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# Changes in the Composition of Circulating CD8+ T Cell Subsets during Acute Epstein-Barr and Human Immunodeficiency Virus Infections in Humans

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In response to viral infection, unprimed naive CD8+, major histocompatibility complex class I–restricted, virus-specific T cells clonally expand and differentiate into memory- and effector-type cells. Changes in CD8+ subset distribution were studied in 17 subjects with acute human immunodeficiency virus type 1 infection and in 14 subjects with acute Epstein-Barr virus (EBV) infection, with combined CD45RO, CD27, and CD28 monoclonal antibodies. A vast expansion of memory-type CD45RO+CD27+CD8+ T cells, with high expression of the cell-cycle marker Ki-67, was observed in both infections. Strikingly, CD45RO+CD27+CD28− cells increased >10-fold in acute viral infection and had high Ki-67 expression. In acute EBV infection, a substantial portion of the expanded T cells were EBV-peptide specific. These cells resided mainly in the CD45RO+CD27+ subpopulation, with most in the CD27+CD28− subpopulation. Content of perforin expression, as a measure of cytotoxic capacity, was relatively low in the CD27+CD28+ T cells and highest in the CD27−CD28− subpopulation.

Within the CD8+ T cell population, unprimed or naive cells that have not yet encountered antigen and primed cells that have encountered antigen can be distinguished. Antigenic challenge induces the formation of effector cells, which exert specific functions, such as cytolysis of virus-infected cells and memory cells, which, on renewed contact with the same pathogen, rapidly induce the generation of novel effector cells and thus provide the basis for immunity [1]. In vitro, cytotoxic T lymphocyte (CTL) effector cells express perforin and can lyse specific targets, without prior activation [2]. Without further activation, memory-type cells do not display cytolytic potential [2–6].

Previously, the frequencies of the antigen-specific T cells were generally estimated by using limiting-dilution assays. Recently, novel methods of estimating frequencies of antigen-specific T cells have been described. These include assays to detect interferon (IFN)–γ release from individual CD8+ T cells after specific stimulation [7] and the use of tetrameric major histocompatibility complex (MHC)–peptide complexes to directly stain T cells of the appropriate specificity [8–10].

CD8+ T cells can be subdivided into naive, effector, and memory cells with a combination of CD45RA and CD27 monoclonal antibodies (MAbs) [11]. On the basis of functional and pheno-

typic characteristics, it was concluded that CD45RA+CD27+ cells represent naive cells [11]. Effector-type cells have a CD45RA+CD27− phenotype and a strong cytolytic activity, without prior in vitro stimulation. As expected, according to their function, CD45RA+CD27− T cells have high perforin and granzyme expression, are the only CD8+ T cells that contain Fas-ligand mRNA, and abundantly produce IFN-γ and tumor necrosis factor (TNF)–α. Memory-type cells can be characterized as CD45RA−CD27+, and they express CD45RO.

It is expected that, during a response to an acute viral infection, the composition of circulating CD8+ subsets will change. Indeed, in acute viral infection, transient increases in the number of CD8+ T cells (mainly activated CD45RO+ T cells) have been reported [12–17]. To investigate changes induced by acute viral infection on subset composition within the CD8+ compartment, we studied subjects with acute human immunodeficiency virus (HIV) type 1 infection or acute Epstein-Barr virus (EBV) infection. HIV-1–infected subjects were studied within 3 weeks after seroconversion, and EBV-infected subjects were studied within 2 weeks after the onset of illness. To dissect the CD8+ subpopulations, we used a combination of CD45RO and CD27, together with the intracellular analysis of perforin [18–20], and the cell-cycle marker Ki-67 [21, 22]. On activation in vivo, T cells lose CD28 [23–26]; thus, this marker was included in the analysis. Furthermore, during acute EBV infection, antigen-specific CD8+ T cell subpopulations were identified by use of tetrameric MHC-peptide complexes.

Our study allows the proposal of a model for differentiation of CD8+ T cells during viral infections. Moreover, we identified the CD8+CD45RO+CD27+CD28− phenotype that may characterize T cells in transition from the proliferative

CD8$^+$CD45RO$^+$CD27$^+$ pool to the nondividing effector population.

## Materials and Methods

*Study population.* Phenotypic characteristics of CD8$^+$ subsets were analyzed in patients with acute HIV or EBV infections. Blood samples were obtained from 17 patients within 3 weeks after seroconversion to HIV-1 [15]. Fourteen EBV-infected patients were selected on the basis of consultation with their general practitioners, whose diagnoses of clinical disease were confirmed by a positive reaction in the Paul-Bunnell test and marked lymphocytosis that consisted of >10% atypical lymphocytes. Blood samples were obtained from EBV patients within 14 days after the onset of illness. Healthy individuals ($n = 22$) served as controls. Assays were done on fresh whole blood or on peripheral blood mononuclear cells (PBMC). The characteristics of the participants are presented in table 1.

*MAbs.* CD8$^+$ T cells were subdivided into naive (CD45RO$^-$CD27$^+$), memory (CD45RO$^+$CD27$^+$ and CD45RO$^+$CD27$^-$), and effector (CD45RO$^-$CD27$^-$) cells. In contrast to another study that used the CD45RA/CD27 combination [11], we used CD45RO combined with CD27. This combination resulted in subset distributions comparable to those in the CD45RA/CD27 combination. We also measured the combined expression of CD27 and CD28. A paucity of material sometimes precluded the simultaneous measurement of all markers. Fluorescein isothiocyanate (FITC)–conjugated CD28 MAb, CD27 MAb, mouse IgG1 control MAb, phycoerythrin (PE)–conjugated mouse IgG2a and IgG2b control MAbs, and biotinylated CD27 MAb were produced at CLB (Amsterdam). PE-conjugated CD28 MAb and peridinin chlorophyll protein (PerCP)–conjugated CD8 MAb were purchased from Becton Dickinson (San Jose, CA). PE- and FITC-labeled CD45RO MAbs were obtained from DAKO (Glostrup, Denmark). FITC-labeled Ki-67 MAb was purchased from Immunotech (Marseilles, France), FITC-conjugated perforin MAb was purchased from Hölzel Diagnostika (Köln, Germany), and allophycocyanin (APC)–labeled streptavidin was from Caltag (South San Francisco, CA).

*Intracellular staining for Ki-67 and perforin.* The nuclear antigen Ki-67, which is expressed in all phases of the cell cycle, is located in the outer parts of the nucleolus and can be used to determine the percentage of proliferating cells [21, 22]. For Ki-67 expression, blood (200 $\mu$L) was surface stained with CD8-PerCP and a combination of CD45RO-PE and biotinylated CD27 or a combination of CD28-PE and biotinylated CD27. Intracellular perforin expression was determined in the CD27 and CD28 subsets. Mouse IgG1–FITC control MAb was used as negative control. After cell-surface staining, we washed cells twice with PBS/0.15% bovine serum albumin (BSA) and incubated them with streptavidin-APC.

Red blood cells were lysed, and lymphocytes were fixed with fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson, San Jose), permeabilized with permeabilization solution (Becton Dickinson, San Jose), and washed with PBS/0.15% BSA. Subsequent staining with Ki-67–FITC or perforin-FITC MAb was done. Cells were washed twice with PBS/0.15% BSA and were resuspended in 150 $\mu$L of fixation buffer (CellFIX; Becton Dickinson, San Jose), after which data acquisition and analyses were done by 4-color flow cytometry (FACSCalibur; Becton Dickinson, Mountain View, CA) with Cellquest software (Becton Dickinson, San Jose). Twenty thousand CD8$^+$ cells were analyzed. During all steps, samples were kept at room temperature; incubation steps were done for 30 min, and fixation and permeabilization were done for 10 min.

Cryopreserved material was available for 9 patients. The staining procedure for PBMC ($1 \times 10^6$ cells) was identical to that described above for whole blood, with the exception that incubation steps were done at 4°C. In a set of preliminary experiments, we showed that cryopreservation did not affect the percentage of Ki-67$^+$ cells (data not shown).

*Cytotoxicity assay.* CTL activity was determined in a CD3 MAb–mediated redirected cytotoxicity assay as described elsewhere [27]. For subset purification, CD8$^+$ T cells were prepared by positive enrichment, using a magnetic activated cell sorting system (Miltenyi Biotec, Bergisch-Gladbach, Germany), as reported elsewhere [11]. Next, cells were stained with PE-conjugated CD28 and FITC-conjugated CD27 and sorted into CD28$^+$CD27$^+$, CD27$^+$CD28$^-$, and CD27$^-$CD28$^-$ populations on a FACStar (Becton Dickinson, Mountain View, CA).

*MHC-peptide tetrameric complexes.* HLA-B8 tetramers complexed with the EBV peptide RAKFKQLL, derived from the lytic protein BZLF1 [28], were provided by A. J. McMichael and M. F. C. Callan (John Radcliffe Hospital, Oxford, UK). We used 4-color staining to measure antigen-specific frequencies. PBMC ($1 \times 10^6$) were stained with APC-conjugated tetrameric complexes in combination with either CD8-PerCP, CD45RO-PE, and CD27-FITC or CD8-PerCP, CD28-PE, and CD27-FITC (at 4°C). After being incubated with FACS lysing solution (at room temperature), cells were washed twice with cold PBS/0.15% BSA and were resuspended in 100 $\mu$L of fixation buffer (Becton Dickinson, San Jose). Time of incubation, data acquisition, and analysis were identical to those described above for Ki-67 analysis.

*Statistical analysis.* Values of the subsets were analyzed between the different study populations, by the Wilcoxon rank sum test.

## Results

*Subsets of CD8$^+$ T cells in acute viral infection.* As expected, the patients with acute HIV-1 and EBV infections had elevated CD8$^+$ T cell numbers, which resulted in inverted CD4/CD8

**Table 1.** Characteristics of study patients with acute human immunodeficiency virus (HIV) or Epstein-Barr virus (EBV) infection.

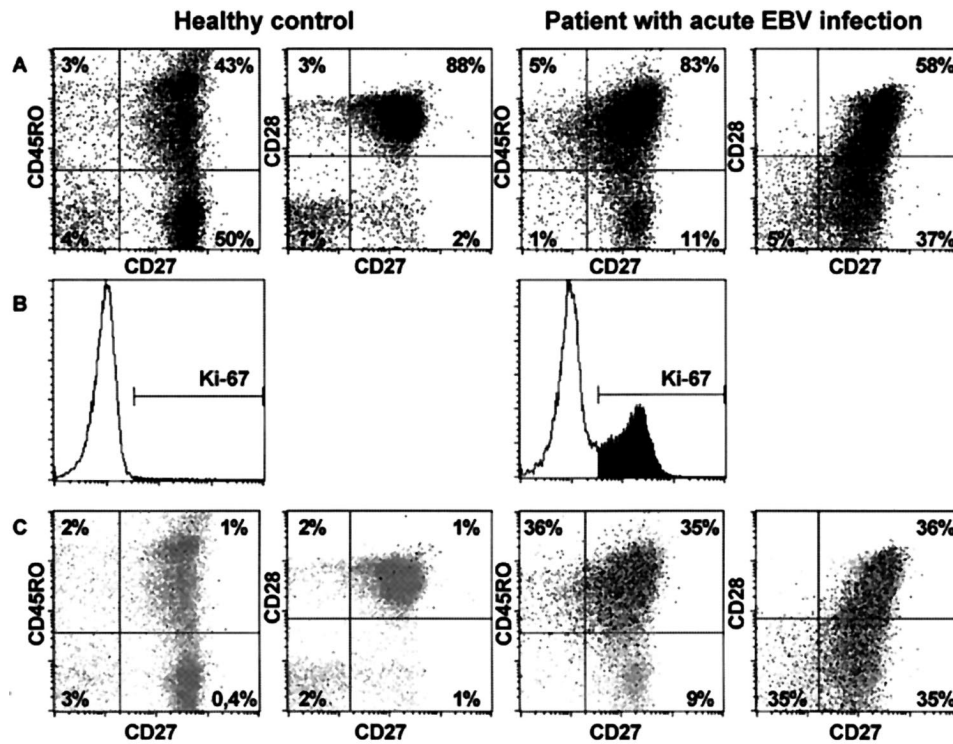| Study group | No. tested | Age, years | CD4$^+$ $\times10^9$/L | CD8$^+$ $\times10^9$/L | CD4/CD8 ratio |
|---|---|---|---|---|---|
| Controls | 22 | 31 (21–60) | 0.87 (0.54–1.72) | 0.52 (0.27–1.01) | 1.76 (0.93–4.33) |
| HIV-1 infected | 17 | 34 (24–56) | 0.45 (0.22–1.64) | 1.56 (0.60–2.94) | 0.36 (0.07–1.15) |
| EBV infected | 14 | 24 (16–57) | 1.08 (0.42–2.37) | 4.80 (1.20–14.6) | 0.20 (0.13–0.41) |

NOTE. Data are median (range).

**Figure 1.** CD8$^+$ cell subset distribution and Ki-67 expression in lymphocytes of a healthy control and a subject with acute Epstein-Barr virus (EBV) infection. *A,* Distribution and percentages of the CD45RO/CD27 and CD27/CD28 subpopulations among the total CD8$^+$ T cell population. *B,* Staining for the intracellular antigen Ki-67 in CD8$^+$ T cells. Values were 1% in the healthy control and 34% in the patient with acute EBV infection. *C,* Distribution of Ki-67$^+$ cells *(in black)* in the total cell population *(in gray)*. Percentages of Ki-67$^+$ cells in the subsets are presented.

ratios (table 1). The increase in CD8$^+$ T cells was caused mainly by an expansion of CD45RO$^+$CD27$^+$ cells and, to a lesser extent, by expansion of CD45RO$^+$CD27$^-$ cells (table 2, figure 1). The CD45RO$^-$ subset did not increase substantially, although the increase in CD45RO$^-$CD27$^+$ cells just reached significance in acute EBV infection. The Ki-67 antigen was expressed mainly in the CD45RO$^+$ subsets. Within the CD45RO$^-$ subsets, the naive cells showed a variable but overall low expression of Ki-67, whereas in CD45RO$^-$CD27$^-$ effector-type cells the Ki-67 antigen was hardly demonstrable, except in 1 patient with EBV infection.

Several reports have documented expansion of the CD8$^+$ CD45RO$^+$CD28$^-$ T cells during viral infections. These cells are present in low numbers in healthy individuals. To investigate the relationship between this population, which has several effector cell–type properties, and the above-defined subsets, the expression of CD28, in relation to CD27, on CD8$^+$ T cells was also analyzed. In healthy controls, expression of CD27 and CD28 appears to be closely linked, resulting in very low CD27$^+$ CD28$^-$ and CD27$^-$CD28$^+$ cell numbers (table 3, figure 1). The acute stage of viral infection was characterized by a considerable expansion of CD27$^+$CD28$^+$ cells, but most striking was the augmentation of the CD27$^+$CD28$^-$ subset. In HIV-1 infection, there was a moderate increase in the number of CD27$^-$CD28$^-$ cells, whereas in EBV infection this fraction was

more markedly increased. All fractions displayed an increase in Ki-67 expression.

*Perforin expression in CD27 and CD28 subsets in acute viral infection.* CTLs contain granules in the cytoplasm that hold several proteolytic enzymes, including perforin and granzymes A and B, which play an important role in the cytotoxic activity of CTLs. As reported elsewhere [11], CD8$^+$CD45RO$^-$CD27$^-$ cells are unique in that they, without additional stimulation in vitro, contain high levels of both proteolytic enzymes. In healthy controls, perforin expression was virtually absent in the CD27$^+$CD28$^+$ subset, intermediately present in the CD27$^+$ CD28$^-$ subset, and primarily present in the CD27$^-$CD28$^-$ fraction (table 4). In the acute stage of HIV-1 infection, although the number of cells in the different subsets clearly increased, the proportion of perforin-positive cells in these subpopulations still was comparable to that in healthy controls. In acute EBV infection, the proportion of perforin-positive cells in the subsets that are CD27$^+$ seemed to be higher than the proportion found in acute HIV infection. However, in each group, only 4 patients were tested and there was a considerable overlap, so the differences failed to reach statistical significance.

*Cytotoxic capacity in acute EBV infection.* Because the proportion of perforin-positive cells in the different subsets during acute EBV infection were increased, we addressed the question of whether these cells behave as CTLs ex vivo. Cytotoxic capacity

**Table 2.** Percentages and absolute numbers of CD8$^+$ subsets defined by CD45RO and CD27 cells and percentage of Ki-67 expression in each subset in healthy controls and patients with acute human immunodeficiency virus (HIV)–1 or Epstein-Barr virus (EBV) infection.

| | Study group | | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| | Controls[a] | | HIV-1[b] | | EBV[c] | |
| CD8$^+$ cell subset | Percentage | Absolute no. $\times 10^9$/L | Percentage | Absolute no. $\times 10^9$/L | Percentage | Absolute no. $\times 10^9$/L |
| CD45RO$^-$CD27$^+$ (naive cells) | 52 (14–74) | 0.23 (0.05–0.55) | 21 (3–35) | 0.30 (0.05–0.59) | 10 (3–24) | 0.44 (0.12–2.63)[d] |
| Ki-67 | 1 (0–14) | | 5 (1–26) | | 16 (0–42) | |
| CD45RO$^+$CD27$^+$ (memory cells) | 33 (5–59) | 0.13 (0.03–0.39) | 61 (26–79) | 0.98 (0.23–2.06)[e] | 63 (25–90) | 3.32 (1.62–9.2)[f] |
| Ki-67 | 2 (0–6) | | 32 (16–69) | | 40 (7–57) | |
| CD45RO$^+$CD27$^-$ (memory/effector cells) | 3 (1–34) | 0.02 (0.01–0.11) | 9 (0–20) | 0.09 (0–0.46)[f] | 14 (2–68) | 1.32 (0.05–6.09)[f] |
| Ki-67 | NA | | 25 (11–59) | | 39 (18–58) | |
| CD45RO$^-$CD27$^-$ (effector cells) | 11.5 (3–48) | 0.04 (0.01–0.20) | 8 (2–28) | 0.10 (0.03–0.38) | 2 (1–10) | 0.10 (0.03–1.46) |
| Ki-67 | 1 (0–6) | | 2 (0–14) | | 6 (2–56) | |

NOTE. Data are median (range). NA, not available because of a very low no. of events.

[a] 20 Subjects tested for absolute no. of subsets/20 tested for Ki-67 expression in each subset.
[b] 17 Subjects tested for absolute no. of subsets/10 tested for Ki-67 expression in each subset.
[c] 13 Subjects tested for absolute no. of subsets/7 tested for Ki-67 expression in each subset.
[d] Comparison of acute stage vs. controls, $P < .05$, by Wilcoxon rank sum test.
[e] Comparison of acute stage vs. controls, $P < .001$, by Wilcoxon rank sum test.
[f] Comparison of acute stage vs. controls, $P < .003$, by Wilcoxon rank sum test.

in freshly purified CD8$^+$ subsets is shown in a CD3 MAb–mediated redirected cytotoxicity assay. As expected, in a healthy control, cytolytic activity was found predominantly in the CD27$^-$CD28$^-$ subset (figure 2A). In agreement with the presence of perforin in the subsets of the patient with acute EBV infection, cytotoxic capacity already could be found in the CD27$^+$CD28$^+$ subpopulation at about a factor 2 level lower than that in the CD27$^+$CD28$^-$ and the CD27$^-$CD28$^-$ fractions (figure 2B).

*Enumeration of T cells specific for EBV by using MHC-peptide tetrameric complexes.* We next analyzed the frequency of T cells specific for the EBV peptide RAKFKQLL, which was derived from the lytic protein BZLF1, using HLA-B8/peptide tetrameric complexes. In healthy-HLA-B8–positive-EBV-seronegative controls, T cells specific for this peptide are virtually absent [28]. Frequencies of antigen-specific T cells were measured for 3 HLA-B8–positive patients with acute EBV infection. Figure 3B shows that almost all tetramer-positive cells reside in the CD45RO$^+$ population, with equal proportions in the CD27$^+$ and CD27$^-$ subsets. Figure 3C shows again that CD27$^-$CD28$^+$ subpopulations are nearly absent, whereas the numbers of CD27$^-$CD28$^-$ cells are very low. From figure 3D, it is clear that the highest proportion of antigen-specific cells can be found in the CD27$^+$CD28$^-$ subpopulation.

It has been suggested that the expansion of CD8$^+$ T cells in various acute viral infections may be wholly accounted for by antigen-specific T cells, and a possible role for bystander cells may have to be rethought [10]. Assuming that the expansion of the CD27$^+$CD28$^-$ subpopulation was due only to antigen-specific cells, then in patient 1 the proportion of antigen-reactive cells in this subpopulation that were recognized by this tetrameric staining was 35% of all EBV antigen–specific cells (figure 3D). By extrapolation, this may imply that only 35% of this patient's EBV-specific cells are recognized by this tetramer (i.e., that the real proportion of EBV-specific cells is a factor 100/

35 greater than that determined by tetrameric staining). In patient 1, the tetramer-positive cells amounted to 31% of CD8$^+$ cells. This means that the real proportion of EBV-positive cells in this patient was $100/35 \times 31\% = 88.6\%$ of CD8$^+$ cells.

From that calculation, it can be determined that 11.4% of the cells are not involved in the immune response against EBV (i.e., $0.114 \times 14.6 \times 10^9$/L = $1.66 \times 10^9$ cells/L. The same calculations for patients 2 and 3 revealed that $1.29 \times 10^9$/L and $0.56 \times 10^9$/L, respectively, of the CD8$^+$ T cells were not involved in the EBV-specific T cell response. CD8$^+$ T cell numbers not involved in EBV are close to the normal range of the number of CD8$^+$ T cells in healthy individuals, which suggests that the expansion is EBV specific and leaves a constant pool of CD8$^+$ T cells uncommitted.

## Discussion

In this study, we documented changes in circulating CD8$^+$ subsets induced by acute HIV-1 or EBV infection. The most striking findings are that (1) CD8$^+$ lymphocytosis, a well-known and common finding in these acute viral infections, is caused by a dramatic expansion of memory-type CD45RO$^+$CD27$^+$ T cells; (2) in virus-infected individuals, the CD27$^+$CD28$^-$ population within the CD8$^+$ memory-type subset expands considerably; (3) the high percentage of Ki-67$^+$ cells in both memory CD27$^+$CD28$^+$ and CD27$^+$CD28$^-$ subpopulations demonstrates that, compared with cells in healthy controls, a great deal of these cells are cycling; (4) in the acute stage, many CD8$^+$ T cells contain perforin and have a high cytolytic capacity in a redirected assay; and (5) during the acute stage, large numbers of antigen-specific T cells, detected by MHC-peptide tetrameric complexes, reside in the memory-type fraction (CD45RO$^+$ CD27$^+$); however, the percentage of tetrameric-positive cells is ~2 times higher in the CD27$^+$CD28$^-$ fraction than in the CD27$^+$ CD28$^+$ fraction.
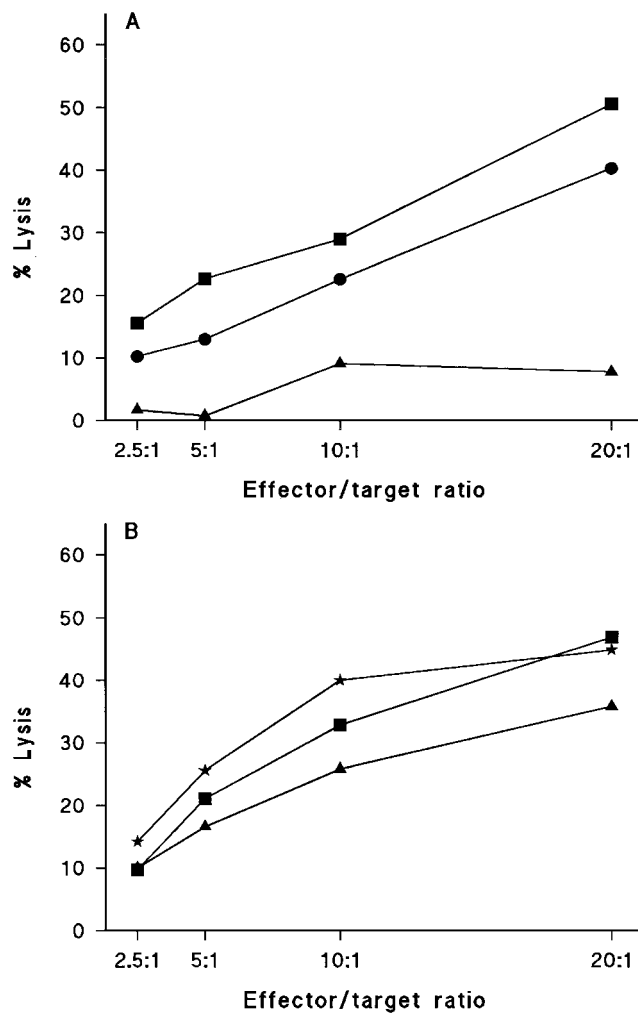
**Figure 2.** Cytotoxic capacity of CD8$^+$ T cell subsets in a CD3 monoclonal antibody–mediated, redirected cytotoxicity assay. Data are for a healthy control *(A)* and a patient with acute Epstein-Barr virus (EBV) infection *(B)*. Fractions tested in the control were CD8$^+$ (●), CD8$^+$CD27$^+$CD28$^+$ (▲), and CD8$^+$CD27$^-$CD28$^-$ (■) cells. Perforin values for these subsets were 21%, 7%, and 55%, respectively. In the patient with acute EBV infection, fractions tested were CD8$^+$CD27$^+$CD28$^+$ (▲), CD8$^+$CD27$^+$CD28$^-$ (★), and CD8$^+$CD27$^-$CD28$^-$ (■) cells. Perforin values for these subsets were 25%, 27%, and 53%, respectively.

Several reports that used only the CD45RA/RO definition of naive and memory cells have documented that the increase in CD8$^+$ cells during the acute stage of EBV and HIV-1 infection occurs mainly in the memory population [16, 17]; however, the validity of CD45RO/RA markers alone for the distinction between naive and memory cells has been questioned [11, 29, 30]. The use of a combination of CD45RA and CD62L has been proposed for a more refined distinction of naive, memory, and effector cells [29]. Mainly for practical reasons, we advocated the combined use of CD45RA and CD27 cells [11]. The

ligand for CD27, a member of the TNF-R family, is CD70. After binding its ligand, CD27 provides a costimulatory signal for T cell proliferation and cytokine production [31]. Next, CD27 is down-regulated, and the complete and irreversible loss of this molecule may mark terminal T cell differentiation [32]. It can be argued that down-regulation of costimulatory molecules could be instrumental in determining the balance between the proliferating memory pool and the nonproliferating effector pool [33].

In another study, we evaluated the correlation between both measurements of naive and memory CD8$^+$ T cells in 122 samples from healthy controls and HIV-1–infected individuals. CD8$^+$ T cell subsets co-expressing CD45RA and either CD62L or CD27 were regarded as truly naive cells, whereas remaining CD8$^+$ T cells, including cells expressing CD45RO and cells expressing CD45RA without CD62L or CD27, were regarded as memory lymphocytes. A good correlation existed between both methods for the enumeration of naive CD8$^+$ T cells (ratio Pearsson [R$p$], 0.96; $P < .005$) and memory CD8$^+$ T cells, (R$p$, 0.98; $P < .005$), and the differences did not systematically vary over the range of measurements [34]. By using these definitions, the expansion of CD8$^+$ T cells in acutely virally infected patients occurs mainly in the CD45RO$^+$CD27$^+$ memory-type cells.

In acutely HIV-1–infected persons, the emergence of CD8$^+$CD28$^-$ T cells has been demonstrated [23–26]. Experiments in vitro showed that CD8$^+$CD28$^-$ cells are derived from CD8$^+$CD28$^+$ precursors [35]. We analyzed the combined expression of CD27 and CD28. At birth, virtually all peripheral CD8$^+$ T cells express both CD27 and CD28 [11, 36–38]. We found that healthy human adults accumulate CD27$^-$CD28$^-$ T cells, whereas the other 2 subsets remain very low. Therefore, it is remarkable that, during acute viral infection, the CD27$^+$

**Table 3.** Absolute numbers of CD8$^+$ subsets defined by CD27 and CD28 monoclonal antibodies, and percentage of Ki-67 expression in healthy controls and in patients with acute human immunodeficiency virus (HIV)–1 or Epstein-Barr virus (EBV) infection.

| | Study group | | |
|---|---|---|---|
| Subset | Controls[a] | HIV-1[b] | EBV[c] |
| CD27$^+$CD28$^+$ | 0.35 (0.11–0.90) | 0.89 (0.26–1.53)[d] | 2.70 (0.66–8.76)[e] |
| Ki-67 | 1 (0–22) | 29 (14–48) | 35 (6–51) |
| CD27$^+$CD28$^-$ | 0.045 (0.02–0.08) | 0.45 (0.15–1.09)[f] | 1.03 (0.42–3.16)[e] |
| Ki-67 | NA | 20 (6–62) | 26 (3–62) |
| CD27$^-$CD28$^+$ | 0.01 (0–0.05) | 0.02 (0–0.11) | 0.29 (0–1.34)[g] |
| Ki-67 | NA | NA | NA |
| CD27$^-$CD28$^-$ | 0.06 (0.02–0.28) | 0.19 (0.09–0.68)[d] | 0.60 (0.10–4.12)[e] |
| Ki-67 | 2 (0–7) | 11 (3–64) | 23 (1–60) |

NOTE. Data for subsets are median (range) $\times 10^9$/L; data for Ki-67 are median (range) percentage of expression in each subset. NA, not available because of a very low number of events.

[a] 22 Subjects tested for absolute no. of subsets/19 tested for Ki-67 expression.
[b] 17 Subjects tested for absolute no. of subsets/11 tested for Ki-67 expression.
[c] 14 Subjects tested for absolute no. of subsets/8 tested for Ki-67 expression.
[d] Comparison of acute stage vs. controls, $P < .005$, Wilcoxon rank sum test.
[e] Comparison of acute stage vs. controls, $P < .003$, Wilcoxon rank sum test.
[f] Comparison of acute stage vs. controls, $P < .001$, Wilcoxon rank sum test.
[g] Comparison of acute stage vs. controls, $P < .02$, Wilcoxon rank sum test.
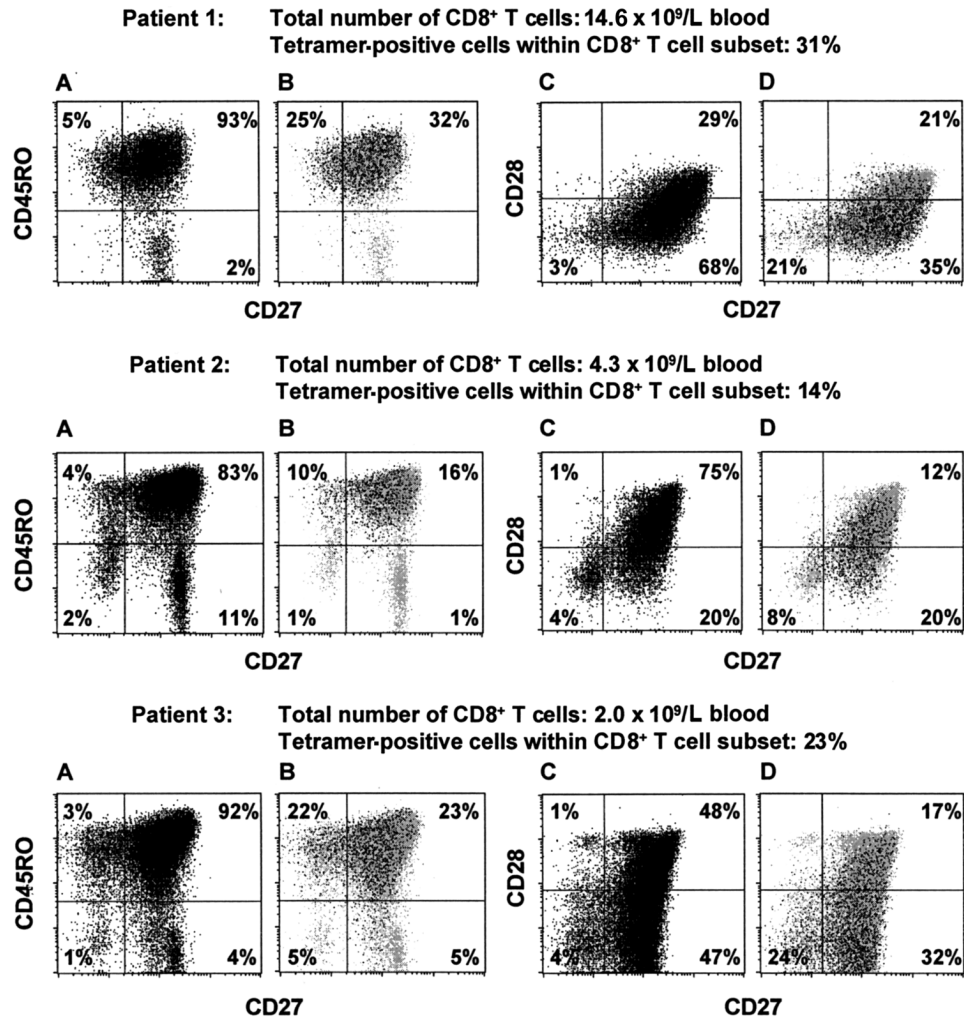
**Figure 3.** HLA-B8/RAKFKQLL–specific cells in 3 patients with acute Epstein-Barr virus (EBV) infection. Distribution and percentage of CD45RO/CD27 *(A)* and CD27/CD28 *(C)* subpopulations are presented among the total CD8+ T cell population. Distribution and percentage of tetramer-positive cells are shown *(in black)* among the CD45RO/CD27 *(B)* and CD27/CD28 *(D)* cell populations *(in grey)*.

CD28⁻ subpopulation emerges. In 3 patients with acute EBV infection, this cell population contains the highest percentage of antigen-specific cells, as determined by tetrameric staining.

It has been proposed that antigen-specific T cells could be wholly accountable for the expansion of CD8+ T cells in various acute viral infections [10, 39, 40]. For this reason, it can be argued that virtually all cells in the CD27+CD28⁻ fraction are antigen specific and that the CD27+CD28+ population contains both antigen-specific cells and cells not involved in the ongoing immune response. On the basis of these assumptions, the number of cells not involved in EBV responses could be calculated and appeared to be within the range of CD8+ T cells found in normal donors. This would mean that the expanded CD8+ T cell population is EBV specific and has little bystander components and that there remains a constant pool of uncommitted CD8+ T cells.

It is not possible to label cells in patients and follow them over time. Nevertheless, we believe that it is defensible to integrate the observations that we made during acute viral infection into a model for CD8+ T cell differentiation in vivo [33]. Viral antigens will induce activation of naive (CD45RO⁻ CD27+CD28+) T cells that convert into the CD45RO+CD27+ CD28+ phenotype [11]. Clonal expansion occurs within this subset, as evidenced by considerable expression of Ki-67. Differentiation toward the effector cell type is accompanied by the successive loss of CD28 and CD27 expression. Cells within this differentiation pathway gain perforin expression but gradually lose the capacity to proliferate as Ki-67 expression successively decreases (table 2). The absence of CD28 and, eventually, other costimulatory molecules probably renders these cells less able to undergo extensive clonal expansion and correlates well with the observation that the mitogenic potential of normal healthy control CD28⁻ T cells in vitro is low [11, 25, 26, 41]. Since the

**Table 4.** Absolute numbers of CD8⁺ subsets defined by CD27 and CD28 monoclonal antibodies and percentage perforin in healthy controls and in patients with acute human immunodeficiency virus (HIV)–1 or Epstein-Barr virus (EBV) infection.

| Subset | Controls[a] | HIV-1[b] | EBV[c] |
|---|---|---|---|
| | Study group | | |
| CD27⁺CD28⁺ | 0.36 (0.18–0.90) | 0.90 (0.26–1.22) | 1.20 (0.66–3.15) |
| Perforin | 1 (0–22) | 7 (0–53) | 60 (25–78) |
| CD27⁺CD28⁻ | 0.04 (0.02–0.07) | 0.34 (0.15–0.81) | 0.74 (0.42–1.23) |
| Perforin | 20 (1–33) | 26 (7–95) | 87 (27–96) |
| CD27⁻CD28⁻ | 0.06 (0.02–0.28) | 0.19 (0.13–0.63) | 0.14 (0.10–0.31) |
| Perforin | 67 (12–90) | 75.5 (57–97) | 92 (53–96) |

NOTE. Data for subsets are median (range) $\times 10^9$/L; data for perforin are percentage expression in each subset.

[a] 11 Subjects tested for absolute no. of subsets/11 subjects tested for perforin expression in each subset.

[b] 4 Subjects tested for absolute no. of subsets/4 subjects tested for perforin expression in each subset.

[c] 4 Subjects tested for absolute no. of subsets/4 subjects tested for perforin expression in each subset.

regulation of CD45 isoform expression appears to be closely regulated by mitogenic T cell activation, this inability to cycle may well explain the reexpression of the CD45RA form on the most differentiated effector cells [11].

This model appears to be supported by recent studies in humans and animals, using tetrameric-MHC class I–peptide complexes [8, 9, 28, 42, 43]. For instance, in a study of humans with acute EBV infection, Callan et al. [28] found that most EBV-specific CD8⁺ cells display the CD45RO⁺ phenotype and have variable CD28 expression [28]. Of importance, in recent studies, it also was demonstrated in asymptomatic EBV carriers that the proportion of antigen-specific T cells expressing CD45RA increases [30, 44]. During acute viral infection, the division time of the virus-specific CD8⁺ T cells shortens substantially [39]. It is conceivable that the higher percentage of Ki-67–positive cells in the CD27⁻CD28⁻ subset in some patients can be explained by proliferating cells that are in the process of losing CD28 and CD27. Likewise, the expression of perforin in CD27⁺CD28⁺ cells may be explained by the fact that cytolytic mediators are among the genes that are rapidly induced in stimulated CD8⁺ T cells [45].

In support of the notion that CD8⁺CD45RA⁺CD27⁻ T cells undergo substantial division before they become terminally differentiated, we recently found that the telomeric restriction fragment (TRF) length of this effector population is ~0.5 kb shorter than that of the CD45RO⁺ memory-type cells and 2.3 ± 1.0 kb shorter than that of the CD45RA⁺CD27⁺ naive-type CD8⁺ cells [46]. This agrees with other reports [35, 38, 47] and with studies that found that, in HIV-1 infection and after bone marrow transplantation, the CD8⁺CD28⁻ cells had a shorter TRF than that of the CD8⁺CD28⁺ cells [38, 48, 49]. This CD28⁻ population overlaps to a large extent with the CD45RA⁻CD27⁻ cells as they were defined in our present study. Persistence of these cells may be of importance in the initial eradication of virus-infected cells and on renewed infection or reactivation [1].

The present report shows part of the maturation and differentiation pathways that CD8⁺ T lymphocytes may undergo during an acute viral infection. It seems that the acute response to HIV-1 does not differ substantially from the response against acute EBV infection. Whether this type of response is universal for all acute viral infections or is limited to infections in which the stage is in the lymphoid system itself has yet to be elucidated. This type of analysis may prove useful to more clearly define correlates of protective immunity not only in viral infection but also in immunization protocols aimed to generate antitumor or antiviral immunity.

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