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 Mikaël J. Pittet,* Danila Valmori,* P. Rod Dunbar,§ Daniel E. Speiser,* Danièle 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 CD45RAhi/RO2 phenotype, whereas variable proportions of Ag-experienced CD45RAlo/RO1 Melan-A–specific cells were observed in the remaining 3 patients. In contrast, ex vivo influenza matrix–specific CTLs from all individuals exhibited a CD45RAlo/RO1 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) gene, which is expressed by normal melanocytes and by most fresh melanoma samples, codes for Ags recognized by tumor-reactive HLA-A*0201–restricted CTLs (4, 5). CTL lines specific for the immunodominant Melan-A26-35 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
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 CD45RAhi population contains cells that express several phenotypic features of primed T cells (16–18). Indeed, CD45RA Ahi cells lacking surface expression of the CD28 costimulatory receptor exhibit direct ex vivo cytotoxic 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 Ag-specific cells in multiparameter analyses including CD28, CD45RA, and CD45RO surface expression and fluorescent tetramers containing the Melan-A26–35 A27L peptide (A2/Melan-A) or the influenza matrix Flu-M A58–66 (A2/Flu-M A) Ag. Since the majority of HLA-A*0201 individuals are sensitized against the latter 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-M A58–66 (GILGFVFTL) immunodominant peptide A2/tyrosinase, A2/Melan-A, and A2/Flu-M A tetramers were used at 20 μg/ml.

Materials and Methods

Cells. Thawed PBMCs were cultured for 16–20 h in IMDM supplemented with 0.55 mM Arg, 0.24 mM Asn, 1.5 mM Glu, 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 stained with A2/ tetramers and FITC and CyChrome mAb conjugates in 50 μl 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. PBMCs 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 μM Melan-A26–35 A27L peptide. At day 13, Ag recognition was assessed using T2 target cells (100 μl) labeled with 35Cr and incubated in the presence or absence of 1 μg/ml of the antigenic Melan-A26–35 A27L peptide for 1 h at 37°C and washed three times. Labeled target cells (105 cells in 50 μl) were then added to effector cells (50 μl) in V-bottomed micro wells (50 μl). The effector cells were preincubated for 20 min at 37°C in the presence of unlabeled K562 cells (5 x 105/well) to eliminate nonspecific lysis due to NK-like effectors. Cr release was measured in supernatant (40 μl) 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 μg/ml peptide and 1.66 × 10^5 PBMCs per well in 200 μl 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-MA A58-66 peptide. The ILKEPVHGVT476-484 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 × 10^5] × (percent 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/10^5 PBMCs (mean = 5 spots/10^5 PBMCs, SD = 4), implying a lower specific detection limit of 1 in ∼60,000 PBMCs.

Since the enumeration of Melan-A–specific lymphocytes with a lower specific detection limit of 1 in 10^5 PBMCs, SD = 21.

Results

Circulating A2/Melan-A^+ CD8^+ T cells are detectable both in melanoma patients and healthy individuals. 11 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 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-MA A58-66 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 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 = 0.363), and <0.02% of CD8^+ T cells with A2/Flu-MA tetramers (cut off = mean + 3 SD = 0.01). 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 = 0.02% of A2/Flu-MA).
Circulating A2/Melan-A^+ 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^hi CD45RAlo CD8^+ phenotype (range: 81–95%), like those found in all healthy donors. However, 3 out of 10 patients either displayed >35% CD28^lo CD45RA^+ CD8^+ (LAU 132 and 240), or >90% CD28^− CD45RA^+ CD8^+ (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-A26–35 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-A26–35 peptide vaccination with GM-CSF (LAU 240) 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

| 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-M | Name | Melan-A | Flu-M | Name | Melan-A | Flu-M | Name | Melan-A | Flu-M |
| 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 | N.D | | | |
| HD 604 | 0.01 | 0.00 | HD 604 | 0.21 | 0.13 | LAU 267 | 0.04 | N.D | | | |

| Name | Melan-A | Flu-M | Name | Melan-A | Flu-M | Name | Melan-A | Flu-M | Name | Melan-A | Flu-M |
|------|---------|-------|------|---------|-------|------|---------|-------|------|---------|-------|
| 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.
A2/Melan-A+ and A2/Flu-MA+ cells in melanoma patients and healthy donors. CD8+ lymphocyte populations were highly purified (>98%) from PBMNCs as illustrated in Fig. 1. The lymphocyte preparations were then stained with A2/Melan-A+, or A2/Flu-MA tetramers together with anti-CD45RA and anti-CD28, and immediately analyzed by flow cytometry. (A) Pattern of expression of CD45RA/RO in total circulating CD8+ T cells (left) from healthy donors (HD 329). They were CD28+CD45RAhi/RO in A2/Melan-A+ gated cells (middle) but CD28+CD45RAlo/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+CD45RAhi/RO phenotype (left). In contrast, tetramer+ cells from 2 out of 10 patients displayed variable proportions of CD45RA hi/RO and CD45RAlo/RO tetramer+ cells (middle) and a CD28+CD45RAint phenotype for 1 out of 10 patients (right). (C) Summary of phenotyping data obtained for melanoma patients and healthy donors. Frequencies of CD45RA+ cells detected in gated A2/Melan-A+ and A2/Flu-MA+ CD8+ T cells were calculated with CellQuest™ software.

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 CD28+CD45RAhi/RO but CD28+CD45RAlo/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+CD45RAhi/RO phenotype (left). In contrast, tetramer+ cells from 2 out of 10 patients displayed variable proportions of CD45RA hi/RO and CD45RAlo/RO tetramer+ cells (middle) and a CD28+CD45RAint phenotype for 1 out of 10 patients (right). (C) Summary of phenotyping data obtained for melanoma patients and healthy donors. Frequencies of CD45RA+ cells detected in gated A2/Melan-A+ and A2/Flu-MA+ CD8+ T cells were calculated with CellQuest™ software.
For LDA, CD45RA<sup>hi</sup> and CD45RA<sup>lo</sup> fractions of CD8<sup>+</sup> T cells were sorted and stimulated twice with Melan-A<sub>26-35</sub> 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<sup>hi</sup> 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<sup>lo</sup> 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<sup>+</sup> lymphocytes. However, LDA underestimated the frequency of Melan-A–specific CTLp by a factor of ~3 for CD45RA<sup>lo</sup> phenotype A2/Melan-A<sup>+</sup> cells (LAU 132), and to a higher extent by a factor of ~13 in average for CD45RA<sup>hi</sup> phenotype A2/Melan-A<sup>+</sup> 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<sup>+</sup> or CD45RA<sup>+</sup> sorted populations, but rather with unsorted PBMCs. As expected for truly naive CD8<sup>+</sup> 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/10<sup>6</sup> CD8<sup>+</sup> 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 H1V-1 Pol<sub>468-476</sub> peptide of PBMCs from the 21 individuals analyzed, was ~90 spots/10<sup>6</sup> CD8<sup>+</sup> 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 naïve CD8<sup>+</sup> 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<sup>+</sup> 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.

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

| Tetramers<sup>*</sup> | LDA<sup>‡</sup> | ELISPOT<sup.§</sup> |
|----------------------|-----------------|-----------------|
|                      | CD45RA<sup>hi</sup> | CD45RA<sup>lo</sup> | CD45RA<sup>hi</sup> | CD45RA<sup>lo</sup> | CD45RA<sup>hi</sup> | CD45RA<sup>lo</sup> |
| HD 329<sup>)*</sup>  | 100             | 7<sup>‡</sup>        | 10              | 0.3             | 1<sup>‡</sup>               |
| HD 604              | 200             | 10<sup>‡</sup>       | 10              | 0.1             | 0.0<sup>‡</sup>               |
| LAU 132             | 40              | 30               | 3               | 9               | 10                             |
| LAU 203             | 70              | 4<sup>‡</sup>        | 10              | 1               | 0.0<sup>‡</sup>               |

All frequencies ×10<sup>-5</sup>.<br>**Values correspond to the number of cells in the A2/Melan-A–CD45RA<sup>hi</sup> and A2/Melan-A–CD45RA<sup>lo</sup> gates divided by the number of CD8<sup>+</sup> lymphocytes.<br>‡Sorted CD45RA<sup>hi</sup> and CD45RA<sup>lo</sup> fractions of CD8<sup>+</sup> T cells were stimulated twice with Melan-A<sub>26-35</sub> 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<sup>hi</sup> and CD45RA<sup>lo</sup> subsets. Thus, the values indicated correspond to the frequency, within CD8<sup>+</sup> T cells, of CD45RA<sup>hi</sup> and CD45RA<sup>lo</sup> Melan-A–specific CTLp, respectively.<br.§Melan-A–specific IFN-γ production and the deduced frequency of Melan-A–specific CTLs in CD8<sup>+</sup> T cells was measured as described in Materials and Methods.<br>§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.<br>‡Frequencies below the lower limit of detection.
m<sub>g</sub>/ml PHA-L, 100 U/ml IL-2, 10 ng/ml IL-7, and 5 × 10<sup>3</sup> cells, while the tetramer<sup>2</sup> fraction contained 0.02% A2/Melan-A<sup>1</sup> cells. As expected, both populations displayed a homogeneous CD45RA<sub>lo</sub> Ag-experienced phenotype (data not shown).

Each cell fraction was subsequently tested for its lytic activity. The polyclonal A2/Melan-A<sup>1</sup> population specifically killed T2 target cells pulsed with the natural or the A27L analogue Melan-A<sub>26–35</sub> peptides, whereas the A2/Melan-A<sup>2</sup> 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<sup>1</sup> population specifically released IFN-γ in ELISPOT assays, whereas the number of IFN-γ spots was insignificant for the A2/Melan-A<sup>2</sup> 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<sup>1</sup> 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).
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-A 26–35, Tyrosinase1–9, Tyrosinase368–376, gp100 280–288, gp100 457–466, and influenza matrix Flu-M58,66 peptide. 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 CD45RAlo phenotype. In marked contrast, 1 mo after the end of the first peptide injections and until the end of the second immunization cycle, half of the tetramer+ cells presented an Ag-experienced CD45RAlo 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 CD45RAloA2/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 CD28hi 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, CD45RAlo-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 CTLp 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-A\(^{2-35}\) healthy individuals is \( \geq 60 \) 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-A\(^{26-35}\) peptide could result from frequent priming events after common subclinical skin injuries in healthy individuals. Alternatively, the Melan-A homologous peptide gC\(^{480-488}\), derived from the unrelated glycoprotein C of the common pathogen HSV-1 (39), could also be responsible for the activation of Melan-A–specific cells in healthy individuals as well as in the majority of melanoma patients other than melanoma (12). In contrast, circulating 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 Melan-A\(^{26-35}\)–specific CTLs 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-A\(^{26-35}\), 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\(^{lo/hi}\)/RO\(^{hi}\)) 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 LN s. 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.

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