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Vaccination with a Melan-A Peptide Selects an Oligoclonal T Cell Population with Increased Functional Avidity and Tumor Reactivity


Both the underlying molecular mechanisms and the kinetics of TCR repertoire selection following vaccination against tumor Ags in humans have remained largely unexplored. To gain insight into these questions, we performed a functional and structural longitudinal analysis of the TCR of circulating CD8+ T cells specific for the HLA-A2-restricted immunodominant epitope from the melanocyte differentiation Ag Melan-A in a melanoma patient who developed a vigorous and sustained Ag-specific T cell response following vaccination with the corresponding synthetic peptide. We observed an increase in functional avidity of Ag recognition and in tumor reactivity in the postimmune Melan-A-specific populations as compared with the preimmune blood sample. Improved Ag recognition correlated with an increase in the $t_{1/2}$ of peptide/MHC interaction with the TCR as assessed by kinetic analysis of A2/Melan-A peptide multimer staining decay. Ex vivo analysis of the clonal composition of Melan-A-specific CD8+ T cells at different time points during vaccination revealed that the response was the result of asynchronous expansion of several distinct T cell clones. Some of these T cell clones were also identified at a metastatic tumor site. Collectively, these data show that tumor peptide-driven immune stimulation leads to the selection of high-avidity T cell clones of increased tumor reactivity that independently evolve within oligoclonal populations.

It is generally agreed that tumor-specific CD8+ CTLs constitute the primary antitumor effector arm of the adaptive immune response. Several lines of evidence indicate that immunity against cancer can spontaneously develop in some patients (1, 2). Numerous Ags recognized by tumor-reactive CTL have been identified. Based on this, a recent approach to cancer vaccination consists of immunizing patients with synthetic peptides recognized by tumor-reactive CTL in the context of autologous MHC class I-presenting molecules. The choice of both antigenic peptides and formulations has been considerably variable in initial trials of peptide vaccination (3, 4). The reported results are complex and often contradictory concerning both the immunogenicity of the peptide/formulation used and the correlation between immunological and clinical end points. However, there is growing evidence that at least some tumor-associated peptide Ags can, upon administration in adjuvant and following intensive immunization schedules, induce vigorous specific CD8+ T cell responses detectable ex vivo in the peripheral blood of some tumor patients (5). Direct evaluation of Ag-specific T cell responses in the above-mentioned trials has been possible thanks to the development of fluorescent MHC class I/peptide multimers, which allow direct visualization of Ag-specific T cells (6). As a result, the complexity of the TCR repertoire elicited in vivo by Ag stimulation has recently become more accessible to direct analysis. Thus, the kinetics of TCR repertoire selection in vivo following challenge with tumor Ags in humans, as well as their functional consequences on tumor recognition, have started to be explored (5, 7–9).

The differentiation Ag Melan-A is selectively expressed by normal cells of the melanocytic lineage including skin melanocytes and by the large majority of malignant melanomas (10). HLA-A2-restricted, Melan-A-specific CD8+ T cells primarily recognize peptides 27–35 and 26–35 (11). The repertoire of Melan-A-specific T cells detectable ex vivo by staining with HLA-A2/Melan-A peptide multimers (referred to as multimers hereafter) in HLA-A2-expressing individuals, including both normal donors and the majority of melanoma patients, is unique, as it comprises $\sim10^{-3}$ of circulating CD8+ T cells phenotypically naive (CD45RA+CCR7+) (12, 13), being therefore the only known human Ag-specific naive repertoire thus far accessible to analysis. We have recently undertaken clinical trials of Melan-A26–35 peptide vaccination in HLA-A2 melanoma patients. In the course of the vaccination, one patient developed a vigorous Ag-specific T cell response as assessed by staining of PBL with HLA-A2/Melan-A peptide multimers (14). In this work, we performed a longitudinal analysis of this response both at the polyclonal and at the monoclonal level in samples encompassing a time period of about 1 year.
after the beginning of vaccination and compared those to both a preimmune sample and a sample containing Ag-specific lymphocytes infiltrating an autologous metastatic lesion.

Materials and Methods

Patient, tissues, cells, and cytotoxicity assay

Detailed clinical history and vaccination protocol for melanoma patient LAU 337 have previously been reported (14). The patient started being vaccinated in April 1999 and received four i.m. injections (at days 1, 28, 56, and 84) of synthetic peptides influenza Matrix$_{a-64}$ (GILGFVFTL) and Melan-A$_{26-35}$ (EAAGIGILTV) (100 μg each at each injection), in 600 μl of adjuvant SB-AS2 (provided by GlaxoSmithKline, Rixensart, Belgium). Four additional intracutaneous injections of the same peptides, but administered s.c. in 600 μl Montanide adjuvant provided by SEPPIC (Paris, France), were given at days 147, 175, 204, and 301. From day 203 to 210 the patient received 5 × 10$^6$ IU of human (h) 3 rIL-2 (Proleukin; Roche, Reinach, Basel, Switzerland), twice a day. A s.c. metastasis of the cheek was surgically removed and then minced with needles in sterile culture medium. The cell suspension was then cultured in medium containing 10% (v/v) Fetal calf serum and 10% (v/v) hirL-2 and 10 ng/ml hIL-2 during 2 wk before analysis. Melan-A-specific CTL clones were derived from tumor-infiltrating lymphocytes (TIL) by limiting dilution cultures. All bulk cultures and clones were subsequently expanded by periodical (3–4 wk) restimulation into microtiter plates together with irradiated feeder cells in the presence of PHA, and hrIL-2. Ag recognition was assessed functionally by chromium release assay as previously described (16). The percentage of specific lysis was calculated as follows: 100 × [(experimental – spontaneous release)/(total – spontaneous release)].

A2-predominant multimers and flow cytometry immunofluorescence analysis

PE-conjugated multimeric HLA-A2-peptide complexes were synthesized as described (6, 17). As the antigenic peptide, the Melan-A$_{26-35}$ A27L analog (ELAAGILTV), which has a higher binding affinity and stability than parental peptide (EAAGIGILTV) (18), was used. Interchangeability of parental Melan-A decapentapeptide and A27L analog in terms of staining specificity has previously been assessed (17). Cell samples were stained with multimers (4.5 μg/ml) in PBS 0.2% BSA during 1 h at room temperature, washed once in the same buffer, stained with mAbs during 30 min at 4°C, washed again, and analyzed by flow cytometry. Anti-CD8 and anti-CD45RA mAbs were purchased from BD Biosciences (San Jose, CA). For TCR β-chain variable segment (BV) repertoire analysis a panel of 21 anti-βV mAbs (purchased from Immunotech, Marseilles, France) was used. Staining was performed as previously described (19). MHC-peptide/TCR dissociation experiments were performed as detailed elsewhere (16) by using an excess of unlabeled multimers to avoid rebinding of PE-labeled multimers after their dissociation from the TCR. Intensity of multimer fluorescence at each time point analyzed was expressed as the natural logarithm of one-normalized fluorescence at t0. Data analysis was performed using CellQuest software (BD Biosciences).

CDR3 size analysis of TCR BV transcripts, sequencing of PCR products, and quantitative assessment of selected Melan-A-specific TCR clonotypes

The complementarity-determining region (CDR3) of the PCR-amplified TCR BV1–24 transcripts was analyzed using a run-off procedure as described previously (20, 21). The run-off products were then run on an automated sequencer in the presence of fluorescent size markers. The length of the DNA fragments and the fluorescence intensity of the bands were analyzed with Base ImagIR software (LI-COR Biotechnology Division, Bad Homburg, Germany). TCR BV-BC PCR products derived from sorted populations were cloned with TOPO TA cloning kit (Invitrogen, Carlsbad, CA). One Shot TOP10 chemically competent Escherichia coli (Invitrogen) were transformed and plated for blue/white color selection on medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Plasmid DNA was extracted from white colonies using the Qiagen Plasmid Mini kit (Qiagen, Hilden, Germany) and sequenced using Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Little Chalfont, U.K.). Quantification of selected T cell clones was performed as previously described (21, 22).

The sequences of clonotypic primers were as follows: BV3, 5'-GTCGCAACCCCGAGAAAACCT-3'; BV5, 5'-GCTGATTGCTGTCCAAAGCTG-3'; BV17, 5'-CCTGCAATTTGCCAGGTATAC-3'; BV14, clone 2C2S-5'-CCGCTGCCAGATAACTC-3', clone ID5, 5'-GGTGTCGGCCGATTAACACT-3', clone 4E5, 5'-AACCCAGGAGCCCTCCAAACT-3', and clone 4C8, 5'-TTCAGTCGCGTATGAGGCT-3'.

Results

Functional avidity maturation of circulating CD8$^+$ A2/Melan-A multimer$^+$ T cells from patient LAU 337 after immunization with peptide Melan-A$_{26-35}$

The clinical course and vaccination protocol for melanoma patient LAU 337 are detailed in Materials and Methods. Blood samples were obtained before peptide vaccination (day = 5) and after two (day 39), four (day 100), six (day 176), and seven (days 211 and 300) injections. The frequency of Melan-A-specific T cells in pre- and postimmune peripheral blood samples was assessed by costaining with anti-CD8 mAbs and multimers (17). Before peptide vaccination (day = 5), we detected a relatively high frequency of multimer$^+$ T cells (0.1% of total CD8$^+$ T cells; Fig. 1) that exhibited a mixed phenotype composed of both CD45RA$^+$bright and CD45RA$^+$low cells. We have previously observed this mixed phenotype in multimer$^+$ T cells from ~30% of HLA-A2 melanoma patients, whereas in the remaining patients (e.g., LAU 203) and in all normal donors analyzed (e.g., HD 604), the majority of multimer$^+$ T cells were CD45RA$^+$bright (12). The percentage of circulating multimer$^+$ T lymphocytes significantly increased already at day 39 after the beginning of vaccination (0.7% of total CD8$^+$ T cells) and accounted for 2.2, 1.8, 1, and 2.3% of total CD8$^+$ T cells at days 100, 176, 211, and 300 postvaccination, respectively. It is of note that the increase was mostly confined to the multimer$^+$ CD45RA$^+$low T cell compartment (Fig. 1).

CD8$^+$ multimer$^+$ T cells from the preimmune and from postimmune samples were purified by cell sorting and expanded in vitro.
by stimulation with PHA as previously described (15). Interestingly, as illustrated in Fig. 2 for the line corresponding to day 211 postimmune and summarized in Table I, lines from postimmune samples recognized Melan-A peptides with higher avidity as compared with the preimmune line. At this time point as well as at day 176, but not at day 39, the hierarchy of peptide Melan-A nonasdecapetide recognition was inverted in the postimmune as compared with the preimmune line (Fig. 2A). Increased functional avidity of Ag recognition resulted in improved lysis of Melan-A-expressing tumor cells (Fig. 2B and Table I). It is of note that, although all the populations tested were stained by multimers with roughly comparable efficiency (Fig. 2C), the TCR complexes formed with the lower-avidity population were less stable than the ones formed with the high-avidity population, as observed following the kinetics of multimer staining decay (Fig. 2D).

### Table I. Relative efficiency of peptide and tumor recognition of pre- and postimmune Melan-A polyclonal nonspecific lines

<table>
<thead>
<tr>
<th>Day</th>
<th>Peptide Recognition&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tumor Recognition&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Melan-A&lt;sub&gt;27-35&lt;/sub&gt;</td>
<td>Melan-A&lt;sub&gt;26-35&lt;/sub&gt;</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>50</td>
</tr>
<tr>
<td>39</td>
<td>0.25</td>
<td>15</td>
</tr>
<tr>
<td>176</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>211</td>
<td>0.4</td>
<td>0.08</td>
</tr>
</tbody>
</table>

<sup>a</sup>Peptide concentration (measured in nanomolar) required for 50% maximum lysis.

<sup>b</sup>Percentage of specific lysis on Me 275 tumor cells at the indicated lymphocyte: target cell ratio.

### Analysis of the postimmune Melan-A-specific T cell repertoire in patient LAU 337 reveals oligoclonal composition and diverse BV chain usage

Due to the high frequency of CD8<sup>+</sup>multimer<sup>+</sup> T cells in postimmune samples, their BV usage could be assessed ex vivo by combining staining with anti-CD8 mAb, multimers, and a panel of mAbs directed against single BV subfamilies as previously described (19). Several CD8<sup>+</sup>multimer<sup>+</sup> T cell subpopulations, each expressing a distinct BV, were detected at each time point analyzed. However, five subpopulations (expressing BV1, BV3, BV5, BV14, or BV17) clearly dominated the response all along the vaccination period. Indeed, the sum of multimer<sup>+</sup> T cells expressing these BV regions accounted for 75–85% of the total multimer<sup>+</sup> T cell population at different time points of the analysis (Fig. 3A). The relative proportion of each subpopulation moderately (e.g., BV17) or significantly (e.g., BV3, BV5.1) varied among the different time points analyzed. To further analyze the clonal composition of the multimer<sup>+</sup> T cell populations along the vaccination period, multimer<sup>+</sup> T cells were isolated ex vivo from postimmune blood samples by multimer-guided cell sorting and directly submitted to analysis of TCR β-chain V segment and CDR3 length by spectratyping (23). In contrast to the bell-shaped pattern characteristic of polyclonal T cell populations that was obtained for multimer negative fractions (for an example, see Fig. 3B, bottom line), CDR3 size profiles obtained for multimer<sup>+</sup> populations displayed prominent peaks that indicated the accumulation of recurrent size transcripts. For each BV a unique peak (e.g., BV3) or multiple peaks (e.g., BV14) were detected. Prominent peaks were detected mostly in the case of dominant populations but also for some of the nondominant ones (e.g., BV2, BV13, and BV16), indicating that clonal expansions, albeit of lower amplitude, could also be present in those latter. Of note, in several instances (e.g., BV3) prominent peaks as well as their CDR3 size profiles were common between some or even all samples analyzed, suggesting the presence of Melan-A-specific T cell clonotypes persisting along the immune response.

### Functional analysis, BV usage, and CDR3 sequencing of Melan-A-specific clonal populations

To analyze the Melan-A-specific response at the clonal level, monoclonal populations were derived from samples corresponding to days 176 and 211 after vaccination, by cloning the sorted CD8<sup>+</sup>multimer<sup>+</sup> T cell fractions under limiting dilution conditions as previously described (24). Sequences of BV, CDR3, and junctional regions of the isolated clones (grouped according to the BV) are reported in Table II. The analysis of the BV used by the isolated clones (a total of 17) confirmed that clones bearing the BV3, BV5.1, BV14, and BV17 were among the most frequently used by multimer<sup>+</sup> T cells. Somewhat surprisingly, no BV1-using clones

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**FIGURE 2.** Functional characterization of Melan-A non-specific polyclonal lines from preimmune and postimmune samples. Multimer<sup>+</sup> polyclonal lines were derived from PMBC (days −5, 39, 176, and 211) by ex vivo multimer-guided cell sorting followed by stimulation in the presence of PHA and IL-2. A. The polyclonal lines were tested for recognition of peptides Melan-A<sub>27-35</sub> and Melan-A<sub>26-35</sub> in a 4-h chromium release assay using T2 cells as targets at a lymphocyte:target ratio of 10:1. Specific lysis was assessed in the presence of graded concentrations of each peptide (Melan-A<sub>27-35</sub>, Circles; Melan-A<sub>26-35</sub>, squares). Results are shown for sample from days −5 (preimmune, open symbols) and 211 (postimmune, filled symbols). B. Tumor recognition was similarly assessed (preimmune, open symbols; postimmune, filled symbols) by using as target cells tumor cell lines Me 275 (HLA-A2, Melan-A<sub>−</sub>, circles) and NA8-MEL (A2<sup>+</sup>Melan-A<sub>+</sub>, squares). C. Polyclonal lines were stained with multimers<sup>+</sup> (1.7 μg/mi) by incubation during 1 h at room temperature. Results are shown for lines from days −5 (preimmune) and 211 (postimmune) and for a control clone of unrelated specificity. D. The kinetic of dissociation of multimers from pre- (open symbols) and postimmune (filled symbols) polyclonal nonspecific lines was measured as detailed in Materials and Methods. Data are shown as the natural logarithm of the percentage of maximum fluorescence (corresponding to mean fluorescence at t<sub>0</sub>) plotted against time.
were isolated. Whereas in the case of BV3 (four clones isolated), BV5 (four clones isolated), and BV17 (two clones isolated) a single clonotype was found, four different clonotypes were identified in the case of BV14. Avidity and fine specificity of Ag recognition of single clonal populations was assessed in a functional chromium release assay in the presence of graded concentrations of either Melan-A\textsubscript{27-35} or Melan-A\textsubscript{26-35} peptides as illustrated in Fig. 4A. In addition, the ability of the clones to specifically lyse Melan-A-expressing tumors was also assessed. Avidity of Ag recognition and tumor reactivity were roughly similar for all isolated clones, irrespective of their BV usage and comparable to that of the corresponding polyclonal monospecific postimmune line (Fig. 4A and data not shown). For internal comparison, clonal populations were also derived from limiting dilution cloning of the preimmune polyclonal monospecific line. Eleven of the 14 Melan-A peptide-reactive clones used BV2 and recognized Melan-A\textsubscript{27-35} much more efficiently than Melan-A\textsubscript{26-35}, and the remaining three used BV13.1 and recognized Melan-A\textsubscript{27-35} slightly better than Melan-A\textsubscript{26-35}. Functional data from one representative clone from each group are shown in Fig. 4B. Consistent with the data obtained on polyclonal monospecific lines, these clones displayed a decreased level of tumor reactivity as compared with the ones derived from postimmune samples (Fig. 4, A and B, and data not shown).

**Analysis of Melan-A-specific T cell clones using clonotypic primers**

The frequency of individual Melan-A-specific T cell clonotypes among the total multimer-sorted population in the different postimmune samples was assessed using the previously described approach with clonotypic primers pairing unique CDR3 (23). Seven clonotypic primers corresponding to distinct CDR3 sequences in Table II were synthesized and used to quantify the
corresponding clonotypes within the total BV-corresponding transcripts. Considering the proportion of T cells expressing that particular BV segment in the multimer$^+$ fraction (Fig. 3A), the proportion of the clone within multimer$^+$ T cells was then extrapolated. Clone 1B7 represented a high proportion (from 32 to 87%) of total BV3 transcripts in all postimmune samples (Fig. 5A). This individual clone was present at a relatively low frequency (<0.1% of total multimer$^+$ cells) at day 39, whereas, at

**Table II. Sequence analysis of Melan-A-specific clones in postimmune samples from melanoma patient LAU 337**

<table>
<thead>
<tr>
<th>Clone</th>
<th>BV</th>
<th>CDR3β (aa)</th>
<th>BJ$^+$</th>
<th>Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B7</td>
<td>BV3</td>
<td>MYLCAS SFLGLGT (9)</td>
<td>FFG (BJ1.1)</td>
<td>8/8</td>
</tr>
<tr>
<td>2A5</td>
<td>MYLCAS SFLGLGT (9)</td>
<td>FFG (BJ1.1)</td>
<td>10/10</td>
<td></td>
</tr>
<tr>
<td>5C6</td>
<td>MYLCAS SFLGLGT (9)</td>
<td>FFG (BJ1.1)</td>
<td>5/5</td>
<td></td>
</tr>
<tr>
<td>6E4</td>
<td>MYLCAS SFLGLGT (9)</td>
<td>FFG (BJ1.1)</td>
<td>5/5</td>
<td></td>
</tr>
<tr>
<td>6C3</td>
<td>BV5</td>
<td>LYLCAS SLDNQ (8)</td>
<td>HFG (BJ1.5)</td>
<td>5/5</td>
</tr>
<tr>
<td>5E6</td>
<td>LYLCA SLDNQ (8)</td>
<td>HFG (BJ1.5)</td>
<td>4/4</td>
<td></td>
</tr>
<tr>
<td>3D9</td>
<td>LYLCA SLDNQ (8)</td>
<td>HFG (BJ1.5)</td>
<td>3/3</td>
<td></td>
</tr>
<tr>
<td>6C7</td>
<td>LYLCA SLDNQ (8)</td>
<td>HFG (BJ1.5)</td>
<td>5/5</td>
<td></td>
</tr>
</tbody>
</table>

$^a$BJ, TCR β-chain join segment.

**FIGURE 4.** Functional characterization of Melan-A-specific clones in PBL from preimmune and postimmune samples and in TIL. Recognition of peptides Melan-A27–35 (AA-GIGILTV) and Melan-A26–35 (EAA-GIGILTV) is shown for two representative clones per group obtained from a postimmune sample (day 211) (A), the preimmune sample (day −5) (B), and TIL (C). Peptide recognition was assessed in a standard 4-h chromium release assay using T2 cells as targets at a lymphocyte:target ratio of 10:1 (left panels). Tumor recognition (right panels) was similarly assessed using NA8-MEL (A2 Melan-A) and Me 275 (A2 Melan-A) tumor lines as target cells in the absence of exogenously added peptide.
the maximum of its expansion (day 176), it represented up to 11.3% (Fig. 5B) of the total multimer+ population, corresponding to an absolute number of ~10^6 cells per liter of blood. The same procedure was used to quantify the remaining clonotypes. Clones 2C2, 4E3, and 4C8 represented, at the maximum of their expansion, ~12-15% of total BV14 transcripts each, i.e., 3.3, 2.7, and 3.4% of multimer+ T cells, respectively. The remaining three clones (6C3, 5B3, and 1D3) were quantitatively minor, detectable only at days 176, 211, and 300, and accounting at most for only 0.2-0.56% of the total multimer+ T cell population (data not shown). Thus, despite the relatively high number of clones isolated and due to the complexity of the populations under study, this analysis only covered ~15% of cells composing the multimer+ population. It is of note that the different clones did not expand simultaneously. Indeed, the proportion of clone 1B7, for example, significantly increased during vaccination, peaking at day 176, and declined thereafter, whereas a significant increase in the proportion of clones 4C8 and 4E3 was measured only at day 300 (Fig. 5B). Thus, whereas some clones progressively disappeared from the circulation after initial expansion, other clones initiated their expansion only late after the beginning of vaccination. It is of note that, using clonotypic primers, we failed to detect the clonotypes isolated from the postimmune samples among both naive and Ag-experienced specific cells in the preimmune sample (data not shown), indicating that, before immunization, those clonotypes would be present in both populations at a frequency <1/10000 cells. This observation is consistent with previous data obtained in a murine model, indicating that the frequency of clones expanded by vaccination was extremely low in the preimmune repertoire (25).

The presence of the clonotypes isolated from the PBL among TIL obtained from a s.c. metastasis of the cheek resected on day 29 was also assessed. TIL were mostly composed of CD3+CD8+ T lymphocytes. Among those, 0.4% specifically stained with multimers (Fig. 6A). Multimer+ T cells in TIL were isolated by sorting and analyzed by spectratyping. Interestingly, the CDR3 size of some prominent peaks was similar in TIL and in PBL from the closest date (day 39), suggesting the presence of identical clonotypes (Fig. 6B). Indeed, as assessed by analysis with clonotypic primers (Fig. 6C), two of the seven clones isolated from the PBL were present in this metastasis. Clone 5B3, which was present at a very low proportion in the PBL, represented 12% of TIL multimer+ cells, whereas clone 1B7, which was prominent among PBL (Fig. 5A), was quantitatively minor in TIL. However, quantitative considerations should be taken with caution, as in vitro culture of TIL could have significantly modified the relative proportion of the clones. Six T cell clones were derived from the TIL multimer+ population. As partially shown in Fig. 4C for two representative clones, Melan-A-specific clones derived from TIL displayed relatively high avidity of Ag recognition and high tumor reactivity. Two clones used BV17. Analysis of the CDR3 revealed that one of those was identical to clone 5B3. The remaining four clones used BV20. In good agreement with this data as well as with the results of spectratyping analysis, costaining of TIL with multimers and anti-BV20 mAb showed that ~21% of multimer+ T cells in TIL used BV20 (Fig. 6A).

**Discussion**

We report in this work a longitudinal analysis of CD8+ Melan-A-specific multimer+ T cells in a melanoma patient who responded vigorously to vaccination with peptide Melan-A26–35 in adjuvant. One major finding was that specific T cell responsiveness to Melan-A peptides significantly increased upon vaccination, resulting in increased tumor recognition, clearly indicating functional avidity maturation during vaccination. Whereas these data are encouraging in the context of the evaluation of clinical trials of cancer vaccination, the underlying molecular mechanisms are unclear. Indeed, even if an increase of functional avidity through rearrangement of surface receptors upon activation of naive T cells as recently proposed (26, 27) could have occurred, several lines of evidence indicate that this phenomenon alone cannot explain the reported data. First, functional avidity maturation did not simultaneously occur toward the nonapeptide and the decapeptide; indeed, even if an increase of functional avidity through rearrangement of surface receptors upon activation of naive T cells as recently proposed (26, 27) could have occurred, several lines of evidence indicate that this phenomenon alone cannot explain the reported data. First, functional avidity maturation did not simultaneously occur toward the nonapeptide and the decapeptide; indeed, even if an increase of functional avidity through rearrangement of surface receptors upon activation of naive T cells as recently proposed (26, 27) could have occurred, several lines of evidence indicate that this phenomenon alone cannot explain the reported data. First, functional avidity maturation did not simultaneously occur toward the nonapeptide and the decapeptide; indeed, even if an increase of functional avidity through rearrangement of surface receptors upon activation of naive T cells as recently proposed (26, 27) could have occurred, several lines of evidence indicate that this phenomenon alone cannot explain the reported data. First, functional avidity maturation did not simultaneously occur toward the nonapeptide and the decapeptide; indeed, even if an increase of functional avidity through rearrangement of surface receptors upon activation of naive T cells as recently proposed (26, 27) could have occurred, several lines of evidence indicate that this phenomenon alone cannot explain the reported data.
FIGURE 6. Analysis of multimer\(^+\) lymphocytes infiltrating a metastatic lesion. A, TIL isolated from a s.c. metastasis of the cheek were stained with anti-CD\(^3\)FITC and anti-CD\(^8\)PE (upper left panel), with anti-CD\(^8\)FITC and multimers PE (upper right panel), and with anti-BV\(^3\)FITC, anti-BV\(^17\)FITC, and anti-BV\(^20\)FITC together with multimers PE and anti-CD\(^8\)PerCP (lower panels). Numbers in quadrants represent the proportion of positive cells. B, Multimer\(^+\) CD\(^8\) TIL were isolated by cell sorting and submitted to immunoscope analysis. C, The proportion of individual clonotypes in multimer\(^+\) TIL was calculated by combining the proportion of clonotype-specific transcripts within the corresponding BV total transcripts together with the percentage of BV using multimer\(^+\) T cells (A).
were identified (Table II), in agreement with our previous finding that the repertoire of Melan-A-specific T cells naturally selected at the tumor site was diverse and mostly nonoverlapping among different individuals (19, 23).

According to the kinetic model of T cell signaling (29–31) the biological consequences of the TCR interaction with MHC/peptide complexes is primarily determined by its duration: only an optimal time of interaction would allow the completion of the multiple steps implicated in full T cell activation, eventually leading to T cell proliferation and clonal expansion. Results in mouse models of CD8+ T cell response to infectious diseases have indeed indicated that specific T cells derived from mice responding to primary infection were characterized by faster TCR/multimer dissociation rates than cells derived from mice responding to recall infection (32, 33). Similarly, in this study, polyclonal monospecific cells from postimmune samples displayed a mean dissociation kinetics significantly slower than that displayed by preimmune cells. However, in contrast to other reports (34), we did not detect significant differences in the multimer staining intensity of these populations. Consistent with these data, by using monoclonal CD8+ T cell populations specific for three tumor Ag-derived peptides including Melan-A, we have observed that the efficiency of staining with the corresponding multimers can considerably vary with staining conditions and does not always correlate with functional avidity of Ag recognition. In contrast, in each case we found a direct correlation between the latter and the stability of the corresponding TCR/multimer complexes (16). These data support the view that maturation of functional avidity during immune responses to cancer would result from the selective clonal expansion of T cells expressing TCR with relatively low dissociation rates from MHC/peptide complexes.

Longitudinal analysis of circulating Melan-A multimer+ populations along the vaccination period revealed that after an initial progressive increase in the frequency of detectable circulating multimer+ cells, this population remained quantitatively stable over time, representing between 1.1 and 2.3% of total circulating CD8+ T lymphocytes. However, the frequency of individual clones within this population considerably and asynchronously varied over time, with some clones declining after initial expansion and others expanding only at late time points. This could be the consequence of either different time of recruitment of multiple T cell clones, each with relatively limited growing potential and life span, and/or of differential trafficking of the latter from and/or to vaccination/tumor sites. Interestingly, in contrast with our results, similar studies of immune responses to SIV- and HIV-derived epitopes have shown that the frequency of some dominant clonal populations can be stable over relatively long periods of time (35, 36). An explanation for this difference could be related to the nature of the immunogen that elicits these CD8+ T cell responses. Indeed, whereas in the case of immune responses to intact pathogens or derived proteins a specific CD4+ Th cell component (which plays a central role in sustaining CD8+ T cell responses) (37) is most likely to be involved, this would in general not be the case upon immunization with short peptides corresponding to well-defined CD8+ T cell epitopes. In this case, only repeated peptide injections would be able to continuously recruit additional specific clonal populations. These observations might provide a rationale for the design of more efficient vaccination schedules.

It is of note that whereas patient LAU 337 and, to a much lesser extent, some other melanoma patients enrolled in the same trial of peptide vaccination mounted a Melan-A-specific response (mostly confined to the CD45RA+ population) concomitantly with peptide vaccination, other patients failed to do so (48). The basis for the heterogeneity of responsiveness to vaccination with Melan-A peptide is not clear at this stage but could be related to the functional state of T cells before vaccination. In this regard, a multimer+ population with an Ag-experienced phenotype accounting for ~50% of the total population was already present in the preimmune sample of patient LAU 337, indicating that in this patient a certain level of activation of Melan-A-specific cells had already occurred spontaneously, most likely in response to the autologous tumor. Further studies directly comparing the T cell repertoire of naive vs Ag experienced Melan-A multimer-positive T cells in responder patients could, in the future, provide more insight into these questions.

Although the vigorous response to peptide vaccination of patient LAU 337 was accompanied by transient regression of some metastases, after 1 year of stable disease a rapid tumor progression occurred. One possible explanation for the apparent dissociation between the observed immunological and clinical responses is that the induced response, although substantial, may be quantitatively insufficient. In humans, the information on the potential levels of tumor-specific CD8+ T cells which may correlate with clinical responses is complex, often contradictory, and difficult to evaluate. Vigorous specific responses accounting for as much as ~3–4% of circulating CD8+ T lymphocytes have been detected following vaccination with a gp100 peptide analog, often in the absence of significant tumor regression (5). In contrast, in other studies, repeated long-term administration of melanoma Ag-derived peptides induced clinical response in the absence of detectable circulating specific T cells (3). High levels of specific CD8+ T cells are detected in immune response to viral infections. For example, as many as 44% of CD8+ T cells specific for an immunodominant EBV-derived epitope can be detected during the acute phase of the viral infection (38). However, a level of ~2% of specific CD8+ T lymphocytes is apparently sufficient to prevent disease progression in individuals infected with HIV (39). Thus, taking into account the limited information presently available, it is difficult to evaluate whether the level of specific CD8+ T cells elicited in patient LAU 337 was quantitatively adequate for controlling tumor growth. Interestingly, some of the clonotypes present in the circulation were retrieved among lymphocytes infiltrating a s.c. tumor metastasis, thus making it unlikely that tumor progression could be due to the inherent inability of tumor-specific circulating cells to reach their target at the tumor site. Alternative explanations for tumor escape could be related to the loss of expression of either tumor Ags or Ag-presenting molecules (40). Indeed, small areas of the s.c. metastasis analyzed in this study showed a clear loss of expression of total MHC class I molecules, although the majority of the tumor tissue analyzed showed normal MHC class I expression and uniformly expressed Melan-A. Clearly, in the absence of a global assessment of MHC class I and tumor Ag expression of massive progressive tumor metastases, the impact of this element is difficult to evaluate. However, it is intuitive that vaccination should be started earlier in the course of diseases, when selective or global down-regulation of HLA and tumor Ag expression is likely to be still modest.

It has been previously suggested that tumor-specific T cells present in the circulation could be, at least in some cases, anergic (41), or that Ag-specific T cells visualized with multimers may include populations displaying low avidity of Ag recognition and therefore not relevant for tumor recognition in physiological conditions (42). Moreover, persistent exposure of T cells to Ags could, under certain circumstances, result in various types of Ag-specific T cell dysfunction (43–46). In a previous study (14) and in this study we have examined the functional state of the Melan-A multimer+ T cells in patient LAU 337. Consistent with a recent report
in the case of patients responding to vaccination with the gp100-derived peptide analog (9), we failed to detect any sign of dys-
cytotoxic T lymphocyte (CTL) recognition directly correlates
with the phenotype of Ag-experienced T cells and not of
effector T cells (47). Consistent with their phenotype these cells
were able to readily secrete IL-2 upon short stimulation (3–6 h)
with Ag. However, they failed to display significant lytic activity
ex vivo but they developed the capacity to efficiently lyse Ag-
expressing target cells upon in vitro expansion following stimu-
lation with either mitogen or Ag (14). Thus, although the relative
in vivo effectiveness of CD8+ T cells at these discrete differentiation
stages for the control of tumor growth is presently unknown, the
design of vaccination strategies aimed at inducing fully differen-
tiated effector CTLs should be attempted. Finding the missing link
between elicitation of tumor Ag-specific T cell responses and in-
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