Melanoma Patients Respond to a Cytotoxic T Lymphocyte-defined Self-Peptide with Diverse and Nonoverlapping T-Cell Receptor Repertoires

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ABSTRACT

HLA-A2* melanoma patients develop naturally a strong CD8+ T cell response to a self-peptide derived from Melan-A. Here, we have used HLA-A2/peptide tetramers to isolate Melan-A-specific T cells from tumor-infiltrated lymph nodes of two HLA-A2* melanoma patients and analyzed their TCR β chain V segment and complementarity determining region 3 length and sequence. We found a broad diversity in Melan-A-specific immune T-cell receptor (TCR) repertoires in terms of both TCR β chain variable gene segment usage and clonal composition. In addition, immune TCR repertoires selected in the patients were not overlapping. In contrast to previously characterized CD8+ T-cell responses to viral infections, this study provides evidence against usage of highly restricted TCR repertoire in the natural response to a self-differentiation tumor antigen.

INTRODUCTION

Self-peptides play a pivotal role in the shaping of the TCR3 repertoire. How TCR selection based on self-recognition can lead to the modeling of immune responses that are effective, but not dangerous to the host, is only partially elucidated. It is generally accepted that central tolerance to self-antigens is incomplete because potentially autoreactive T cells can be frequently found in the blood not only of patients suffering from auto-immune diseases but also of cancer patients and even normal individuals (1–3). Peripheral tolerance mechanisms also contribute to the shaping of TCR repertoire of autoreactive T cells. Whereas autoreactive T cells recognizing antigens presented by thymic antigen-presenting cells (APC) are clonally deleted during thymic selection (central tolerance), autoreactive T cells recognizing peripherally expressed antigens not present in the thymus can be either deleted, functionally anergicized, or simply remain “ignorant.” Thus, it is considered that self-peptide/MHC complexes select a set of mature self-reactive T cells with a certain level of tolerance that can be broken by “danger” signals (4). However, the actual complexity of the human TCR repertoire against defined self-antigens, as well as the variability of such a repertoire in different individuals, remains an unresolved question.

Most studies on the human TCR repertoire have relied on the analysis of antigen-specific T-cell clones specific either for viral or tumor-derived antigens (5–9). Although informative, these studies provide only partial insight into the diversity of the immune TCR repertoire among different individuals. A more global assessment of TCR repertoire diversity in T cells present in normal and disease-associated lymphoid cell populations has been attempted through the analysis of TCR β chain V segment and CDR3 length by spectratyping (10). However, this approach has been, thus far, mostly applied to the analysis of polyclonal T-cell populations of undefined antigen specificity, such as those found in tumor infiltrating lymphocytes (for an example see Ref. 11). The development of fluorescent MHC-class I/peptide tetrameric complexes containing antigenic peptides has recently enabled the direct detection and isolation of antigen-specific CD8+ T cells. Thus, it is now possible to combine these two powerful techniques in a single procedure that allows direct analysis of the diversity of polyclonal but antigen-monospecific CD8+ T-cell populations.

In this study, we focused on the analysis of the TCR repertoire that is specific for the HLA-A2-restricted immunodominant epitope derived from the melanoma-associated antigen Melan-A/MART/1 (Melan-A, hereafter; Refs. 12, 13). Melan-A is a self protein of unknown function that is expressed by both melanocytes and the majority of malignant melanoma cells. HLA-A2-restricted Melan-A-specific CTLs have been shown to recognize primarily Melan-A peptide [26]27–35 (9, 14). Using tetramers containing the Melan-A antigenic peptide, we have recently documented the presence of high numbers of Melan-A-specific CD8+ T lymphocytes with an activated/memory phenotype in TILNs of HLA-A2* melanoma patients (15).

In this study, we isolated Melan-A-specific CD8+ T cells present in TILNs from two patients by tetramer-guided cell sorting and analyzed their TCR β chain V segment and CDR3 length by spectratyping. Analysis of the TCR displayed by the two Melan-A-specific CD8+ T cell populations revealed no restriction in BV usage. Despite the presence of dominant clones, many distinct TCR clonotypes were identified in each population. In addition, we failed to detect any common CDR3 sequence between the two populations, which indicated that the immune Melan-A-specific TCR repertoires in the two patients were not overlapping. Thus, in contrast to previously characterized CD8+ T-cell responses to viral infections, our study indicates that a large repertoire of specific CD8+ T cells may be selected in response to a self-differentiation tumor antigen.

MATERIALS AND METHODS

Tissues and Cells. Surgically resected tumor infiltrated lymph node were finely minced with needles in sterile RPMI 1640 supplemented with 10% FCS. Cell suspensions were placed in 24-well tissue culture plates (Costar, Cambridge, MA) in 2 ml of Iscove’s Dulbecco medium (Life Technologies, Basel, Switzerland) supplemented with 0.24 mM Asn, 0.55 mM Arg, 1.5 mM Gln, 8% pooled human A+ serum (CTL medium), 100 units/ml IL-2, and 10 ng/ml IL-7. TILNs were cultured two weeks prior to analysis. Melan-A-specific CTL clones were derived from TILNs from limiting dilution cultures in the presence of irradiated allogeneic peripheral blood mononuclear cells, EBV-transformed B lymphocytes, PHA, and rIL-2 as described previously (16). They were subsequently expanded by periodic (every 3–4 weeks) restimulation into microtiter plates, together with irradiated feeder cells in the presence of PHA and rIL-2.
Tetramers and Flow Cytometry Immunofluorescence Analysis. Complexes were synthesized as described (15, 17). As the antigenic peptide, the Melan-A 26–35 A27L analogue (EAAAGILTV), which has a higher binding affinity and stability than the natural Melan-A decapeptide (EAAAGILTV) or the nonapeptide (AAGIGILTV; Ref. 12) was used. Interchangeability of parental Melan-A decapeptide and A27L analogue in terms of staining specificity has previously been assessed (15). TILNs were stained with tetramers in 20 μl of PBS-2% BSA during 1 h at room temperature; then 20 μl of anti-CD8FITC mAbs (Becton Dickinson; 1:100 final dilution) were added and incubated for 30 min at 4°C. Cells were washed once in the same buffer and analyzed by flow cytometry. Anti-Vβ antibodies were purchased from Immunotech (Beckman-Coulter, Marseille, France). Anti-Vβ3 mAb was FITC conjugated. Anti-CD8PerCP was from Becton Dickinson (San Jose, CA). Staining and washing was performed in PBS-2% BSA. For labeling, cells were: (a) incubated with tetramers (0.2 μg/sample in 20 μl) during 20 min at room temperature; (b) washed with purified anti-BV3FITC mAbs during 30 min at 4°C; and (c) washed with anti-CD8PerCP-labeled mAbs during 30 min at 4°C. Cells were then washed once and analyzed immediately in a FACS Calibur (Becton Dickinson). Data analysis was performed using Cell Quest software.

Cytotoxicity Assays. Cytotoxic activity was measured using chromium-release assays. Briefly T2 cells (HLA-A*0201) were labeled for 1 h at 37°C with Na2Cr, washed, and then added (1000 cells in 100 μl) to varying numbers of effector cells (100 μl) in the presence or in the absence of synthetic peptide Melan-A 26–35 (1 μM) in V-bottomed microc wells. Chromium release was measured in supernatant (100 μl) harvested after 4 h of incubation at 37°C. The percentage of specific lysis was calculated as:

\[
\text{Percentage specific lysis} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Total release} - \text{spontaneous release}} \times 100
\]

The background lysis obtained in the absence of peptide was subtracted from the values obtained in the presence of peptide.

CDR3 Size Analysis of TCR BV Transcripts. The CDR3 region of the PCR-amplified TCR BV1–24 transcripts was analyzed using a run-off procedure, as described previously (10, 19). Briefly, total RNA was prepared from total TILNs or sorted fractions (2 × 106 cells/sample) using TRIzol (Life Technologies, Paisley, United Kingdom) and converted to cDNA by standard methods using reverse transcriptase and an oligo(dT) primer. These cDNA were amplified using a panel of validated 5' sense primers specific for the 24 BV subfamilies and one 3' antisense primer specific for the BC gene segment (20). Aliquots (2 μl) of BV1–24/BC PCR products were subjected to one cycle run-off reactions, using dye-labeled oligonucleotide primers, specific for the BC segment. 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 Immunoscope software (developed by C. Pannetier, Paris, France).

Sequencing of PCR Products. TCR BV-BC PCR products derived from sorted populations were cloned into pBS-SK+ vector (Stratagene, La Jolla, CA). Competent XL-1 blue Escherichia coli (Stratagene) were transformed and plated for blue/white color selection on media containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Plasmid DNA was extracted from white colonies using the Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany) and sequenced using the Dye Terminator Cycle Sequencing kit (ABI PRISM; Perkin-Elmer, Foster City, CA) according to the manufacturer’s instructions.

Quantitative Assessment of Selected Melan-A-specific TCR Clonotypes. Quantification of selected T cell clones was performed as described previously (19, 21). Briefly, clonotypic primers hybridizing with the CDR3 region of the selected clone were synthesized and labeled with a 6-Fam fluorophore (GENSET, Paris, France). The sequences of clonotypic primers were: 5'-GAACCCCCCCGCTACCTCTCT-3' for the recurrent BV20 sequence in LAU-203; 5'-GGTGTTTCCCAACCCTGTCAG-3' for clone LAU 203/1.5 (BV13); 5'-GAACCCCCCCGCTACCTCTCT-3' for clone LAU 181/1.3 (BV3); 5'-ATGCAACACTTCTGAT-3' for the recurrent BV20 sequence in LAU 203; 5'-ACCCAGGCCTCGGGACT-3' and 5'-CCCGAGCCCCCTGAT-3' for the two BV20 recurrent sequences in LAU 181; and 5'-ATCTCCGTGACCAACCTGAGC-3' for the BV14 recurrent sequences in LAU 181. cDNA samples were amplified using the appropriate BV and a BC primer; then aliquots of the BV-BC PCR products were subjected to an elongation (one cycle) with either a 6-Fam-labeled and nested BC primer (10) or with the clonotypic primer. The two run-off products were then mixed in equal amounts and size-fractionated in the automated sequencer. The proportion of the specific sequence in the total BV mRNA population was calculated by dividing the area under the curve (AUC) obtained with the clonotypic primer by the sum of the AUC obtained with the BC primer. The ratio was corrected for the relative specific activity of the clonotypic primer, which was calculated by dividing the AUC obtained with the clonotypic primer by the AUC obtained with the BC primer when amplifying plasmid DNA encoding the relevant BV sequence. Overall, this method determines the respective proportion of a unique recurrent transcript in the total BVx mRNAs.

RESULTS

A2/Melan-A Peptide Tetramer-guided Isolation of Antigen-monospecific T Cells from TILNs. We have previously shown that high numbers of CD8+ T cells specific for the Melan-A peptide [26]27–35 are frequently found in short-term cultured TILNs from HLA-A2+ melanoma patients (15). TILN cell populations from two such patients were stained with A2/Melan-A tetramers and anti-CD8 mAb and analyzed by flow cytometry. As shown in Fig. 1A, TILNs

Fig. 1. A2/Melan-A peptide tetramer-guided isolation of antigen-monospecific T cells from TILNs. A. TILN cell suspensions prepared from metastatic lymph nodes excised from patients LAU 203 and LAU 181 were cultured in complete medium supplemented with IL-2 and IL-7 during 2 weeks. Cells were double stained with anti-CD8FITC and A2/Melan-A tetramers as detailed in “Materials and Methods.” A2/Melan-A tetramer versus CD8 dot plot is shown for TILN LAU 203 for gated live lymphocytes. TILN LAU 203 contained 97% CD8bright lymphocytes, 4.2% of which were A2/Melan-A tetramer+. TILN LAU 203 contained 54% CD8bright lymphocytes, 9.6% of which were A2/Melan-A tetramer+. \( R \), CD8bright lymphocytes from TILN were sterile sorted into A2/Melan-A tetramer+ and tetramer- populations and analyzed immediately after sorting as shown here for TILN LAU 203. Tetramer+ and tetramer- populations from TILN LAU 203 were 99.7% and 94.4% pure, respectively. Tetramer+ and tetramer- populations from TILN LAU 181 were 98.4% and 96.7% pure, respectively. C, sorted tetramer+ and tetramer- populations were expanded for 2 weeks in the presence of irradiated allogeneic peripheral blood mononuclear cells and PHA and tested for their lytic activity against chromium-labeled T2 cells (HLA-A2+). The percentage lysis obtained on T2 alone was subtracted from the percentage lysis obtained in the presence of Melan-A 26–35 peptide (EAAAGILTV, 1 μM). This value is indicated as percentage specific lysis. D, the same cell fractions assayed in C were also assayed for their lytic activity against the autologous tumor cell line Me 290, derived from the same TILN cell populations.
from patient LAU 203 contained 4.2% CD8^+ A2/Melan-A tetramer^+ T cells. Similarly, TILNs from patient LAU 181 contained 9.6% CD8^+ A2/Melan-A tetramer^+ cells (data not shown). To analyze the TCR BV repertoire displayed by the CD8^+ A2/Melan-A tetramer^+ T cells, CD8^+ tetramer^+ and CD8^+ tetramer^- populations were isolated by flow-cytometry cell sorting. Immediate reanalysis of the separated populations showed a high degree of purity (Fig. 1B). The sorted populations were expanded by mitogen-driven stimulation during 2 weeks and then assayed for cytolytic activity. As shown in Fig. 1C, cultured CD8^+ A2/Melan-A tetramer^+ T cells specifically lysed peptide-loaded T2 cells, whereas no significant specific lysis was observed with cultured CD8^+ Melan-A tetramer^- cells. In addition, cultured CD8^+ A2/Melan-A tetramer^- cells specifically lysed autologous tumor cells. Autologous tumor cells were also lysed by CD8^+ A2/Melan-A tetramer^- cells, which indicated the presence of tumor-reactive cells of undefined specificity within this population (Fig. 1D).

Similar results were obtained with TILN cells from patient LAU 181 (data not shown).

**TCR BV Repertoire of CD8^+ A2/Melan-A Tetramer^+, CD8^+ A2/Melan-A Tetramer^+, and Unseparated TILN Populations.** As a first step to determine the repertoire of Melan-A-specific T cells present in TILN samples from patients LAU 203 and LAU 181, we analyzed the CDR3 size variability of amplified TCR BV gene segment transcripts in sorted CD8^+ A2/Melan-A tetramer^+ and CD8^+ A2/Melan-A tetramer^- populations as well as in unseparated TILN cells (Fig. 2). Melan-A-specific CD8^+ T cells from both patients (Rows A) expressed many distinct BV gene segments. In contrast to the pattern generally observed for polyclonal T-cell populations (10), the CDR3 size profiles of the majority of BV subfamilies were generally not bell-shaped but displayed prominent peaks that indicated the accumulation of recurrent size transcripts. Significant restriction in the CDR3 size variability was also found in the sorted CD8^+ A2/Melan-A tetramer^- population (Rows B). As expected from the prevalence (>90%) of non-Melan-A-specific T cells in the analyzed TILN samples, the CDR3 size profiles of the unseparated TILNs (Rows C) were very similar to those obtained with the CD8^+ A2/Melan-A tetramer^- populations. A striking exception was the BV3 profile obtained for patient LAU 181, which was almost identical in unseparated TILNs and in the CD8^+ A2/Melan-A tetramer^+ population. This is explained by the fact that 88% of the CD8^+ A2/Melan-A tetramer^- cells expressed BV3 in contrast to only 0.3% of BV3^- cells among CD8^+ A2/Melan-A tetramer^- cells as quantitated by staining with A2/Melan-A tetramers and BV3-specific mAbs (data not shown).

Prominent peaks as well as their CDR3 size profiles were clearly different in CD8^+ A2/Melan-A tetramer^+ and CD8^+ A2/Melan-A tetramer^- populations in most cases (Fig. 2). There were a few exceptions in which overwhelming peaks were detected in both populations (i.e., BV1 and -12 for LAU 203; BV14 for LAU 181). Several possibilities can be considered to explain the presence of common CDR3 size peaks. Because of the high sensitivity of the RT-PCR-based analysis, some peaks observed for the tetramer^- population could be attributable to a few contaminating Melan-A-specific T cells that were not stained by tetramers. Alternatively, common CDR3 size peaks could correspond to TCR with identical CDR3 size albeit of distinct sequences.

**The Repertoire of Melan-A-specific T Cells Is Distinct in TILN Samples from Patients LAU 203 and LAU 181.** To more precisely appraise the TCR BV repertoire of Melan-A-specific populations, we directly compared the CDR3 size profiles of A2/Melan-A tetramer^+ populations from patients LAU 203 and LAU 181 (Fig. 3). The majority of prominent CDR3 size peaks were distinct between the two populations, which indicated usage of different TCR BV repertoires. However, prominent peaks with the same CDR3 size were found for some BV (i.e., BV14 and BV20) in both populations. To assess whether this was attributable to the presence of Melan-A-specific T cells with identical or similar TCR, the BV14 and BV20 PCR products were sequenced. As illustrated in Table 1, a single recurrent sequence with a 10-amino acid CDR3 size was found in the BV14 transcripts obtained from patient LAU 181, whereas, in agreement with the BV14 profile, seven different sequences with various CDR3 sizes were found for patient LAU 203. Moreover, the three sequences with a 10-amino acid CDR3 size in the population from patient LAU 203 were different from the 10-amino acid CDR3 sequence found in the sample from patient LAU 181. With the exception of two clones (H6 and H7), each of the others expressed different TCR BJ segments. Certain conserved structural features were observed in the CDR3 regions such as a Leu residue at position 96 in three of eight clonotypes and a Gly residue at position 97 in four of eight clonotypes (Table 1).

The analysis of BV20 transcripts produced reciprocal results to those found with BV14 transcripts. Indeed, a unique 9-amino acid CDR3 recurrent sequence was identified in the BV20 transcripts obtained from patient LAU 203 (Table 2). In contrast, in the BV20 PCR products obtained from patient LAU 181, and in agreement with the corresponding BV20 CDR3 size profile, sequences with CDR3 sizes of 9 amino acids (19 of 29) and 11 amino acids (10 of 29), respectively, were identified. Moreover, the 7 different sequences with a 9-amino acid CDR3 size, identified in the population from patient LAU 181, were clearly distinct from the recurrent 9-amino acid CDR3 sequence found in the population from patient LAU 203. In addition and remarkably, the CDR3 sequences found in the population from patient LAU 181 exhibited a number of features all suggestive of the presence of TCR structural constraints. Eighteen of 29 BV20 chains analyzed were recombined with BJ1.5, a gene segment that is not among the most frequently used (22). In addition, among the 19 sequences with a 9-amino acid CDR3, both conservative and nonconservative substitutions were identified, leading to an overall impressive similarity in the different CDR3 sequences. Together, these results indicate that expanded Melan-A-specific T-cell populations can include (for a given BV) either a single TCR clonotype or multiple TCR clonotypes.

It is noteworthy that none of the sequences identified in the CD8^+ A2/Melan-A tetramer^- population from patient LAU 181 was found in the corresponding population from patient LAU 203 and vice versa. To exclude the possibility that some of the prevalent clonotypes found in one patient could be present in low proportion in the other patient, four clonotypic primers (indicated in Tables 1 and 2) were synthesized. As illustrated in Fig. 4, run-off analysis of cells from patient LAU 181 with clonotypic primers for recurrent BV20 sequences selected in patient LAU 203 produced no detectable signals (the estimated detection limit of this method being 1 in 10^5 cells, (23). Reciprocally, similar negative results were obtained with cells from patient LAU 203 when tested for BV14 sequences selected in patient LAU 181 (data not shown).

**Quantitation of Prevalent Melan-A-specific TCR Clonotypes.** Melan-A-specific T-cell clones LAU 203/1.5 (BV13) and LAU 181/1.3 (BV3) were derived from sorted A2/Melan-A tetramer^+ populations obtained from the corresponding TILN samples. To estimate their frequencies in the Melan-A-specific populations, TCR BV CDR3 regions were sequenced (Fig. 5, A and B) and clonotypic primers were synthesized and used to quantitate the corresponding clonotypes. Clone LAU 203/1.5 (CDR3 12 amino acid) represented ~30% of the total BV13 mRNA in the tetramer^+ population, whereas it was undetectable in the tetramer^- fraction (Fig. 5A). Because the proportion of BV13 T cells in the tetramer^- fraction was 13% (as
determined by anti-BV13 mAbs and A2/Melan-A peptide tetramer double staining) it could be estimated that this clone accounted for 4% of the tetramer population and 0.13% of CD8 T cells in TILNs from patient LAU 203. Clone LAU 181/1.3 represented 70% of the total BV3 mRNA in the tetramer population (Fig. 5B). A small peak was observed in the tetramer population, most likely as the result of a few contaminating cells. Because 88% of tetramer+ cells in the TILN sample from patient LAU 181 expressed BV3 (data not shown), it could be estimated that this single BV3 T-cell clone accounted for ~62% of the Melan-A-specific population and about 6% of CD8+ T cells. Clone LAU 203/1.5 was not detected in either population from patient LAU 181. Conversely, clone LAU 181/1.3 was not detected in

![Graphs showing CDR3 size distributions](image.png)
either population from patient LAU 203 (Fig. 5 and data not shown). These results underline the observation that despite the diversity of Melan-A-specific repertoires in the tetramer+ TILN fraction of each patient, highly dominant clonotypes can also be found.

**DISCUSSION**

This report represents the first comprehensive analysis of TCR β chain usage by human CD8+ T cells directed against a self-differentiation tumor antigen. Our results indicate that the repertoire of Melan-A-specific T cells present in TILNs is highly diverse in terms of both BV usage and clonal composition. In addition, no common TCR sequences were observed in two different cancer patients.

**MHC/Peptide Tetramer-guided Analysis of Antigen-specific TCR Repertoire.** We have previously used A2/Melan-A tetramers to enumerate and characterize Melan-A-specific CD8+ T cells in metastatic lymph nodes of HLA-A2 melanoma patients (15). To determine the individual variability of Melan-A-specific TCR repertoire at the molecular level, we have combined: (a) isolation of Melan-A-specific CD8+ T cells by A2/Melan-A tetramer-guided cell sorting and (b) analysis of the CDR3-size variability of amplified TCR BV gene segment transcripts of the sorted populations. It is noteworthy that this approach, when coupled with the use of double staining with anti-BV mAb and A2/peptide tetramers, and analysis of individual TCRs with clonotypic primers, enables a detailed and quantitative clonal description of polyclonal but antigen-monospecific responses. Therefore, it offers the possibility not only of characterizing total T-cell responses to a given antigen but also of evaluating the relative contribution of individual clonotypes to the total response.

**High Diversity and Individual Variability of Melan-A-specific TCR Repertoire.** The first observation was the nonrestricted usage of BV genes by Melan-A-specific T cells. Although dominant clonotypes were identified, a significant signal was obtained in almost all of the BV-BC PCR products from both tetramer-sorted populations. Furthermore, CDR3 size profiles indicated a larger TCR diversity, with more than 50 individual peaks in each sample. This diversity was further increased by the observation that a defined peak can reflect the accumulation of transcripts with the same CDR3 length but different sequences (as BV14 in LAU 203 and BV20 in LAU 181). Together, these data confirmed and extended our previous observations showing that Melan-A-specific CD8+ T cells expressed different BV chains, as assessed by a multiparametric flow cytometry analysis based on the use of anti-BV mAb and A2/Melan-A tetrarsers (24). A study of multiple s.c. metastases from two patients by RT-PCR and denaturing gradient gel electrophoresis revealed the presence from 40 to

<table>
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<th>CDR3β (nt)</th>
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* Arrows indicate the clonotypic primer used for experiments in Fig. 5.

aa, amino acid; nt, nucleotide.
sequences among the total BV20 samples.

T-cell clones that were highly expanded in one individual were either identified in both of the Melan-A tetramer populations, they did not include common BV sequences. Overall, although we cannot exclude the presence of a small number of shared TCRs, the present data indicate that >99% of Melan-A-recognizing TCRs are different in two individuals with melanoma.

This remarkable level of variability does not preclude that Melan-A-specific TCRs are selected according to structural criteria. Indeed, when individual BV families were analyzed, some conserved structural features emerged: in the case of BV14-bearing T cells, a Leu residue at position 96 was conserved in three of eight clonotypes as well as in four of six TCR BV14 Melan-A-specific clones previously described (9, 26). In addition, for patient LAU 181, conserved BJ usage as well as conserved CDR3 features were identified among Melan-A-specific clonotypes using BV20. These results suggest that, despite the lack of restricted BV gene segment usage, some structural constraints in the CDR3 region could indeed characterize Melan-A-specific TCRs within a given BV family.

The large and diverse TCR repertoire exhibited by Melan-A-specific CTLs is in clear contrast with the highly restricted TCR repertoire generally described for CTL specific for viral-derived antigens. Indeed, conserved TCR chain variable segment and especially BV usage are conserved in melanoma patients with melanoma.

Melan-A-specific TCR repertoires in two HLA-A2 melanoma patients were largely not overlapping, as supported by several lines of evidence. First, the direct comparison of CDR3 size profiles in both of the Melan-A tetramer populations revealed different prominent peaks in the different BV PCR products. This indicates that the vast majority of Melan-A-specific TCR clonotypes were different in the two patients, even if it is difficult to have a quantitative estimate of the single TCR specificities included in the different peaks. Indeed, sequence data obtained here and elsewhere (10, 19, 25), showed that a single CDR3 peak can correspond either to a recurrent single TCR BV or to TCR BVs with the same CDR3 length but different sequences. Second, the Melan-A-specific T-cell clones that dominated the response in one patient were not detectable in TILNs or highly enriched circulating CD8+ T cells of the other patient (data not shown), which indicated that T-cell clones that were highly expanded in one individual were either absent or present at a frequency <105 CD8+ T cells in the other one. Third, even when BV transcripts containing CDR3 peaks of identical size were identified in both of the Melan-A tetramer populations, they did not include common BV sequences. Overall, although we cannot exclude the presence of a small number of shared TCRs, the present data indicate that >99% of Melan-A-recognizing TCRs are different in two individuals with melanoma.

<table>
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<td>2/29</td>
</tr>
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<td>AGT GTA GGA</td>
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<td>GGA ACA GGA</td>
<td>7/29</td>
</tr>
<tr>
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<td>GTUVPSGAHL</td>
<td>BJ2.6</td>
<td>GGA ACA GGA</td>
<td>3/29</td>
</tr>
</tbody>
</table>

* Nonconservative substitutions are underlined, and conservative substitutions are indicated in bold. Clonotypic primers used for subsequent experiments described in Fig. 5 are indicated by arrows.

aa, amino acid; nt, nucleotide.

Fig. 4. Recurrent BV20 sequences in the A2/Melan-A tetramer population from one individual are not detectable in the corresponding population from the other patient. Clonotypic primers (indicated in Tables 1 and 2) were used to detect the recurrent BV20 sequences included in A2/Melan-A tetramer populations of LAU 203 and LAU 181. Rates (%) beside the profiles, the proportion of the recurrent sequences among the total BV20 samples.
the individual variability in the clonal composition of the CD8 immune TCR repertoire in individual mice is strongly limited by only known example of an antigen-specific naive repertoire quantification of TCR repertoire usage thus far reported concerns EBV-specific CTLs. Indeed, highly conserved or even identical EBV-derived antigens, Melan-A-specific CD8 T cells, under physiological circumstances, would insure peripheral tolerance.

The present study shows the existence of an unprecedented level of complexity, diversity, and individual variability in the TCR immune repertoire specific for an immunodominant tumor-associated self-antigen. Whether this large diversity simply reflects a large set of naive precursors and/or originates from low-affinity antigen recognition remains to be elucidated. Future experiments should provide insights into the dynamics of Melan-A-specific TCR repertoire, by comparing the repertoire in naive and memory populations and by studying its evolution along disease progression or regression and therapeutic interventions.

**Fig. 5. Quantitation of Melan-A-specific T-cell clones using clonotypic primers.**

Melan-A-specific T-cell clones LAU 203/1.5 (BV/3) and LAU 181/1.3 (BV/3) were derived from sorted A2/Melan-A tetramer negative populations. Their TCR \( \beta \) chain was sequenced, and their presence in different samples was assessed using clonotypic primers (arrow under each TCR sequence), as detailed in “Materials and Methods”). A, the BV13 Melan-A-specific T-cell clone was found only in the LAU 203 A2/Melan-A tetramer negative population, whereas no signal was detected in LAU 203 A2/Melan-A tetramer positive and LAU 181 tetramer positive populations. The same procedure was used to assess the presence and the proportion of the BV3 T cell clone in different samples (B).

**Why Is the Melan-A-specific TCR Repertoire Large and Diverse?** A recent study (29) suggests that the diversity of the immune TCR repertoire in individual mice is strongly limited by the size of the antigen-specific naive precursors’ pool. Therefore, the individual variability in the clonal composition of the CD8 T cells directed against a given peptide could reflect large differences in the corresponding TCR naive repertoires among individuals. In this regard, it is noteworthy that, using A2/Melan-A tetramers, we recently observed high frequencies of naive Melan-A-specific T cells (>1 in 2500 CD8 T cells) in blood lymphocytes from the majority of HLA-A2 melanoma patients as well as in 6 of 10 HLA-A2 healthy individuals (3). To our knowledge, this is the only known example of an antigen-specific naive repertoire quantitatively sufficient for tetramer detection ex vivo. Indeed, parallel analysis of the same blood samples using tetramers containing Flu MA peptide 58–66 revealed that, although Flu MA-specific CD8 T cells exhibiting a memory phenotype were readily detectable in the majority of HLA-A2 individuals, naive Flu MA-specific CD8 T cells were generally below the limit of detection of tetramer staining. Similarly, naive T cells specific for other known HLA-A2-restricted epitopes were generally undetectable in blood (30, 31).

Why naive T cells specific for Melan-A are much more frequent than for other epitopes is unknown. One explanation could be related to the singularity of its highly hydrophobic amino-acid composition. Indeed, the localization of the Melan-A immunodominant peptide in the transmembrane region greatly limits the diversity of the amino acids included in its sequence. This hypothesis is supported by the observation that Melan-A immunodominant peptide-homologous sequences are frequently found among other self-proteins as well as pathogen-derived proteins (32). Thus, it is possible that the existence of numerous weakly cross-reactive self-sequences together with the absence of the nominal antigen during thymic selection would result in the large number of Melan-A-specific naive precursors detected in the periphery. As suggested by recent studies (33, 34), it is conceivable that a similar set of peptides would be involved in the maintenance of Melan-A-specific naive precursors in the periphery, whereas the localization of the nominal antigen in body tissues that are not accessible to naive T cells, under physiological circumstances, would insulate peripheral tolerance.

The availability of a large-sized Melan-A-specific CD8 T-cell precursor pool could explain, at least in part, the TCR diversity of Melan-A-specific CD8 T cells found in tumor infiltrated lymph nodes. Additional factors could contribute to the TCR diversity. In particular, it is noteworthy that, in contrast to high-affinity antigen recognition commonly exhibited by CD8 T cells specific for viral-derived antigens, Melan-A-specific CD8 T cells, as well as most CD8 T cells specific for nonmutated self-derived antigens, generally recognize natural peptides with relatively low affinity (16, 18). It is conceivable that the structural constraints imposed by low-affinity antigen recognition would be less stringent than the ones required for high-affinity antigen recognition, thus allowing the selection of a larger number of different specific \( \alpha/\beta \) TCR primary structures. Concomitantly, contraction of TCR repertoire and predominance of high-affinity clones along with the development of immune responses could be less pronounced than for high-affinity antigen recognition.

Finally, the recent finding that thymic functions are maintained late in life (35) supports the view that the TCR repertoire that is specific for a given antigen should be considered as a dynamic process that can show profound changes during life. This observation could be particularly relevant in the case of an antigen-specific naive precursor pool of large size because the TCR diversity in the naive pool was recently reported to be much higher than that of the memory compartment (36).

The present study shows the existence of an unprecedented level of complexity, diversity, and individual variability in the TCR immune repertoire specific for an immunodominant tumor-associated self-antigen. Whether this large diversity simply reflects a large set of naive precursors and/or originates from low-affinity antigen recognition remains to be elucidated. Future experiments should provide insights into the dynamics of Melan-A-specific TCR repertoire, by comparing the repertoire in naive and memory populations and by studying its evolution along disease progression or regression and therapeutic interventions.
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