The Journal of Immunology

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J Immunol 2001; 166:7634-7640; ;
doi: 10.4049/jimmunol.166.12.7634
http://www.jimmunol.org/content/166/12/7634

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Ex Vivo IFN-γ Secretion by Circulating CD8 T Lymphocytes: Implications of a Novel Approach for T Cell Monitoring in Infectious and Malignant Diseases[1]

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To elucidate the functional heterogeneity of Ag-specific T lymphocyte populations, we combined labeling of lymphocytes with MHC/peptide tetramers and a cell surface affinity matrix for IFN-γ. Magnetic cell sorting of IFN-γ-positive lymphocytes allowed the selective enrichment and identification of live Ag-specific cytokine-secreting cells by flow cytometry. Naive, memory, and effector Ag-specific populations were evaluated in healthy HLA-A2 individuals. Significant fractions of influenza- and CMV-specific cells secreted IFN-γ upon challenge with cognate peptide, consistent with an effector/memory status. The sensitivity of the approach allowed the detection of significant numbers of CMV-specific IFN-γ-secreting cells ex vivo (i.e., without Ag stimulation). This was not apparent when using previously described assays, namely, ELISPOT or intracellular IFN-γ staining (cytospot). CD8$^+$ T cells specific for the melamoma-associated Ag Melan-A/MART-1 did not produce IFN-γ upon challenge with cognate peptide, reminiscent with their naive functional state in healthy individuals. In contrast, CD45RA^{low} Melan-A/MART-1 tumor-specific cells from three of three melanoma patients presented levels of activity similar to those found for influenza- or CMV virus-specific lymphocytes, compatible with a functional differentiation into competent effector/memory T lymphocytes in vivo. Notably, a sizable fraction of Melan-A/MART-1-specific cells from a patient secreted IFN-γ ex vivo following peptide-based vaccination. Thus, the high sensitivity of the assay provides a valuable tool to monitor effector T cell responses in different clinical situations. ***The Journal of Immunology*****, 2001, 166: 7634–7640.**

The CD8$^+$ T cells are crucial for the host defense against pathogens and tumors (1). Upon Ag recognition, CD8$^+$ T lymphocytes may proliferate and acquire effector functions. Immune protection is mediated by the specific killing of Ag-bearing cells via the perforin- and Fas- dependent pathways and/or by the release of different cytokines (2). Cytokines such as IFN-γ may contribute to the host defense by initiating a potent local inflammatory response. Secretion of IFN-γ by effector cells mediates recruitment and activation of macrophages as well as induction of increased MHC I and MHC II expression on macrophages and infected cells. Several procedures have been designed to assess IFN-γ production by T lymphocytes at the single-cell level. In ELISPOT assays, the release of IFN-γ by Ag-specific cells allows the generation of discrete spots reflecting the number of cytokine-secreting cells (3–6). Intracellular IFN-γ staining (Cytospot assay) enables the identification of IFN-γ-positive cells by flow cytometry (7, 8). Finally, a third approach allows the detection of IFN-γ on the surface of cytokine-secreting cells by using a cell surface affinity matrix (9). Secreted IFN-γ molecules are retained on the cell surface by means of a bispecific Ab recognizing 1) the IFN-γ molecule and 2) the CD45 molecule that is widely expressed by leukocytes. The captured IFN-γ is detected by a fluorochrome-labeled secondary Ab, permitting efficient visualization of IFN-γ-secreting cells by flow cytometry (10, 11). Of interest, subsequent magnetic labeling and sorting of IFN-γ$^+$ cells allows the isolation and characterization of live Ag-specific cells.

Studies of CTL clones (12–14) and ex vivo polyclonal monospecific populations (15–18) have shown that only a fraction of lymphocytes produce IFN-γ upon stimulation, raising questions on the functional capacity of single lymphocytes and the functional heterogeneity of whole Ag-specific populations. Hence, it becomes particularly interesting to combine cytokine-based assays with the tetramer technology. Previously used tetrameric approaches integrated intracellular staining that requires permeabilization of the cell membrane (15–18) and does not allow the isolation of live Ag-specific cells. In this study, we developed a new assay that combines a cell surface affinity matrix for IFN-γ and MHC/peptide tetramers. By gating on Ag-specific tetramer$^+$ lymphocytes, we could precisely assess the cytokine secretion of tumor (Melan-A/MART-1)- and virus (influenza and CMV)-specific T cells. This approach revealed two major advantages: first, the sensitivity of the assay was increased as compared with the previously described techniques and, second, the assay allowed to sort out and visualize functionally active, Ag-specific cells.

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Received for publication February 23, 2001. Accepted for publication April 13, 2001.

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[1] This work was in part supported by Grant KFS 633-2-1998 from the Swiss Cancer League and by the Leenards Foundation. A.Z. was supported by Grant Zi685/1-1 from the Deutsche Forschungsgemeinschaft.

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0022-1767/01/$02.00

Materials and Methods

Cells

Twenty-eight healthy blood donors registered at the blood transfusion center in Lausanne, Switzerland, and three melanoma patients with advanced tumor stage (LAU 156, LAU 337, and LAU 465) were included in this study. All individuals were selected on the basis of HLA-A2 expression as assessed by flow cytometry of PBMC stained with allele-specific mAb BB7.2 (19). Cord blood lymphocytes from healthy newborns were obtained at the Maternity Service, University Hospital (Lausanne, Switzerland). PBMC and cord blood cells were prepared by standard Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden), cryopreserved in RPMI 1640/40% FCS/10% DMSO, and stored in liquid nitrogen.

Tetramers and mAbs

Synthesis of PE- and allophycocyanin-labeled HLA-A2/peptide tetrameric complexes was performed as previously described (20, 21). One peptide was the Melan-A_{26-35} A27L analog (ELAGIGILTV), which has a higher binding stability to HLA-A*0201 and a higher T cell antigenicity and immunogenicity than the natural Melan-A decapeptide EAAGIGILTV or the nonapeptide AAGIGILTV (22). Other peptides were the Flu-MA_{58-66} (GILGFVFTL) peptide from the influenza matrix protein (23), and the $pp65_{495-503}$ (NLVPMVATV) peptide from the CMV structural protein pp65 (24).

Synthetic peptides

Peptides were synthesized by standard solid-phase chemistry on a multiple peptide synthesizer (Applied Biosystems, Foster City, CA) by using F-moc for transient NH_2-terminal protection and were analyzed by mass spectrometry. All peptides were >90% pure as indicated by analytical HPLC. Lyophilized peptides were diluted in DMSO and stored at −20°C.

FACS staining and phenotype analysis

$CD8^+$ lymphocytes were purified from PBMC by magnetic cell sorting using a MiniMACS device (Miltenyi Biotec, Bergisch Gladbach, Germany). To assess the frequency of Ag-specific $CD8^+$ lymphocytes, cells were stained with the appropriate PE- and APC-labeled tetramers at room temperature for 1 h before incubating with $CD8^{PerCP}$ mAb (Becton Dickinson, San Jose, CA). The observed frequencies of A2/tetramer$^+$ cells in $CD8^+$ cells were identical using either PE- or APC-labeled complexes. For surface phenotype analysis, cells were incubated with 1) PE- or APC-labeled tetramers for 60 min at room temperature; 2) anti-CCR7 rat IgG mAb 3D12 (25) (kindly provided by M. Lipp and R. Forster, Max Delbrück Institute, Berlin, Germany) for 20 min at 4°C and washed; 3) goat anti-rat IgG (H + L)^{FITC} (Southern Biotechnology Associates, Birmingham, AL) for 20 min at 4°C and washed; and 4) anti-$CD8^{PerCP}$ mAb along with either anti-$CD28^{PE}$ (Becton Dickinson), anti-$CD27^{PE}$ (PharMingen, San Jose, CA), or anti-$CD45RA^{APC}$ (Caltag, Burlingame, CA) mAbs for 20 min at 4°C. Cells were then washed once and immediately analyzed on a FACSCalibur (Becton Dickinson) using CellQuest software.

IFN-γ secretion assay

Measurement of IFN-γ secretion by Ag-specific cells was performed when the frequency ex vivo of A2/tetramer$^+$ cells was ≥0.04% of $CD8^+$ T cells and when sufficient cell numbers were available. This was done in replicates by two independent examiners. Fresh or previously frozen PBMC ($1–10 \times 10^6$ cells) were resuspended in culture medium consisting of IMDM supplemented with 0.55 mM Arg, 0.24 mM Asn, 1.5 mM Gln, and 8% pooled human A^+ serum (complete medium). Cells were incubated for 4 h either with 1 μg/ml cognate peptide or $Pol_{476-484}$ (ILKEPVHGV) peptide from the reverse transcriptase of HIV-1 (negative control; all subjects in this study were HIV-1 seronegative). Cell surface detection of IFN-γ-secreted molecules was performed as follows: cells were labeled for 5 min at 4°C with an IFN-γ-specific high-affinity capture matrix (Miltenyi Biotec), i.e., a bispecific Ab-Ab conjugate directed against CD45 and IFN-γ. Afterward, cells were transferred into 37°C warm medium for 45 min to permit secretion of IFN-γ, washed, and stained for 30 min at 4°C with anti-$CD8^{FITC}$ mAb (Becton Dickinson), PE-labeled anti-IFN-γ mAb (Miltenyi Biotec), and APC-labeled tetrameric complexes. Cells to be activated were stained with tetramers for 30 min at 37°C before activation. Otherwise, TCR down-regulation induced by the stimulation (26) greatly reduces the tetramer staining (15, 18). Half of each cell sample was then immediately analyzed by flow cytometry (nonpurified fraction), while the remaining cells were washed and magnetically labeled for 15 min at 4°C with anti-PE Ab microbeads (Miltenyi Biotec). PE-labeled IFN-γ^+ cells were enriched in two rounds of positive selection by magnetic cell sorting (purified fraction) and immediately analyzed by flow cytometry. The vast majority (90–98%) of positively sorted cells was generally composed of dead cells, as measured by staining with 1 μg/ml propidium iodide (data not shown). However, selective analysis of small living lymphocytes by forward and side scatter gating and exclusion of propidium iodide binding particles allowed a specific detection of $CD8^+$ T lymphocytes.

IFN-γ Cytospot assay

Measurement of intracellular IFN-γ production by Ag-specific lymphocytes was performed in replicates by two independent examiners. Thawed PBMC ($1–10 \times 10^6$) were resuspended in complete medium and incubated for 4 h either with 1 μg/ml cognate peptide or irrelevant HIV-1 $Pol_{476-484}$ peptide. After 1 h, 10 μg/ml brefeldin A (Sigma, St. Louis, MO) was added. After 3 additional hours, cells were incubated with 0.8 ml Orthopermeafix (Ortho Diagnostic Systems, Raritan, NJ) for 40 min at room temperature, washed, and stained for 40 min at 4°C with PE-labeled tetramers along with anti-IFN-γ^{FITC} (PharMingen) and $CD8^{PerCP}$ (Becton Dickinson). Cells to be activated were stained with PE-labeled tetramers for 30 min at 37°C before activation to reduce TCR down-regulation. Cells were immediately analyzed on a FACSCalibur (Becton Dickinson).

IFN-γ ELISPOT assay

The IFN-γ ELISPOT assay was performed in replicates by two independent examiners, as previously described (4). PBMC ($1–10 \times 10^6$) were thawed and cultured overnight in 6 ml of complete medium to ensure good viability. ELISPOT plates (Millipore, Bedford, MA) were coated with Ab to human IFN-γ (Diaclone, Besançon, France) and washed six times. One microgram per milliliter peptide and 1.66×10^5 PBMC/well in 100 μl of complete medium were added and incubated for 20 h at 37°C. Assays were performed in six replicates either with the cognate peptide or the irrelevant HIV-1 $Pol_{476-484}$ peptide. Cells were removed and plates were developed with a second Ab to human IFN-γ (biotinylated) and streptavidin-alkaline phosphatase (Diaclone). The deduced frequency of peptide-specific $CD8^+$ cells was calculated by subtracting the mean number of nonspecific IFN-γ spots in the control sample from the mean number of specific IFN-γ spots in the peptide-stimulated sample.

Results

Frequency and phenotype analysis ex vivo of A2/Melan-A-, A2/Flu-MA-, and A2/CMV-specific $CD8^+$ T cells in HLA-A2 healthy individuals

We investigated the frequency ex vivo of circulating $CD8^+$ T lymphocytes specifically recognizing Melan-A_{26-35}, Flu-MA_{58-66}, or CMV $pp65_{495-503}$ peptides in a group of HLA-A2 healthy individuals. Significant numbers (4) of A2/Melan-A^+, A2/Flu-MA^+, and A2/CMV^+ cells were detected in 18 of 25 (72%), 25 of 26 (96%), and 13 of 26 (50%) individuals with a mean frequency of 0.06, 0.13, and 3.2% of $CD8^+$ T cells, respectively (data not shown). As illustrated in Fig. 1, A2/Melan-A^+, A2/Flu-MA^+, and A2/CMV^+ $CD8^+$ T cells were evaluated for CD45RA and CCR7 expression (27). A2/Melan-A^+ cells homogeneously presented a $CD45RA^{high}CCR7^+$ phenotype in four of four individuals analyzed (Table I). Independent analyses performed on other HLA-A2 individuals revealed that A2/Melan-A^+ cells were also $CD45RA^{high}CD45RO^-CCR7^+CD27^+CD28^+CD57^-CD62\ L^+$ phenotype (data not shown), indicating that Melan-A tetramer$^+$ cells exhibit a naive phenotype. In marked contrast, A2/Flu-MA^+ cells often segregated into two populations, namely $CD45RA^{low}CCR7^+$ (central memory) and $CD45RA^{low}CCR7^-$ (effector memory) cells (Table I). A2/Flu-MA^+ cells were also mainly $CD45RO^+CD27^+$ $CD28^+$ (data not shown). As an exception, 90% of A2/Flu-MA^+ cells from HD 7833 were $CD45RA^{high}CCR7^-$ (Table I) and mostly $CD27^+CD28^-$ (data not shown). Finally, A2/CMV^+ cells presented various CD45RA phenotypes among individuals but were consistently $CCR7^-$ (Table I). A detailed analysis of A2/CMV^+ cells from HD 2709, 2713, 4474, 7833, and 7847 revealed that ~65% were $CD28^-$ and that ~50% were $CD27^-$ (data not shown). Together, the pattern of cell

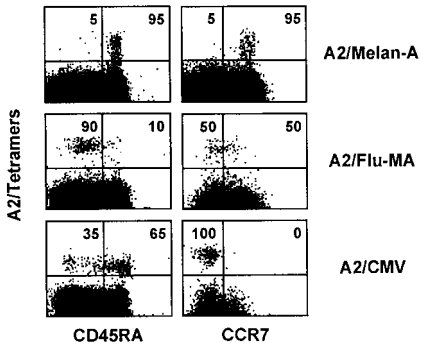

FIGURE 1. Surface phenotype ex vivo of circulating Melan-A-, Flu-MA-, and CMV-specific $CD8^+$ T cells from three representative individuals. $CD8^+$ lymphocyte populations were highly purified from PBMC (>98%), stained with the appropriate PE-labeled tetramers and anti-$CD8^{PerCP}$ mAb, along with anti-$CD45RA^{APC}$ mAb or rat anti-CCR7 mAb labeled with goat anti-rat IgG^{FITC}, and then analyzed by flow cytometry. Dot plots are shown for gated $CD8^+$ cells. Numbers in the *upper quadrants*, Percentages of A2/$tetramer^+$ cells with the corresponding phenotype.

surface marker expression is compatible with an effector phenotype.

Cytokine secretion by single Ag-specific cells

The capacity to secrete effector cytokines such as IFN-γ following short-term stimulation is a typical feature of Ag-experienced T cells. We thus assessed IFN-γ secretion by Melan-A-, Flu-MA-, and CMV-specific $CD8^+$ T cells by using the IFN-γ secretion assay (9–11). IFN-γ production was observed in a sizable fraction of ex vivo $CD8^+$ T cells (mean, 0.3% of $CD8^+$ T cells; SD, 0.3%, data not shown), preventing the analysis of rare Ag-specific populations (as low as 0.04% of $CD8^+$ lymphocytes). We surmised that integrating the tetramer technology in this assay would allow us to limit the analysis to Ag-specific A2/$tetramer^+$ populations, thus overcoming the high IFN-γ background (Fig. 2*A*). Using this approach, A2/Melan-A $tetramer^+$ cells did not detectably secrete IFN-γ either upon exposure to the control HIV-1 peptide (mean, 0.4% of A2/$tetramer^+$ cells; SD, 0.5%) or upon stimulation with Melan-A peptide (mean, 0.3%; SD, 0.5%) in seven of seven individuals analyzed (Table I). In contrast, subpopulations of A2/Flu-MA^+ cells (mean, 25%; SD, 23%) secreted IFN-γ upon stimulation with Flu-MA peptide in six of seven individuals, whereas no significant secretion (mean, 0.2%; SD, 0.6%) was noted upon exposure to irrelevant peptide (Table I). Again, the Flu-MA-specific cells from HD 7833 behave exceptionally since they did not secrete IFN-γ (Table I). Large numbers of A2/CMV^+ cells (mean, 56%; SD, 20%) secreted IFN-γ upon stimulation with CMV peptide in nine of nine individuals, whereas no or relatively low proportions of IFN-γ-secreting cells (mean, 1.0%; SD, 1.0%) were observed upon exposure to irrelevant peptide (Table I).

Cytospot and ELISPOT assays

IFN-γ production by Melan-A-, Flu-MA-, and CMV-specific $CD8^+$ T cells was independently assessed by intracellular staining of tetramer-labeled cells (Cytospot assay) (15–18) and by ELISPOT assays (3–6). As presented in Fig. 3, the frequencies of IFN-γ^+ cells within A2/$tetramer^+$ lymphocytes measured with the secretion assay correlated with the corresponding frequencies measured by Cytospot ($p < 0.0001$) and ELISPOT assays ($p < 0.001$). In accordance with the IFN-γ secretion assay, A2/Melan-A $tetramer^+$ cells did not detectably produce IFN-γ upon stimulation with Melan-A peptide in seven of seven individuals in both Cytospot and ELISPOT assays. Furthermore, significant fractions of A2/Flu-MA^+ cells were IFN-γ^+ upon stimulation with Flu-MA peptide in six of seven individuals. As already observed when using the IFN-γ secretion assay, Flu-MA-specific cells from HD 7833 did not produce IFN-γ in response to challenge with Flu-MA peptide. Likewise, large numbers of A2/CMV^+ cells were IFN-γ^+ upon stimulation with the CMV peptide in seven of seven individuals. Notably, no significant IFN-γ production by A2/Melan-A^+, A2/Flu-MA^+, and A2/CMV^+ $CD8^+$ T cells was detectable by intracellular staining upon exposure with irrelevant HIV-1 peptide (percentage IFN-γ^+ cells in A2/$tetramer^+$ cells: mean, 0.6%; SD, 0.4%).

Table I. *Frequency, surface phenotype, and IFN-γ secretion by Ag-specific $CD8^+$ T lymphocytes*

Individual	Frequency[a]	CD45RA[b]	CCR7[b]	Secretion Assay[c] HIV-1 Ag	Secretion Assay[c] Cognate Ag
Melan-A					
HD 0009	0.18	100	ND	1	0
HD 2706	0.04	90	95	0	0
HD 2710	0.05	95	90	0	0
HD 2711	0.05	90	ND	0	0
HD 2713	0.04	85	ND	0	0
HD 7590	0.09	95	95	1	1
HD 7834	0.10	100	100	1	1
Mean	0.08	95	95	0	0
Influenza					
HD 2714	0.13	20	55	0	8
HD 2715	0.23	10	5	0	62
HD 4296	0.17	10	50	0	14
HD 4469	0.15	0	30	0	49
HD 4470	0.07	25	40	ND	27
HD 7590	0.09	35	85	ND	15
HD 7833	0.48	90	10	1	0
Mean	0.19	25	40	0	25
CMV					
HD 2709	1.9	65	0	0	66
HD 2713	4.5	5	10	2	79
HD 4469	0.9	65	5	ND	25
HD 4470	1.5	50	10	0	45
HD 4474	1.4	15	5	0	32
HD 7517	2.9	35	5	1	58
HD 7524	12.2	55	5	1	72
HD 7833	1.4	5	5	3	77
HD 7847	1.1	70	15	0	46
Mean	3.2	40	5	1	56

[a] $CD8^+$ lymphocyte populations were highly purified from PBMC (>98%) and stained with the appropriate PE-labeled tetramers along with anti-$CD8^{PerCP}$ mAb. Values are the percentage of A2/$tetramer^+$ cells in gated $CD8^+$ cells.

[b] $CD8^+$ cells were also stained with anti-$CD45RA^{APC}$ mAb or rat anti-CCR7 mAb labeled with goat anti-rat IgG^{FITC}. Values are the percentage of A2/$tetramer^+$ cells with the corresponding phenotype.

[c] PBMC were stimulated for 4 h with 1 μg/ml irrelevant HIV-1 peptide or cognate peptide and stained with the appropriate APC-labeled A2/tetramers. Cell surface detection of IFN-γ-secreted molecules was performed by using an IFN-γ-specific affinity matrix and subsequent labeling with PE-conjugated anti-IFN-γ mAb. Values are the percentage of IFN-γ^+ cells in gated A2/$tetramer^+$ $CD8^+$ cells.

Increasing the sensitivity for the detection of IFN-γ secretion by Ag-specific cells

The IFN-γ secretion assay allows the selective enrichment of PE-labeled IFN-γ^+ cells using anti-PE magnetic microbeads (10, 11). PBMC challenged with either irrelevant HIV-1 peptide or cognate peptide were stained with anti-$IFN\text{-}\gamma^{PE}$ mAb and subsequently isolated by magnetic sorting with anti-PE microbeads (Fig. 2*B*). As shown in Fig. 4, purification of PE^+ cells upon challenge with

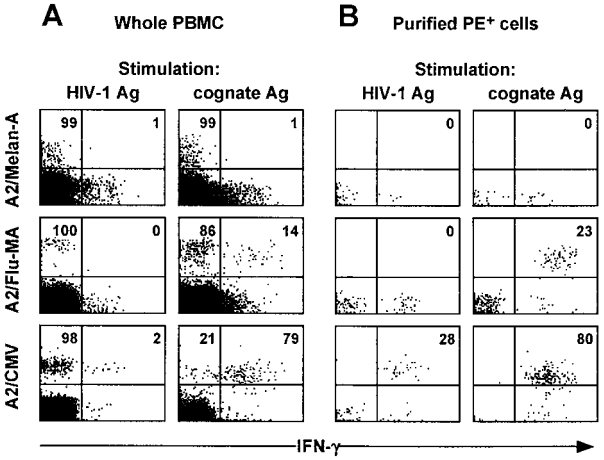

FIGURE 2. IFN-γ secretion based detection and enrichment of Melan-A-, Flu-MA-, and CMV-specific CD8$^+$ T cells from three representative individuals. *A*, PBMC were stimulated for 4 h with 1 μg/ml irrelevant HIV-1 peptide or cognate peptide, labeled as described in *Materials and Methods*, and then analyzed by flow cytometry. Dot plots show staining with PE-conjugated anti-IFN-γ mAb and appropriate APC-labeled A2/tetramers. Numbers in the *upper quadrants*, Percentages of A2/tetramer$^+$ cells with the corresponding phenotype. *B*, PE-labeled IFN-γ$^+$ cells were enriched in two rounds of positive selection by magnetic cell sorting and analyzed by flow cytometry. Numbers in the *upper quadrants*, Percentages of IFN-γ$^+$ A2/tetramer$^+$ cells within purified PBMC. All dot plots are shown for gated CD8$^+$, propidium iodide-negative cells.

cognate peptide (*D*) confirmed the results obtained with nonpurified lymphocytes (*B*). Indeed, we could not purify IFN-γ$^+$ Melan-A-specific cells, whereas significant numbers of A2/Flu-MA$^+$ and A2/CMV$^+$ IFN-γ$^+$ cells were sorted in six of seven and nine of nine individuals, respectively. As an exception, A2/Flu-MA$^+$ IFN-γ$^+$ cells were not detected in PBMC from HD 7833, confirming that A2/Flu-MA$^+$ cells remained IFN-γ$^-$ in this individual. In marked contrast, purification of PE$^+$ cells upon exposure to irrelevant HIV-1 peptide (*C*) brought additional information, as compared with the results completed with nonpurified lymphocytes (*A*). Indeed, enrichment of PE$^+$ cells allowed the detection of A2/CMV$^+$ IFN-γ$^+$ cells in high numbers in two individuals (HD 2713 and 7524) and to a lesser extent in three others (HD 4470, 7517, and 7833), whereas these cells were not or only poorly detectable within nonpurified lymphocytes and when using ELISPOT and Cytospot assays. Enrichment of PE-labeled IFN-γ$^+$ cells, however, did not allow the detection of A2/Melan-A$^+$ and A2/Flu-MA$^+$ IFN-γ$^+$ cells in the same experimental settings.

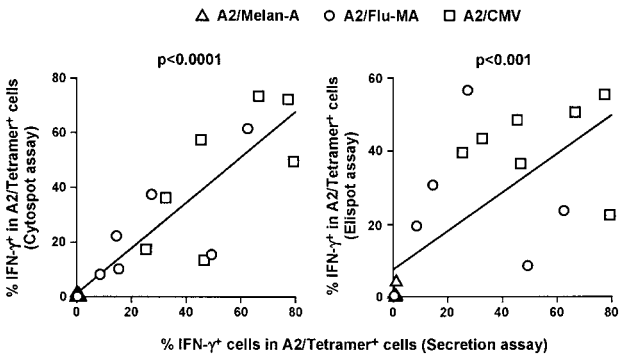

FIGURE 3. Correlation of A2/tetramer$^+$ T cell frequencies producing IFN-γ obtained from secretion assay (*x*-axis) vs Cytospot assay (*y*-axis, *left*) or ELISPOT assay (*y*-axis, *right*). For secretion or Cytospot assays, PBMC were incubated for 4 h with 1 μg/ml cognate peptide, labeled as described in *Materials and Methods*, and then analyzed by flow cytometry. Values are the percentage of IFN-γ$^+$ cells in gated A2/tetramer$^+$ CD8$^+$ cells. For ELISPOT assays, PBMC were incubated for 20 h with 1 μg/ml cognate peptide and treated as described in *Materials and Methods*. The number of IFN-γ-specific spots in PBMC were related to the frequency of A2/tetramer$^+$ CD8$^+$ cells in PBMC independently measured by flow cytometry. This enables us to calculate the frequency of IFN-γ$^+$ cells within peptide-specific CD8$^+$ cells. In the Cytospot assay, peptide-specific CD8$^+$ T cells did not detectably produce IFN-γ upon stimulation with irrelevant HIV-1 peptide (percentage of IFN-γ$^+$ cells in A2/tetramer$^+$ cells: Mean, 0.6%; SD. 0.4%). Correlation of IFN-γ production obtained from the different assays was measured by linear regression analysis.

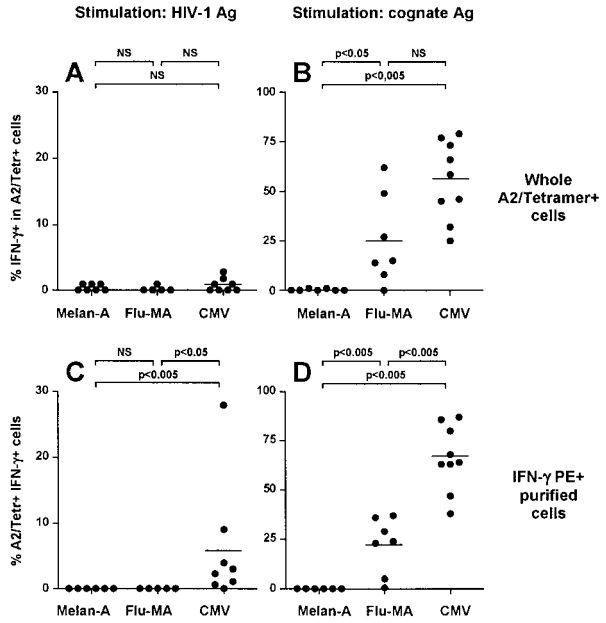

FIGURE 4. IFN-γ secretion-based detection and enrichment of Melan-A-, Flu-MA-, and CMV-specific CD8$^+$ T cells. PBMC were incubated for 4 h with irrelevant HIV-1 peptide (*A* and *C*) or cognate peptide (*B* and *D*), labeled as described in *Materials and Methods*, and then directly analyzed by flow cytometry (*A* and *B*) or magnetically enriched for PE-labeled IFN-γ$^+$ cells (*C* and *D*). Numbers in the *top panels*, Percentages of IFN-γ$^+$ cells in gated A2/tetramer$^+$ lymphocytes. Numbers in the *bottom panels*, Percentages of IFN-γ$^+$ A2/tetramer$^+$ cells within purified PBMC. Percentages of IFN-γ-secreting cells found for the different Ag-specific populations were compared using the Wilcoxon test (NS, $p \geq 0.05$).

To rule out unspecific labeling of the used mAbs, PE sorting on CFSE-labeled cord blood cells was performed. As expected for naive T lymphocytes, CD3$^+$ cord blood cells remained IFN-γ$^-$ and were not purified in two independent experiments (Fig. 5). To test the possibility that a fraction of A2/tetramer$^+$ cells may also catch IFN-γ molecules secreted by unspecific cells (bystander effect), we diluted ~10% naive CFSE-labeled cord blood cells in ~90% PBMC (HD 7833), known to contain elevated numbers (1%) of CD3$^+$ IFN-γ$^+$ cells ex vivo. IFN-γ secretion assay upon challenge with irrelevant HIV-1 peptide and subsequent purification of PE$^+$ cells allowed the enrichment of IFN-γ$^+$ PBMC, but not of IFN-γ$^+$ cord blood cells in two independent experiments (Fig. 5). In contrast, cord blood cells were efficiently labeled when adding exogenous IFN-γ, ruling out lack of binding of the bispecific reagent to cord blood lymphocytes. Furthermore, PE$^+$ PBMC (HD 7833) were not purified when labeling with IFN-γ catch reagent was omitted (data not shown). These results excluded a bystander IFN-γ$^+$ staining and confirmed the high specificity of the reagents used.

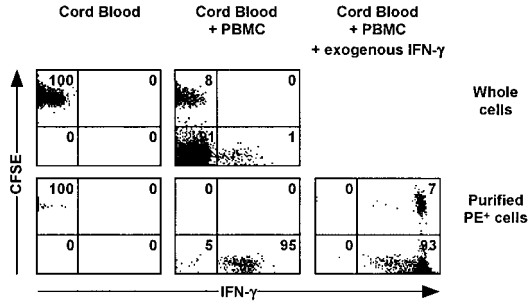

FIGURE 5. Specificity of the IFN-γ secretion assay. Cord blood lymphocytes were labeled with CFSE and incubated for 4 h with irrelevant HIV-1 peptide either alone (*left*), in the presence of PBMC (*middle*), or in the presence of PBMC plus exogenous IFN-γ (*right*). The IFN-γ secretion assay was investigated either directly (*top*) or after enrichment of PE-labeled IFN-γ^+ cells (*bottom*). Numbers, Percentages of cells in the respective quadrants. Dot plots are shown for gated $CD3^+$, propidium iodide-negative cells.

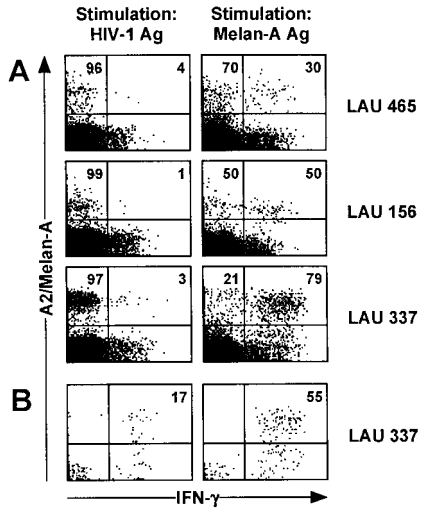

FIGURE 6. IFN-γ secretion-based detection and enrichment of Melan-A-specific $CD8^+$ T cells in patients with malignant melanoma. *A*, PBMC from patients LAU 465, LAU 156, and LAU 337 were stimulated for 4 h with 1 μg/ml irrelevant HIV-1 peptide or cognate peptide, labeled as described in *Materials and Methods*, and then analyzed by flow cytometry. Dot plots show staining with PE-conjugated anti-IFN-γ mAb and APC-labeled A2/Melan-A tetramers. Numbers in the *upper quadrants*, Percentages of A2/tetramer$^+$ cells with the corresponding phenotype. *B*, PE-labeled IFN-γ^+ cells from patient LAU 337 were enriched in two rounds of positive selection by magnetic cell sorting and analyzed by flow cytometry. Numbers in the *upper quadrants*, percentages of IFN-γ^+ A2/tetramer$^+$ cells within purified PBMC. All dot plots are shown for gated $CD8^+$, propidium iodide-negative cells.

Analysis of Melan-A-specific $CD8^+$ T cells in melanoma patients

Others and we have observed that tumor-specific $CD8^+$ T cells in cancer patients may expand in vivo and develop an Ag-experienced phenotype. To rule out functional unresponsiveness (17), we assessed the capacity of Melan-A-specific $CD8^+$ T cells to secrete IFN-γ by using the mentioned IFN-γ secretion assay in three melanoma patients with different clinical conditions. Patient LAU 465 did not receive any therapy at the time of blood collection; patient LAU 156 was treated with IFN-α and developed vitiligo 5 years before blood collection; patient LAU 337 received repeated vaccination with Melan-A_{26-35} peptide plus SBA-S2 adjuvant and concomitantly developed a systemic $CD8^+$ T cell response against Melan-A_{26-35} (18, 28). A2/Melan-A^+CD8^+ cells were mostly $CD45RA^{low}CD28^-$ in patient LAU 156, $CD45RA^{low}CD28^+$ in LAU 465, and $CD45RA^{low}CD28^-CCR7^-$ in LAU 337. As shown in Fig. 6*A*, large amounts of A2/Melan-A^+ cells (30–80%) secreted IFN-γ upon stimulation with Melan-A peptide in three of three melanoma patients. Notably, purification of PE^+ PBMC from LAU 337 allowed the detection of A2/Melan-A^+ IFN-γ^+ $CD8^+$ cells even without in vitro stimulation (Fig. 6*B*). This demonstrates that a fraction of Melan-A-specific cells in this individual secreted IFN-γ molecules ex vivo, similar to the ex vivo activity apparent for A2/CMV^+ cells in some healthy individuals.

Discussion

We studied the functional heterogeneity of cytokine secretion in Ag-specific $CD8^+$ T cells. We focused on circulating Melan-A-, influenza-, and CMV- specific lymphocytes representative of different stages of in vivo $CD8^+$ T cell differentiation. The experimental approach described here combines the advantage of directly quantifying and phenotyping Ag-specific T cells using MHC/peptide tetramers with the analysis of their functional capacity with a surface affinity matrix for IFN-γ. In marked contrast to the absence of IFN-γ secretion by naive Melan-A-specific $CD8^+$ T cells, significant fractions of effector/memory virus-specific T cells from healthy individuals produced IFN-γ either spontaneously and/or after antigenic stimulation. As illustrated by the observed IFN-γ secretion by Ag-experienced Melan-A-specific T cells in melanoma patients, this approach provides precise information on the differentiation and/or development of effector function in vivo at the single Ag-specific cell level.

The characterization of the different functional stages of $CD8^+$ T cells, from naive to memory, has become essential to study and monitor in vivo immune responses. In humans, the expression of the chemokine receptor 7 (CCR7) (29) along with CD45 isoforms (30–32) has recently been used to define four subsets of $CD8^+$ T lymphocytes with different homing and effector capacities (27). Indeed, $CCR7^+$ T cells may contain both naive ($CD45RA^{high}$) and central memory ($CD45RA^{low}$) cells, while $CCR7^-$ cells may be composed of effector memory ($CD45RA^{low}$) and terminally differentiated effector ($CD45RA^{high}$) cells. Analysis of 28 healthy individuals revealed that Melan-A-, influenza-, and CMV-specific $CD8^+$ T cells presented three distinct phenotypes. As recently reported (4, 33), Melan-A-specific cells consistently expressed a naive $CD45RA^{high}CCR7^+$ phenotype, whereas influenza- and CMV-specific cells had an Ag-experienced phenotype, compatible with the notion that most individuals have been previously exposed to the corresponding viruses. Notably, influenza-specific cells were composed of two distinct subsets, namely, central memory ($CD45RA^{low}CCR7^+$) and effector memory ($CD45RA^{low}CCR7^-$) lymphocytes, while CMV-specific cells displayed a more fully differentiated ($CD45RA^{low/high}CCR7^-$) phenotype. Further investigation of CMV- and influenza-specific lymphocytes revealed that down-regulation of the differentiation markers CD27 (34) or CD28 (35) only occurred in a fraction of $CCR7^-$ cells and was absent in $CCR7^+$ cells (data not shown). This was also confirmed at the whole $CD8^+$ T cell level (data not shown). Consequently, the CCR7 surface marker is more accurate than CD27 or CD28 to segregate the naive from the Ag-experienced subsets of $CD8^+$ T lymphocytes.

Rapid IFN-γ production can be induced by short-term peptide stimulation and has been shown to be a typical feature of Ag-experienced T cells (34). To assess cytokine secretion by single Ag-specific cells, others and we have previously reported the combination of tetramers with intracellular staining of IFN-γ (15–18).

We developed here a technique that creates an affinity matrix for IFN-γ on the surface of tetramer$^+$ cells. This allows us to visualize functionally active, Ag-specific cells without permeabilization and fixation. Subpopulations of influenza- and CMV-specific cells were observed to secrete IFN-γ after 4-h stimulation with cognate peptide, but not after incubation with an irrelevant HIV-1 peptide. In contrast, IFN-γ secretion was absent in the analyzed Melan-A-specific CD8$^+$ T cells from all healthy donors, consistent with their naive CD45RA^{high} CCR7$^+$ surface phenotype. The experimental approach described here shows the heterogeneous capacity of effector/memory Ag-specific populations to secrete inflammatory cytokines. This was not detectable when using tetramers alone or cytokine assays alone. Importantly, varying concentrations of added peptide (from 1 ng/ml to 10 μg/ml) did not affect the results (data not shown). IFN-γ^- cells may possibly exhibit distinct functions, such as active proliferation, production of other cytokines such as IL-4, or cytolytic activity. Alternatively, these cells may present functional defects leading to anergy in vivo (17). Due to the limited amount of cells available, we did not further investigate the function of lymphocytes in this study. Nevertheless, the possible enrichment of live lymphocytes may help in further addressing these questions.

Within Ag-experienced T cells, lack of CCR7 expression has been correlated with secretion of inflammatory cytokines (27). In agreement, Melan-A-, influenza-, and CMV-specific lymphocytes were CCR7$^+$, CCR7^{int}, and CCR7$^-$, respectively, while on average 0, 25, and 56% of these cells secreted IFN-γ upon antigenic stimulation. Nevertheless, we noticed discrepancies between CCR7 expression and IFN-γ cytokine secretion in some individuals. For instance, only 25–80% of CCR7$^-$ CMV-specific CD8$^+$ T cells secreted IFN-γ upon stimulation in all subjects tested. More strikingly, although influenza-specific CD8$^+$ T cells from HD 7833 displayed a terminally differentiated effector phenotype (CCR7$^-$CD45RA^{high}), they did not produce IFN-γ upon peptide stimulation. This cannot be explained by a general failure of CD8$^+$ T cells to secrete IFN-γ since 1) 1% of CD8$^+$ T cells spontaneously secreted IFN-γ ex vivo and 2) 77% of the A2/CMV$^+$ CD8$^+$ cells secreted IFN-γ upon stimulation with the cognate peptide. Because phenotype analyses may not reveal the true function of Ag-specific cells, the accuracy of the functional assay described in this study becomes advantageous to directly evaluate the effectiveness of CD8$^+$ T cell responses.

Cytokine-producing cells can be highly enriched by coupling the cell surface affinity matrix technology with magnetic cell sorting. This selective enrichment reveals two major advantages: First, we could sort out and visualize significant numbers of Ag-specific cytokine-secreting cells by flow cytometry; these cells have been shown to exhibit significant cytolytic activity after expansion in cell culture (10, 11). Second, the increase of sensitivity allowed a clear detection of CMV-specific cells producing IFN-γ ex vivo in five of eight healthy donors. This was not detectable without magnetic purification or when using previously described assays. Because the ELISPOT assay cannot be combined with tetramer identification of specific T cells, IFN-γ^+ spots ex vivo do not reflect cytokine secretion by CMV-specific lymphocytes but reflect IFN-γ production from all PBMC. Moreover, because IFN-γ molecules are labeled intracellularly, the Cytospot assay does not allow further sorting of IFN-γ^+ cells. The lack of this enrichment step may mainly explain the lower sensitivity and the absence of detectable IFN-γ-secreting CMV-specific cells ex vivo. Interestingly, the IFN-γ secretion assay did not reveal an ex vivo cytokine release by influenza-specific cells in any of the individuals tested. Although these cells may not be visualized due to their lower frequency, they display a memory state after influenza virus infection (4) and may therefore lack effector function without previous restimulation. In contrast, CMV manages to escape the immune response leading to latency and continued reactivation (36). This would explain the observed differences in the surface phenotype of influenza- and CMV-specific cells and the selective IFN-γ secretion ex vivo by CMV-specific cells. Importantly, A2/CMV$^+$ IFN-γ^+ cells detected upon exposure with HIV-1 peptide did not correspond to cross-reactive T cell populations. The frequencies ex vivo of A2/CMV$^+$ IFN-γ^+ cells from HD 2713 as well as of A2/Melan-A$^+$ cells from LAU 337 were identical whether exposed to HIV-1 peptide or not exposed to any peptide. Similarly, CMV-specific clones derived from HD 2709, 7519, and 7524 were not reactive to HIV-1 peptide, as assessed by cytokine secretion assay (data not shown). Because T cells undergo rapid on/off cycling of cytokine production (37), it is possible that CMV-specific CD8$^+$ T cells may have recently encountered viral antigenic ligand in these clinically disease-free individuals and switched on cytokine production. Likewise, the 0.3% of CD8$^+$ IFN-γ^+ T cells that are found on average upon ex vivo assays in healthy individuals likely reflect recent reactivation of effectors by Ags from clinically "silent" pathogens.

The differentiation state of Melan-A-specific cells in HLA-A2 healthy individuals remains under debate. The mean frequency of these cells comprises ~0.06% of CD8$^+$ T cells, which is at least 50–200 times higher than the frequency currently estimated for naive Ag-specific lymphocyte precursors in the periphery. However, Melan-A-specific T lymphocytes exhibit a naive CD45RA^{high}CD28$^+$CCR7$^+$ phenotype ex vivo and do not secrete detectable amounts of IFN-γ in the 20-h ELISPOT assay (4, 33). Here, Melan-A-specific cells from healthy individuals did not produce IFN-γ after short-term peptide stimulation when using the cytokine secretion assay. More strikingly, no IFN-γ^+ Melan-A-specific cells were detectable following magnetic enrichment. This substantiates the notion that these cells are not Ag experienced. The mechanisms involved in selecting and maintaining a high frequency of Melan-A-specific CD8$^+$ T cells in the circulation without undergoing significant differentiation needs to be further investigated.

The use of MHC/peptide tetramers has allowed quantitation of CD8$^+$ T cell responses against various tumor Ags in cancer patients, both at the tumor site and in the periphery. The function of activated tumor-specific lymphocytes, however, remains controversial. In many cases, these cells were described as functional memory cells with proliferative potential and cytolytic function. In contrast, they may also become functionally unresponsive, as described for tyrosinase-specific CD8$^+$ T cells in one melanoma patient (17). We evaluated here the ability of Ag-experienced Melan-A-specific cells from three melanoma patients to produce IFN-γ by applying the cytokine secretion assay. Melan-A-specific CD8$^+$ T cells in all three patients presented levels of activity similar to those found for influenza- or CMV virus-specific lymphocytes, showing a functional differentiation in vivo into effector/memory lymphocytes. The systemic response against Melan-A$_{26–35}$ observed in patient LAU 337 occurred concomitantly with repeated Melan-A peptide administration (18). The number of circulating Melan-A-specific cells increased >20-fold within a period of 3 mo, reaching >2% of the CD8$^+$ T cell pool. Some lung and s.c. metastases showed signs of regression during the period of treatment while other lesions remained stable. Using the outlined assay, significant numbers of Melan-A-specific cells spontaneously secreted IFN-γ in this individual, compatible with a strong level of activity in vivo. Further analyses with cell samples from other patients that received the same treatment will help in assessing the potential role of this immune therapy.

It is essential to define mechanisms of protective immunity in infectious and malignant diseases. There is increasing awareness that T cells exist in a large variety of activation/differentiation stages, of which only some are involved in protective immunity. The high sensitivity of the assay described in this study provides a unique tool to further characterize single Ag-specific T cells and to monitor immunity in experimental and clinical situations. It may represent a valuable technology to promote future development of specific immunotherapy of cancer and persistent viral infections such as HIV.

Acknowledgments

We thank Dr. Martin Lipp and Dr. Reinold Forster for providing the anti-CCR7 Ab and Dr. Immanuel Lüscher for tetramer synthesis. We gratefully acknowledge the help of Dr. Pascal Batard and the excellent technical assistance of Céline Baroffio, Susanne Hoher, Renate Milesi, Andrée Porret, and Martine van Overloop.

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