere lengths, and chronic activation due to persistent Ag exposure (7–9). Our data clearly show that CD57 is expressed to a greater degree on more differentiated CCR7⁻ effector CD4⁺ T cells than on CCR7⁺ naive and Tcm cells in both seronegative and HIV-1-infected subjects. Telomere lengths of CCR7⁻CD45RA⁺ T cells are the shortest of the memory subsets, suggesting that they are the most differentiated (33), and increased frequencies of CCR7⁻, CD45RA⁺ virus-specific CD4⁺, and CD8⁺ T cells have been correlated with control of viral replication (33, 34). In this study, we observed increased CD57 expression on CCR7⁻CD45RA⁺CD4⁺ T cells (Temtd) from untreated subjects with progressive disease (compared with seronegative subjects) and found a positive correlation between the percentage of peripheral blood CD57⁺CD4⁺ Temtd and HIV-1 viral load ($r = 0.53$; $p = 0.02$; data not shown). These findings demonstrate that there are distinct phenotypic and functional changes within the effector memory CD4⁺ T cell population in subjects with untreated HIV-1 infection that may play a role in the loss of virological control. Support for this comes from the finding that hepatitis B- and C-specific CD8⁺ T cells share the same effector-memory phenotype

(CCR7⁻, CD45RA⁻) but have striking functional differences and are associated with different clinical outcomes (35). HBV-specific CD8⁺ T cells have high perforin content and expand vigorously, whereas HCV-specific CD8⁺ T cells do not. Interestingly, increased frequencies of CCR7⁻, CD45RA⁺ virus-specific CD4⁺, and CD8⁺ T cells have been correlated with control of viral replication (33, 34). These findings suggest that this important effector T cell population may be functionally impaired in untreated HIV-1-infected subjects and may play a role in the loss of virological control.

The finding that CD57 is mainly expressed on terminally differentiated memory CD4⁺ cells suggests CD57 expression may be used to identify “exhausted” cells. The lack of proliferation also suggests that these cells may have reached the point of replicative senescence. Very low TCR rearrangement excision circle levels and shortened telomere lengths in CD57⁺CD8⁺ T cells demonstrate that these cells have undergone extended rounds of replication. CD57⁺CD8⁺ T cells are also highly susceptible to activation-induced cell death (8). In concordance with the CD8⁺ T cell data, we found that CD57⁺CD4⁺ T cells display a higher rate of spontaneous apoptosis and activation-induced apoptosis than CD4⁺ T cells not expressing CD57. The finding that CD57⁺CD4⁺ T cells cannot proliferate, exhibit increased rates of apoptosis, and are found mainly in the Temtd memory subset indicate that, like CD57⁺CD8⁺ T cells, these cells have reached the end of their life span. The fact that increased percentages of CD57⁺CD4⁺ T cells were seen in untreated HIV-1-infected subjects is also in accord with the finding that there is significantly higher CD4⁺ T cell turnover in HIV-1-infected subjects (36, 37).

The impact that increased percentages of CD57⁺CD4⁺ T cells have on HIV-1 pathogenesis is unclear. Although CD4⁺ T cells that express CD57 are unable to proliferate or produce significant amounts of IL-2, they do produce IFN- γ and may be capable of cytolytic activity based on perforin production (7). It has been suggested that cytolytic CD57⁺CD4⁺ T cells are byproducts of inflammation and elevated activation, exhibiting increasing characteristics of replicative senescence and demonstrating cytotoxic potential for reasons still unclear (38). The question remains as to which type of HIV-1-specific CD4⁺ T cell response is most important for the control of chronic viral infection; one that provides help to virus-specific CD8⁺ T cells in the form of IL-2 and has the ability to self-renew, or one that directly kills virus-infected cells. The strongest evidence to date shows that HIV-1-specific CD4⁺ T cells that produce both IL-2 and IFN- γ correlate with virological control, whereas CD4⁺ T cells that produce only IFN- γ do not (4, 6, 39). Recently, it was shown that HIV-1-specific CD8⁺ T cell proliferation in chronically infected subjects was restored in vitro by the addition of autologous IL-2-producing HIV-specific CD4⁺ T cells (40). Our findings demonstrate a strong correlation between the percentage of CD57⁺ HIV-1-specific CD4⁺ and CD8⁺ T cells in the peripheral blood. One possible explanation for this association is that increased senescence in the CD8 compartment may result from an increased proportion of virus-specific CD57⁺CD4⁺ T cells that produce only IFN- γ and not IL-2. These findings suggest that HIV-1-specific CD4⁺CD57⁺ T cells that produce only IFN- γ may not provide adequate help to CD8⁺ T cells needed to mount a more effective CTL response.

Taken together, these data demonstrate that CD57 expression can be used to identify proliferation-incompetent CD4⁺ T cells, which these cells tend to accumulate in subjects with untreated HIV-1 disease, and that they persist after treatment. Elevated percentages of CD57⁺ T cells are found in the CCR7⁻ effector populations of HIV-1-infected subjects, and expression is increased on IFN- γ -producing cells that have lost the ability to produce IL-2, suggesting that these cells may arise from chronic Ag exposure. HIV-1 and CMV-specific

IFN- γ -producing CD4⁺ T cells express similar levels of CD57, indicating that up-regulation of this marker may be physiological and used to identify exhausted cells. CD57⁺ T cells may contribute to HIV-1 disease pathogenesis, and the examination of this population may be useful for determining the functional competence of the CD4⁺ T cell compartment during HIV-1 disease.

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Disclosures

The authors have no financial conflict of interest.

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