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IL-2 Production by Virus- and Tumor-Specific Human CD8 T Cells Is Determined by Their Fine Specificity

Eric Mallard,* Frédérique Vernel-Pauillac,* Thierry Velu,† Frédéric Lehmann,† Jean-Pierre Abastado,§ Margarita Salcedo,§ and Nadège Bercovici²*

Memory CD8 T cells mediate rapid and effective immune responses against previously encountered Ags. However, these cells display considerable phenotypic and functional heterogeneity. In an effort to identify parameters that correlate with immune protection, we compared cell surface markers, proliferation, and cytokine production of distinct virus- and tumor-specific human CD8 populations. Phenotypic analysis of epitope-specific CD8 T cells showed that Ag specificity is associated with distinct CCR7/CD45RA expression profiles, suggesting that Ag recognition drives the expression of these molecules on effector/memory T cells. Moreover, the majority of central memory T cells (CD45RAlowCCR7null) secreting cytokines in response to an EBV epitope produces both IL-2 and IFN-γ, whereas effector memory CD8 cells (CD45RAnullCCR7−) found in EBV, CMV, or Melan-A memory pools are mostly composed of cells secreting exclusively IFN-γ. However, various subsets, including Melan-A-specific effector memory cells differentiated in cancer patients, display similar Ag-driven proliferation in vitro. Our findings show for the first time that human epitope-specific CD8 memory pools differ in IL-2 production after antigenic stimulation, although they display similar intrinsic proliferation capacity. These results provide new insights in the characterization of human virus- and tumor-specific CD8 lymphocytes. The Journal of Immunology, 2004, 172: 3963–3970.

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The increased size of Ag-specific T cell pool and the selection of T cells with higher affinity contribute to the efficiency of secondary immune responses (2, 3). On a per-cell basis, memory CD8 T cells may have enhanced capacity to proliferate and improved cytokine production and cytotoxic activity in response to Ag compared with naïve CD8 T cells (4, 5). Overall, the pool of human memory T cells circulating in peripheral blood contains subsets with distinct functional potentials (6). Obviously, this observation may be different when looking at T cells with defined Ag specificity. Some T cell clones might represent a readily available population of effector cells, displaying inflammatory and cytotoxic functions, whereas others may persist as progenitor cells, homing to lymph nodes, helping B cells and dendritic cells (DC), and generating new waves of effector cells. CCR7 and CD62L molecules are expressed on central memory T cells (T_{CM} cells) but not on effector memory T cells (T_{EM} cells) and may confer distinct patterns of migration and homing after resolution of a primary immune response (6). In line with this model, memory T cell subsets may also differ in terms of survival or proliferation potential, as central memory cells are supposed to expand upon secondary challenge to renew the memory T cell pool. Even though the role in vivo of these different subsets remains controversial, the use of these markers has triggered discussions about T lymphocyte subsets and differentiation pathways.

One important step to move forward was to identify the proposed memory subsets for T cells with known antigenic specificity. Recently, different groups have initiated a characterization of virus-specific CD8 T cells, combining tetramers to CCR7 and CD45RA markers and in vitro functional assays such as assessment of cytokine production (7–11). These studies have underlined the heterogeneity of epitope-specific CD8 T cells in terms of cell surface and effector molecules. In particular, recent reports have provided evidence that both CCR7null and CCR7− CD8 T cells recognizing the same epitope can secrete IFN-γ ex vivo (12–14) in contrast with previous experiments (6, 7). Thus, further investigations are clearly required to identify and classify Ag-specific memory pools.

The diversity of cytokines produced by human memory CD8 T cells remains poorly described (10), notably the production of cytokines such as IL-2. Interestingly, Wherry et al. (15) reported recently that only T_{CM} CD8 cells produced IL-2 following a short peptide stimulation in lymphocytic choriomeningitis virus (LCMV) mouse model. The proliferation potential of various human memory CD8 subsets is also not clear. Initial experiments

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2 Abbreviations used in this paper: DC, dendritic cell; T_{EM} cell, effector memory T cell; T_{CM} cell, central memory T cell; LCMV, lymphocytic choriomeningitis virus; PF, precursor frequency; PMD, precursor mean division; MFI, mean fluorescence intensity.
conducted by Sallusto et al. (6) did not provide evidence for strong differences in the proliferation of human T\textsubscript{CM} and T\textsubscript{EM} CD4 cells, although T\textsubscript{EM} cells consistently demonstrated a slightly higher proliferative response. Further studies conducted with HIV-specific CD8 T cells in particular, showed that CCR7\textsuperscript{+} cells contained a higher fraction of cells engaged in cell cycle, suggesting that T\textsubscript{EM} cells were more prone to proliferate (16). In contrast, van Leeuwen et al. (17), by measuring proliferation with CFSE labeling, have provided evidence that CMV-specific T\textsubscript{EM} cells can expand massively in vitro upon viral challenge. Nevertheless, no quantitative assessment of proliferation potentials has been conducted in parallel in different CD8 T cell subsets upon in vitro stimulation. Recent work on cohorts of patients infected by HIV showed that proliferation of memory CD8 T cells specific for HIV was reduced in vitro in HIV progressors compared with nonprogressors, suggesting that expansion potential of memory CD8 T cell subsets may constitute a key issue to control pathogens (18).

Regarding tumor-specific T cells, CCR7\textsuperscript{+} CTL have been identified among Melan-A, MAGE-3, gp100, NY-ESO-1, or tyrosinase-specific CD8 populations whether they have differentiated spontaneously or after active immune therapies in melanoma patients (19–22). In particular, Valmori et al. (23) have shown that CD8 PBLs specific for a tyrosinase-derived epitope display a CCR7\textsuperscript{+}CD45RA\textsuperscript{+} profile. Even if the number of reports focusing on the phenotype of tumor-specific T cells is limited, to our knowledge, T\textsubscript{CM} CD8 cells specific for tumor epithiles have been detected in tumor-free and also in some tumor-invaded lymph nodes (22) but not in peripheral blood. Functional analyses have shown that tumor-specific T cells can exert ex vivo functions: cytokine secretion (19, 24) as well as cytotoxic activity (23). By contrast, Lee et al. (25) have also characterized unresponsive tyrosinase-specific T cells. Few studies have compared tumor-specific T cells with virus-specific T cells (21, 22), although of great interest to estimate the extent of effector functions needed by tumor-specific T cells to be fully protective.

The present work compares different populations of memory CD8 T cells specific for viral epitopes in their capacity to proliferate and to produce IFN-\gamma and IL-2 ex vivo. We asked whether particular T cell subsets can be identified and how these relate to the CCR7 and CD45RA expression profiles. We further extended this analysis to memory CD8 T cells specific for the differentiation Ag Melan-A in two stage IV melanoma patients to identify potential differences between virus- and tumor-specific CD8 T cell populations.

Materials and Methods

Peptides and tetramers

The following different peptides, presented by HLA-A*0201 molecules, were used; CMV pp65 (NLVPVMATV, 495–503; Nesyosystem, Strasbourg, France), Melan-A (ELAGIGILTV, 26–35 (27L); Nesyosystem), EBV BMLF1 (GLCTLVAML, 280–288; Cybergene, Huddinge, Sweden), EBV LMP2a (CGLGLLTMV, 426–434; Cybergene), and influenza matrix protein (GILGFVFTL, 58–66; Cybergene). PE-labeled HLA-A*0201 tetramers contained the following peptides: Melan-A 26–35 (27L), EBV BMLF1 280–288, EBV LMP2a 426–434, CMV pp65 495–503 and influenza matrix protein 58–66, and were purchased from Biolegend (San Diego, CA), and Beckman Coulter Immunobics (San Diego, CA), as well as PE-labeled A*0201/HIV gag (SYNTIYATTL) tetramers, used as a negative control.

Healthy volunteers and patients samples

Apheresis were performed from HLA-A*0201 healthy volunteers recruited at Cochin Hospital (Paris, France) through advertisement. Melanoma patients were included in a phase II/II clinical trial (V. Baron-Bodo, S. Mas-sicard, E. Mallard, A. Lim, D. Duriau, F. Lehmann, P. Vereecken, T. Velu, P. Kourilsky, J. P. Abastado, et al., manuscript in preparation).

DC differentiation

DC were differentiated using the VacCell processor (Immunod差-Related Molecules, Paris, France) as previously described (26). Briefly, PBMC were differentiated from monocytes with a 7-day in vitro culture in serum-free Immunod-Related Molecules VacCell medium (Life Technologies, Paisley, U.K.) supplemented with 500 U/ml GM-CSF (Novartis Pharma, Basel, Switzerland) and 50 ng/ml IL-13 (Sanofi-Synthelabo, Paris, France). DC were then isolated by elutriation. Purity ranged from 80 to 99%; viability was >95%. DC were frozen in 4% human serum albumin with 10% DMSO (Sigma-Aldrich, St. Louis, MO) and stored in liquid nitrogen until use.

CD8\textsuperscript{+} cell isolation

CD8\textsuperscript{+} cells were purified from PBMC by positive selection using CD8\textsuperscript{+} microbeads (Miltenyi Biotec, Paris, France) according to the manufacturer’s instructions. Purity determined by flow cytometry was >90% CD3\textsuperscript{+}CD8\textsuperscript{+} among alive cells. Purified CD8\textsuperscript{+} cells were frozen in FCS with 10% DMSO and stored in liquid nitrogen until use.

Tetramer staining and immune phenotyping

Staining for analysis by flow cytometry was performed in FACS buffer (PBS containing 2% FCS and 0.02% NaN\textsubscript{3}). Cells were incubated with tetramers for 20 min at 37°C, and fixed for another 10 min at 4°C with the following mAbs: anti-CD3 conjugated with allophycocyanin (UCHT-1; Immunotech, Marseille, France); anti-CD4 FITC (13B8.2; Immunotech) or PerCP (SK3; BD Biosciences, San Jose, CA); anti-CD8 FITC, PE (B9.11; Immunotech); or PerCP (SK1; BD Biosciences); anti-CD14 allophycocyanin (RM052; Immunotech); anti-CD19 allophycocyanin (J4119; Immunotech); anti-CD68 allophycocyanin (22-11001; Immuno-Bio); or anti-CD54RA FITC (ALB11; Immunotech) or allophycocyanin (MEM56; Caltag, Burlingame, CA); or matched isotype controls. For CCR7 staining, tetramer-stained cells were incubated with 3 μg of purified anti-CCR7 mAb (mouse IgG1 clone 6B3; ebioscience, San Diego, CA) for 20 min at 4°C. After washing, cells were incubated for 15 min at 4°C with a goat anti-mouse IgG1 FITC mAb (Southern Biotechnology Associates, Birmingham, AL), washed, and stained for an additional 15 min at 4°C with anti-CD8 PerCP and anti-CD45RA allophycocyanin mAb or their isotype controls. Cells were then fixed in FACS buffer after final washing and stained with 3 ml TM TO-PRO-3 (Molecular Probes, Leiden, The Netherlands) when cells were not stained with Ab conjugated with allophycocyanin. At least 200,000 CD8\textsuperscript{+} lymphocytes were acquired on a FACSCalibur with CellQuestPro software (BD Biosciences). The specificity of tetramer staining was controlled with HIV gag tetramer. Cells stained by HIV gag tetramer represented always <0.02% of alive CD8\textsuperscript{+} lymphocytes.

Intracellular cytokine staining

CD8\textsuperscript{+} cells were thawed and left overnight at 37°C in Iscove’s medium, supplemented with 10% human AB serum, 1% penicillin-streptomycin, amino-acids l-glutamine (BioWhittaker, Verviers, Belgium), l-arginine, and l-asparagine (Sigma-Aldrich). On the day of the assay, 10\textsuperscript{5} CD8\textsuperscript{+} cells were stimulated with the appropriate peptide (10 μg/ml) or in absence of any additive, at a final concentration of 5 × 10\textsuperscript{5} cells/ml, in 5-ml polypropylene tubes, at 37°C, with 10 μg/ml brefeldin A (Sigma-Aldrich) added after 90 min. After a 6-h stimulation, nonstimulated CD8\textsuperscript{+} cells were stained with the relevant tetramer, followed by staining with anti-CD8 PerCP. Then, cells were washed in FACS buffer, fixed, and permeabilized with 50 μl of FACS permeabilizing solution 2 (BD Biosciences) for 10 min at room temperature. The cytokine staining was performed for 25 min at room temperature with anti-IFN-\gamma FITC (clone B27), anti-IL-2 allophycocyanin (clone MQ1-17H12), or their corresponding isotype controls (BD Pharmingen, San Diego, CA); cells were washed and fixed with 1% paraformaldehyde (Prolabo, Nogent-sur-Marne, France). Cells were analyzed by flow cytometry the day after.

To cope with the low frequency of secreting cells, we delineate the IFN-\gamma- IL-2\textsuperscript{+}, and IFN-\gamma–IL2\textsuperscript{+} populations with three gates set on the nonstimulated sample. For the double-negative IFN-\gamma–IL-2\textsuperscript{+} cells, the gate was delineated as follows: the x- and y-axes were successively defined in order to select the negative IFN-\gamma population (and then IL-2\textsuperscript{+} population) represented 99% of the total CD8\textsuperscript{+} cells. The square, thus defined, was corrected to exclude the highest fluorescent cells from the double-negative IFN-\gamma–IL-2\textsuperscript{+} population, merging into the gate.

The gate for the single-negative IFN-\gamma– (or respectively IL-2\textsuperscript{+}) populations was delineated as follows: y-axis (respectively x-axis) was the border of the double-negative IFN-\gamma–IL2\textsuperscript{+} population; the other axis was defined in order that the single-positive IFN-\gamma– (respectively IL-2\textsuperscript{+})...
population represented at most 0.01% of total CD8\(^+\) cells of the nonstimulated sample.

In that way, we consider detecting the following: 1) a single-secreting population when its frequency among CD8 cells is >0.02%; and 2) a double-secreting population when it represents >5% of total single-positive cells, once background is subtracted.

**PKH dilution assay**

DC were thawed in VacCell medium supplemented with 1% penicillin-streptomycin and pulsed with the appropriate peptide (10 \mu g/ml) and \(\beta_2\)-microglobulin (5 \mu g/ml; Sigma-Aldrich) or in the absence of any additive. After loading overnight, DC were treated for 30 min at 37°C with 50 \mu g/ml mitomycin C (Sigma-Aldrich) and washed carefully. Purified CD8\(^+\) cells were labeled with PKH67 (Sigma-Aldrich) according to manufacturer’s instructions. Labeled cells were cultured with unpulsed or peptide-pulsed DC in the presence of exogenous cytokines IL-2 (10 U/ml) and IL-7 (5 ng/ml; R&D Systems, Abingdon, U.K.). On day 7, cultured CD8 cells were harvested, stained with tetramers, anti-CD8 mAb, and TO-PRO-3, and analyzed by flow cytometry. The precursor frequencies (PF) and precursor mean divisions (PMD) among viable tetramer CD8\(^+\) cells were calculated with ModFit software (Verity Software House, Topsham, ME), according to the following formulas:

\[
PF = \sum_{k=0}^{T_{\text{pf}}} \frac{T_{k}}{T_{7}}, \quad \text{PMD} = \sum_{k=0}^{T_{\text{pf}}} \frac{T_{k}^{2}}{T_{7}}
\]

where \(k\) represents the number of divisions accomplished at day 7, and \(T_{k}\) represents the number of tetramer CD8\(^+\) cells detected in the generation \(k\).

**Results**

**CCR7 and CD45RA expression by epitope-specific CD8 T cells**

We first determined CCR7 and CD45RA expression by CD8 T cells specific for viral epitopes in different healthy volunteers. CD8 cells were isolated from peripheral blood and stained with MHC class I tetramers, CCR7 and CD45RA mAbs. We used HLA-A*0201 tetramers folded with peptides derived from CMV (peptide NLVPMAVT from pp65 protein; referred to as CMV1), EBV (peptide GLCTLVAML from BMLF1 and peptide CLGGLLTM from LMP2A; EBV1 and EBV2, respectively). In addition, HLA-A*0201/Melan-A tetramers, folded with ELA GIGILTV peptide (referred to as MEL1), were used to compare expression profiles of experienced and naive CD8 T cells. Representative examples of CCR7/CD45RA expression patterns, by total CD8 pool or tetramer-positive gated populations, found in the different donors are shown in Fig. 1, A and B, respectively.

As shown in Fig. 1A, we identified a total of three subsets of CD8\(^+\) cells: CD45RA\(^{\text{high}}\)/CCR7\(^{\text{high}}\), CD45RA\(^{\text{low}}\)/CCR7\(^{\text{null}}\), and CCR7\(^{\text{null}}\) with intermediate-to-high expression of CD45RA. CD8\(^+\) populations specific for given viral epitopes were homogeneous and remarkably conserved among the analyzed volunteers, as shown in Fig. 1B. CD8 T cells specific for EBV1 exhibited a CCR7/CD45RA\(^{\text{null}}\) profile. The profile of CD8 T cells specific for CMV1 was similar, with a higher expression of CD45RA molecules. CD8 T cells specific for EBV2 display a CD45RA\(^{\text{low}}\)/CCR7\(^{\text{null}}\) profile. By contrast, CD8 T cells specific for MEL1 were CD45RA\(^{\text{high}}\)/CCR7\(^{\text{null}}\). Thus, EBV1-, CMV1-, and EBV2-specific CD8\(^+\) T cells consistently expressed distinct levels of CCR7 and CD45RA. The mean fluorescence intensity (MFI) of CCR7- and CD45RA-stained molecules of individual epitope-specific CD8 cells are shown in Fig. 1C for the different donors tested. In particular, we observed that CD45RA MFI of CMV1-specific T cells (477 ± 220) was consistently higher than MFI of EBV1-specific CD8 T cells (163 ± 40). Altogether, these results underline that CD8 T cells specific for distinct viral epitopes show highly conserved CCR7/CD45RA expression patterns from one healthy donor to another but modulate differently the expression of these molecules. CD8 T cells specific for EBV2 were thus used as a model of epitope-specific T\(_{\text{CM}}\) as compared with EBV1- and CMV1-specific T\(_{\text{EM}}\) CD8 cells.

**Ex vivo cytokine production after Ag-specific recognition**

The first experiments that initiated the model of central and effector memory cells have shown segregation in the secretion of IL-2 and IFN-\(\gamma\) in response to anti-CD3 and anti-CD28 mAbs (6). Therefore, we tested whether an epitope-specific CD8 T cells, with a given CCR7/CD45RA profile, could secrete a particular pattern of cytokines in a short-term peptide stimulation assay. We selected healthy volunteers with detectable epitope-specific CD8 T cells and performed intracellular detection of IL-2 and IFN-\(\gamma\) after a 6-h stimulation with synthetic peptide. In parallel, nonstimulated CD8 T cells were stained with tetramers to assess the frequency of epitope-specific CD8 T cells in the sample. The frequencies of CD8\(^+\) cells producing IL-2 and/or IFN-\(\gamma\) correlated with the percentage of CD8 cells stained with tetramers before stimulation and

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**FIGURE 1.** CD45RA and CCR7 expressed by various epitope-specific CD8 T cells. PBMC from healthy volunteers were stained with tetramers, anti-CD8, -CD45RA, and -CCR7 mAb. A and B, CD45RA vs CCR7 staining is shown, after gating on CD8\(^+\) cells (A) or tetramer CD8\(^+\) cells that are specific for EBV1, EBV2, CMV1, or MEL1 epitopes (B). In A, squares delineate three distinct CD8 subsets: CD45RA\(^{\text{high}}\)/CCR7\(^{\text{high}}\), CD45RA\(^{\text{low}}\)/CCR7\(^{\text{null}}\), and CCR7\(^{\text{null}}\) with intermediate-to-high expression of CD45RA. C, MFI of CD45RA and CCR7, analyzed in different donors, are plotted for each epitope-specific population.
represented at least 70% of CD8\(^+\) cells stained by tetramers (data not shown). Fig. 2A shows representative patterns of IFN-\(\gamma\) and IL-2 staining on gated CD8\(^+\) cells after stimulation with EBV1, EBV2, and CMV1. IFN-\(\gamma\) synthesis was detected in all three epitope-specific CD8 subsets. By contrast, CD8\(^+\) cells producing IL-2 without IFN-\(\gamma\) were not detectable. CD8\(^+\) cells producing both IL-2 and IFN-\(\gamma\) were detected in different proportions among CD8 subsets. In this representative experiment, when stimulated with EBV2 peptide, 0.05% of CD8 T cells produce both cytokines, whereas 0.03% secrete only IFN-\(\gamma\). Thus, \(\sim 70\%\) of EBV2-reactive CD8 cells produce IL-2 together with IFN-\(\gamma\). In contrast, the vast majority of CD8 T cells secreting cytokines in response to EBV1 or CMV1 produce only IFN-\(\gamma\), and only 15% produce both IFN-\(\gamma\) and IL-2. IL-2 was completely blocked by preincubating cells with 0.3 \(\mu g\) of nonconjugated anti-IL-2 mAb demonstrating specificity of IL-2 staining (data not shown). A 16-h stimulation did not enhance the production of IL-2 cytokines, nor did the use of peptide-pulsed DC instead of free peptide (data not shown). Fig. 2B plots the ratio between double IL-2*IFN-\(\gamma\)* cells and single IFN-\(\gamma\)* cells, calculated with experiments conducted in different healthy volunteers with the three epitopes. The ratio is always \(< 0.5\) after stimulation with EBV1 peptide (mean \(\pm SD\), 0.24 \(\pm\) 0.08) and after stimulation with CMV1 peptide (mean \(\pm SD\), 0.30 \(\pm\) 0.14). On the contrary, the ratio is always \(>1\) after stimulation with EBV2 peptide (mean \(\pm SD\), 1.66 \(\pm\) 0.63). Thus, these data show that CD8 cells specific for EBV2 are more prone to secrete IL-2 together with IFN-\(\gamma\) in short-term antigenic stimulation than the two other CD8 populations specific for EBV1 and CMV1 epitopes. These results suggest that \(T_{EM}\) and \(T_{CM}\)-like memory subsets differ in their cytokine profiles.

**Proliferation potential of epitope-specific CD8 T cells**

As memory CD8 cells may persist with different capacity to expand following antigenic stimulation, we compared the proliferation potential of the three epitope-specific populations. In addition, our observation that EBV2-specific CD8 cells produce IL-2 in different proportions compared with the two other memory CD8 populations studied, suggested that the production of IL-2 might influence, in an autocrine way, proliferation of CD8 memory subsets. CD8\(^+\) cells were purified from healthy volunteers, labeled with the vial PKH67 fluorescent dye, and stimulated in vitro with unloaded or peptide-loaded autologous DC. After 7 days of culture, CD8 cells were stained with tetramers and analyzed by flow cytometry. Peptide-loaded DC, but not unloaded DC, induced massive expansion of epitope-specific CD8\(^+\) cells, as shown by the high frequency and the decreased PKH fluorescence intensity of tetramer-positive cells at day 7 (Fig. 3A). However, the response of tetramer-positive cells to the peptide stimulation was heterogeneous: a proportion of the cells did not divide. We noticed that a fraction of tetramer-negative cells also divided but in similar proportion in cultures with peptide-loaded and unloaded DC, indicating that this proliferation was not peptide specific.

To quantify the proportion of precursor T cells that have divided and the number of cell divisions, we performed deconvolution analysis using the ModFit software (Fig. 3B). CD8 T cells stimulated with peptide-loaded DC have undergone up to nine divisions. This approach enabled us to draw on one hand the distribution of day 7 tetramer-positive cells among generations, and in contrast, the day 7 distribution of cells as they were at day 0, to follow the fate of precursors. To rule out any donor-specific behavior, two epitope-specific CD8 T cell populations were analyzed for each volunteer. As shown in Fig. 4A, the distribution of \(T_{CM}\) and \(T_{EM}\) CD8 cells specific for EBV1 and \(T_{CM}\) cells specific for EBV2 was similar. The majority of cells stained by tetramers at day 7 have undergone five to six divisions. However, for both epitopes, these cells are the progeny of only 25% of ex vivo tetramer-positive cells (15 and 10% have divided five or six times, respectively), the majority of these precursors dividing less than twice (Fig. 4B). For the different donors tested, the proliferation potential could be described by two parameters: the mean division of dividing precursors (PMD) and the percentage of precursors dividing at least twice (PF). Data obtained for EBV1-, EBV2- and CMV1-specific CD8 T cells are summarized in Fig. 4. C and D. PMD was relatively constant among volunteers and among the different CD8 T cell subsets, with a mean division number of 4 to 5 (Fig. 4C). The recruitment was more heterogeneous, varying from 20 to 70% of initial specific CD8\(^+\) cells (Fig. 4D). Despite this heterogeneity, the three CD8 T cell subsets contained similar proportion of precursors able to proliferate; most variations were found among donors, not among volunteers.
epitopes. Altogether, these results show that the three epitope-specific CD8 populations display similar capacity to be recruited and proliferate after antigenic stimulation in vitro.

**MEL1-specific T_{EM} CD8 cells in cancer patients**

We investigated in two cancer patients how tumor-specific CD8 T cells that have experienced Ag recognition in vivo during tumor progression can be compared with memory CD8 T cells specific for viral epitopes. We based the comparison on the CCR7/CD45RA expression profile when specific T cells were detectable ex vivo and evaluated their capacity to produce cytokine and expand in response to antigenic stimulation. Both patients were stage IV melanoma patients, with peripheral blood MEL1-specific CD8 T cells secreting IFN-γ ex vivo as assessed by ELISPOT assay (V. Baron-Bodo, S. Massicard, E. Mallard, A. Lim, D. Duriau, F. Lehmann, P. Vereecken, T. Velu, P. Kourilsky, J. P. Abastado, et al., manuscript in preparation). In patient P08, MEL1-specific CD8 cells were also detectable by tetramers directly ex vivo (1.5% among CD8 T cells), as well as CD8 cells specific for EBV1 (2%) and influenza matrix peptide FLU1 (0.05%). No EBV2-specific CD8 cells could be detected ex vivo in this patient, nor in patient P05 in whom no EBV cells at all could be detected ex vivo (data not shown). CD8 T cells isolated from peripheral blood of patient P08 were stained ex vivo with tetramers, CCR7, and CD45RA Abs. As shown in Fig. 5A, MEL1-specific CD8 T cells display a CD45RA_{high}CCR7_{low} expression pattern, contrasting with the naive phenotype found in healthy volunteers (Fig. 1B). The profile of EBV1-specific cells was comparable with that of healthy donors with CD45RA_{high}CCR7_{low} expression pattern typical of T_{EM} cells (Figs. 1B and 5A). Although both MEL1- and EBV1-specific CD8^+ cells look very similar, the level of CD45RA was significantly higher on EBV1-specific population (p < 0.001, Kolmogorov-Smirnov test).

We then analyzed IL-2 and IFN-γ produced by tumor- and virus-specific CD8 cells after a 6-h stimulation in vitro with the relevant peptides. In parallel, nonstimulated CD8 cells were stained with tetramers to assess the frequency of epitope-specific CD8 T cells in the samples. We first observed that, in contrast to healthy donors, the frequencies of CD8^+ cells stained with MEL1 or EBV1 tetramers before stimulation and represented only ~30% of tetramer^+ cells (data not shown). As shown in Fig. 5B, IFN-γ synthesis was detected in both epitope-specific CD8 subsets. CD8^+ cells producing IL-2 without IFN-γ...
were not measurable. When stimulated with EBV1 peptide, 0.16% of CD8 cells produced both cytokines, whereas 0.54% secreted only IFN-γ. Thus, as in healthy donors, ∼20% of EBV1-responding CD8 cells produced both IFN-γ and IL-2 (Figs. 2A and 5B). In contrast, the proportion of CD8+ cells producing these two cytokines was lower among MEL1-specific TEM cells, representing only 5% of secreting CD8 cells (Fig. 5B). These data show that, although MEL1- and EBV1-specific CD8 T cells are similar in terms of acquisition of effector memory phenotype, MEL1-specific CD8 T cells are skewed toward IFN-γ production without IL-2.

Because cell proliferation increases the frequency of rare proliferating precursors, we could characterize the proliferation capacity of MEL1-specific CD8 T cells in patient P08 and also in patient P05, although MEL1-specific CD8 cells were undetectable ex vivo with tetramers in the latter. In parallel, we have followed the proliferation of FLU1-specific CD8 T cells detectable in both patients. The proliferation profile at day 7 of MEL1- and FLU1-specific CD8 T cells in patient P05 is shown in Fig. 6A. MEL1-specific CD8 T cells expanded well in response to peptide-loaded DC (Fig. 6A). As described above in healthy donors (Fig. 4C), the mean division number of precursors in the two melanoma patients was ∼4–5 for both MEL1- and FLU1-specific CD8 cells (Fig. 6B).

A similar proportion of precursors was recruited to proliferate in both pools of memory CD8 cells, although we observed that the recruitment was in general lower than in experiments conducted in healthy volunteers (10–30% of initial specific CD8 cells; Fig. 6C). Altogether, these data were typical of those found in healthy donors and show that MEL1-specific CD8 cells that have been differentiated in vivo during disease progression in these two patients can be recruited and proliferate in vitro as well as CD8 T cells specific for viral Ags.

**Discussion**

Memory CD8 T cells play a key role in the control of persistent viruses. We hypothesized that such experienced T cell populations have acquired potential to protect the host, and therefore, comparison with tumor-specific CD8 T cells differentiated in cancer patients may reveal critical parameters required to turn on protective immunity against cancer. Our results show that virus-specific CD8 T cells, irrespective of their CCR7/CD45RA profiles, can similarly expand in vitro. However, TEM-like CD8 cells that compose the pool of EBV-specific T cells include a higher proportion of cells producing both IL-2 and IFN-γ than TEM CD8 cells in short-stimulation assay ex vivo. We also underlined that some tumor-specific CD8 T cells differentiate into TEM cells in cancer patients, produce IFN-γ without IL-2, and can be expanded in vitro similarly to virus-specific CD8 memory cells.

Human T cells specific for viral Ags express different levels of CCR7 and CD45RA molecules (27) and can be considered as models of protective CD8 T cells. We observed that CD8 T cells
specific for epitopes derived from lytic (BMLF1) and latent (LMP2A) viral proteins of EBV or derived from the lower matrix protein pp65 of CMV express distinct patterns of CD45RA and CCR7, which are conserved among various individuals (Fig. 1). This is in agreement with other studies showing that CD45RA and CCR7 expression profiles acquired by CD8 cells during resolution of EBV infection appear to be driven by the epitope specificity (28). The context in which the epitope is recognized (i.e., the APC, the Ag load) and the kinetics of Ag presentation may imprint the pattern of CCR7 and CD45RA expression. Although we show that these markers are homogeneously expressed on CD8 cells specific for a defined viral epitope, other activation/differentiation markers like CD27 and CD28 (29–31) may be regulated differently.

We wondered whether a defined phenotypic profile could correlate with a given ex vivo cytokine secretion pattern. We first observed that the ability to produce IFN-γ ex vivo is shared by CCR7+ and CCR7− CD8 T cells (Fig. 2). This is in contrast to results obtained on pools of CD8 T cells of unknown specificity (6). Our results confirm recent findings in both human (12, 14) and mouse (13) showing that TCM cells produce a significant amount of IFN-γ ex vivo. Indeed, human TCM CD8 cells are more heterogeneous than initially thought (32). A subset of these cells would secrete IFN-γ ex vivo, whereas others would display a type 2 cytokine profile upon polyclonal stimulation. Thus, it is possible that the capacity of TCM CD8 cells to secrete IFN-γ ex vivo varies with epitope specificity.

A second finding lies in the ability of CD8 memory subsets to produce both IL-2 and IFN-γ in different proportions following a short antigenic stimulation ex vivo. We found that most EBV2-specific CD8 cells produce both IL-2 and IFN-γ, whereas most CMV1- and EBV1-specific CD8 cells produce IFN-γ but no IL-2 (Fig. 2). This study is, to our knowledge, the first showing in humans such bivalent virus-specific CD8 T cells. Analysis of other epitope-specific memory cells, in particular those having a TCM phenotype like EBV2-specific T cells, will clarify whether this is a general distinction between TCM and TEM memory subsets. Our finding extends the work conducted in mice where LCMV-specific memory CD8 cells, expressing CD62L, have been shown to secrete higher amounts of IL-2 than CD62L− cells (15). Although it could be argued that in vitro cytokine assays may not reflect the in vivo situation, the dual production of IL-2 and IFN-γ ex vivo at the single-cell level was also shown to be typical from late memory CD8 cells, but not from recently activated naive ones (4). It would be interesting to determine whether these bivalent cells display any selective advantage. One possibility would be that IL-2 production would have an autocrine effect, favoring expansion and renewal of the memory pool. Alternatively, the higher expression of IL-2-Rβ chain by TEM CD8 cells (32) may increase responsiveness to IL-2 and compensate for the lower production of IL-2.

ME1-specific TEM cells in patient P08 did not produce IL-2 (Fig. 5). In parallel, EBV1-specific CD8 cells in this patient looked similar to that observed in healthy donors. We therefore believe that the lack of IL-2 production by ME1-specific T cells is related to this particular epitope and does not reflect any general compromised immune status. Characterization of other tumor-specific T cells in conjunction with Ag load is required to further document the cytokines produced by subsets of memory cells and clarify what could be the impact on immune protection.

One important feature of a memory pool is rapid expansion upon secondary challenge. Several parameters determine the expansion potential of a T cell pool: the recruitment of Ag-specific CD8 T cells, the extent of proliferation, the survival of proliferating cells but also CD4 help (33, 34). We assessed the first two parameters using a novel assay based on fluorescent dye dilution (35, 36). Interestingly, in our experimental conditions, we did not observe any differences between the various T cell subsets (Fig. 4). This is in contrast with the better in vitro and in vivo expansion of CD8 TCM cells specific for gp33 epitope from LCMV in mice, compared with TEM ones, and associated with a better immune protection (15). Interestingly, Wherry et al. also detected a higher IL-2 production by TCM cells similar to our own finding for EBV2-specific CD8 cells.

Alternatively, the use of DC in our system, regarding their cytokine production and expression of costimulatory molecules, may have masked differences that could exist in vivo, considering in particular that TEM cells, localized in nonlymphoid tissues, are more susceptible to interact with epithelial cells. Thus, further investigation may be required to state whether differences in Ag-driven proliferation exist between these CD8 memory subsets and to what extent the microenvironment influences their in vivo expansion.

In patients P05 and P08, MEL1- and FLU1-specific CD8 T cells display similar proliferation capacity (Fig. 6). Although MEL1-specific CD8 cells were not detectable by tetramers ex vivo in patient P05, this T cell pool is mostly composed of memory/effector T cells, corresponding to a single clone and able to secrete IFN-γ ex vivo (V. Baron-Bodo, S. Massicard, E. Mallard, A. Lim, D. Duriau, F. Lehmann, P. Vereecken, T. Velu, P. Kourilsky, J. P. Abastado, et al., manuscript in preparation). Our results indicate that some tumor-specific T cells already primed during immune surveillance and tumor progression are not compromised in their proliferation potential and do not differ intrinsically from virus-specific CD8 T cells that contribute to immune protection. Obviously, it would be interesting to extend this study to T cells specific for other tumor Ags than MEL1, because the frequency of T cell clones specific for this epitope is particularly high. In addition, characterization of other patients with already primed tumor-specific T cells is of great importance to determine whether clusters of patients can be identified, depending on the proliferation potential of antitumor effector/memory CD8 cells. For instance, in HIV-infected individuals, HIV-specific CD8 T cells in nonprogressors have been shown to better proliferate compared with progressors (18).

In summary, we have pointed out differences in IL-2 production by Ag-specific CD8 populations with no evidence for distinct intrinsic proliferation potential in response to antigenic stimulation. Other investigations are needed to extend the panel of virus- and tumor-specific CD8 populations characterized, to further document what advantage, in terms of protective immunity, IL-2 or any other molecule can confer to a memory CD8 T cell pool.

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