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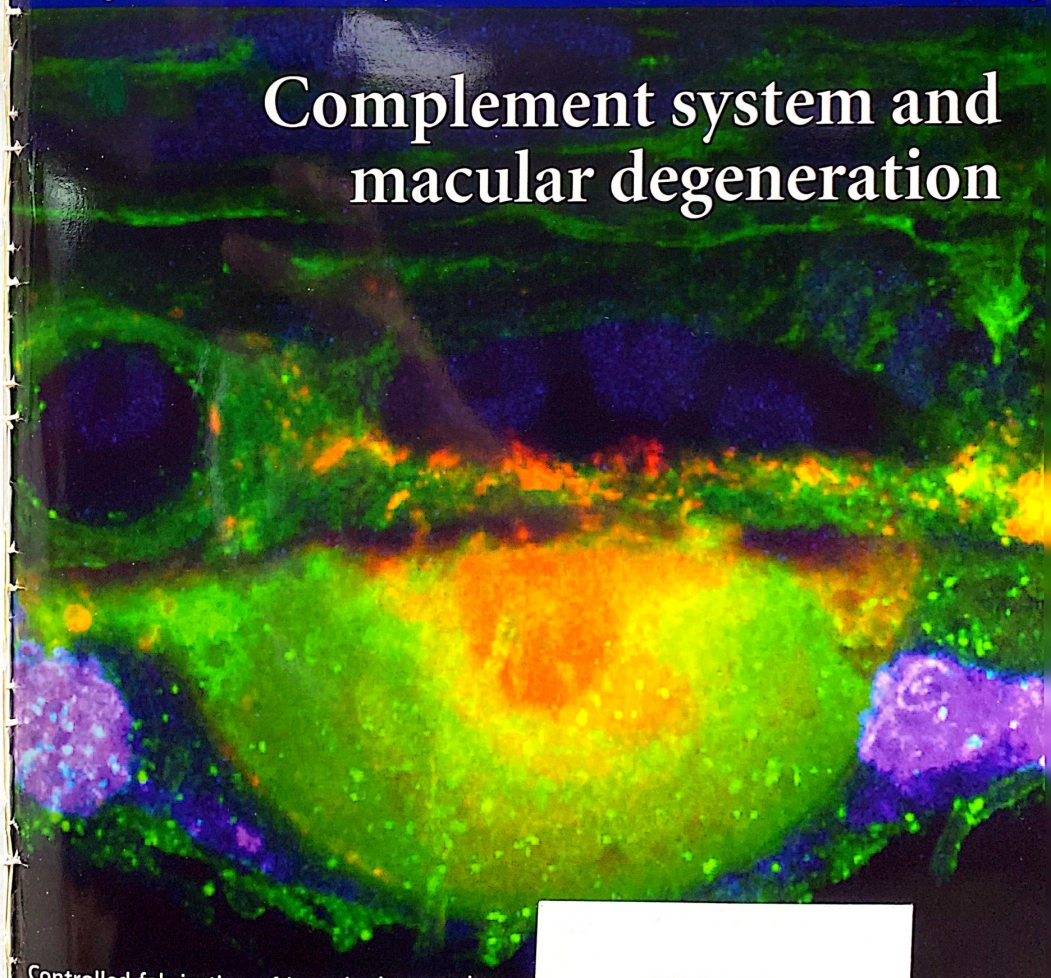
Complement system and macular degeneration



Controlled fabrication of branched nanotubes

3D structures of protein reaction pathway

Assessing success of vertebrate invaders

Gene flow and mutation accumulation

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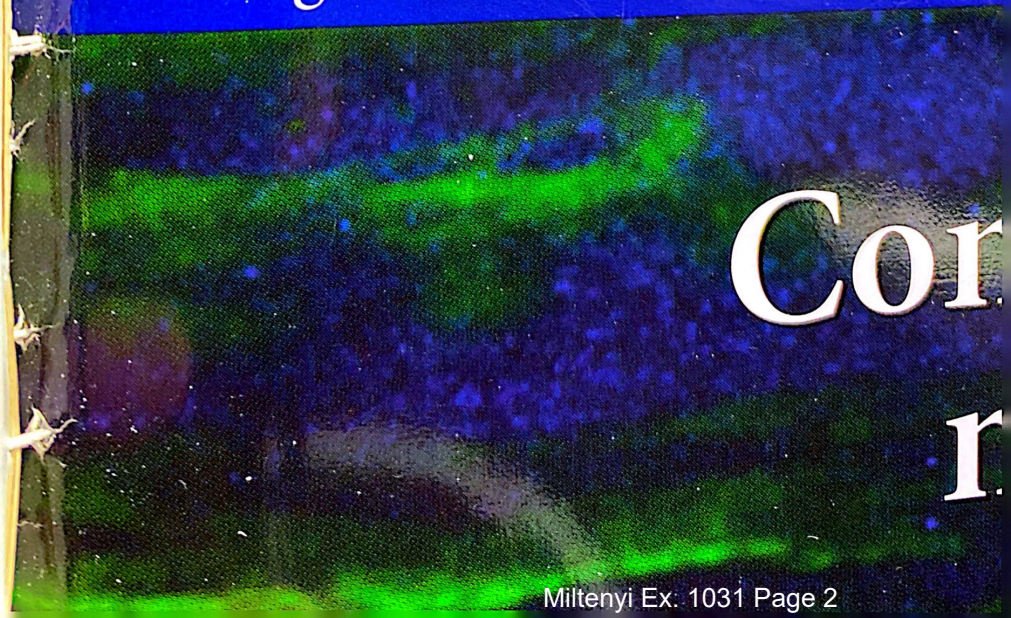

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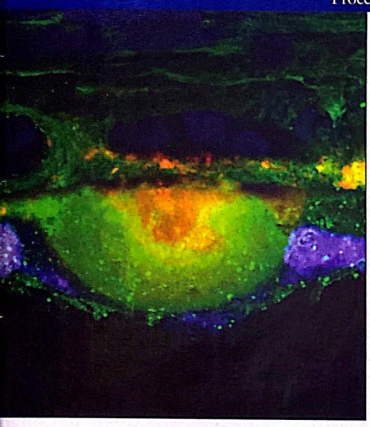

Cover image: Laser scanning confocal image of an ocular druse, the hallmark lesion associated with age-related macular degeneration. The complement system protein 5b-9 is shown in orange and red, and factor H, which inhibits the complement pathway, is shown in green. The retinal pigment epithelium is shown in purple. Genetic variation in the factor H gene is a major contributor to age-related macular degeneration. See the article by Hageman *et al.* on pages 7227–7232. Image courtesy of Patrick Johnson (Center for the Study of Macular Degeneration, University of California, Santa Barbara).

From the Cover

Contents

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HIV-1-specific IFN-γ/IL-2-secreting CD8 T cells support CD4-independent proliferation of HIV-1-specific CD8 T cells

Simone C. Zimmerli, Alexandre Harari, Cristina Cellerai, Florence Vallelian, Pierre-Alexandre Bart, and Giuseppe Pantaleo*

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Functional and phenotypic characterization of virus-specific CD8 T cells against cytomegalovirus, Epstein–Barr virus, influenza (flu), and HIV-1 were performed on the basis of the ability of CD8 T cells to secrete IFN-γ and IL-2, to proliferate, and to express CD45RA and CCR7. Two functional distinct populations of CD8 T cells were identified: (*i*) dual IFN-γ/IL-2-secreting cells and (*ii*) single IFN-γ-secreting cells. Virus-specific IFN-γ/IL-2-secreting CD8 T cells were CD45RA$^-$CCR7$^-$, whereas single IFN-γ CD8 T cells were either CD45RA$^-$CCR7$^-$ or CD45RA$^+$CCR7$^-$. The proportion of virus-specific IFN-γ/IL-2-secreting CD8 T cells correlated with that of proliferating CD8 T cells, and the loss of HIV-1-specific IL-2-secreting CD8 T cells was associated with that of HIV-1-specific CD8 T cell proliferation. Substantial proliferation of virus-specific CD8 T cells (including HIV-1-specific CD8 T cells) was also observed in CD4 T cell-depleted populations or after stimulation with MHC class I tetramer–peptide complexes. IL-2 was the factor responsible for the CD4-independent CD8 T cell proliferation. These results indicate that IFN-γ/IL-2-secreting CD8 T cells may promote antigen-specific proliferation of CD8 T cells even in the absence of helper CD4 T cells.

CD8 T cells play a critical role in the control of viral infections (reviewed in ref. 1). Several studies have shown a wide heterogeneity of memory CD8 and CD4 T cells with multiple phenotypes and functions in response to virus infections (2–7). Functionally distinct populations of CD8 T cells can be defined by the expression of CD45RA and CCR7 (8) and are able to proliferate and/or to secrete cytokines such as IL-2, IFN-γ, and TNF-α after antigen (Ag)-specific stimulation (9–11). The determination of quantitative and qualitative changes of virus-specific CD8 T cells in rapidly controlled acute, more slowly controlled or uncontrolled chronic infections showed that high load of lymphocytic choriomeningitis virus resulted in the progressive diminution of the ability of CD8 T cells to produce IL-2, TNF-α, and IFN-γ (9). Of interest, the capacity to secrete cytokines could be restored if the viral load was brought under control (9).

IL-2 production from virus-specific CD8 T cells has been the object of few studies in humans. Recent studies have shown that a variable percentage of cytomegalovirus (CMV)- and Epstein–Barr virus (EBV)-specific CD8 T cells were able to secrete IL-2 (10, 11), whereas IL-2 was not produced by melanoma-1-specific CD8 T cells obtained from patients with stage IV melanoma (10). With regard to HIV-1 infection, no studies have investigated the ability of HIV-1-specific CD8 T cells to secrete IL-2. However, it has been shown that HIV-1-specific CD8 T cells of HIV-1-infected subjects with nonprogressive disease, i.e., long-term nonprogressors (LTNPs), had greater proliferation capacity as compared with HIV-1-specific CD8 T cells from progressors (12), and this finding was associated with a better ability to control virus replication (12). A recent study has shown that the loss of HIV-1-specific CD8 T cell proliferation was associated with the loss of HIV-1-specific helper CD4 T cells and has proposed a critical role of HIV-1-specific helper CD4 T cells in sustaining Ag-specific CD8 T cell proliferation (13).

Recent studies (14–16) investigating antiviral memory CD4 T cell responses have shown that the combined assessment of IL-2 and IFN-γ is instrumental to distinguish functionally distinct populations of memory CD4 T cells and patterns of antiviral immune responses associated with different conditions of virus persistence and control.

In the present study, we have performed functional and phenotypic characterization of antiviral CD8 T cell responses specific for HIV-1, CMV, EBV and influenza (flu) on the basis of their ability to proliferate, to secrete IL-2 and IFN-γ, and to express CD45RA and CCR7. Our results indicate: (*i*) a wide heterogeneity of antiviral CD8 T cell immune responses under different conditions of virus persistence; (*ii*) a combined loss of virus-specific IFN-γ/IL-2-secreting and -proliferating CD8 T cells in progressive HIV-1 infection; (*iii*) a typical phenotype of effector cells, i.e., CD45RA$^-$CCR7$^-$, for the IFN-γ/IL-2-secreting CD8 T cells; (*iv*) a correlation between the proportion of virus-specific IL-2-secreting and -proliferating CD8 T cells; and (*v*) the occurrence of Ag-specific CD8 T cell proliferation also in experimental conditions, excluding the involvement of Ag-specific helper CD4 T cells.

Materials and Methods

Study Groups. The 21 subjects with progressive chronic HIV-1 infection enrolled in this study were naïve to antiviral therapy, with CD4 T cell counts of >250 cells per microliter (mean ± SE: 810 ± 39) and plasma viremia counts of ≥5,000 HIV-1 RNA copies per ml (mean ± SE: 41,854 ± 12,339). Five HIV-1-infected patients with nonprogressive disease, i.e., LTNPs, as defined by documented HIV-1 infection for >14 years, stable CD4 T cell counts of >500 cells per microliter (mean ± SE: 912 ± 125) and plasma viremia of <1,000 HIV-1 RNA copies per ml (mean ± SE: 97 ± 38) were also included. Patient 1010 has a documented HIV-1 infection since March 1999. He was treated with antiviral therapy at the time of primary infection and remained on antiviral therapy for 18 months. He interrupted therapy spontaneously in December 2000. During the last 4 years, he constantly had levels of viremia of <50 HIV-1 RNA copies per ml and CD4 T cell count in the range of 1,400 cells per microliter. In addition, blood from 28 HIV-negative subjects was obtained from the local blood bank or from laboratory coworkers. The studies were approved by the Institutional Review Board of the Centre Hospitalier Universitaire Vaudois.

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Abbreviations: EBV, Epstein–Barr virus; CMV, cytomegalovirus; Ag, antigen; LTNP, long-term nonprogressor; CFSE, carboxyfluorescein succinimidyl ester; SEB, staphylococcal enterotoxin B.

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Synthetic Peptides and Tetramers. The following individual peptides were used: A2-restricted CMV pp65 (amino acids 495–503: NLVPMVATV) peptide (17), B7-restricted CMV pp65 (amino acids 415–429: TPRVTGGGAM) peptide (17), A2-restricted EBV BMLF1 (amino acids 259–267: GLCTLVAML) peptide (18), B8-restricted EBV EBNA3A (amino acids 325–333: FLRGRAYGL) peptide (18), B8-restricted EBV BZLF1 (amino acids 190–197: RAFKQLL) peptide (18), A2-restricted flu matrix 1 (amino acids 58–66: GILGFVFTL) peptide (19), A2-restricted HIV-1 pol (amino acids 476–484: ILKEPVHGV) (20), A2-restricted HIV-1 gag (amino acids 77–85: SLYNTVATL) (21), B8-restricted HIV-1 gag (amino acids 259–267: GEIYKRWII), (22) or B8-restricted HIV-1 nef (amino acids 89–97: FLKEKGGL) (23) peptides. Cells were stimulated with HIV-1 (strain IIIB) peptide pools. Each pool consisted of 50–62 15-mers peptides overlapping by 11 amino acids (Synpep, Dublin, CA). Pools 1–6 spanned the gag, pol, and nef sequence; pool 1: amino acids 1–230; pool 2: amino acids 220–432; pool 3: amino acids 421–655; pool 4: amino acids 645–879; pool 5: amino acids 871-1103; and pool 6: amino acids 1043–1326. CMV-, EBV-, or flu-derived peptides were used either all in a pool or grouped as virus-specific pools (24).

For tetramer stimulations, A2- and B7-restricted class I peptide tetramers were produced as described (25, 26).

Detection of IFN-γ and IL-2 Secretion. Cell stimulations were performed as described (14). For stimulation of CD8 T cells, individual peptides (5 μg/ml) or peptide pools (1 μg/ml for each peptide) were used. Cells were then stained with CD8-PerCP-Cy5.5, CD69-FITC, IFN-γ-APC and IL-2-PE (Becton Dickinson, Franklin, NJ). For phenotypic analysis, the following Abs were used in combination: Rat anti-human CCR7 (Becton Dickinson) followed by goat anti-rat IgG(H+L)-APC (Caltag, Burlingame, CA), CD8-Pacific blue (DAKO, Glostrup, Denmark), CD45RA-Biotin followed by anti-Streptavidin-PercP, anti-CD69-APC-Cy7, anti-IL-2-PE, and anti-IFN-γ-FITC (Becton Dickinson). Data were acquired on a FACScalibur or an LSR II and analyzed by using CELLQUEST and DIVA software (Becton Dickinson). The number of nongated events ranged between 10^5 and 10^6 events.

***Ex Vivo* Proliferation Assay.** After an overnight rest, cells were washed twice, resuspended at 1×10^6 ml in PBS, and incubated for 7 min at 37°C with 0.25 μM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes). The reaction was quenched with 1 volume of FCS, and cells were washed and cultured in the presence of anti-CD28 Ab (0.5 μg/ml) (Becton Dickinson). Cells were either stimulated with HIV-1 peptide pools (1 μg/ml of each peptide), individual peptides (5 μg/ml), or tetramers (0.31 μg/ml). Staphylococcal enterotoxin B (SEB) stimulation (200 ng/ml) served as positive control. Where indicated, 10% exogenous IL-2 (Roche, Basel) was added 48 h after peptide stimulation. For neutralization experiments, anti-IL-2-neutralizing Ab or isotype control Ab (Becton Dickinson) were added at 10 μg/ml. At day 5, cells were harvested and stained with CD4-PE-Cy5 (Becton Dickinson) and CD8-APC (Becton Dickinson). Cells were fixed with CellFix (Becton Dickinson) and acquired ($1–8 \times 10^5$ nongated events) on a FACScalibur (Becton Dickinson).

CD4 T Cell Depletion. CFSE-labeled cells were stained with CD4-APC and sorted by using a FACS Vantage (Becton Dickinson). The purity of the CD4-depleted cell populations was 99%.

Statistical Analysis. Statistical significance (P values) of the results was calculated by using a two-tailed Student t test. A two-tailed P value of <0.05 was considered significant. The correlations among variables were tested by simple regression analysis.

Results

Distinct Cytokine Secreting Populations of Virus-Specific CD8 T Cells. We used different models of virus-specific CD8 T cell responses, including HIV-1-, CMV-, EBV-, and flu-specific CD8 T cell responses. Based on the observation that functionally distinct Ag-specific CD4 T cell populations are defined by the secretion of IL-2 and IFN-γ (14–16), we performed functional characterization of virus-specific CD8 T cell responses by simultaneous assessment of IFN-γ and IL-2 secretion after Ag-specific stimulation. Representative examples obtained from the analysis of 21 HIV-1-infected progressors and 28 HIV-negative blood donors in whom CMV-, EBV-, or flu-specific CD8 responses were detected are shown in Fig. 1*A*. The dual IFN-γ/IL-2-secreting T cells were absent in HIV-1-specific CD8 T cells, whereas they were found within CMV-, EBV-, and flu-specific CD8 T cells (Fig. 1*A*). These observations were confirmed by the analysis of a larger number of subjects. A significant difference was found between the percentage of HIV-1-specific IFN-γ/IL-2-secreting cells in progressive HIV-1 infection and that found in the virus-specific IFN-γ/IL-2-secreting CD8 T cells ($P < 0.05$) of the other virus infections (Fig. 1*B*). We also evaluated the proportion of IL-2-secreting cells within IFN-γ-secreting CD8 T cells. Cumulative data of this analysis are shown in Fig. 1*C*. The proportion of CMV-specific ($12.7 \pm 1.8\%$, $n = 11$) and EBV-specific ($19.2 \pm 3.2\%$, $n = 10$) IL-2-secreting CD8 T cells was significantly higher ($P < 0.05$) compared with that of HIV-1-specific IL-2-secreting CD8 T cells ($2.3 \pm 0.6\%$, $n = 21$) (Fig. 1*C*). The proportion ($25.6 \pm 3.6\%$, $n = 7$) of flu-specific IL-2-secreting CD8 T cells was significantly higher ($P < 0.05$) compared with that

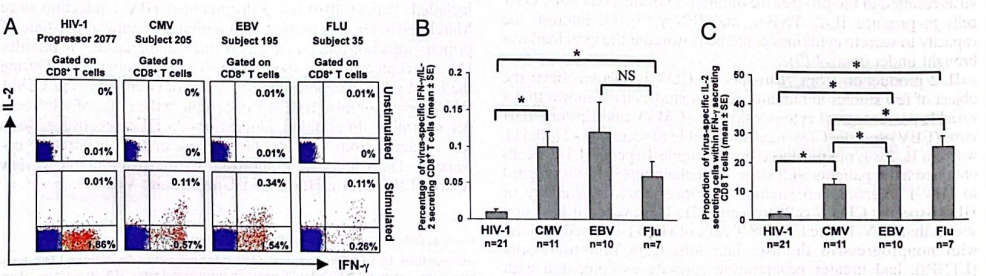

Fig. 1. Analysis of different virus-specific IFN-γ- and IL-2-secreting CD8 T cells after stimulation with single peptides. (*A*) Distribution of IFN-γ- and IL-2-secreting virus-specific CD8 T cells. Cells were stimulated with single peptides. One representative profile is shown for HIV-1-, CMV-, EBV-, or flu-specific CD8 T cell responses. The cluster of events shown in red corresponds to the responder CD8 T cells, i.e., secreting IFN-γ or IL-2, and the blue clusters correspond to the nonresponder cells. (*B*) Cumulative data on the percentage (mean ± SE) of IFN-γ/IL-2-secreting cells within the different virus-specific CD8 T cell responses. (*C*) Cumulative data on the proportion (mean ± SE) of IL-2-secreting cells within IFN-γ-secreting CD8 T cells. *, $P < 0.05$.

of HIV-1- and CMV-specific but not with that of EBV-specific IL-2-secreting CD8 T cells (Fig. 1*C*). Finally, the proportion of EBV-specific IL-2-secreting cells was also significantly higher compared with that of CMV-specific IL-2-secreting CD8 T cells ($P < 0.05$) (Fig. 1*C*). CMV-, EBV-, and flu-specific CD8 T cell responses were also studied in HIV-1-infected individuals either by using peptides specific to CMV and EBV ($n = 7$) and flu ($n = 6$) or a pool of 21 CMV-, EBV-, and flu-derived peptides in 30 HIV-1-infected subjects. The proportion of CMV-, EBV-, or flu-specific IL-2-secreting CD8 T cells in HIV-1-infected subjects was not significantly different from that observed in HIV-negative subjects ($P > 0.05$).

To exclude the possibility that the lack of detection of HIV-1-specific IFN-γ/IL-2-secreting CD8 T cells was specific of the response to certain peptides, we performed stimulation with peptide pools spanning gag, pol, and nef proteins of HIV-1. A representative flow cytometry profile of one (of 21) HIV-1-infected subjects with progressive disease (progressors) is shown in Fig. 2*A*. Despite the presence of HIV-1-specific IFN-γ-secreting CD8 T cells after stimulation with different HIV-1 peptide pools, IL-2-secreting CD8 T cells were not detected (Fig. 2*A*).

Previous studies (12) have shown that HIV-1-specific CD8 T cells of LTNPs, but not of progressors, proliferated in response to Ag-specific stimulation (12). The evaluation of the presence of HIV-1-specific IFN-γ/IL-2-secreting CD8 T cells in three of five representative LTNPs showed variable intensities of the response to the different peptide pools (Fig. 2*B*). HIV-1-specific IFN-γ-secreting CD8 T cells were detected consistently after stimulation with different peptide pools (Fig. 2*B*), and a substantial percentage of dual IFN-γ/IL-2-secreting cells was also found after stimulation with peptide pools 1 and 2 (Fig. 2*B*). The percentage (0.13 ± 0.04, $n = 5$) of IFN-γ/IL-2-secreting cells in LTNPs was significantly different ($P = 0.0003$) compared with progressors (0.01 ± 0.002, $n = 21$).

Phenotypic Analysis of Cytokine-Secreting Virus-Specific CD8 T Cells. Previous studies in humans and mice have shown that IL-2-secreting CD8 T cells were contained within the $CCR7^+$ central memory CD8 T cell population, whereas the IFN-γ-secreting CD8 T cells were contained within the $CCR7^-$ effector CD8 T cells (8, 27). Blood mononuclear cells of LTNPs and HIV-negative donors with known HIV-1, flu, or CMV CD8 T cell responses were stimulated with the appropriate virus-derived peptides, and cells were stained with CD8, CD45RA, CCR7, IL-2, IFN-γ, and CD69 Abs. The results obtained indicated that the virus-specific IFN-γ/IL-2 CD8 T cells were contained within the $CD45RA^-CCR7^-$ effector cell population and the IFN-γ-secreting CD8 T cells within the $CD45RA^-CCR7^-$ and $CD45RA^+CCR7^-$ effector cell populations (Fig. 3). These results were representative of the analysis of two LTNPs and seven HIV-negative subjects.

Proliferation Capacity of Virus-Specific CD8 T Cells. Recent studies (12, 13) have shown the loss of proliferation capacity of HIV-1-specific CD8 T cells of subjects with progressive disease, whereas HIV-1-specific CD8 T cell proliferation was retained in CD8 T cells of LTNPs. Based on these observations, it has been proposed that Ag-specific CD8 T cell proliferation represents a characteristic of effective and protective immune response (12). Furthermore, it has been proposed that the loss of HIV-1-specific CD8 T cell proliferation depended on the loss of HIV-1-specific CD4 helper T cells (13). In the present study, we decided to investigate (*i*) the correlation between the ability of virus-specific CD8 T cells to secrete IL-2 and their proliferation capacity and (*ii*) the potential mechanism responsible for Ag-specific CD8 T cell proliferation. Representative examples of the proliferation capacity of CMV-, EBV-, flu-, and HIV-1-specific CD8 T cells after virus-specific stimulation are shown in Fig. 4 *A–C*. Cells were labeled with CFSE, stimulated for 5 days with virus-derived peptides, and virus-specific CD8 T cell

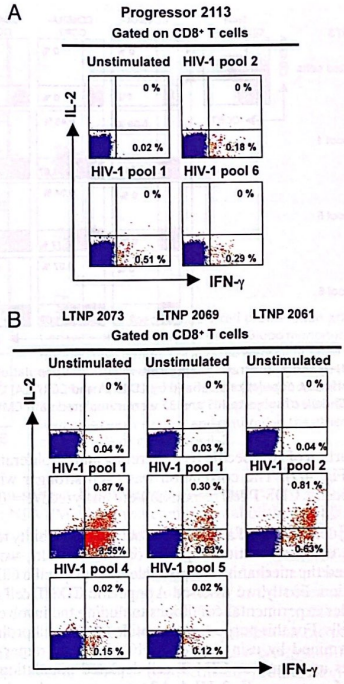

Fig. 2. Analysis of HIV-1-specific IFN-γ- and IL-2-secreting CD8 T cells in progressors and LTNPs after stimulation with peptide pools. Flow cytometry profiles of IFN-γ- and IL-2-secreting HIV-1-specific CD8 T cells of progressor 2113 (*A*) and three different LTNPs (*B*) after stimulation of blood mononuclear cells with different peptide pools spanning gag, pol, and nef proteins.

proliferation was measured by the loss of CFSE in the dividing CD8 T cells. A substantial proportion of CD8 T cells of subject 248 proliferated after stimulation with CMV- and Flu-derived peptides (Fig. 4A). Similarly, CD8 T cells of subject 359 proliferated after stimulation with two different EBV-derived peptides (Fig. 4*A*). We then determined the proliferation of HIV-1-specific CD8 T cells after stimulation with HIV-1-derived peptide pools in progressors ($n = 9$) and LTNPs ($n = 5$). HIV-1-specific CD8 T cell proliferation was barely detected or was absent in these two representative progressors [two of nine patients each tested with one to three pools (16 responses were tested in total)] (Fig. 4*B*). However, CD8 T cells of progressors were able to proliferate after SEB stimulation (Fig. 4*B*), thus indicating a selective loss of HIV-1-specific proliferation. Consistent with results previously shown by Migueles *et al.* (12), vigorous HIV-1-specific CD8 T cell proliferation was observed in two of five representative LTNPs (Fig. 4*C*). The mean ± SE percentage of HIV-1-specific CD8 T cell proliferation in progressors was 0.45 ± 0.16 compared with 6.88 ± 1.69 in LTNPs ($P < 0.00001$).

We then determined the correlation between the proportion of Ag-specific proliferating CD8 T cells and the proportion of IL-2-secreting CD8 T cells within IFN-γ-secreting cells. This analysis was performed by pooling together 32 individual determinations from 21 subjects of Ag-specific CD8 T cell-proliferating and IL-2-secreting CD8 T cells. We found a significant correlation between

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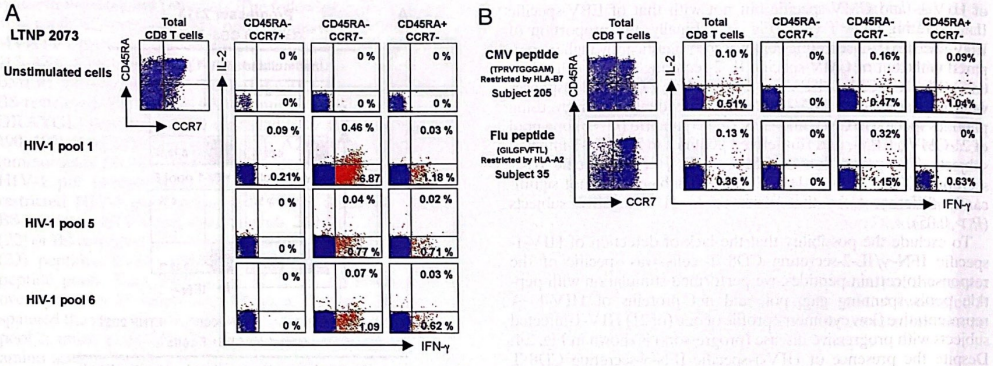

Fig. 3. IFN-γ- and IL-2-secreting CD8 T cells in different populations defined by CD45RA and CCR7. Shown is the distribution of IFN-γ- and IL-2-secreting CD8 T cells in different populations defined by CD45RA and CCR7. (*A*) Cells of LTNP 2073 were stimulated with different peptide pools spanning gag, pol, and nef proteins. (*B*) Cells of subjects 205 and 35 were stimulated with CMV or flu peptides, respectively.

the proportion of Ag-specific IL-2-secreting and -proliferating CD8 T cells (Fig. 4*D*). The correlation was even stronger when only HIV-1-specific CD8 T cell responses were analyzed ($R = 0.53, P < 0.01, n = 24$).

Having demonstrated a correlation between the ability to secrete IL-2 and the proliferation capacity of CD8 T cells, we further investigated the mechanism responsible for Ag-specific CD8 T cell proliferation. Firstly, we assessed Ag-specific CD8 T cell proliferation under experimental conditions excluding the involvement of CD4 T cells. For this purpose, Ag-specific CD8 T cell proliferation was determined by using either MHC class I tetramer–peptide complexes as stimuli or CD4 T cell-depleted populations in the absence of exogenous IL-2. HLA-A2 tetramer complexed with flu- and CMV-derived peptides induced vigorous Ag-specific proliferation of CD8 T cells of subjects 172 and 180 (Fig. 5*A*). It is important to underscore that no CD4 T cell proliferation was observed (Fig. 5*A*), thus indicating that Ag-specific CD8 T cell proliferation was not associated with the stimulation of Ag-specific helper CD4 T cells. Consistent with the observations previously reported (12, 13), HIV-1-specific CD8 T cell proliferation was barely detected in progressors after stimulation with the HLA-A2 tetramer complexed with an HIV-1 pol ILKEPVHGV-derived peptide (20) (Fig. 5*B*). Of interest, in agreement with the work of Lichterfeld *et al.* (13), HIV-1-specific CD8 T cell proliferation was recovered in the presence of exogenous IL-2 (Fig. 5*B*). No proliferation was observed in CD4 T cells after MHC class I tetramer–peptide complex

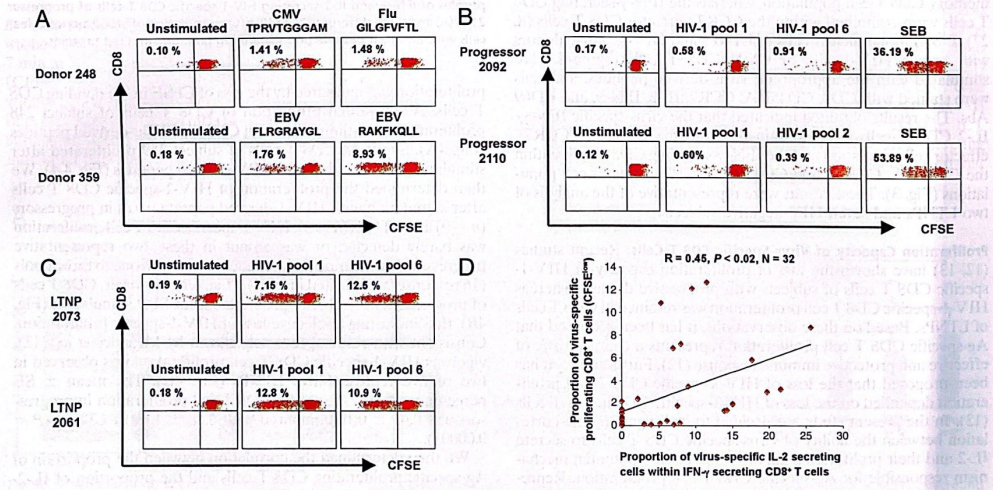

Fig. 4. Virus-specific CD8 T cell proliferation after stimulation with single peptides or peptide pools. (*A*) CFSE-labeled cells of HIV-negative donors 248 and 359 were stimulated with CMV-, flu-, or EBV-derived peptides. Profiles of proliferating cells, i.e., CFSE low cells, are gated on CD8 T cells. (*B*) HIV-1-specific CD8 T cell proliferation in HIV-1 progressors after stimulation with different HIV-1 peptide pools or SEB. (*C*) HIV-1-specific CD8 T cell proliferation in LTNPs after stimulation with different HIV-1 peptide pools. (*D*) Correlation between the proportion of IL-2-secreting and -proliferating virus-specific CD8 T cells.

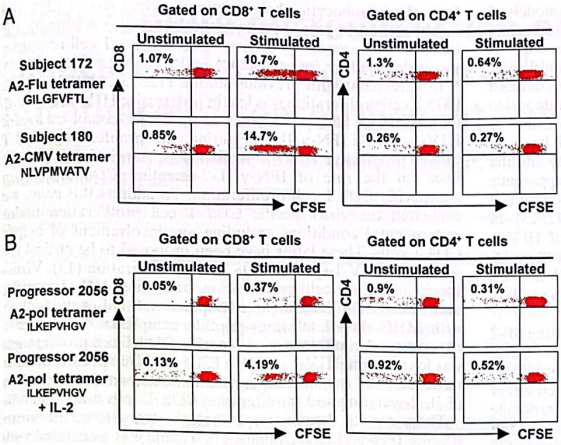

Fig. 5. Virus-specific CD8 T cell proliferation after stimulation with HLA class I tetramers. (*A*) Blood mononuclear cells of HIV-negative donors 172 and 180 were stimulated with A2-flu or -CMV tetramers, respectively. Flow cytometry profiles of proliferating CD8 (*Left*) and CD4 (*Right*) T cells are shown. (*B*) Blood mononuclear cells of progressor 2056 were stimulated with an A2-pol tetramer and cultured in the absence or presence of 10% of exogenous IL-2.

stimulation (Fig. 5*B*). To further confirm the hypothesis that HIV-1-specific CD8 T cell proliferation was independent of CD4 helper T cells, we compared the HIV-1-specific CD8 T cell proliferation in response to the p24-derived GPGHKARVL peptide that has been previously characterized as a CD8 epitope (17) restricted by HLA-B7. Unfractionated blood mononuclear cells or CD4 T cell-depleted populations of patient 1010 with chronic HIV-1 infection were stimulated with the peptide GPGHKARVL. As reported in *Materials and Methods*, patient 1010 had constantly controlled viremia since 4 years after interruption of antiviral therapy. A large percentage (59%) of HIV-1-specific CD8 T cells proliferated after stimulation of unfractionated cell populations with the p24 peptide (Fig. 6*A*). Substantial HIV-1-specific CD8 T cell proliferation (32.7%) occurred also in the CD4 T cell-depleted populations although it was reduced (45% reduction) compared with the cell cultures containing CD4 T cells (Fig. 6*A*). It is important to underscore the fact that the CD8 T cell proliferation in the CD4-depleted cell populations was not due to contaminating CD4 T cells because CD4 T cells were almost absent (0.6%) in the CD4-depleted cell populations at day 5 (Fig. 6*A*). The experiments shown in Fig. 6*A* were performed in the absence of exogenous IL-2. Secondly, Ag-specific CD8 T cell proliferation was assessed in the presence of anti-IL-2 Ab. The substantial proliferation of CD8 T cells from subject 180 observed after stimulation with the CMV tetramer NLVPMVATV was completely abolished (95% inhibition of proliferation) in the presence of anti-IL-2 Ab (Fig. 6*B*). Therefore, virus-specific CD8 T cell proliferation, including HIV-1-specific proliferation, depends on IL-2 and on the presence of the IFN-γ/IL-2 CD8 T cells, and may occur in the absence of helper CD4 T cells. The finding that CD8 T cell proliferation was independent of CD4 T cell help and dependent on the presence of IFN-γ/IL-2-secreting CD8 T cells was also confirmed for CMV- and EBV-specific CD8 T cell-mediated proliferation in three HIV-negative subjects (data not shown).

Discussion

In the present study, we have investigated the function and phenotype of memory CD8 T cells in different models of virus-specific T cell responses, including HIV-1, CMV, EBV, and flu. HIV-1-specific CD8 T cell responses were studied in subjects with progressive and nonprogressive infection who were naïve to therapy. The other virus-specific CD8 T cell responses were analyzed in HIV-negative donors. Functional characterization was performed by the measurement of the ability of CD8 T cells to proliferate and to secrete IFN-γ and IL-2 after Ag-specific stimulation.

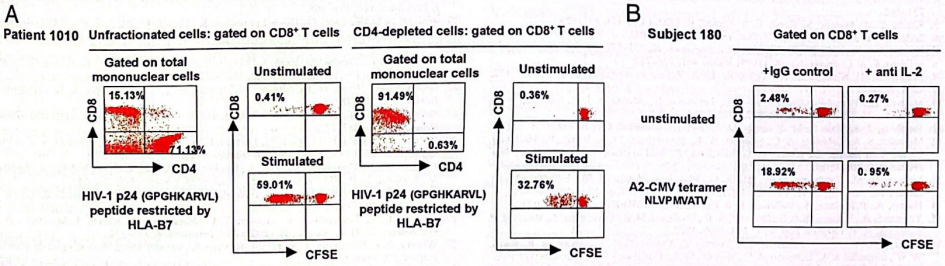

Fig. 6. Virus-specific CD8 T cell proliferation in CD4-depleted cells or after neutralization of IL-2. (*A*) CD8 T cell proliferation was evaluated in CD4 T cell-depleted populations stimulated with HIV-1-derived peptide. The purity of the sorted $CD4^-$ T cell populations was 99%. (*B*) Inhibition of virus-specific CD8 T cell proliferation with anti-IL-2 Ab. Cells of subject 180 were stimulated with an A2-restricted CMV tetramer and cultured in the presence of anti-IL-2 or isotype control Abs.

Most studies performed on CD8 T cells in different models of antiviral responses in both mice and humans were predominantly focused on the characterization of effector functions such as perforin and granzyme expression or secretion of IFN-γ and TNF-α (9–11). Recently, a series of studies have shown the importance of investigating other functions such as the ability to proliferate and to secrete IL-2 (14–16) that have generally been the object of extensive investigation in CD4 T cells. With regard to CD8 T cells, it has been shown that the preservation of the proliferation capacity and the ability to secrete IL-2 were generally associated with an apparently effective immune response because virus replication was controlled in both mouse and human models of virus infection (12, 28). In addition, a recent study has shown a paralleled loss of HIV-1-specific helper CD4 T cells and HIV-1-specific CD8 T cell proliferation, and concluded that HIV-1-specific helper CD4 T cells are critical for the maintenance of HIV-1-specific proliferating CD8 T cells (13).

This is the first study, to our knowledge, investigating IL-2 secretion in HIV-1-specific CD8 T cells. In addition, it compares the function of HIV-1-specific CD8 T cells with that of CMV-, EBV-, and flu-specific CD8 T cells that are able to keep either on check (CMV and EBV) or clear (flu) the virus. The rationale for studying antiviral CD8 T cell responses in different models of virus persistence resides on recent studies (28) performed in mice, demonstrating that the function of CD8 T cells was modulated by different conditions of Ag levels and/or persistence. HIV-1 infection in subjects with progressive disease corresponded to the model of immune failure with Ag persistence and high Ag levels. CMV, EBV, and HIV-1 infection in subjects with nonprogressive disease corresponded to the model of immune control with protracted virus persistence and low Ag levels and flu to the model of Ag clearance. Our results demonstrated the presence of an Ag-specific IFN-γ/IL-2-secreting CD8 T cell population in the models of virus infections associated with resolved virus infection or with virus control, i.e., CMV, EBV, and nonprogressive HIV-1 infection or virus clearance, i.e., flu. This cell population was absent in progressive HIV-1 infection. Therefore, we provided evidence for (*i*) a loss of IFN-γ/IL-2-secreting CD8 T cells in progressive HIV-1 infection and (*ii*) a skewed representation of functionally distinct memory HIV-1-specific CD8 T cells in progressive HIV-1 infection. The present results showed that the same pathogen, i.e., HIV-1, can be associated with substantially different CD8 T cell responses in progressive and nonprogressive infection where the major difference between these two conditions was indeed represented by Ag levels. Therefore, along with the observation from the lymphocytic choriomeningitis virus model (28), our results rather supported the hypothesis that also in humans the functional heterogeneity of virus-specific CD8 T cell responses was influenced by Ag persistence and Ag levels.

In agreement with previous studies (12, 13), HIV-1-specific CD8 T cell proliferation was lost in progressive HIV-1 infection. Of interest, we have provided evidence for the combined loss of HIV-1-specific IFN-γ/IL-2-secreting and -proliferating CD8 T cells in progressive HIV-1 infection. This association raised the issue on the role of IFN-γ/IL-2-secreting CD8 T cells in Ag-specific CD8 T cell proliferation. To address this issue, we evaluated the virus-specific CD8 T cell proliferation under experimental conditions excluding any involvement of helper CD4 T cells. These latter have been proposed to be critical for sustaining HIV-1-specific CD8 T cell proliferation (13). Virus-specific CD8 T cell proliferation, including HIV-1-specific, occurred in CD4 T cell-depleted populations or after stimulation with MHC class I tetramer–peptide complexes. Under these experimental conditions, virus-specific CD8 T cell proliferation was found in the HIV-1-, CMV-, EBV- and flu-specific immune responses, and a significant correlation between the proportion of IL-2-secreting and -proliferating CD8 T cells was observed.

These results demonstrated that the persistence of virus-specific IFN-γ/IL-2-secreting CD8 T cells was associated with the persistence of CD8 T cell proliferation. Virus-specific CD8 T cell proliferation was supported by IL-2 because it was completely abolished in the presence of the anti-IL-2 Ab. Therefore, taken together, they indicate that IFN-γ/IL-2-secreting CD8 T cells are able to promote CD8 T cell proliferation through the secretion of IL-2 even in the absence Ag-specific helper CD4 T cells. Despite the demonstration *in vitro* of a CD4-independent CD8 T cell proliferation, it is important to underscore that Ag-specific helper CD4 T cells are crucial *in vivo* for the maintenance and for preventing impairment of optimal CD8 T cell function (29). Of interest, this CD4-indepndent proliferation capacity was present in the effector, i.e., CD45RA$^-$CCR7$^-$ cell population. The importance *in vivo* of this CD4-independent proliferation capacity of effector CD8 T cells during the expansion phase of the immune response remains to be determined.

These results represent a further step in the understanding of the functional characterization of virus-specific CD8 T cell responses and in the understanding of the impairment of CD8 T cell functions in progressive HIV-1 infection.

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