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Characterization of CD4⁺ CTLs Ex Vivo¹

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The cytotoxic potential of CD8⁺ T cells and NK cells plays a crucial role in the immune response to pathogens. Although in vitro studies have reported that CD4⁺ T cells are also able to mediate perforin-mediated killing, the in vivo existence and relevance of cytotoxic CD4⁺ T cells have been the subject of debate. Here we show that a population of CD4⁺ perforin⁺ T cells is present in the circulation at low numbers in healthy donors and is markedly expanded in donors with chronic viral infections, in particular HIV infection, at all stages of the disease, including early primary infection. Ex vivo analysis shows that these cells have cytotoxic potential mediated through the release of perforin. In comparison with more classical CD4⁺ T cells, this subset displays a distinct surface phenotype and functional profile most consistent with end-stage differentiated T cells and include Ag experienced CD4⁺ T cells. The existence of CD4⁺ cytotoxic T cells in vivo at relatively high levels in chronic viral infection suggests a role in the immune response. The Journal of Immunology, 2002, 168: 5954–5958.

The CD4⁺ T cells are widely known for their capacity to produce cytokines, such as IL-2 (Th1) and IL-4 (Th2), providing helper functions in the immune response to pathogens, and have often been referred to as Th cells (1). Over the past 2 decades, the ability of CD4⁺ T cells to display cytotoxic potential has been reported in isolated, but repeated, reports in both mouse and human (2–6). However, the observation of such cytotoxic activity in CD4⁺ T cells has usually been restricted to cell lines and CD4⁺ T cell clones generated by long term in vitro culture and has therefore been considered by some authors to be an artifact (7). Accordingly, the in vivo existence and significance of CD4⁺ CTLs have been disputed or disregarded. Studies of clones and cell lines suggest that CD4⁺ CTLs use the perforin-dependent cytotoxic mechanism, rather than the Fas-dependent pathway (8–12). Perforin is a 70-kDa protein found in lytic granules of lymphocytes. It forms pores in target cell membranes, enabling the entry of granzymes that activate an apoptotic cascade resulting in cell death (13, 14). The expression of perforin and granzymes is usually said to be restricted to circulating CD8⁺ T cells and NK cells (15), but has recently also been shown in CD4⁺ CD8⁻ double-positive T cells (6).

By studying perforin expression in CD4⁺ T cells, we present here for the first time a detailed ex vivo identification and characterization of CD4⁺ cytotoxic T cells. These cells display a distinct functional phenotype in comparison with more classical CD4⁺ T cells. Although found at a low level in healthy donors, the CD4⁺ CTL subset is expanded from the early stages of HIV infection. The presence of CD4⁺ CTLs appears to be associated with inflammatory conditions, which suggests a role for these cells in the immune response.

Materials and Methods

Patients

Samples were taken from volunteers attending clinics in London, U.K., or Sydney, Australia. The study was approved by the relevant local institutional review boards and ethical committees. HIV-infected patients were classified into four different groups: primary HIV-1 infection (as previously described in Ref. 16), chronic untreated or undergoing treatment with ART (patients infected for >3 yr and displaying signs of progression with increasing viral load and decreasing CD4⁺ T cell counts), and nonprogressor (patients with no sign of progression and CD4⁺ counts >500 cells/ml for a median of 13 yr without therapy). EBV-infected patients were identified in primary infection as symptomatic and IgM positive by serology for EBV (16). Blood samples were generally used fresh within 8 h, or PBMCs were separated from heparinized blood and cryopreserved for subsequent studies.

FACS analysis and in vitro stimulation

Cell surface and intracellular staining was generally conducted directly on whole blood. In the studies performed in Oxford, a panel of titrated anti-human Abs was added to 150 μl heparinized blood for 15 min at room temperature. The lymphocytes were then fixed, and the RBCs were lysed using FACS lysis solution (BD Biosciences, San Diego, CA). Cells were washed and permeabilized in FACS permeabilization buffer (BD Biosciences). After washing, intracellular staining was performed for 15 min at room temperature in the dark using titrated concentration of Abs. Cells were then washed and stored in Cell Fix buffer (BD Biosciences) at 4°C until flow cytometric analysis was performed. GMP-17 or CCR7 staining was followed by addition of anti-mouse Ig Abs (FITC) and staining for cell surface markers. Samples were analyzed on a FACScalibur (BD Biosciences) after compensation was checked using freshly stained PBMCs. Essentially identical methods were used in studies performed in Sydney, as previously described (16), and analyzed on an EPICS XL (Coulter, Hialeah, FL).

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For IL-2, TNF-α, and IFN-γ staining, cells were first stimulated for 6 h with staphylococcal enterotoxin B (SEB; 5 μg/ml; Sigma, St. Louis, MO), PMA (50 ng/ml; Sigma, St. Louis, MO), and ionomycin (1 μg/ml; Sigma, St. Louis, MO) in the presence of brefeldin A (10 μg/ml; Sigma). CFSE labeling was performed by incubating PBMCs with 5 μM CFSE (Molecular Probes, Eugene, OR) in RPMI for 10 min at 37°C before quenching with ice-cold 10% FCS RPMI and washing. The cells were then incubated with SEB (5 μg/ml) or immobilized OKT3 (10 μg/ml) for 5 days before staining (neither anti-CD28 Abs nor IL-2 were added). The following Abs were used in different combinations. Anti-CD4 (PerCP), anti-CD7 (FITC or allophycocyanin), anti-CD27 (FITC or allophycocyanin), anti-CD45RO (FITC or PE), anti-CD45RA (FITC), anti-granzyme A (FITC), anti-IL-2 (PE), anti-IFN-γ (allophycocyanin), anti-TNF-α (FITC), and anti-CCR7 (purified) Abs were purchased from BD Pharmingen; anti-GMP-17 Abs (purified or PE) were obtained from Immunotech (Marseilles, France); and anti-CD45RB (FITC) and isotype controls were purchased from DAKO (Glostrup, Denmark).

**Cytotoxic assay**

EBV-transformed autologous B cell lines were used as target cells in a modified 51Cr release CTL assays. 51Cr labeling was performed for 1 h, following which cells were pulsed for 1 h in the presence of SEB (5 μg/ml; Sigma) and washed twice in RPMI medium. Controls included target cells incubated with medium or 5% Triton X-100 only. CD4+ T cells were positively selected from freshly isolated PBMCs by means of anti-CD4 MACS beads (Miltenyi Biotec, Auburn, CA; the positively selected population was 99% pure) and added to the targets at different E:T cell ratios in duplicate. Inhibition of perforin-mediated cytotoxicity was obtained by incubating the CD4+ T cells for 2 h with 100 nM concanamycin A (CMA; Sigma). Specific 51Cr release was calculated from the following equation: 

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\text{Specific} \text{ } 51\text{Cr} \text{ release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100\%.
\]

**Results**

**Identification of CD4+ perforin+ T cells**

We analyzed the expression of perforin in CD4+ T cells (gated on CD3-positive and CD8-negative or -dim) present in PBMC samples freshly obtained from volunteers. As expected, the numbers of perforin-expressing CD4+ T cells in healthy donors was generally low (mean ± SE, 2.2 ± 0.6% of CD4+ T cells; Fig. 1A); however, outliers with obvious perforin expression in the CD4+ T cell population could be found, as exemplified in Fig. 1B.

To characterize perforin-expressing CD4+ cells, we performed detailed phenotypic analyses using a large panel of markers. The CD4+ perforin+ T cells displayed a distinct cell surface phenotype independently of the donors: negative for the costimulatory receptors CD27 and CD28 and the chemokine receptor CCR7, mainly positive for the CD45 isoforms RO and RB, but RA negative, and expressing high levels of the integrins CD11a and CD11b (Fig. 2A). CD4+ perforin+ T cells did not show any signs of activation or proliferation; they were CD38low, CD69−, Bcl-2high, Ki67− (Fig. 2B). These cells did not appear to be regulatory T cells, being CD25−, nor did they belong to the recently described NK T cell family (which can express CD4 molecules) (17), expressing no or dim levels of CD16, CD56, and CD161 (Fig. 2C).

The pattern of marker expression is consistent with a phenotype of fully differentiated T cells (7, 18–21). Perforin expression was most closely associated with the loss of CD28 on the cell surface.

**Expansion of the CD4+ CTL subset in chronic viral infections**

We compared the levels of perforin+ CD4+ T cells in healthy donors with levels in groups of HIV-infected patients at various stages of the disease as well as with those in EBV-infected individuals. HIV-infected individuals exhibited significantly higher numbers of perforin-expressing CD4+ T cells (Fig. 3). This increase appeared early in the disease process, even at the earliest stages of primary HIV-1 infection, while CD4+ perforin+ T cells reached higher numbers in chronically infected patients. A trend toward lower CD4+ perforin+ T cell numbers was observed in treated chronic HIV-infected patients and nonprogressors with good viral control compared with untreated, chronic HIV-infected patients (Fig. 3). However, in both cases, levels were significantly higher than those in healthy controls (p < 0.001). An increase in the size of this population was not restricted to HIV infection, as it was also seen in individuals with acute EBV infection; however,

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*Abbreviations used in this paper: SEB, staphylococcal enterotoxin B; CMA, concanamycin A.*
the increase was less marked (Fig. 3). A similar phenotype was observed regardless of whether the cells were from healthy or HIV-infected donors and was essentially independent of disease stage or therapeutic intervention. In the case of those subjects studied in the first 2 wk of symptomatic primary HIV-1 infection, the phenotype was consistent with that described previously, except that these cells coexpressed higher levels of CD38 and Ki67, indicative of recent activation and proliferation (data not shown).

**Functional characterization of CD4$^+$ perforin$^+$ T cells**

Following stimulation with the superantigen SEB or PMA/ionomycin, CD4$^+$/perforin$^+$ T cells produced the effector cytokines TNF-$\alpha$ and IFN-$\gamma$. In contrast, the expression of IL-2 was limited to perforin-negative cells (Fig. 4A).

CD4$^+$ perforin$^+$ T cells also contained high levels of the granule membrane protein (GMP-17) and granzyme A (Fig. 4B), indicating that the cells possessed both lytic granules and the factors necessary to complete the perforin-dependent cytolytic pathway. Furthermore, using a redirected killing assay dependent upon superantigen SEB cross-linking T cells expressing V$\alpha$ families to targets expressing HLA class II molecules, we were able to demonstrate ex vivo target cell killing by CD4$^+$ T cells positively selected from PBMC of a donor exhibiting 19.3% CD4$^+$ perforin$^+$ T cells reacting to SEB (Fig. 4C). The cytotoxic potential of the CD4$^+$ T cells was significantly reduced in the presence of CMA, an inhibitor of perforin-mediated killing (22), indicating that the cytotoxic activity was due to the CD4$^+$ perforin$^+$ T cells (Fig. 4C). This ex vivo lytic activity exhibited by CD4$^+$ T cells strongly supports the presence of cytotoxic CD4$^+$ T cells in vivo.

CD4$^+$ cytotoxic T cells (identified according to CD28 expression) displayed lower proliferative potential compared with the other CD4$^+$ T cells. Although both subsets of CD4$^+$ T cells were activated, displaying clear up-regulation of CD69 within 6 h of stimulation by immobilized anti-CD3 Ab or SEB (Fig. 4D), only the CD4$^+$/CD28$^+$ T cell subset was able to undergo cell division (Fig. 4E). The CD4$^+$ CD28$^-$ population, which included all the CD4$^+$/perforin$^+$ cells, exhibited little or no proliferation in response to either stimuli despite expressing the activation marker CD69. In fact, in this assay the CD4$^+$/CD28$^-$ T cell number appeared to decrease over time following activation, suggesting that these cells underwent activation-induced cell death following stimulation.

**FIGURE 3.** Perforin expression in CD4$^+$ T cells in virus-infected donors. The expression of perforin in CD4$^+$ T cells is shown in all donors (left panel), and means and 95% confidence intervals are presented (right panel). Statistical differences between groups were determined with the Mann-Whitney nonparametric test.

**FIGURE 4.** Functional characteristics of CD4$^+$ T cells. A, Staining for intracellular perforin, IFN-$\gamma$, and IL-2 was conducted following stimulation using SEB or PMA/ionomycin. Cells are gated on the CD4$^+$ population for control and SEB stimulation or on lymphocytes for PMA stimulation. B, Staining for intracellular perforin, GMP-17, and granzyme A was conducted ex vivo, directly on whole blood. Cells are gated on the CD4$^+$ population. C, Lysis of chromium-loaded target cells pulsed (○) or non-pulsed (●) with SEB in the presence of positively selected CD4$^+$ T cells at several E:T cell ratios for 4 h. Filled lozenges indicate the presence of CMA, an inhibitor of perforin-mediated cytotoxicity. D and E, Following stimulation with anti-CD3 Abs or SEB, PBMCs were stained for CD4 and CD28 and analyzed for CD69 expression after 6 h (D) and for CFSE labeling after 5 days (E). Cells are gated on the CD4$^+$ population. Representative results from two independent experiments are shown.
CD4<sup>+</sup> perforin<sup>+</sup> T cells represent a subset of Ag-experienced cells

When the TCR repertoire was examined by flow cytometry, perforin expression was restricted to a limited number of CD4<sup>+</sup> V<sub>B</sub> subfamilies, e.g., V<sub>B2</sub> (Fig. 5A), indicating that CD4<sup>+</sup> CTLs represent a population of expanded cells. The full range of the antigenic specificity of this population remains undefined. However, CMV-responsive cells, detected by means of IFN-γ intracellular staining following stimulation with a CMV lysate, were found within but represented only a fraction of this population (Fig. 5B–D). Conversely, in each individual tested, the CMV-specific CD4<sup>+</sup> perforin<sup>+</sup> cells represented only a fraction of the total CD4<sup>+</sup> population that responded to CMV in cytokine secretion assays (0–82%). The sizes of both fractions varied between donors. On occasion, HIV-infected individuals demonstrated CD4<sup>+</sup> perforin<sup>+</sup> T cells reacting to HIV Ags (p24 whole protein) by cytokine secretion, but this number was generally much smaller than of CMV-specific CD4<sup>+</sup> CTLs (data not shown).

In vitro, cytotoxic CD4<sup>+</sup> T cell clones have been grown from a range of hosts, with specificities for a variety of Ags, including HIV, EBV, dengue, and melanoma-derived Ags, as well as autoantigens (12, 23–26). Similarly, the specificity of CD4<sup>+</sup>CD27<sup>+</sup> T cells in PBMC has been ascribed to a range of Ags, including tetanus toxoid and various allergens (27). Our observations combined with those from the literature suggest that CD4<sup>+</sup> cytotoxic cells represent a subpopulation of Ag-experienced cells.

**Discussion**

We have been able to identify and characterize CD4<sup>+</sup> CTLs ex vivo. The combination of surface and intracellular proteins along with the pattern of cytokine secretion suggest a unique phenotype for this CD4<sup>+</sup> T cell subset. These cells have a surface phenotype consistent with certain subpopulations of CD4<sup>+</sup> cells previously characterized, but in which cytotoxic potential was not considered. Two previous studies have demonstrated that CD4<sup>+</sup>CD27<sup>+</sup> cells are relatively poor in providing help for B cell differentiation (28, 29), which is consistent with their inability to produce IL-2 (30). CD4<sup>+</sup> CTLs do not appear to belong to the CD4<sup>+</sup> regulatory or CD4<sup>+</sup> NK T cell subsets, but seem rather to represent highly differentiated, Ag-experienced (memory) CD4<sup>+</sup> T cells. The phenotype of these cells is consistent with an end-stage cell (CD28<sup>-</sup>) resulting from chronic stimulation (CD27<sup>-</sup>) (20, 21). On the basis of their phenotype, cytokytoxic function, and cytokine production profile these CD4<sup>+</sup> cytotoxic T cells are remarkably similar to late differentiated CD8<sup>+</sup> T cells (31, 32). Differences in the relative proportions of subpopulations within the memory CD4<sup>+</sup> T cell subset may explain discrepancies between IFN-γ production and proliferative capacity often observed in the study of Ag-specific CD4<sup>+</sup> T cells (33).

Expansions of CD4<sup>+</sup> CTLs appear to be related to chronic virus infections, such as HIV-1, EBV, and CMV infections. The presence of similar cells has also been reported in a subset of patients diagnosed with rheumatoid arthritis (9). Taken together these observations suggest that the presence of such cells may be associated with general inflammatory conditions. Under certain chronic inflammatory conditions, increasing numbers of CD4<sup>+</sup> T cells may undergo progressive differentiation, during which cells lose expression of the costimulatory molecules CD28 and CD27 and gain expression of intracellular cytotoxic granules and perforin, as observed with CD8<sup>+</sup> T cells (31, 32). A model for this pathway of differentiation is shown in Fig. 6. Although these cells are present in increased numbers at all stages of HIV infection, the initial expansion of this subset occurs very early in the infection, as indicated by the increased level of CD38 and Ki-67 during this period. The expansion of this subset occurs concurrently with the
increase in CD28 /CD27 /CD8 T cell numbers that is characteristic of HIV infection (33). One possible explanation for the increase in CD4+ CTL numbers is that the same factors that drive CD8 T cell differentiation drive the increase in this CD4+ perforin+ T cell subset. One candidate for this would be IL-15, which is known to increase CD8 T cell proliferation and differentiation and which is present at abnormally elevated levels in several pathologies, including rheumatoid arthritis and HIV infection (34). Preliminary experiments have shown that short-term (24- to 48-h) incubation with IL-15 increases the proportion of CD4+ T cells expressing the cytotoxic granule protein (GMP-17) and perforin in PBMC from both healthy and HIV-infected donors (J. J. Zaunders, unpublished observations). Once produced, these cells, with their limited proliferative capacity, may be long-lived (Bcl-2high) cells with very low turnover (Ki-67).

This study demonstrates that the existence of CD4+ cytotoxic T cells is not merely an in vitro artifact and requires more consideration. Previous observations of the emergence of such cells following prolonged in vitro culture may give insight into the particular conditions favoring this pathway of differentiation. Although a relative increase in the number of these cells appears to be the result of chronic stimulation, the driving force for the expansion of this subset of CD4+ T cells begins at the earliest stages of HIV infection. One possibility is that they may play a role in containing viral infections tropic for HLA class II+ cells, such as EBV in B cells or HIV-1 in activated CD4+ T cells, monocytes, and dendritic cells, although the mechanism of Ag presentation is at present unknown. It is also worth noting that HIV, EBV, and CMV prevent normal MHC class I expression as part of their strategies of immune evasion (35), which may result in preferential targeting of the class II pathway. The study by Khanna and colleagues (36), showing recognition of EBV-infected Burkitt’s lymphoma cells (with MHC class I down-regulation) by EBV-specific CD4+ CTL clones, supports this hypothesis. The presence of CD4+ CTLs in vivo is intriguing, and their exact role remains to be understood.

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