

High Frequencies of Naive Melan-A/MART-1-specific CD8⁺ T Cells in a Large Proportion of Human Histocompatibility Leukocyte Antigen (HLA)-A2 Individuals

By Mikael J. Pittet,* Danila Valmori,* P. Rod Dunbar,[§]
Daniel E. Speiser,* Danielle Liénard,*[‡] Ferdy Lejeune,[‡]
Katharina Fleischhauer,^{||} Vincenzo Cerundolo,[§]
Jean-Charles Cerottini,* and Pedro Romero*

From the *Division of Clinical Onco-Immunology, Ludwig Institute for Cancer Research, Lausanne Branch, and the [‡]Multidisciplinary Oncology Center, Centre Hospitalier Universitaire Vaudois, 1011 Lausanne, Switzerland; the [§]Institute of Molecular Medicine, Nuffield Department of Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, United Kingdom; and the ^{||}Tissue Typing Laboratory, Department of Biology and Biotechnology (DIBIT), Istituto Scientifico H.S. Raffaele, 20132 Milano, Italy

Summary

Using fluorescent HLA-A*0201 tetramers containing the immunodominant Melan-A/MART-1 (Melan-A) tumor-associated antigen (Ag), we previously observed that metastatic lymph nodes of melanoma patients contain high numbers of Ag-experienced Melan-A-specific cytolytic T lymphocytes (CTLs). In this paper, we enumerated and characterized ex vivo Melan-A-specific cells in peripheral blood samples from both melanoma patients and healthy individuals. High frequencies (≥ 1 in 2,500 CD8⁺ T cells) of Melan-A-specific cells were found in 10 out of 13 patients, and, surprisingly, in 6 out of 10 healthy individuals. Virtually all Melan-A-specific cells from 6 out of 6 healthy individuals and from 7 out of 10 patients displayed a naive CD45RA^{hi}/RO⁻ phenotype, whereas variable proportions of Ag-experienced CD45RA^{lo}/RO⁺ Melan-A-specific cells were observed in the remaining 3 patients. In contrast, ex vivo influenza matrix-specific CTLs from all individuals exhibited a CD45RA^{lo}/RO⁺ memory phenotype as expected. Ag specificity of tetramer-sorted A2/Melan-A⁺ cells from healthy individuals was confirmed after mitogen-driven expansion. Likewise, functional limiting dilution analysis and interferon γ ELISPOT assays independently confirmed that most of the Melan-A-specific cells were not Ag experienced. Thus, it appears that high frequencies of naive Melan-A-specific CD8⁺ T cells can be found in a large proportion of HLA-A*0201⁺ individuals. Furthermore, as demonstrated for one patient followed over time, dramatic phenotype changes of circulating Melan-A-specific cells can occur in vivo.

Key words: melanoma • tetramer • influenza matrix • immunotherapy • tumor immunity

Accumulating evidence supports the importance of Ag-specific cytolytic T lymphocyte (CTL) responses in tumor immunity. Moreover, the molecular identification of CTL-defined tumor-associated Ags has prompted the design of novel Ag-specific cancer vaccines (1–3). Indeed, numerous phase I clinical trials are underway aimed at inducing CTL responses against defined tumor Ags. In particular, the Melan-A/MART-1 (Melan-A)¹ gene, which is expressed by normal melanocytes and by most fresh mel-

noma samples, codes for Ags recognized by tumor-reactive HLA-A*0201-restricted CTLs (4, 5). CTL lines specific for the immunodominant Melan-A_{26–35} Ag derived from peripheral blood of both melanoma patients and healthy individuals were obtained after repeated antigenic stimulation in vitro (6–8). There has been suggestive evidence that Melan-A-specific cells may present distinct naive and memory phenotypes in healthy individuals and melanoma patients, respectively (8, 9). However, the lack of direct assays had prevented a precise assessment of CTLs ex vivo (10, 11). The development of HLA-A*0201 tetrameric complexes containing melanoma-derived antigenic peptides has enabled direct enumeration and phenotyping of tumor-specific CTLs. For instance, we recently reported

¹Abbreviations used in this paper: CTL, cytolytic T lymphocyte; CTLp, cytolytic T lymphocytes precursor(s); LDA, limiting dilution analysis; Melan-A, Melan-A/MART-1.

the presence of high numbers of Ag-experienced tumor-specific CTLs in metastatic LNs of melanoma patients (12).

In this study, the characterization of HLA-A*0201-restricted Melan-A-specific CD8⁺ T cells has been extended to the peripheral blood compartment from both melanoma patients and healthy individuals. Cells were analyzed for surface expression of naive-, effector-, and memory-type-associated markers. In humans, the CD45RA and CD45RO surface Ags have been used to identify naive and memory T cells, respectively (13–15). However, these two Ags may be insufficient, as the CD45RA^{hi} population contains cells that express several phenotypic features of primed T cells (16–18). Indeed, CD45RA^{hi} cells lacking surface expression of the CD28 costimulatory receptor exhibit direct ex vivo cytolytic activity (19, 20), and abundantly express Fas ligand transcripts as well as perforin and granzyme B (21, 22), thereby displaying the attributes of effector CTLs (22, 23). Accordingly, a model has been proposed in which naive-, effector-, and memory-type CD8⁺ T cells could be distinguished based on surface expression of CD28 and CD45RA (24). We have applied this model on the level of single Ag-specific cells in multiparameter analyses including CD28, CD45RA, and CD45RO surface expression and fluorescent tetramers containing the Melan-A_{26–35} A27L peptide (A2/Melan-A) or the influenza matrix Flu-MA_{58–66} (A2/Flu-MA) Ag. Since the majority of HLA-A*0201 individuals are sensitized against the latter immunodominant peptide (25), it served as a source of well-defined memory-type, epitope-specific CTL populations in circulating lymphocytes (26).

Ex vivo tetramer staining generally revealed high frequencies of A2/Melan-A⁺CD8⁺ T cells. Surprisingly, in the majority of individuals these cells displayed a naive CD28⁺CD45RA^{hi}/RO⁻ phenotype, in contrast to the CD28⁺CD45RA^{lo}/RO⁺ memory phenotype exhibited by most A2/Flu-MA⁺ cells. Both specificity and phenotype of A2/Melan-A⁺ cells were independently confirmed by functional assays.

Materials and Methods

Cells. 13 patients (22–75 yr old) with advanced stage malignant melanoma and 10 healthy donors (22–73 yr old) were selected for this study on the basis of HLA-A2 Ag expression as assessed by flow cytometry of PBMCs stained with allele-specific mAb BB7.2 (27). Molecular HLA-A*02 subtyping performed by PCR-sequence-specific oligonucleotide probe (SSOP) (28) revealed that all HLA-A2⁺ individuals were A*0201. None of them had two different A*02 subtypes. PBMCs from healthy blood donors were obtained from buffy coats provided by the blood transfusion center in Lausanne, Switzerland. PBMCs were separated from heparinized blood diluted 1:2 with PBS by centrifugation over Ficoll-Paque (Pharmacia), washed three times, and cryopreserved in RPMI 1640/40% FCS/10% DMSO. Vials containing 10⁷ PBMCs were stored in liquid nitrogen.

Tetramers. Tetrameric complexes were synthesized as previously described (12, 26). In brief, purified HLA heavy chain and β 2-microglobulin were synthesized using a prokaryotic expression system (pET; R&D Systems, Inc.). The heavy chain was

modified by deletion of the transmembrane cytosolic tail and COOH addition of a sequence containing the BirA enzymatic biotinylation site. Heavy chain, β 2-microglobulin, and peptide were refolded by dilution. The 45-kD refolded product was isolated by fast protein liquid chromatography, then biotinylated by BirA (Avidity) in the presence of biotin, adenosine 5'-triphosphate, and Mg²⁺ (all from Sigma Chemical Co.). Streptavidin-PE conjugate (Sigma Chemical Co.) was added in a 1:4 molar ratio, and the tetrameric product was concentrated to 1 mg/ml. Tetramers were synthesized around two tumor antigenic peptides recognized by HLA-A*0201-restricted CTLs, both of which derive from melanocyte lineage-specific proteins. One peptide was the natural tyrosinase_{368–376} epitope (the N370D variant, YMD-GTMSQV, generated by Ag processing [29]); the other was a modification of the Melan-A_{26–35} epitope (30). This modified epitope, ELAGIGILTV, carrying a substitution of Ala for Leu at position 2 from the NH₂ terminus (hereafter A27L), forms relatively stable complexes with HLA-A*0201 and is a more potent immunogen than is the natural Melan-A peptide (31, 32). Experiments have shown that tetramers synthesized around the A27L-modified epitope generally stained polyclonal and monoclonal Melan-A-specific CTL populations (data not shown and reference 12). However, we can not totally exclude the possibility that some T cell populations might not have been stained with the A27L peptide analogue containing tetramer. Finally, a third tetramer was synthesized around the HLA-A*0201-restricted influenza matrix Flu-MA_{58–66} (GILGFVFTL) immunodominant peptide. A2/tyrosinase, A2/Melan-A, and A2/Flu-MA tetramers were used at 20 μ g/ml.

mAbs. Anti-CD3^{PE}, CD8^{FITC}, PerCP, and CD45RA^{Cyc} were obtained from Becton Dickinson; anti-CD45RO^{FITC} was from DAKO Corp.; and anti-CD28^{FITC} was from Immunotech.

Flow Cytometry Analysis. Thawed PBMCs were cultured for 16–20 h in IMDM supplemented with 0.55 mM Arg, 0.24 mM Asn, 1.5 mM Gln, and 8% pooled human A⁺ serum (complete medium). CD8⁺ lymphocytes were then purified from PBMCs in two rounds of positive selection by magnetic cell sorting using a MiniMACS device (Miltenyi Biotec Inc.) The resulting cells were >98% CD3⁺CD8⁺, and 10⁶ were stained with A2/tetramers and FITC and Cychrome mAb conjugates in 50 μ l of PBS, 2% BSA, and 0.2% azide for 40 min at 4°C. CD8 enrichment did not disturb the detection of tetramer⁺ cells, either in terms of frequency or phenotype (data not shown). Cells were then washed once in the same buffer and analyzed immediately in a FACSCalibur[®] (Becton Dickinson).

Limiting Dilution Analysis. CD45RA^{lo} and CD45RA^{hi}CD8⁺ T cells subsets from healthy donors HD 329 and HD 604 and from melanoma patients LAU 132 and LAU 203 were sorted using a FACStar[®] (Becton Dickinson). These subpopulations were cultured at 10,000, 5,000, 2,500, and 1,250 cells/well (24 wells per condition) in complete medium plus 100 U/ml recombinant human IL-2, and stimulated at days 0 and 7 by autologous CD8⁻ PBMCs (10⁵/well) pulsed with 1 μ M Melan-A_{26–35} A27L peptide. At day 13, Ag recognition was assessed using T2 target cells (100 μ l) labeled with ⁵¹Cr and incubated in the presence or absence of 1 μ g/ml of the antigenic Melan-A_{26–35} A27L peptide for 1 h at 37°C and washed three times. Labeled target cells (10³ cells in 50 μ l) were then added to effector cells (50 μ l) in V-bottomed microwells (50 μ l). The effector cells were preincubated for 20 min at 37°C in the presence of unlabeled K562 cells (5 \times 10⁵/well) to eliminate nonspecific lysis due to NK-like effectors. Cr release was measured in supernatant (40 μ l) harvested after 4 h of incubation at 37°C. The percentage of specific lysis and the de-

duced frequency of CTL precursors (CTLp) present in each subset was calculated as previously described (33).

IFN- γ ELISPOT Assay. ELISPOT plates (Millipore) were coated overnight with antibody to human IFN- γ (Mabtech) and washed six times. 10 μ g/ml peptide and 1.66×10^5 PBMCs per well in 200 μ l Iscove's medium/8% human serum were added and incubated for 20 h at 37°C. Assays were performed in six replicates with either the Melan-A₂₆₋₃₅ A27L or the Flu-MA₅₈₋₆₆ peptide. The ILKEPVHGV Pol₄₇₆₋₄₈₄ peptide from the reverse transcriptase of HIV-1 was also included as a negative control. (All subjects in this study were HIV seronegative.) Cells were removed, and plates were developed with a second antibody to human IFN- γ (biotinylated) and streptavidin-alkaline phosphatase (Mabtech). The deduced frequency of peptide-specific CTLs in CD8⁺ T cells was calculated as: (mean no. of specific spots) / [(1.66 $\times 10^5$) \times (percentage of CD3⁺CD8⁺ cells in PBMCs)], where the percent of CD3⁺CD8⁺ cells in PBMCs was determined by flow cytometry on the same batch of cryopreserved cells. The baseline number, or cut off value, of nonspecific IFN- γ spots was calculated as the mean number of spots in the presence of the control HIV-1 peptide in 21 individuals + 3 SD. This value was 17 spots/10⁶ PBMCs (mean = 5 spots/10⁶ PBMCs, SD = 4), implying a lower specific detection limit of 1 in ~60,000 PBMCs. Since the enumeration of Melan-A-specific lymphocytes with tetramers was directly performed on gated CD8⁺ lymphocytes, the cut off value and the frequencies determined by IFN- γ ELISPOT were all adjusted to reflect the percentage of CD8⁺ lymphocytes determined in the same PBMC batch by flow cytometry. This was performed to enable comparison with tetramer frequency values. The CD8-adjusted baseline spot value was 87 spots/10⁶ CD8⁺ PBMCs (mean = 25 spots/10⁶ CD8⁺ PBMCs, SD = 21).

Results

Circulating A2/Melan-A⁺CD8⁺ T Cells Are Detectable both in Melanoma Patients and Healthy Individuals. 13 HLA-A2⁺ patients with advanced stage malignant melanoma and 10 HLA-A2⁺ healthy donors were randomly selected for this study. Among melanoma patients, three presented concurrent vitiligo that developed either after a systemic treatment with intravenous IL-2 + Cis Platinum + IFN- α (patient LAU 155), during IFN- α therapy (LAU 156), or after isolated limb perfusion with high dose of TNF- α + melphalan (LAU 269).

Highly enriched circulating CD8⁺ T lymphocytes (>98% CD3⁺CD8⁺) from each individual were stained for flow cytometry with different A2/tetramers, two synthesized around melanoma-associated Ags, namely the Melan-A₂₆₋₃₅ A27L analogue and tyrosinase₃₆₈₋₃₇₆, and one around the viral influenza matrix Flu-MA₅₈₋₆₆ peptide. As illustrated in Fig. 1, circulating A2/Melan-A⁺ and A2/Flu-MA⁺CD8⁺ cells were detected both in melanoma patients and healthy donors. In contrast, the frequency of A2/tyrosinase⁺ cells was generally too low for direct ex vivo detection. However, we observed that a short in vitro Ag-driven expansion was sufficient to detect A2/tyrosinase⁺ cells in the majority of A2/melanoma patients, confirming the presence of circulating tyrosinase specific CTLp (33a).

To determine the levels of nonspecific A2/tetramer

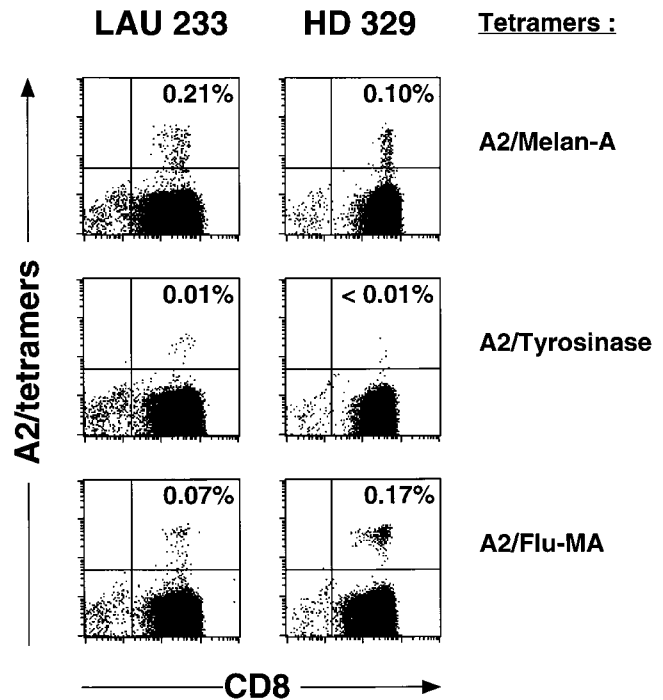


Figure 1. Detection of ex vivo circulating A2/Melan-A⁺ and A2/Flu-MA⁺, but not A2/Tyrosinase⁺CD8⁺ T cells by flow cytometry, in a melanoma patient (LAU 233) and a healthy donor (HD 329). Highly homogeneous CD8⁺ lymphocyte populations (>98%) were obtained from PBMCs of patient LAU 233 or from healthy donor HD 329, by two rounds of positive selection with magnetic cell sorting. The lymphocyte preparations were then stained with A2/Melan-A, A2/Tyrosinase, or A2/Flu-MA tetramers together with anti-CD8^{FITC} mAb, and analyzed immediately by flow cytometry. The frequency of up to 0.01% of A2/tyrosinase⁺ cells was below the limit of detection ($\geq 0.04\%$ for A2/Melan-A; $\geq 0.02\%$ for A2/Flu-MA).

staining of circulating CD8⁺ T cells, a series of nine blood samples of randomly selected HLA-A2⁻ blood donors was analyzed (Table I). Although this approach does not provide direct insight on the level of nonspecific epitope-based A2/tetramer staining in HLA-A2⁺ individuals, it allowed us to define a lower detection limit for tetramer staining in A2⁺ individuals. This lower detection limit was ~0.04% of CD8⁺ T cells with A2/Melan-A tetramers (cut off = mean + 3 SD = 0.036), and <0.02% of CD8⁺ T cells with A2/Flu-MA tetramers (cut off = mean + 3 SD = 0.011). These detection limits for staining with A2/Melan-A tetramers are clearly lower for circulating cells than for tumor-infiltrated LNs (~0.25%; calculated previously; reference 12). According to these limits, ex vivo circulating A2/Melan-A⁺CD8⁺ cells were found in significant numbers in 10 out of 13 melanoma patients, and in 6 out of 10 healthy donors. As we previously reported (34), the frequency of A2/Melan-A⁺ cells was generally very high in melanoma patients with concurrent vitiligo (mean = 0.23% of CD8⁺ cells). In contrast, the frequency of CD8⁺ cells stained with A2/Melan-A tetramers was comparable between melanoma patients without vitiligo and healthy donors (mean =

Table I. Percentage of Circulating A2/Melan-A⁺ and A2/Flu-MA⁺ Subsets in CD8⁺ T Cells*

HLA-A2-negative healthy donors			HLA-A*0201 healthy donors			HLA-A*0201 melanoma patients			HLA-A*0201 melanoma patients + vitiligo		
Name	Melan-A	Flu-MA	Name	Melan-A	Flu-MA	Name	Melan-A	Flu-MA	Name	Melan-A	Flu-MA
HD 001	0.01	0.01	HD 006	0.05	0.11	LAU 50	0.03 [‡]	0.41	LAU 155	0.12	0.07
HD 004	0.00	0.00	HD 007	0.01 [‡]	0.07	LAU 56	0.08	0.13	LAU 156	0.40	0.18
HD 048	0.01	0.00	HD 008	0.06	0.02	LAU 97	0.07	0.10	LAU 269	0.16	0.02
HD 108	0.01	0.00	HD 099	0.08	0.07	LAU 132 [§]	0.07	0.05			
HD 143	0.03	0.00	HD 301	0.02 [‡]	0.09	LAU 198	0.02 [‡]	1.65			
HD 222	0.02	0.00	HD 304	0.03 [‡]	0.05	LAU 203	0.07	0.03			
HD 299	0.01	0.00	HD 329	0.10	0.17	LAU 212	0.03 [‡]	0.05			
HD 347	0.01	0.00	HD 421	0.11	0.03	LAU 233	0.21	0.08			
HD 408	0.01	0.00	HD 422	0.02 [‡]	0.49	LAU 240	0.04	ND			
			HD 604	0.21	0.13	LAU 267	0.04	ND			
Mean	0.014	0.001	Mean	0.07	0.12	Mean	0.07	0.31	Mean	0.23	0.09
SD	0.008	0.003	SD	0.06	0.14	SD	0.06	0.55	SD	0.15	0.08
C.O.	0.036	0.011									

*The percentages of tetramer⁺ cells were determined as illustrated in Fig. 1. Values are the mean of duplicates.

[‡]Percentage values below the lower limit of detection, as determined with the nine HLA-A2-negative healthy donors (cut off [C.O.] = mean + 3 SD).

[§]Analysis of LAU 132 was performed with sample from September 1997.

0.07%) (Table I). Likewise, the frequency of A2/Flu-MA⁺ cells was globally equivalent in all groups, with the exception of LAU 198, who had 1.65% of CD8⁺ cells stained with tetramers.

Circulating A2/Melan-A⁺ and A2/Flu-MA⁺ CD8⁺ T Cells in Healthy Individuals Present Naive and Memory Phenotypes, Respectively. In humans, cell surface expression of the CD45RA and CD45RO isoforms have been used to identify naive and memory T cells, respectively (13–15). As circulating CD28⁺CD8⁺ T cells present direct ex vivo cytolytic activity (19, 20) and were recently proposed to correspond to effector-type CTLs (22–24), individuals presenting significant amounts of circulating A2/Melan-A⁺ and A2/Flu-MA⁺ cells were phenotyped for CD28, CD45RA, and CD45RO surface expression by three-color flow cytometry analyses.

Clearly, circulating A2/Melan-A⁺ and A2/Flu-MA⁺ CD8⁺ T cells from healthy donors displayed distinct phenotypes (as illustrated for one donor in Fig. 2 A and summarized in Fig. 2 C). Practically all of the A2/Melan-A⁺ cells were CD28⁺CD45RA^{hi}/RO⁻ (range: 84–95%), corresponding to a naive phenotype. In marked contrast, most of A2/Flu-MA⁺ cells were CD45RA^{lo}/RO⁺ (range: 83–97%). Thus, the phenotype of A2/Flu-MA⁺ cells corresponded to Ag-experienced memory T cells, compatible with the notion that the Flu-MA_{58–66} peptide probably represents a recall Ag in these HLA-A2⁺ individuals. In addition, ~20% of CD45RA^{lo}/RO⁺A2/Flu-MA⁺ cells from HD 099 and HD 604 presented a CD28⁻ phenotype (data not shown).

Circulating A2/Melan-A⁺ CD8⁺ T Cells of Some Melanoma Patients Consist of Variable Proportions of Naive and Ag-experienced Phenotypes. In contrast to healthy individuals, the phenotype of A2/Melan-A⁺ cells was heterogeneous in melanoma patients (Fig. 2 B). In most of them (7 out of 10), A2/Melan-A⁺ cells presented a uniformly naive CD28⁺CD45RA^{hi}/RO⁻ phenotype (range: 81–95%), like those found in all healthy donors. However, 3 out of 10 patients either displayed >35% CD45RA^{lo}/RO⁺ (LAU 132 and 240), or >90% CD28⁻CD45RA^{int} (LAU 156) A2/Melan-A⁺ cells (Fig. 2 C). It is not possible with the current data to determine whether such phenotypic changes in Melan-A-specific lymphocytes may have occurred in response to peptide-based vaccination (LAU 132 received five rounds of vaccination with the Melan-A_{26–35} peptide plus other melanoma-associated Ags with GM-CSF), or simply reflect non-Ag-specific changes after administration of cytokines such as GM-CSF or IFN- α (LAU 132 received GM-CSF concomitant with peptide administration, and LAU 156 was treated with IFN- α). Moreover, other melanoma patients included in this study who also received Melan-A_{26–35} peptide vaccination with GM-CSF (LAU 269) or IFN- α therapy (LAU 267) did not present memory phenotype A2/Melan-A⁺ cells in the circulating compartment.

Altogether, phenotype and frequency of A2/Melan-A⁺ cells were generally not correlated, since (a) memory phenotype cells detected in patients LAU 132 and 240 were not found at increased frequencies (0.07 and 0.04% of CD8⁺ T cells, respectively); and (b) high frequencies of

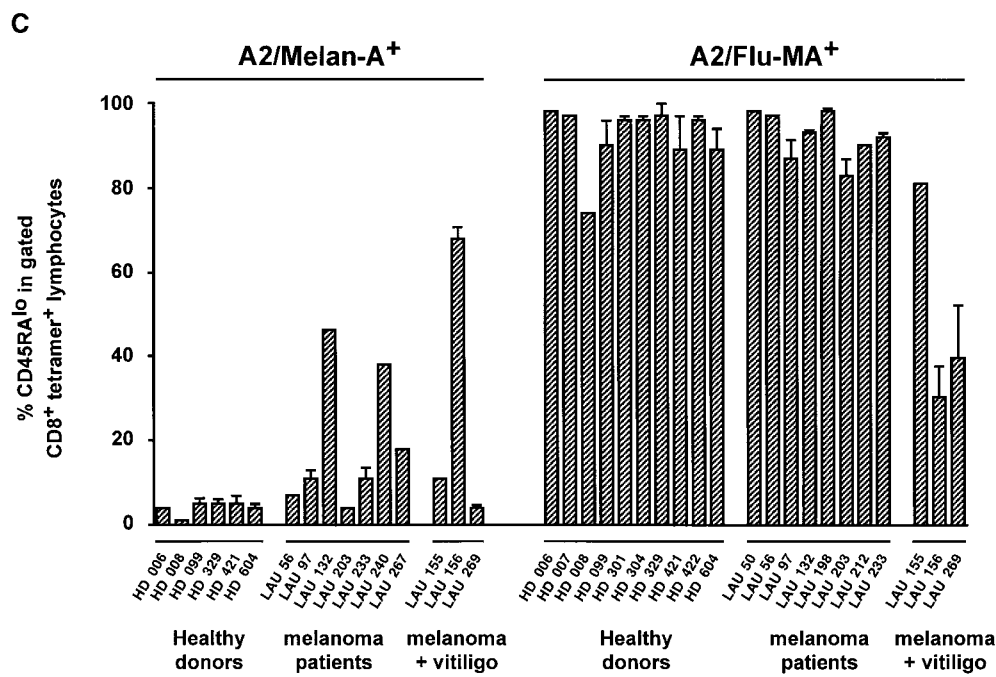
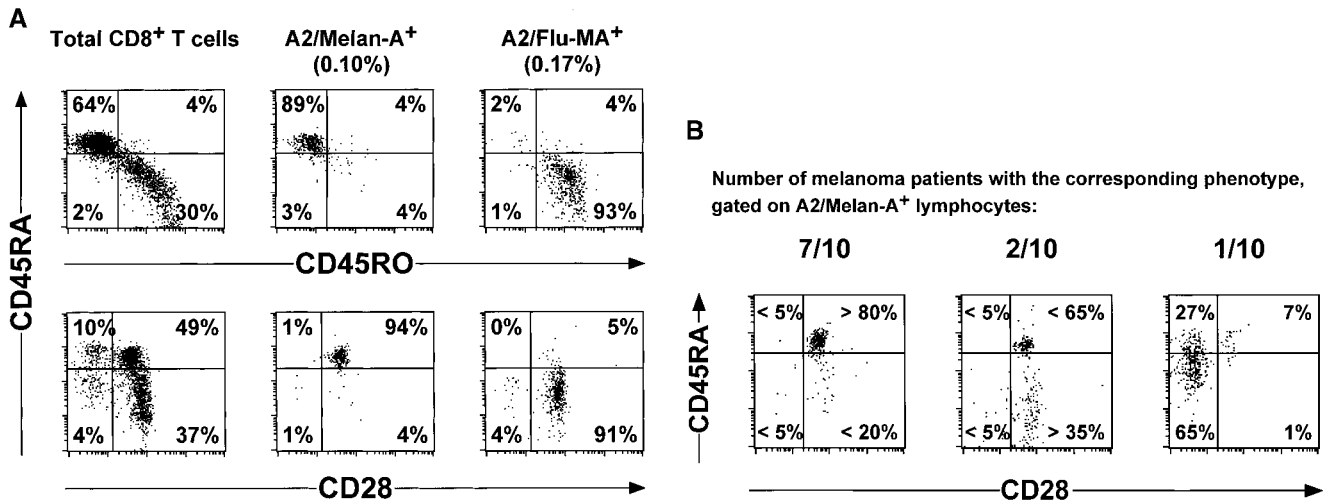


Figure 2. Phenotypic analysis of ex vivo circulating A2/Melan-A⁺ and A2/Flu-MA⁺ cells in melanoma patients and healthy donors. CD8⁺ lymphocyte populations were highly purified (>98%) from PBMCs, as illustrated in Fig. 1. The lymphocyte preparations were then stained with A2/Melan-A, or A2/Flu-MA tetramers together with anti-CD45RA^{CYC} together with either anti-CD45RO^{FITC} or anti-CD28^{FITC} mAbs, and immediately analyzed by flow cytometry. (A) Pattern of expression of CD45RA/RO (top) or CD45RA/CD28 (bottom) in total circulating CD8⁺ T cells (left) from healthy donors (shown is HD 329). They were CD28⁺CD45RA^{hi}/RO⁻ in A2/Melan-A⁺ gated cells (middle) but CD28⁺CD45RA^{lo}/RO⁺ in A2/Flu-MA⁺ gated cells (right). (B) Circulating A2/Melan-A⁺CD8⁺ T cells detected in the majority (7 out of 10) of melanoma patients presented a

CD28⁺CD45RA^{hi}/RO⁻ phenotype (left). In contrast, tetramer⁺ cells from 2 out of 10 patients displayed variable proportions of CD45RA^{hi}/RO⁻ and CD45RA^{lo}/RO⁺tetramer⁺ cells (middle) and a CD28⁻CD45RA^{int} phenotype for 1 out of 10 patients (right). (C) Summary of phenotyping data obtained for melanoma patients and healthy donors. Frequencies of CD45RA^{lo} cells detected in gated A2/Melan-A⁺ and A2/Flu-MA⁺CD8⁺ T cells were calculated with CellQuest™ software.

A2/Melan-A⁺ cells (>0.2% CD8⁺ T cells) in patients LAU 155, 233, and 269 as well as in healthy donor HD 604 were not of the memory phenotype. As an exception, LAU 156 presented high frequency of CD28⁻A2/Melan-A⁺ cells.

The vast majority of A2/Flu-MA⁺ cells displayed a memory-like CD28⁺CD45RA^{lo}/RO⁺ phenotype in 11 out of 13 patients, as well as in all healthy donors (Fig. 2 C). However, the A2/Flu-MA⁺ cells presented both CD28⁻CD45RA^{int} (60%) and CD28⁺CD45RA^{int} (30%) phenotypes in LAU 156, and a CD28⁺CD45RA^{int} phenotype in LAU 269 (CD28 phenotype not shown).

Confirmation of Ag-specificity and Functional Status of A2/Melan-A⁺ Cells by Limiting Dilution Analysis and IFN-γ ELISPOT Assays. Initially, two healthy donors (HD 329 and 604) and two melanoma patients (LAU 132 and 203) were selected according to the phenotype of A2/Melan-A⁺ cells: the vast majority of A2/Melan-A⁺ cells from HD 329, HD 604, and LAU 203 were CD45RA^{hi} (95, 95, and 94%, respectively); on the other hand, 46% of those from LAU 132 presented a CD45RA^{lo} phenotype (Table II). These phenotypes were independently examined by two functional assays, limiting dilution analysis (LDA) and IFN-γ ELISPOT.

Table II. Frequency of Melan-A-specific Cells within CD8⁺ T Cells Measured by Tetramer Staining, LDA, and IFN- γ ELISPOT

	Tetramers*		LDA [‡]		ELISPOT [§]
	CD45RA ^{hi}	CD45RA ^{lo}	CD45RA ^{hi}	CD45RA ^{lo}	total
HD 329	100	7 [¶]	10	0.3	1 [¶]
HD 604	200	10 [¶]	10	0.1	0.0 [¶]
LAU 132	40	30	3	9	10
LAU 203	70	4 [¶]	10	1	0.0 [¶]

All frequencies $\times 10^{-5}$.

*Values correspond to the number of cells in the A2/Melan-A⁺CD45RA^{hi} and A2/Melan-A⁺CD45RA^{lo} gates divided by the number of CD8⁺ lymphocytes.

[‡]Sorted CD45RA^{hi} and CD45RA^{lo} fractions of CD8⁺ T cells were stimulated twice with Melan-A₂₆₋₃₅ A27L peptide at limiting dilution conditions, then CTL activity was measured as described in Materials and Methods. The frequency of CTLp in each fraction was calculated using LDA analysis software (33), then normalized to the percentages of the respective CD45RA^{hi} and CD45RA^{lo} subsets. Thus, the values indicated correspond to the frequency, within CD8⁺ T cells, of CD45RA^{hi} and CD45RA^{lo} Melan-A-specific CTLp, respectively.

[§]Melan-A-specific IFN- γ production and the deduced frequency of Melan-A-specific CTLs in CD8⁺ T cells was measured as described in Materials and Methods.

^{||}Two healthy donors (HD 329 and 604) and two melanoma patients (LAU 132 and 203) were selected. Analysis of LAU 132 was performed with samples from September 1997. Values are the mean of duplicates, except for tetramer analysis of LAU 132 and 203, and are rounded to one significant figure.

[¶]Frequencies below the lower limit of detection.

For LDA, CD45RA^{lo} and CD45RA^{hi} fractions of CD8⁺ T cells were sorted and stimulated twice with Melan-A₂₆₋₃₅ A27L peptide at limiting dilution conditions. After 13 d, the large majority of microcultures displaying Melan-A-specific CTL activity in HD 329, HD 604, and LAU 203 were detected in the progeny from the CD45RA^{hi} subset (98, 99, and 90% of positive microcultures, respectively). In contrast, 73% of positive microcultures in LAU 132 were detected in the cells expanded from the CD45RA^{lo} subset. Thus, the distribution of CTLp among the naive and memory subsets evaluated by functional LDA assays parallels that observed by flow cytometry phenotypic analysis, confirming the Ag specificity of the relatively low numbers of tetramer⁺ lymphocytes. However, LDA underestimated the frequency of Melan-A-specific CTLp by a factor of ~ 3 for CD45RA^{lo} phenotype A2/Melan-A⁺ cells (LAU 132), and to a higher extent by a factor of ~ 13 in average for CD45RA^{hi} phenotype A2/Melan-A⁺ cells (Table II).

In parallel, the phenotype of Melan-A-specific cells was indirectly assessed by a 20-h IFN- γ ELISPOT assay. Given the limited number of cells available, this assay was not performed on CD45RA^{+/−} sorted populations, but rather with unsorted PBMCs. As expected for truly naive CD8⁺ T cells, ex vivo Melan-A-specific IFN- γ producing cells were undetectable in PBMCs from HD 329, HD 604, and LAU 203 (Table II). In contrast, for LAU 132, we found ~ 100 IFN- γ -specific spots/10⁶ CD8⁺ T cells, which represented a significant frequency above background levels (calculated as described below). To further investigate ex vivo IFN- γ production in response to challenge with Ag, both Melan-A- and Flu-MA-specific cells from all healthy donors and melanoma patients (except LAU 240 and 267)

were analyzed (Fig. 3). First, we determined a lower detection limit (cut off) for this assay. This cut off, based on the number of nonspecific spots obtained after stimulation with the irrelevant HIV-1 Pol₄₆₈₋₄₇₆ peptide of PBMCs from the 21 individuals analyzed, was ~ 90 spots/10⁶ CD8⁺ T cells (cut off = mean + 3 SD = 87, see Materials and Methods for details). With this detection limit, the frequencies of Flu-MA-specific IFN- γ -producing cells (Fig. 3 A) reached significant levels for 17 out of 21 individuals. Moreover, these frequencies correlated well with those calculated by tetramers ($P < 0.0001$, linear regression analysis), but were systematically underestimated (median, 3 times; min, 1.5 times; max, 15 times). In marked contrast, ex vivo Melan-A-specific cells generally did not produce IFN- γ , as expected for naive CD8⁺ T cells (Fig. 3 B). Therefore, the apparent frequency of Melan-A-specific IFN- γ -producing cells was generally much lower than that obtained by tetramer staining (median, 30 times; min, 4 times; max, infinite). It is worth noting that, as some patients had a considerable fraction of A2/Melan-A⁺ cells with an Ag-experienced phenotype (patients LAU 132 and LAU 156, filled symbols in Fig. 3 B), the frequencies of IFN- γ -producing cells upon stimulation with the Melan-A peptide analogue were less underestimated (seven and four times, respectively), when compared with direct counting with A2/Melan-A tetramers.

Naive A2/Melan-A⁺ Cells Are Ag-specific in Lytic Assays. To rule out the possibility that the relatively low numbers of Melan-A⁺ lymphocytes detected in A2⁺ individuals was the result of some flow cytometry artifacts, circulating A2/Melan-A^{+/−} CD8⁺ T cells from a healthy donor (HD 604) were directly sorted into tetramer⁺ and tetramer[−] populations. After 15 d of mitogen-driven polyclonal expansion (1

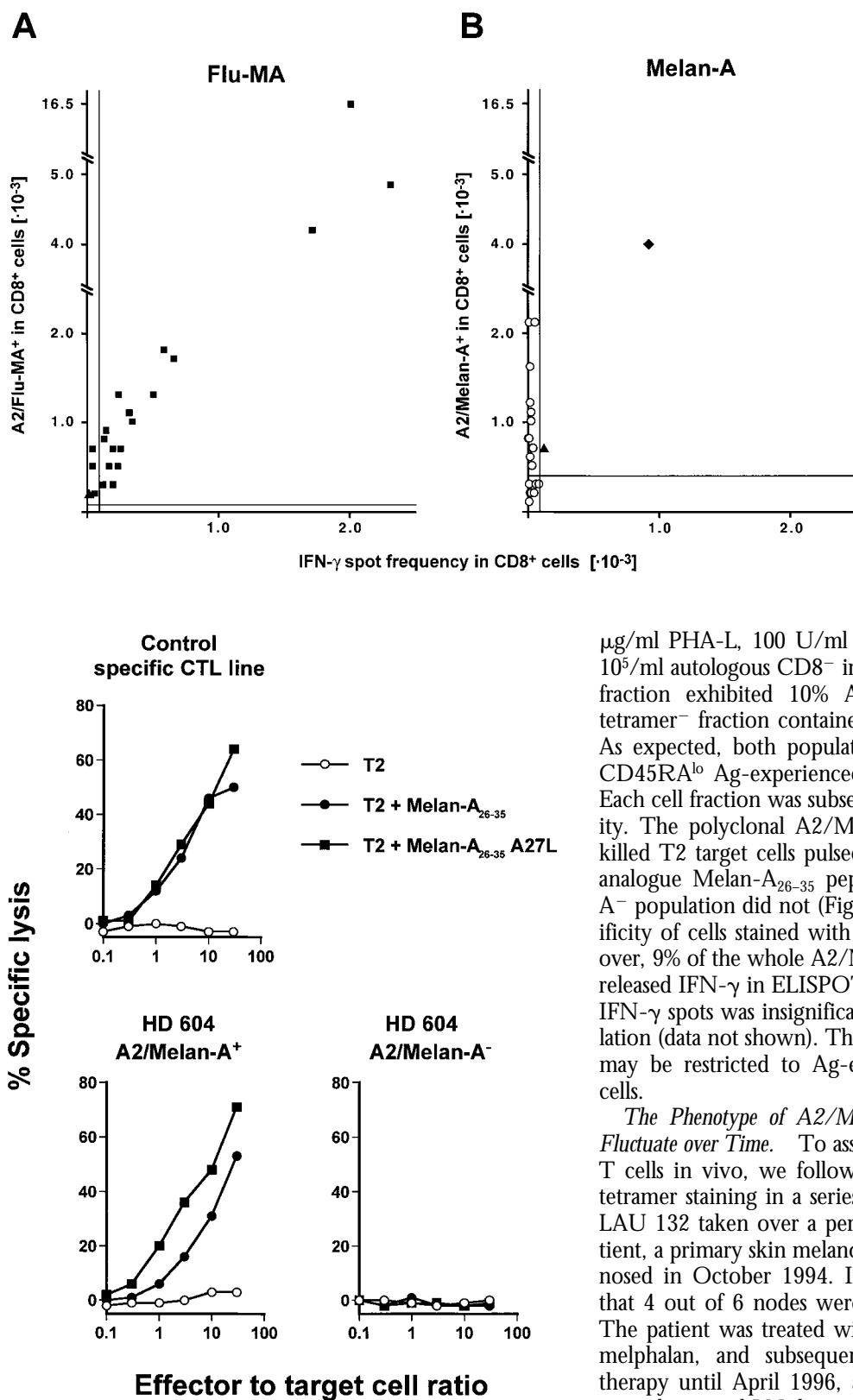


Figure 4. Functional activity of PBMCs sorted according to their tetramer staining phenotype correlates with Ag specificity. Ex vivo CD8⁺ PBMCs from a healthy donor (HD 604) were sorted into A2/Melan-A tetramer⁺ and tetramer⁻ populations. After 2 wk in the presence of irradiated autologous PBMCs and PHA, each cell fraction and a specific CTL

$\mu\text{g/ml}$ PHA-L, 100 U/ml IL-2, 10 ng/ml IL-7, and $5 \times 10^5/\text{ml}$ autologous CD8⁻ irradiated PBMCs), the tetramer⁺ fraction exhibited 10% A2/Melan-A⁺ cells, while the tetramer⁻ fraction contained $<0.02\%$ A2/Melan-A⁺ cells. As expected, both populations displayed a homogeneous CD45RA^{lo} Ag-experienced phenotype (data not shown). Each cell fraction was subsequently tested for its lytic activity. The polyclonal A2/Melan-A⁺ population specifically killed T2 target cells pulsed with the natural or the A27L analogue Melan-A₂₆₋₃₅ peptides, whereas the A2/Melan-A⁻ population did not (Fig. 4). This indicates the Ag specificity of cells stained with A2/Melan-A tetramers. Moreover, 9% of the whole A2/Melan-A⁺ population specifically released IFN- γ in ELISPOT assays, whereas the number of IFN- γ spots was insignificant for the A2/Melan-A⁻ population (data not shown). This confirms that release of IFN- γ may be restricted to Ag-experienced phenotype specific cells.

The Phenotype of A2/Melan-A⁺ Cells Can Dramatically Fluctuate over Time. To assess the fate of Melan-A-specific T cells in vivo, we followed Ag-specific lymphocytes by tetramer staining in a series of blood samples from patient LAU 132 taken over a period of 2 yr (Fig. 5). In this patient, a primary skin melanoma of the lower limb was diagnosed in October 1994. Inguinal LN dissection revealed that 4 out of 6 nodes were infiltrated by melanoma cells. The patient was treated with isolated limb perfusion with melphalan, and subsequently received adjuvant IFN- α therapy until April 1996, at which time he underwent a second inguinal LN dissection (15 out of 16 positive LNs).

line was tested for its lytic activity against Cr-labeled T2 target cells pulsed with different peptides: T2 cells alone (○), T2 + Melan-A 26-35 (●); and T2 + Melan-A 26-35 A27L (■).

Figure 3. IFN- γ ELISPOT assay confirms the naive status of most Melan-A-specific cells. Frequency of Flu-MA- (A) and Melan-A-specific (B) CTLs in CD8⁺ cells from 10 healthy donors and 11 melanoma patients (LAU 240 and 267 excepted) was measured by both IFN- γ ELISPOT assay (x-axis) and tetramer staining (y-axis), as described in Materials and Methods. The phenotypes of tetramer⁺ cells are as follows: CD28⁺CD45RA^{hi} (○), CD28⁺CD45RA^{lo} (■), 30–50% CD28⁺CD45RA^{lo} (▲) and $>60\%$ CD28⁻ (◆). The bars indicate the lower detection limits for both techniques. These were 0.4×10^{-3} for A2/Melan-A, 0.1×10^{-3} for A2/Flu-MA (see footnote to Table I for details), and 0.09×10^{-3} for IFN- γ ELISPOT (see Materials and Methods for details).

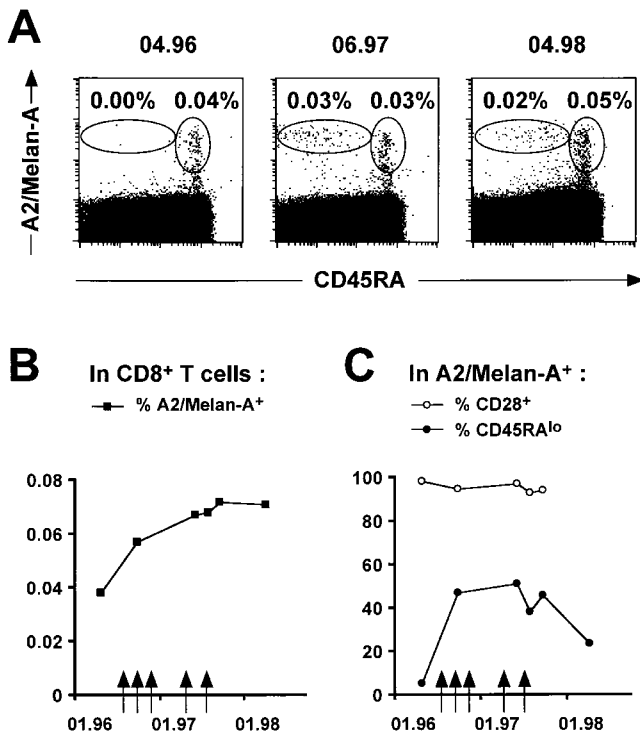


Figure 5. Time course analysis of PBMCs from immunized melanoma patient LAU 132 shows dramatic phenotype changes of A2/Melan-A⁺CD8⁺ T cells. CD8⁺ lymphocyte populations from PBMCs of patient LAU 132 collected at different time points were highly purified (>98%), as illustrated in Fig. 1. The lymphocyte preparations were then stained with A2/Melan-A tetramers together with anti-CD45RA^{CYC} and anti-CD28^{FITC} mAbs and analyzed immediately by flow cytometry. (A) The patient received five immunization cycles, consisting of subcutaneous injections of Melan-A₂₆₋₃₅, Tyrosinase₁₋₉, Tyrosinase₃₆₈₋₃₇₆, gp100₂₈₀₋₂₈₈, and gp100₄₅₇₋₄₆₆ tumor-associated Ags, and influenza matrix Flu-MA₅₈₋₆₆ peptide. All cycles with the exception of the first included treatment with GM-CSF. CD45RA phenotype of A2/Melan-A⁺ cells was analyzed before the first immunization cycle (left), during immunizations (middle), and at the end of immunizations (right). (B) Percentage of A2/Melan-A⁺ cells in CD8⁺ T cells was followed over time. Each arrow represents an immunization cycle. (C) Proportions of CD28⁺ (○) and CD45RA^{lo} (●) in gated A2/Melan-A⁺ cells were followed over time.

The patient was tumor free from May 1996, then developed a brain metastasis diagnosed in December 1998. Immunization with melanoma-specific peptides was begun in June 1996; he received a first immunization cycle consisting of three or four weekly subcutaneous injections of 100 μg of each of the peptides Melan-A₂₆₋₃₅, Tyrosinase₁₋₉, Tyrosinase₃₆₈₋₃₇₆, gp100₂₈₀₋₂₈₈, gp100₄₅₇₋₄₆₆, and influenza matrix Flu-MA₅₈₋₆₆ in PBS. Four additional immunization cycles were given in August and October 1996, then in March and June 1997. All cycles with the exception of the first included treatment with GM-CSF (daily subcutaneous injections of 75 μg, starting 4 d before peptide injection and covering the whole 3-wk immunization period).

Before the first immunization cycle, A2/Melan-A⁺ cells (0.04% of CD8⁺ T cells) presented a naive CD45RA^{hi} phenotype. In marked contrast, 1 mo after the end of the first peptide injections and until the end of the second im-

munization cycle, half of the tetramer⁺ cells presented an Ag-experienced CD45RA^{lo} phenotype. This was accompanied by a small increase in the frequency of A2/Melan-A⁺ cells (from 0.04 to 0.07% of CD8⁺ T cells). During the next year, the proportion of CD45RA^{lo}A2/Melan-A⁺ cells gradually decreased (from 51 to 23% of A2/Melan-A⁺ cells), while the frequency of total A2/Melan-A⁺ cells remained constant (~0.07%). Moreover, the vast majority of A2/Melan-A⁺ cells continuously displayed a CD28⁺ phenotype over time. Enumeration of tyrosinase-specific CD8⁺ lymphocytes using an available tetramer made with the Tyrosinase₃₆₈₋₃₇₆ peptide failed to reveal significant levels of positive cells in the samples tested (September 1996, June 1997, July 1997, and April 1998).

Discussion

Using tetrameric complexes (35), we have directly enumerated and phenotyped ex vivo melanoma-specific CD8⁺ T cells present in peripheral blood. This study reveals that circulating Melan-A-specific CD8⁺ T cells are generally present in high numbers both in melanoma patients and healthy individuals. These cells present a naive phenotype in healthy individuals, but may develop an Ag-experienced phenotype in some melanoma patients. Furthermore, Ag-specificity and phenotype of A2/Melan-A⁺ cells were independently confirmed by functional assays. As recorded for one patient immunized with a Melan-A peptide, marked and reversible shifts in the proportion of memory-type circulating Melan-A-specific CTLs occurred in vivo. In contrast, circulating influenza virus-specific CTLs in most of the same individuals display a homogeneous memory phenotype.

Our findings confirm and extend previous reports on the presence of circulating Melan-A-specific cells both in melanoma patients and healthy individuals (6–9, 36). However, the necessity of stimulating CTLp with Ag in order to detect Melan-A-specific cells had previously prevented a precise assessment of their frequency and phenotype ex vivo. We find here that circulating Melan-A-specific cells are indeed present in a large proportion (60%) of healthy individuals. Although this prevalence is in agreement with that measured in the previous studies (20–75% of healthy individuals), tetramer staining reveals for the first time that the frequency of circulating Melan-A-specific cells is much higher than had been anticipated (mean = ~1/1,500 of CD8⁺ T cells) both in healthy individuals and melanoma patients. These frequencies are underestimated consistently by indirect assays, as demonstrated in this study by LDA and IFN-γ ELISPOT. Phenotypic analyses of A2/Melan-A⁺ cells reveal that underestimation reflects the failure to detect primarily, although not exclusively, CD45RA^{hi}-specific cells. Thus, naive phenotype cells are less efficiently stimulated during in vitro Ag-driven differentiation to effectors, as has been shown in experiments involving naive phenotype cells challenged by Ag-independent TCR cross-linking (37, 38).

The frequencies of naive single epitope-specific CTLs have been estimated at $\leq 1/100,000$ CD8⁺ T cells. Thus, it is striking that the mean frequency of circulating Melan-A-specific cells from HLA-A2⁺ healthy individuals is ≥ 60 times higher, in fact attaining the range of frequencies for single epitope-specific memory CTLs. In this regard, since Melan-A is a melanocyte lineage protein, it might be argued that enrichment of cells specific for the Melan-A₂₆₋₃₅ peptide could result from frequent priming events after common subclinical skin injuries in healthy individuals. Alternatively, the Melan-A homologous peptide gC₄₈₀₋₄₈₈, derived from the unrelated glycoprotein C of the common pathogen HSV-1 (39), could also be responsible for the activation of Melan-A cross-reactive cells. However, the observation that ex vivo A2/Melan-A⁺ cells from healthy individuals are of the naive CD28⁺CD45RA^{hi}/RO⁻ phenotype does not support these hypotheses. It remains possible that CD28/CD45RA surface Ags do not reflect true human Melan-A-specific naive T lymphocytes. For instance, reversion of CD45RO⁺ T cells to CD45RA⁺ T cells after prolonged in vitro culture periods has been observed (40, 41). Although Flu-MA-specific CD8⁺ T cells from virtually all individuals studied displayed a memory CD45RO⁺ phenotype, it remains to be ascertained whether such a conversion may have taken place in Melan-A-specific CD8⁺ T cells in vivo. Nevertheless, our results in no way rule out the possibility that Melan-A-specific cells in healthy individuals as well as in the majority of melanoma patients represent either truly naive T cells or cells that have been partially activated (42, 43). This raises the question as to how such relatively large repertoire of naive T cells is maintained in the periphery. For instance, what are the ligands and/or cytokines involved? Further phenotype and functional characterization of these cells are required to clarify these issues.

Does tetramer staining of Melan-A-specific cells reveal differences between melanoma patients and healthy individuals? In terms of frequencies, Melan-A-specific cells were generally found in high numbers in patients, but these numbers were comparable with those measured in healthy individuals. In contrast, and as previously reported (34), the frequency of Melan-A-specific cells was even higher in patients undergoing vitiligo. Previous reports have shown that development of vitiligo is more frequent in melanoma patients (44), and that vitiligo may be associated with an ongoing immune response directed against melanoma cells (45, 46). This evidence suggests that melanocyte-specific CTLs can play a role in melanocytic destruction. From a clinical standpoint, the presence of high numbers of circulating Melan-A-specific cells found in the majority of HLA-A2⁺ melanoma patients represents a unique opportunity for vaccination protocols aimed at harnessing the po-

tential power of these CTLs to migrate to and destroy tumors. In addition, polyclonal-specific T cell populations could easily be obtained by tetramer-guided sorting and used for adoptive transfer therapy (47).

The tetramer technology not only allows frequency analyses, but opens the possibility to characterize phenotypes corresponding to various differentiation stages of T cells. We observed that the majority of influenza virus-specific CTLs were CD45RA^{lo}, whereas the melanoma-specific CTLs were largely CD45RA^{hi}. Finally, CD45RA^{lo} melanoma-specific CTLs were only observed in 3 out of 10 melanoma patients. The different expression of CD45 isoforms probably corresponds to different cellular activation status. Therefore, the fact that some patients, but no healthy individuals, had CD45RA^{lo}/RO⁺ Melan-A-specific cells may suggest that CTLs have been activated in vivo as part of immune activation against melanoma cells.

When tracking a patient's immune response over the course of an immunotherapeutic treatment with Melan-A₂₆₋₃₅, we recorded a marked but reversible shift in the proportion of memory-type Melan-A-specific cells. However, no changes in frequency or phenotype of Melan-A-specific cells were observed after a similar immunization schedule on a second melanoma patient (LAU 269). It is not possible at this time to establish whether peptide vaccination and/or GM-CSF administration were responsible for the phenotype shifts observed in the former patient. A larger group of vaccinated patients will be analyzed, as it is possible that this vaccination procedure is not efficient. Immunization with potent adjuvants and Melan-A peptide analogues with enhanced immunogenicity will be tested.

Direct detection of tumor-specific lymphocytes in the periphery and in tumor-infiltrated LNs allowed us to obtain a better appraisal of a tumor-specific response in vivo. In tumor-infiltrated LNs, Melan-A-specific cells were enriched with frequencies ranging from 1/400 to 1/30 CD8⁺ LN cells and displayed an Ag-experienced (CD45RA^{lo}/RO⁺) phenotype in most cases, as compared with noninfiltrated adjacent LNs or LNs from patients with forms of cancer other than melanoma (12). In contrast, circulating Melan-A-specific cells were generally not enriched in melanoma patients, as compared with healthy individuals and with normal LNs. Moreover, they infrequently presented Ag-experienced phenotypes. It is tempting to speculate that the differences observed in both frequencies and surface phenotype of Melan-A-specific CTLs are the consequence of selective in vivo activation of these cells at infiltrated LNs. It is also conceivable that primed cells are selectively accumulated at infiltrated LNs. Together, our findings emphasize the need to monitor both the tumor sites and the periphery to thoroughly evaluate the impact of natural or vaccine-induced tumor-specific CTL responses.

We would like to give special thanks to all of the patients for their enthusiastic collaboration. We gratefully acknowledge Renate Milesi, Nicole Montandon, Danielle Minaïdis, and Christian Knabenhans for excellent technical assistance. We also thank John Gordan for critical reading of this manuscript.

This work was supported by the grant KFS 633-2-1998 from the Swiss Cancer League.

Address correspondence to Pedro Romero, Division of Clinical Onco-Immunology, Ludwig Institute for Cancer Research, Centre Hospitalier Universitaire Vaudois – BH 19-602, 1011 Lausanne, Switzerland. Phone: 41-21-314-01-78; Fax: 41-21-314-74-77; E-mail: pedro.romero@isrec.unil.ch

Submitted: 1 April 1999 Revised: 30 June 1999 Accepted: 6 July 1999

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