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Large and Dissimilar Repertoire of Melan-A/MART-1-Specific CTL in Metastatic Lesions and Blood of a Melanoma Patient

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It is widely accepted that the repertoire of Melan-A-specific T cells naturally selected in melanoma patients is diverse and mostly nonoverlapping among different individuals. To date, however, no studies have addressed the TCR profile in different tumor sites and the peripheral blood from the same patient. We compared the TCR usage of Melan-A-specific T cells from different compartments of a single melanoma patient to evaluate possible expansion or preferential homing over a 4-mo follow-up period. Using HLA-A2 peptide tetramers, CD8⁺ T cells recognizing the modified Melan-A immunodominant ELAGIGILTV peptide were isolated from four metastatic lesions resected from a single melanoma patient, and their TCR repertoire was studied. A panel of T cell clones was generated by cell cloning of tetramer-positive cells. Analysis of the TCR β-chain V segment and the complementarity-determining region 3 (CDR3) length and sequence revealed a large diversity in the TCR repertoire, with only some of the clones showing a partial conservation in the CDR3. A similar degree of diversity was found by analyzing a number of T cell clones obtained after sorting a Melan-A-specific population derived from PBLs of the same patient after in vitro culture with the immunodominant epitope. Moreover, clonotypes found at one site were not present in another, suggesting the lack of expansion and circulation of one or more clonotypes. Taken together, these results buttress the notion that the CTLs recognizing the immunodominant Ag of Melan-A comprise a different repertoire of clonotypic TCR, of which only some exhibit common features in the CDR3.

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3 Abbreviations used in this paper: TIL, tumor-infiltrating lymphocytes; CDR3, complementarity-determining region 3; rIL, human rIL; TCRBV, TCR β-chain V gene segment.

Materials and Methods

Tumor samples and cells

This study was approved by the local Human Ethics Committee and performed following the patient’s informed consent. Fresh melanoma lesions were received as biopsy material immediately after surgery. In a follow-up period of 4 mo, four metastatic melanoma lesions were excised from a single HLA-A2⁺ patient at the following anatomical sites: nape (lesion A), arm muscle (lesion B), left arm (lesion C), and right arm (lesion D). Each lesion was divided into three parts: one was fixed in formaldehyde and...
processed for routine histopathology, one was snap frozen in liquid nitrogen and stored at −80°C until use for RNA extraction to ascertain Melan-A expression by RT-PCR, and the last was used for in vitro culture. To obtain TIL, biopsy material was finely minced with scalpels and then dissociated into a single cell suspension by incubation for 2 h at 37°C in sterile IMDM (Life Technologies, Paisley, Scotland) supplemented with DNsase (30 U/ml), collagenase (1 mg/ml), and hyaluronidase (0.1 mg/ml). Cell suspensions were placed in tissue culture flasks (Falcon BD Labware, Franklin Lakes, NJ) containing IMDM supplemented with 0.24 mM Asn, 0.35 mM Arg, 1.5 mM Gln, 10% human pooled serum, 10% human linsl (Austria) (referred to as culture medium), and 200 U/ml human rIL-2 (rHIL-2), and cultured for 2–3 wk.

Melen-A-specific CTL clones were derived from TILs after cell sorting. Cells stained by Melan-A/A2 tetramer, CD3, and CD8 mAbs were seeded at 1 cell/well in round-bottom 96-well microplates. They were then stimulated by adding irradiated (50 Gy) allogeneic PBMC (8 × 10^5/well) as feeder cells as well as irradiated (100 Gy) allogeneic HLA-A2 EBV-transformed B cells (2 × 10^5/well) incubated with the Melan-A/A2b35 analog peptide ELAGIGILTV (10 μM) and washed. Culture medium contained rHIL-2 (100 U/ml), rHIL-4 (PeproTech EC, London, U.K.) (10 ng/ml), and rHIL-7 (PeproTech EC) (10 ng/ml). The CTL clones were restimulated each week by the addition of feeder cells and peptide-pulsed HLA-A2 EBV-B cells, in medium with rHIL-2 (50 U/ml); after ~3 wk, they were transferred into 2-ml wells and maintained with weekly restimulations, alternating allogeneic HLA-A2 PBMC and HLA-A2 EBV-B cells as peptide-presentation cells.

In vitro stimulation of PBMC

Two weeks after the last metastasis resection, PBMC were separated from heparinized blood, diluted 1/2 with RPMI 1640 medium, by centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden), washed, and cryopreserved in IMDM supplemented with 10% human serum and 10% DMSO in liquid nitrogen. To use, PBMC were thawed and 5 × 10^6/ml were incubated for 1 h at room temperature with 1% human serum and 5 μM Melan-A/A2b35 analog peptide. Cells were then washed and plated at 4 × 10^5/ml in culture medium with rHIL-2 (20 U/ml), rHIL-4 (10 ng/ml), and rHIL-7 (10 ng/ml) in 24-well tissue culture plates. On day 7, the lymphocytes were incubated with the peptide as on day 1. Cells were transferred in fresh culture medium. After 14 days of culture, lymphocytes were used for cytometric analysis and cell sorting. Melen-A2-specific CTLs were described as above.

Immunohistochemical analysis of Ag expression in tumor biopsies

Staining for Melan-A (Novocastra Laboratories, Newcastle upon Tyne, U.K.), CD20 (clone L26; DAKO, Glostrup, Denmark), CD3 (clone PS1; Novocastra Laboratories), CD4 (clone 1F6; Novocastra Laboratories), and CD8 (clone C8/144B; DAKO) was performed by a peroxidase-labeled avidin-biotin method on an automated stainer (DAKO autostainer). Paraffin tissue sections were mounted on amino-lysines-lined glass slides and heated to optimize Ag retrieval. Endogenous peroxidase activity was blocked by incubation in hydrogen peroxide/methanol. Immunohistochemical staining involved the sequential application of diluted primary Ab, biotinylated goat secondary Ab, and then peroxidase-labeled avidin. The Ags were visualized by incubation with aminochromic/carbazole substrate in the presence of hydrogen peroxide. Nonimmune mouse IgG in place of specific Ab was used for negative control sections.

Tetramers, flow cytometry, immunofluorescence analysis, and sorting

Soluble HLA-peptide tetramers were produced using a method similar to that described previously (10). Briefly, rHLA-A*0201 H chain, with a 15-aa substrate peptide for BirA-dependent biotinylation at its C terminus, and human β2-microglobulin were produced as inclusion bodies in Escherichia coli cells transformed with the expression plasmid PET23a–HLA-A*0201 and pPHN1 + β2m-microglobulin (kindly provided by F. Marincola, Clinical Research Center, National Institutes of Health, Bethesda, MD) and peptide Melan-A2b35, A27L analog (ELAGIGILTV). The inclusion bodies were purified and dissolved in urea-denaturing buffer, as described (11). Monomeric MHC-peptide complexes were formed by combining the HLA-A*0201, β2-microglobulin, and the peptide in an arginine-rotating buffer. The refolding reaction was dialyzed, and concentrated on a Superdex 75 gel filtration column to verify the folded tetramer (Amersham Pharmacia Biotech). Soluble purified complexes were biotinylated using BirA enzyme (Avidity, Denver, CO). PE-labeled tetramers were produced by mixing the biotinylated complexes with Extravidin-PE (Sigma–Aldrich, St. Louis, MO), and were validated by staining CTL clones with the appropriate specificity. Each tetramer batch was titrated and used at the optimum concentration, ranging from 20 to 40 nM HLA-A2 H chain.

For surface Ag staining, lymphocytes were washed, resuspended in PBS with 1% human serum, and incubated for 15 min at room temperature with the HLA-A*0201 tetramer. Anti-CD3 Abs coupled to FITC (SK7; BD Biosciences, San Jose, CA) and anti-CD8 Abs coupled to PerCP (SK1; BD Biosciences) were then added, and after a further 15-min incubation at 4°C, the cells were washed and analyzed on an EPICS XL flow cytometer (Coulter Electronics, Hialeah, FL). Cell sorting was performed with a FACSVantage SE (BD Biosciences).

Cytotoxicity assay

Cytotoxic activity was measured using a chromium-release assay (12). Briefly, T2 cells (HLA-A*0201) were labeled with Na251CrO4 for 1 h at 37°C, washed, then pulsed with synthetic peptide ELAGIGILTV (2 μM) for 30 min at 37°C. After washing, the cells were added to varying numbers of effector cells in V-bottom microwells. Chromium release was measured in the supernatant harvested after 4 h of incubation at 37°C.

Analysis of TCRBV transcripts and sequencing of PCR products

Total RNA from CTL clones or from sorted populations was extracted using the Rneasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was performed at 42°C for 1 h with 200 U Moloney murine leukemia virus reverse transcriptase (Life Technologies) mixed with 4 μl 5′ first strand buffer (Life Technologies), 2 μl 20 μM oligo(dT)12,18 (Promadin, Madison, WI), 20 U RNasin (Promadin), 2 μl 100 μM dTT (Life Technologies), 1 μl each dNTP at 25 mM each (Amersham Pharmacia Biotech), and 2 μl pyrococcus-treated water, in a total volume of 20 μl. TCRBV usage was assayed by RT-PCR and sequencing. cDNA served as template for PCR amplification using panels of BV-specific upstream primers and one downstream BC primer, chosen on the basis of described panels of TCRBV region oligonucleotides (13) and alignments of TCR sequences available at the International Immunogenetics Database of M. P. Lefranc (Montpellier, France) (http://imgt.cines.fr). TCR BV-BC PCR products derived from clones or bulk cultures of sorted populations were purified with the Qiaquick PCR purification kit (Qiagen) and sequenced using the dye terminator cycle sequencing kit (ABI–PRISM; PerkinElmer, Foster City, CA), according to the manufacturer’s instructions, to obtain a complete identification of the CDR3.

Results

Identification of Melan-A-specific T cells in tumor lesions of a melanoma patient

In a follow-up period of 4 mo, four metastatic lesions were resected from a single HLA-A2-positive patient. The first two lesions were resected from the nape of the neck and an arm muscle on the same day, and the other two from the right and the left arms 4 mo later (Table I).

Immunohistochemical techniques disclosed that most of the tumor cells showed a strong immunoreactivity for Melan-A (Fig. 1, A and B). Analysis of the lymphocytic infiltration revealed the predominance of CD3+ cells (data not shown). T cell immunophenotyping showed that CD4+ lymphocytes were localized predominantly at the periphery of tumor nodules, while the CD8+ lymphocytes were closely intermixed with the tumor cells (Fig. 1, C and D).

For in vitro culture, all four tumor lesions were dissociated into single cell suspensions, and nonadherent cells were cultured in complete medium supplemented with rHIL-2. TIL expansion was performed without Ag-specific stimulation, but only in the presence of residual tumor cells. After 2–3 wk of cell culture, the TIL populations were analyzed by flow cytometry to determine the percentage of Melan-A/A2–recognizing T cells. For this purpose, we used a tetramer folded with an antigenic peptide, ELAGIGILTV, containing a modification of the natural Melan-A26–35 epitope, substituting Leu for Ala at position 2 from NH2 terminus (hereafter called Melan-A/A2 peptide); this epitope forms relatively stable complexes with HLA-A*0201 and is a more potent immunogen than the natural Melan-A peptide (14). In addition,
interchangeability between the tetramer folded with the Melan-A/A2 analog and the natural peptide was previously demonstrated using tumor-specific CD8 T cells derived from melanoma patients (15). TILs were stained with anti-CD8 and anti-CD3 mAbs together with the Melan-A/A2 tetramer. As shown in Fig. 2, all TIL populations contained CD3+/CD8+/tetramer− cells ranging from 2 to 11% in the four samples.

Functional analysis of Melan-A/A2 tetramer-derived CTL specificity

To confirm the specificity of tetramer-positive cells, CD3+/CD8+/tetramer− cells were isolated by flow cytometry sorting. Cells gated in Fig. 2 were sorted according to tetramer staining and immediately cloned; they were stimulated with irradiated HLA-A2+ EBV-transformed B cells that had been incubated with the Melan-A/A2 peptide, in the presence of irradiated allogeneic PBMC as feeder cells, rhIL-2, and rhIL-4. A total of 56 T cell clones were derived that showed growth in long-term cultures, and were stained by the Melan-A/A2 tetramer (Table I). These clones were also tested for their ability to lyse specific target cells pulsed with the relevant peptide, as well as melanoma cell lines expressing the Melan-A/A2 Ag. The results obtained with 10 representative clones sorted from the TILs derived from the nape of the neck (A) and the arm muscle (B) are shown in Fig. 3. All the clones efficiently lysed T2 cells pulsed with the Melan-A/A2 peptide, but did not recognize T2 cells alone; they also recognized the melanoma cell line PDO-35-MEL HLA-A2+ expressing the Melan-A gene, albeit less efficiently than the peptide-pulsed T2 cells and with a certain clone-to-clone variability. Similar results were obtained with the other CD3+, CD8+, and tetramer− clones thus derived by cell sorting from lesions C and D (data not shown). Taken together, these findings provide functional evidence that the CTL clones derived from the TILs are Melan-A/A2-specific cells endowed with cytotoxic activity against tumor cell lines.

Analysis of the TCRBV repertoire of the Melan-A/A2-specific CTL clones derived from the TILs

As a first step to determine the repertoire of Melan-A/A2-specific CD8+ cells present in the TIL populations after in vitro expansion, we extracted RNA from the sorted Melan-A/A2 tetramer+ T cell

Table I. Characteristics of biological samples analyzed

<table>
<thead>
<tr>
<th>Biological Sample</th>
<th>Location/Tissue of Origin</th>
<th>Sampling Date (days)a</th>
<th>Melan-A Expressionb</th>
<th>HLA-A2 Expressionb</th>
<th>No. of CTL Clones Obtained</th>
<th>No. of CTL Clones Sequenced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesion A</td>
<td>Nape of neck, dermal</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
<td>Lesion B</td>
<td>Right arm, i.m.</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>17</td>
<td>6</td>
</tr>
<tr>
<td>Lesion C</td>
<td>Right arm, i.m.</td>
<td>106</td>
<td>+</td>
<td>+</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>Lesion D</td>
<td>Left arm, i.m.</td>
<td>106</td>
<td>+</td>
<td>+</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Blood</td>
<td></td>
<td>119</td>
<td>ND</td>
<td>ND</td>
<td>53</td>
<td>37</td>
</tr>
</tbody>
</table>

a Day 0 was chosen as the day in which the first metastatic lesion was biopsied.
b As determined by RT-PCR.

FIGURE 1. Section of a melanoma biopsy resected from the left arm immunostained with anti-Melan-A (A, ×80; B, ×120), anti-CD8 (C, ×60 and ×120), and anti-CD4 mAbs (D, ×60).
populations derived from lesions A and B and performed RT-PCR amplification using a panel of subfamily-specific oligonucleotide primers complementary to the V and C regions of the β-chain of the TCR. This analysis showed the presence of multiple clonotypic transcripts covering the majority of the BV families (data not shown). To examine the TCRBV repertoire more closely, we next analyzed the CDR3 at the clonal level using the RNA obtained from CTL clones derived from TILs after sorting the tetramer+ cells. Only 13 of the 56 clones analyzed showed long-term expansion sufficient to obtain enough RNA for the genetic analysis. RT-PCR was followed by sequence analysis of the PCR product to determine the sequence of the CDR3 of the β-chain of the TCR. Among the sequenced clones, three came from lesion A, six from lesion B, and four from lesions C and D (Table I). This analysis revealed a wide diversity of BV usage by Melan-A/A2-specific CD8+ cells not only among the four different lesions, but also at the level of each single lesion. This diversity was further confirmed when the CDR3 was analyzed: all the clones differed from one CTL to another in terms of their amino acid composition. We also observed that 8 of the 12 sequences analyzed were recombined with the J1-5*01 segment, and that the sequences sharing this J segment showed an overall CDR3 similarity, despite the usage of different BV regions (Fig. 4A). In fact, besides the four identical amino acids derived from the J segment and contributing to the CDR3, these clonotypes shared from one to four other identical amino acid residues, and six of these eight sequences had the same CDR3 length. Moreover, the clonotypes sharing the J1-5*01 segment came from three different lesions. Concerning the clonotypes recombined with a different J segment, the similarities in terms of amino acid composition were more limited.

None of the sequences identified in one lesion was found in the other populations, thus indicating that multiple clonotypic and nonoverlapping TCR contribute to the repertoire of the immunodominant Ag of Melan-A.

**TCRBV repertoire of Melan-A/A2-specific CTL clones derived from blood**

To compare the repertoire of CTL directed against the Melan-A/A2 Ag present at the tumor site with that circulating in the peripheral blood, we stimulated 4 × 10⁶ PBMC from the same melanoma patient with the Melan-A/A2 peptide in the presence of rhIL-2, rhIL-4, and rhIL-7. PBMC were collected 2 wk after the last metastasis excision. On day 15, after a second stimulation with the Ag under the same conditions, we labeled the cells with the Melan-A/A2 tetramer and identified 16% tetramer+ cells in the CD3+ CD8+ fraction (data not shown). Triple-labeled cells were sorted, seeded at one cell/well, and restimulated as above. We obtained 53 clones that were stained by the Melan-A/A2 tetramer; the clones proved to be Ag specific as they lysed T2 target cells pulsed with the Melan-A/A2 peptide as well as a melanoma cell line expressing the Melan-A/A2 Ag, but they did not recognize T2 cells alone (data not shown). We next extracted RNA from 37 clones that showed long-term expansion for which we had enough RNA for genetic analysis, and determined the CDR3 of the TCR β-chain, as described above. The nucleotide and predicted protein sequences spanning the VDJ regions of these clones are shown in Fig. 4B. Among the 37 clones, we found 14 different clonotypes; some were found in different copy number indicating that some clones expanded in vitro. The 14 clonotypes showed an extensive usage of the TCRBV region as well as recombination with several J regions; moreover, they differed in CDR3 length, which ranged from 9 to 13 aa. Interestingly, usage of BV and BJ segments was rather different compared with that observed in the clones derived from the metastatic lesions (Fig. 4A); moreover, none of the clonotypes matched those derived from the TILs.

**Discussion**

This study compared the TCR repertoire against a melanoma differentiation Ag in four different metastatic lesions and in the blood of a single melanoma patient during the natural history of the disease. Previous studies suggested that the repertoire of CTL clones directed against the major Ag of Melan-A was rather large (1, 6, 7, 15–17). CTL reactivity to the Melan-Aβ15–26 Ag was frequently observed in TILs and could be readily elicited from the PBMC of HLA-A2+ melanoma patients (18, 19). The advent of tetramer technology revealed important points about this tumor Ag that were not previously appreciated. One of its main characteristics is the presence of a large number of CTL precursors in the blood of melanoma patients and also healthy persons; this enables Melan-A/A2 tetramer+ cell detection in the peripheral blood of ~50% of healthy donors (20), as it comprises ~10−3 of circulating phenotypically naive CD8+ T cells (20, 21). To date, this is the only known tumor Ag that can be detected ex vivo by tetramers, without a round of in vitro stimulation (22). These cells present a naïve phenotype in healthy individuals, but may develop an Ag-experienced one in some melanoma patients (15, 20). Staining with tetramer revealed that the repertoire against this Ag not only has a higher frequency compared with other known tumor Ags, but it is also highly diversified, as tetramer+ cells are also stained by most of the Abs directed against the different Vβ-chain families (6).

Our findings indicate a further level of complexity, because a high degree of TCR diversity emerged from an extensive analysis of the clonotypes recognizing this tumor Ag in different metastatic lesions and the blood of our patient. Moreover, among the different clonotypes, none was present in any other lesion or in the blood. A broadening of the TCR repertoire was also demonstrated for a
different tumor Ag, gp100, in melanoma patients; in this case, repeated exposure to the modified gp100:209–217 epitope enhanced immune competence by increasing the frequency of immunogen-specific precursor T cells through selection of higher affinity CTL (23). It is quite striking to observe that the repertoire directed against a single epitope includes such a high number of different TCRs. However, a closer look at the CDR3s showed an overall similarity among most of the clones derived from the TILs, while a more heterogeneous situation was observed among the Melan-A/A2-specific clones derived from blood. If we analyze the CDR3s in terms of hydrophobicity, it appears that the majority of these sequences, from both blood and metastases, include a central portion of variable length containing a 3-aa motif based on a G-L-G sequence or amino acid substitutions with the same hydrophobic characteristics, surrounded by two hydrophilic regions (Fig. 4, boxed residues). This central pattern might be important for the recognition of the antigenic peptide, which, being hydrophobic, will probably contact amino acids by hydrophobic interactions. Another important amino acid in the CDR3 is a glutamic acid (Q), which was present in all but one of the clones derived from TILs, and in 9 of 14 clones obtained from blood (Fig. 4, boxed residues). However, an identification pattern of the Ag cannot easily be defined. Indeed, preliminary findings obtained with a few of these clones in the CDR3 of the /H9251-chain showed an elevated heterogeneity. Moreover, some of the clones possessed two productively rearranged /H9251-chains composed of different TCR AV and AJ segments (data not shown) that would have increased the level of complexity of the clones recognizing a single Melan-A/A2 epitope if they are, in fact, both expressed on the cell surface.

FIGURE 3. Evaluation of functional activity of CTL clones recognizing the Melan-A_{26-35} analog epitope A27L. The figure shows the cytotoxic activity of 10 CTL clones derived from TILs obtained from the lesions at the nape (lesion A) and arm muscle (lesion B) (top of each panel) and the staining with tetramer Melan-A/A2 (bottom of each panel). The first letter of each clone identifies the lesion from which it is derived. The specificity of peptide Ag recognition by the 10 CTL clones was assayed on chromium-labeled T2 target cells (○), T2 cells pulsed with peptide ELAGIGILTV (□), or melanoma cell line PDO-35-MEL (∆) at the indicated lymphocyte to target cell ratios. CTL clones were stained with soluble Melan-A/A2 tetramer coupled to PE.
FIGURE 4. Nucleotide and amino acid sequence of the TCR of CTL clones derived from TILs (A) and blood (B) of the same melanoma patient and recognizing the Melan-A/A2 epitope. The first letter of the clones derived from TILs identifies the lesion from which they are derived (A, lesion at the nape; B, lesion of the arm muscle; C, lesion of the right arm; and D, lesion of the left arm). Only the last three residues of the V region are shown, followed by the CDR3 defined according to Chothia et al. (26), by the J region, and by the first two residues of the C region. The TRCBV CDR3 length is defined according to Moss and Bell (27). TCR J segment residues contributing to CDR3 are underlined.
The fact that clonotypes found at one site were not present at another argues against the possibility that Melan-A/A2-specific CTL might be expanded in vivo as a consequence of tumor-induced activation. The high frequency of precursors generally observed in blood might determine the presence of numerous different CTL against the immunodominant epitope of Melan-A in the metastatic lesions. To explain the high precursor frequency in blood, Loftus, Rivoltini and colleagues (24) advanced that the peptide sequence recognized by the CTL might resemble sequences occurring frequently in proteins of different origin. It was demonstrated that peptide sequences derived from viral, bacterial, and human proteins that conform to the HLA-A2-binding motif and possess features essential to recognition by anti-Melan-A/2 CTL do indeed exist, therefore implying that epitope mimicry might play a role in modulating the CTL response against this Ag. A recent study traced the origin of these cells back to thymic selection and demonstrated that they are mostly generated by thymic output of a high number of precursors that undergo limited expansion in the periphery and remain functionally naive T cells in normal donors (25). In contrast to healthy individuals, the vast majority of melanoma patients has detectable accumulations of memory/effector melan-A-specific T cells in metastatic tumor lesions (6, 15, 25), but only after vaccination with the melan-A peptide it is possible to observe the expansion of some dominant clonotypes and the maturation of their functional avidity (8), thus suggesting that Melan-A-specific CTL can undergo clonal expansion upon appropriate stimulation.

Although our results are based only on the analysis of one patient, they provide direct evidence that a large melan-A-specific T-cell pool may also be maintained in the presence of high Ag load associated with tumor progression. The anti-Melan-A/A2 CTLs derived from our patient’s blood and metastatic lesions specifically recognized the exogenously supplied modified peptide as well as the endogenously processed natural peptide, thus excluding that tetramer-guided isolation of T cells might modify the natural T-cell repertoire. However, as we did not determine the affinity of these CTL clones, we cannot rule out the possibility that those present within the metastatic lesions have a higher affinity. Moreover, the finding of tumor-specific CTLs at the tumor site does not reflect an ongoing cytotoxic response, irrespective of CTL affinity. In this regard, it would have been important to analyze the lymphocytes for surface expression of naive, effector, and memory-associated markers; however, the number of cells in the metastatic lesions was too small to be properly analyzed, and therefore culture in vitro was a mandatory step. To understand the meaning of tumorspecific CTLs at the tumor site, we would have to find one or more identical clonotypes in different sites in a single patient, thus demonstrating activation, proliferation, and circulation in the body. Although we cannot rule out the possibility that such CTL clones were present in our patient and that we lost them in culture as a result of an in vitro selection, it is clear that if such clones existed, they were not present in high copy number.

In conclusion, the original finding in this study is that a large pool of melan-A-specific T cells, with a diverse TCRBV usage and a few common features in the CDR3, was present in different peripheral compartments of a single melanoma patient without evidence of Ag-driven selection or preferential homing at the metastatic site; the relevance of this diverse T repertoire to the antitumor response remains to be elucidated.

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References

generation of peptide-specific CTLs but does not overcome tumor escape from immune surveillance in metastatic lesions. J. Exp. Med. 190:651.


