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Predominant Involvement of CD8$^+$CD28$^-$ Lymphocytes in Human Immunodeficiency Virus-Specific Cytotoxic Activity

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Distinct functional CD8$^+$ T-cell populations have been observed during human immunodeficiency virus (HIV) infection. One of these functions is the inhibition of viral replication by a noncytotoxic mechanism, which was shown to be mediated by the CD8$^+$CD28$^+$ subpopulation. On the other hand, CD8$^+$ T cells exert an HIV-specific cytotoxic activity. The present study shows that CD8$^+$CD28$^-$ lymphocytes display this HIV-specific cytotoxic activity, which is detectable immediately after the cells are purified from peripheral blood. The CD28$^-$ population is also able to proliferate and to retain its cytotoxic activity after in vitro restimulation with autologous blast cells. Finally, HIV-specific cytotoxic T cells can be obtained in vitro from the CD8$^+$CD28$^+$ population.

The CD28 molecule is present on about 95% of CD4$^+$ T lymphocytes and is the principal costimulatory molecule involved in T-cell activation (11). Only 50% of CD8$^+$ T cells are CD28$^+$ (2), and the functional differences between these two CD8$^+$ populations are not clearly understood (1, 6, 7, 9, 27). The CD8$^+$ population appears to have two functions in human immunodeficiency virus (HIV) infection. One is an intense, highly polymorphic cytotoxic T-lymphocyte (CTL) activity (15), and the other is an antiviral, noncytotoxic activity that consists of inhibition of HIV replication (29). The CD28$^+$ cells seem to be involved in this noncytotoxic antiviral activity (17). On the other hand, recent reports indicate that the CD8$^+$CD28$^-$ population gradually expands in the peripheral blood and lungs of HIV-infected subjects (4, 5, 21, 25). An involvement of these cells in the clinical evolution of AIDS has been suggested, especially since these cells could be terminally differentiated (4, 18).

The CTL response is generated soon after HIV infection (3, 16, 24) and remains vigorous during the asymptomatic stage (15). It can be detected in freshly isolated peripheral blood mononuclear cells (PBMC), i.e., without in vitro reactivation (28). This characteristic is specific to the HIV system and is probably due to a large burst of immune response which is maintained by a high viral replication rate (8, 20). This has allowed us to study directly ex vivo the specific cytotoxic capacities of CD8$^+$CD28$^-$ and CD8$^+$CD28$^+$ cells from several HIV-seropositive subjects, whose clinical and phenotypic characteristics are given in Table 1. The expression of CD28 on CD8$^+$ PBMC was analyzed by standard flow cytometry, as previously described (19). Most of the subjects (7 of 10) had below-normal percentages of CD28$^+$ cells in their CD8$^+$ populations.

A first set of experiments was carried out to analyze directly the participation of CD28$^+$ and CD28$^-$ subsets in the cytotoxic activity. These cells were positively or negatively selected from PBMC by using goat anti-mouse immunoglobulin-coated magnetic beads (BioMag goat anti-mouse immunoglobulin G; PerSeptive Diagnostics). CD4$^+$ and CD8$^+$ cells selected by using the same experimental approach were used as controls. Briefly, 30×10^6 cells were incubated for 25 min at 4°C with 30 μg of either OKT4 or OKT8 monoclonal antibody (MAb) (Orthodiagnostic Systems) or with 50 μg of anti-CD28.2 MAb, which was chosen for its high affinity (19). The cells were washed twice and incubated with beads coated with goat anti-mouse immunoglobulin G (15 beads per cell) for 30 min at 4°C. The suspension was placed in a magnetic separator for 10 min, and the supernatant was carefully removed and put through a second magnetic separation. The selected cells (CD4$^+$, CD8$^+$, and CD28$^+$) were eluted from the beads by incubation overnight at 37°C and 5% CO_2 in culture medium (RPMI 1640 supplemented with 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] buffer and 10% fetal calf serum). Uncoated cells were recovered from the enrichment experiments and called the negative selected population (CD4$^-$, CD8$^-$, and CD28$^-$). These cells were also incubated overnight.

Selected cells were used as effector cells in a chromium release test performed as previously described (15). Since the yield of purified effector cells was low, only Gag and Pol proteins were tested, as they are the proteins most frequently recognized by HIV-specific CTLs and there is no interference with antibody-dependent cell cytotoxicity due to circulating antibodies directed against the Env protein (23). The results for subject P1 (stage IV) show that CD4$^+$ cells were not cytotoxic, as expected (15), and that CD8$^+$ cells retained their Gag-specific cytotoxicity after positive selection with beads (Fig. 1). Purified CD28$^-$ PBMC displayed a Gag-specific cytotoxic activity equivalent to that of CD8$^+$ or CD4$^-$ cells, while CD28$^+$ PBMC were essentially inactive. Some NK activity was observed on K562 cells with all of the selected effector populations (11 to 25% for an effector/target cell ratio of 90:1). However, the cytotoxic activity observed with CD8$^+$ and

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TABLE 1. Phenotype analysis of seropositive and control subjects[a]

Subject(s)	Clinical stage	CD4 cells[b]	CD8 cells[b]	CD4/CD8 ratio	% CD28 on CD8 cells
Controls[c]		970 ± 200	644 ± 150	1.6	58 ± 18
P6	II	513	500	1	29
Z87	II	1,127	1,072	1	46
Z43	II	646	931	0.7	41
Z59	II	654	935	0.7	29
Z44	II	467	1,177	0.4	56
Z18	II	416	1,774	0.2	26
Z16	III	912	1,488	0.6	31
P2	III	515	1,324	0.4	26
P11	III	637	2,591	0.2	20
P1	IV	210	1,639	0.1	16

[a] The MAbs used were fluorescein isothiocyanate- or phosphatidylethanolamine-conjugated anti-CD3, anti-CD4, or anti-CD8 (Coulter) and phosphatidylethanolamine-conjugated anti-CD28 (Becton Dickinson).

[b] Absolute number per cubic millimeter.

[c] PBMC were obtained from 21 seronegative donors to the Hôpital Cochin blood bank.

$CD8^+CD28^-$ cells on autologous lymphoblastoid target cells was probably mediated not by NK cells but by major histocompatibility complex (MHC)-restricted CTLs, since it was specific to target cells expressing HIV antigens and was not detectable with the cell population depleted only of $CD8^+$ cells. Similar results were obtained for subject Z44, who was still asymptomatic (Table 1). Since the CD28 molecule is expressed by almost all $CD4^+$ cells, the following experiments were performed by removing $CD4^+$ cells before purifying the $CD28^+$ cells in order to concentrate the $CD8^+CD28^+$ cells. This approach confirmed that the $CD28^-$ cells were the main HIV-specific cytotoxic population of PBMC from subjects P6, P2, and P11, as illustrated in Fig. 2 with Gag-specific CTLs, and also from subject Z59. Overall, the mainly cytotoxic activity of $CD8^+CD28^-$ cells detected directly ex vivo had been found in six HIV-seropositive subjects.

The process that induces the development of $CD8^+CD28^-$ cells in HIV-seropositive subjects is still unclear (5, 21, 25). Our results suggest that this expansion is correlated with the activation of HIV-specific CTLs. A similar expanded population has also been observed during chronic infection of an allograft recipient with cytomegalovirus (14) and could corre-

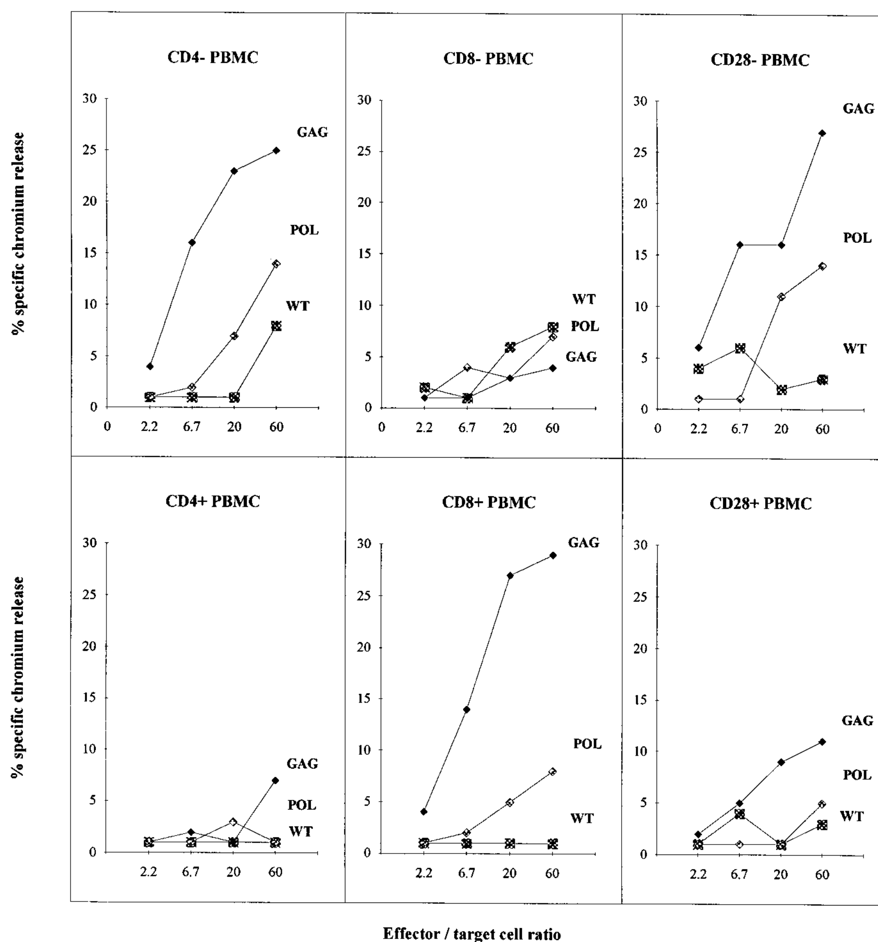

FIG. 1. Ex vivo cytotoxic activities of CD4-, CD8-, and CD28-selected PBMC from subject P1. Cytotoxic activities of positive ($CD4^+$, $CD8^+$, and $CD28^+$) and negative ($CD4^-$, $CD8^-$, and $CD28^-$) purified PBMC were tested immediately after purification. The target cells were autologous lymphoblastoid cell lines obtained by transforming PBMC with Epstein-Barr virus (EBV-LCL) and infected for 18 h with 5 PFU of the wild-type vaccinia virus (WT) (Copenhagen strain) or recombinant vaccinia virus encoding the Gag or Pol protein of the HIV LAI isolate per ml (18). The $CD4^-$ population contained 1% $CD4^+$ cells and 61% $CD8^+$ cells (65% of them were $CD28^+$), the $CD8^-$ population contained 60% $CD4^+$ cells and 9% $CD8^+$ cells, the $CD28^-$ population contained 8% $CD4^+$ cells and 70% $CD8^+$ cells (8% of them were $CD28^+$), the $CD4^+$ population contained 60% $CD4^+$ cells and 20% $CD8^+$ cells, the $CD8^+$ population contained 7% $CD4^+$ cells and 80% $CD8^+$ cells (70% of them were $CD28^+$), and the $CD28^+$ population contained 42% $CD4^+$ and 40% $CD8^+$ cells (95% of them were $CD28^+$).

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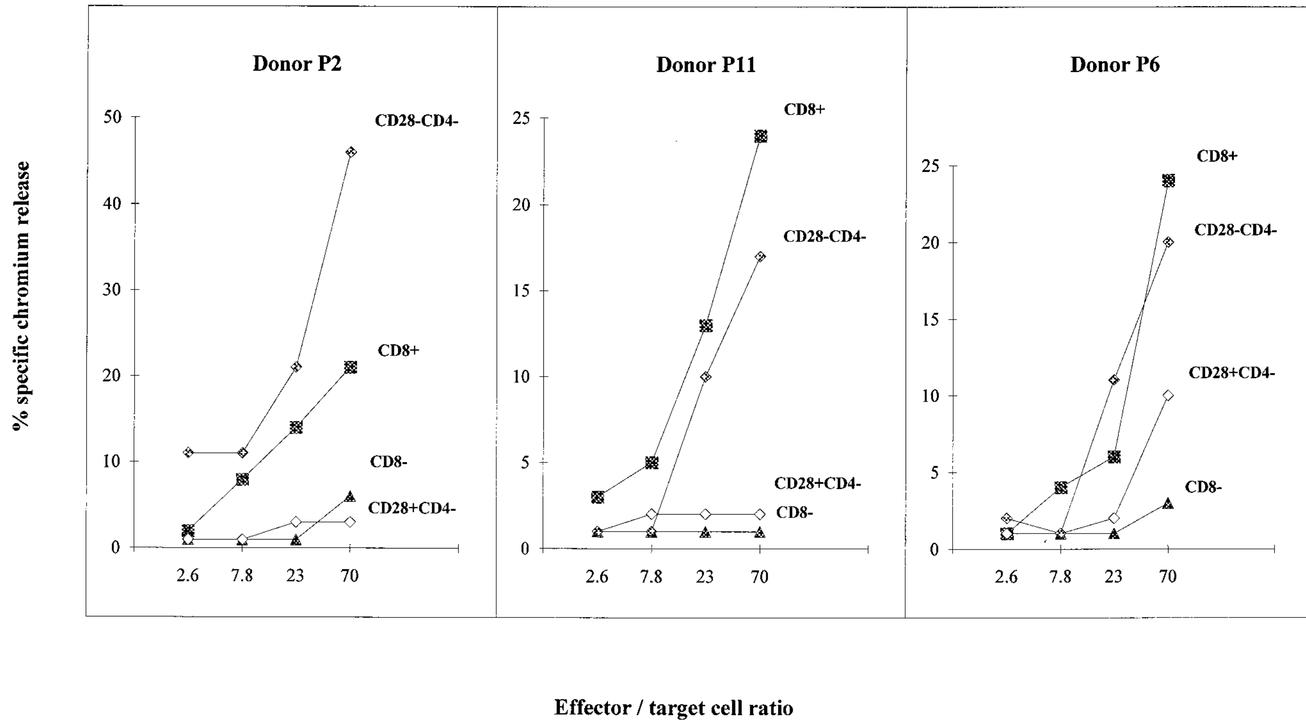

FIG. 2. Ex vivo cytotoxic activities of selected PBMC from subjects P2, P11, and P6. Gag-specific cytotoxic activities of positive ($CD8^+$ and $CD28^+CD4^-$) and negative ($CD8^-$ and $CD28^-CD4^-$) selected PBMC were tested on autologous EBV-LCL infected with recombinant vaccinia virus encoding the Gag protein of HIV LAI. The phenotypes of the $CD8^+$ and $CD28^-$ populations were very similar to those from subject P1 illustrated in Fig. 1. The $CD28^-CD4^-$ population of subject P2 contained 8% $CD4^+$ cells and 62% $CD8^+$ cells (2% of them were $CD28^+$), and the $CD28^+CD4^-$ population consisted of 5% $CD4^+$ cells and 63% $CD8^+$ cells (97% of them were $CD28^+$). The $CD28^-CD4^-$ population from subject P11 contained 1% $CD4^+$ cells and 66% $CD8^+$ cells (1% of them were $CD28^+$), and the $CD28^+CD4^-$ population consisted of 5% $CD4^+$ cells and 65% $CD8^+$ cells (96% of them were $CD28^+$). The $CD28^-CD4^-$ population from subject P6 consisted of 5% $CD4^+$ cells and 40% $CD8^+$ cells (9% of them were $CD28^+$), and the $CD28^+CD4^-$ population consisted of 6% $CD4^+$ cells and 50% $CD8^+$ cells (94% of them were $CD28^+$).

spond to CTLs specific for this virus. $CD8^+CD28^-$ cells are not frequent in cord blood (2), but they have been detected in the peripheral blood of adults and elderly subjects, and their number is correlated with continuous exposure to antigen (22). The expansion of these cells in the course of an HIV infection may also be due to the progressive destruction of lymph nodes (8, 20), with a consequent reduction in the number of antigen-presenting cells expressing molecules of the B7 family. However, there is the question of whether the $CD8^+CD28^-$ cytotoxic cells can really control the viral infection in vivo. Despite their presence in the peripheral blood, it has not yet been established that they are also present in other tissues, such as lymphoid organs, where viral replication is known to be particularly important (8, 20). Furthermore, their capacity to lyse naturally infected cells needs to be assayed. Previous reports have suggested that the functional integrity of $CD8^+CD28^-$ cells is impaired in vivo (4) and that they are cytotoxic only in short-term cultures (2). Analysis of the proliferative responses of $CD8^+$ lymphocytes subsets from HIV-seropositive and -seronegative subjects showed that $CD8^+CD28^+$ cells proliferate better than $CD8^+CD28^-$ cells in response to phytohemagglutinin, staphylococcal enterotoxin, or OKT3 after the addition of interleukin-2 and that they secrete more interleukin-2 after these stimulations than do $CD28^-$ cells (4, 5). The $CD8^+CD28^-$ cells also have more DNA fragmentation than do $CD8^+CD28^+$ cells (18). In spite of this, our results clearly show that circulating $CD8^+CD28^-$ cells display an integral cytotoxic ability ex vivo.

To assay the in vitro stability of these $CD28^-$ CTLs and the possibility of in vitro expansion of HIV-specific CTLs within the $CD28^-$ and/or the $CD28^+$ population, cell lines were generated by in vitro restimulation of PBMC from six HIV-seropositive subjects. Autologous phytohemagglutinin-activated PBMC were used as stimulating cells (15), since they are naturally HIV antigen-presenting cells. Both $CD8^+CD28^-$ and $CD8^+CD28^+$ cells expanded in parallel in these cultures, since the percentages of $CD28^-$ cells in the $CD8^+$ population were equivalent before (about 65%) and after (about 64%) stimulation. By contrast, the $CD8^+CD28^+$ population was expanded preferentially in cultures of PBMC from seronegative subjects stimulated in vitro under the same conditions (only 24% of $CD8^+$ cells were $CD28^-$ after stimulation, while this value was about 49% in PBMC). A significant proliferation of the $CD8^+CD28^-$ population was also observed in anti-EBV and anti-influenza virus cell lines generated with PBMC from subjects responding to these viruses, after in vitro stimulation performed as described previously (10), suggesting that these cells can proliferate in vitro only when they are specifically restimulated with the appropriate antigen (not shown). Most of the HIV-specific cytotoxic activity was found in the $CD28^-$ population purified after in vitro stimulation and just before the chromium release test, but specific activity was also present in the $CD28^+$ population, as illustrated with Gag-specific CTLs from subjects Z16 and Z18 (Fig. 3a). Similar results were obtained with other HIV proteins (Env, Pol, and Nef). This cytotoxic activity was likely MHC restricted, since it was not observed with MHC-mismatched target cells and was inhibited by a MAb directed against class I MHC molecules (data not shown). The cytotoxic capacity of the $CD28^-$ cells was always higher than that of the $CD28^+$ cells: two to five times more

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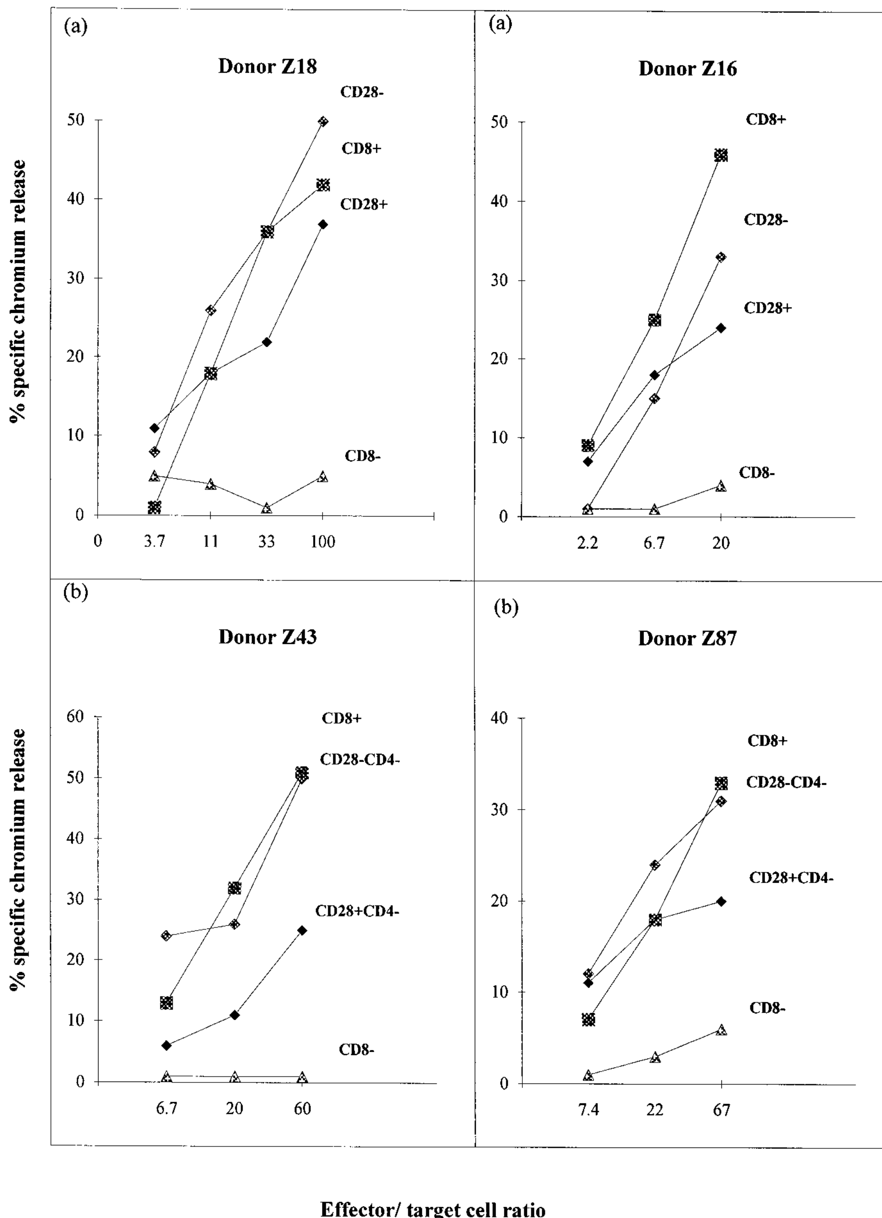

FIG. 3. Cytotoxic activities of $CD8^+CD28^+$ and $CD8^+CD28^-$ cells after in vitro restimulation. PBMC from subjects Z16, Z18, Z43, and Z87 were cocultivated with autologous phytohemagglutinin-activated PBMC, as previously described (15). Gag-specific cytotoxic activity was tested on autologous EBV-LCL after 14 days in culture. Positive and negative selections of $CD8^+$ populations were performed from the total T-cell lines for the four subjects tested. $CD28^+$ or $CD28^-$ cells were selected from the total T-cell lines (subjects Z18 and Z16) (a) or from the $CD4^-$-preselected T-cell lines (subjects Z43 and Z87). The phenotypes of the selected populations were very similar to those given in the legends to Fig. 1 and 2.

$CD28^+$ effector cells than $CD28^-$ effector cells were required to obtain the same cytotoxic activity, even when the $CD8^+CD28^+$ population was enriched by removing $CD4^+$ cells before $CD8^+$ purification, as illustrated with subjects Z43 and Z87 in Fig 3b and also observed with subjects Z44 and Z59. Furthermore, addition of an anti-CD28 MAb (or an anti-CD4 MAb) at the time of the chromium release test did not affect the cytotoxic activity of $CD8^+CD28^+$ cells, while an anti-CD8 MAb significantly diminished this activity (not shown).

In order to analyze whether the cytotoxic activity of $CD28^-$ or $CD28^+$ cells could be independently expanded after selection of these cells before in vitro reactivation, $CD28^-$- and $CD28^+$-selected PBMC were separately restimulated, as described for the polyclonal cell lines. Again, these two selected populations were able to grow in vitro and show Gag- or Pol-specific cytotoxic activity when tested after 2 weeks in culture (not shown). The $CD28^-$ cells also seemed to proliferate as well as $CD28^+$ cells after this type of in vitro stimulation; e.g., cells from donor Z44 had multiplication indexes of 10.2 ($CD28^+$) and 7.8 ($CD28^-$) after 10 days in culture. Together, our results show that the $CD28^-$ CTLs are probably the mature CTL population present in vivo and can multiply in vitro in response to specific antigen.

The analysis of the HIV system has allowed the correlation of the two $CD8^+$ cell subsets ($CD28^+$ and $CD28^-$) with two functions. On the one hand, HIV-specific cytotoxic activity is

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mainly mediated by $CD8^+CD28^-$ cells, as demonstrated here for the first time in a natural infection. This observation is in agreement with the presence of intracellular cytotoxic markers in $CD8^+CD28^-$ cells from HIV-seropositive and healthy individuals (4). The low frequency of circulating active CTLs in other viral infections prevents their ex vivo analysis. However, we have evidence that most of the anti-EBV or anti-influenza virus CTLs obtained after in vitro stimulation are also essentially $CD8^+CD28^-$ (unpublished data). This cell population also exerts the cytotoxic activity specific for lymphocytic choriomeningitis virus in $CD28^{-/-}$ mutant mice (26). Hence, there is considerable evidence that $CD8^+CD28^-$ cells play an important part in antiviral CTL activity. However, this does not exclude a role for the CD28 molecule in the activation of the CTLs, as suggested for nonviral systems (1, 9, 27), and their ability to differentiate into $CD28^-$ functional CTLs. On the other hand, inhibition of HIV replication is controlled by $CD8^+CD28^+$ cells (17). There have also been reports that $CD8^+$ cells inhibit the replication of simian immunodeficiency virus (12) and *Toxoplasma gondii* (13), but the participation of $CD28^+$ cells in these antigenic systems remains to be confirmed. We suggest that these phenotypically and functionally distinct $CD8^+$ cell populations, which are present in equal numbers in normal subjects, may be involved in the response to infections by other microorganisms.

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