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Tetramer-Guided Analysis of TCR β-Chain Usage Reveals a Large Repertoire of Melan-A-Specific CD8$^+$ T Cells in Melanoma Patients[1]

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The assessment of the TCR repertoire expressed by tumor-specific CD8$^+$ T lymphocytes has been hampered to date by the difficulty of targeting the analysis to lymphocytes directed against a single epitope. In the present study we have used fluorescent A2/Melan-A tetramers in conjunction with anti-CD8 and anti-TCR β-chain variable (BV) mAbs to analyze by flow cytometry the BV segment usage by Melan-A-specific CD8$^+$ T cells in tumor-infiltrated lymph nodes (TILN) and tumor-infiltrating lymphocytes (TIL) from A2 melanoma patients. Analysis of TILN populations revealed small proportions of A2/Melan-A tetramer$^+$ cells expressing many different BV together with over-representation of A2/Melan-A tetramer$^+$ cells expressing certain BVs. The BV usage by A2/Melan-A tetramer$^+$ lymphocytes in TIL was more restricted than that in TILN. Moreover, the predominant BV segments were quite distinct in populations derived from different patients. A2/Melan-A tetramer$^+$ cells expressing the dominant BVs found in TILN could also be found in the corresponding peptide-stimulated autologous PBMC, although A2/Melan-A tetramer$^+$ lymphocytes expressing additional BVs were also identified. Together, these results suggest that a large and diverse repertoire of Melan-A-specific T cells using different BV TCR segments is available in A2 melanoma patients. ***The Journal of Immunology,*** **2000, 165: 533–538.**

Tumor-reactive lymphocytes can often be isolated from both primary and metastatic melanoma lesions or melanoma-infiltrated lymph nodes (TIL[3] and TILN) and expanded by in vitro culture in the presence of exogenously added cytokines. Although TIL and TILN may contain both CD4$^+$ and CD8$^+$ T lymphocytes, tumor-specific cytolytic activity is generally exerted by CD8$^+$ CTL, whose presence appears to correlate with favorable clinical outcomes (1–3).

T cell Ag recognition is based on the interaction of a clonotypic TCR-αβ with antigenic peptides presented by a given HLA class I or class II molecule. Thus, it is conceivable that the structural constraints imposed by the recognition of immunodominant tumor Ags in TIL(N) would select a limited number of TCR that exhibit at least some common features. Previous attempts to demonstrate restricted TCR V gene segment usage by tumor-reactive TIL have yielded contrasting results. In some studies, limited TCR V gene segment usage was indeed found (4, 5), while other reports suggested a more diverse TCR V gene repertoire (6). The main difficulty in interpreting the results from these studies was that even in those cases where antitumor reactivity and HLA restriction of the analyzed populations were demonstrated, no information was available concerning the Ag(s) recognized. Thus, the search for correlations between TCR V gene segment usage and tumor Ag-specific recognition has been limited to the analysis of few available Ag-specific T cell clones (7–11).

Overall, these studies have been greatly limited by the difficulty of identifying tumor-reactive CD8$^+$ T cells of defined single Ag specificity within polyclonal polyspecific T cell populations. We have recently shown that direct visualization of CD8$^+$ T cells specific for the immunodominant epitope from the melanoma-associated Ag Melan-A can be achieved through staining with fluorescent HLA-A*0201/Melan-A peptide tetramers (A2/Melan-A tetramers thereafter) (12). Staining with A2/Melan-A tetramers is highly sensitive, allowing the identification of Melan-A-specific cells at frequencies as low as 0.2% in CD8$^+$ lymph node cells (12) and even lower in CD8$^+$ circulating lymphocytes (~0.04%) (13). We have previously shown that the frequency of A2/Melan-A tetramer$^+$ cells detected in bulk cultures directly correlates with peptide-specific cytotoxicity (14). The specificity of A2/tetramer$^+$ staining was further validated by assessment of the functional specificity of sorted A2/Melan-A tetramer$^+$ populations (12, 14). In the present study we used A2/Melan-A tetramers in combination with anti-CD8 and a panel of anti-BV mAb to directly and quantitatively analyze the TCR BV region usage by Melan-A-specific CTL in TILN and TIL as well as in peptide-stimulated PBMC cultures from HLA-A2 melanoma patients.

The results of these analyses revealed that small proportions of A2/Melan-A tetramer$^+$ CD8$^+$ cells expressing several different BV can be found in a single sample, thus showing the existence of

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[3] Abbreviations used in this paper: TIL, tumor-infiltrating lymphocytes; TILN, tumor-infiltrated lymph nodes; LN, lymph node; BV, β-chain variable.

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Table I. *Clinical status of metastatic melanoma patients and enumeration of A2/Melan A tetramer binding lymphocytes in TIL(N)*

Patient Code[a]	Age (yr)	Tumor Stage (AJCC)[b]	Sample[a]	%CD8$^+$ in Lymphocyte Gate[c]	%CD8$^+$ A2/Melan-A Tetramer$^+$
LAU 156	50	T3N0M1	Paravertebral metastasis	97	9.7
LAU 156	51	T3N0M1	Paravertebral metastasis	85.4	14.7
LAU 212	71	pTXN2cM0	Subcutaneous metastasis	92	3.9
LAU 337	25	pT3aN2M1	Soft tissue metastasis	34.2	0.3
LAU 321	65	pT2N2cM0	Subcutaneous metastasis	65.5	0.2
LAU 343	78	pT3aN2cM0	Subcutaneous metastasis	63.6	15.5
LAU 253	61	pT3aN2M0	Tumor-infiltrated lymph node	62.3	21.8
LAU 50	65	pT4aN2cM0	Tumor-infiltrated lymph node	47.5	14.5
LAU 233	75	pT4N2cM0	Tumor-infiltrated lymph node	87.4	24.4
LAU 203	66	pTxN2cM0	Tumor-infiltrated lymph node	98	4.9
LAU 314	42	pT2N0M1	Tumor-infiltrated lymph node	67.7	<0.01
LAU 198	71	pT3aN1M0	Tumor-infiltrated lymph node	52.8	<0.01

[a] All samples included in this series were collected by surgical dissection, finely minced with needles and cultured for 2–3 wk in complete medium supplemented with hrIL-2 and hrIL-7.

[b] AJCC, American Joint Committee on Cancer.

[c] Aliquots of TIL(N) were analyzed by two-color flow cytometry after staining with anti-CD8 and A2/Melan-A tetramers. CD8^{bright} cells were gated, and the percentage of CD8$^+$ A2/Melan-A tetramer$^+$ cells was calculated with CellQuest software.

a large repertoire of Melan-A-specific T cells. However, enrichment of A2/Melan-A tetramer$^+$ CD8$^+$ T cells expressing certain BV was also found. A2/Melan-A tetramer$^+$ CD8$^+$ T cells expressing BV14 were over-represented in the majority of the samples analyzed, while over-representation of other BV appeared to be characteristic of single samples. These findings suggest that the repertoire of Melan-A-specific T cells available in different A2$^+$ melanoma patients could be only partially overlapping.

Materials and Methods

Tissues and cells

Melanoma patients subjected to therapeutic surgical resection of lymph node (LN) or metastatic lesions were selected for this study on the basis of HLA-A2 Ag expression as assessed by HLA typing. Individual samples collected by surgical dissection were finely minced with needles in sterile RPMI 1640 supplemented with 10% FCS. Cell suspensions from each sample were placed in 24-well tissue culture plates (Costar, Cambridge, MA) in 2 ml of IMEM (Life Technologies, Basel, Switzerland) supplemented with 0.24 mM Asn, 0.55 mM Arg, 1.5 mM Gln, 10% pooled human A$^+$ serum (CTL medium), 100 U/ml IL-2, and 10 ng/ml IL-7. Cells were cultured 2–3 wk before FACS analysis. For peptide stimulation experiments of PBMC, CD8$^+$ lymphocytes were positively selected by magnetic cell sorting from PBMC of HLA-A*0201 melanoma patients using a MiniMACS device (Miltenyi Biotec, Sunnyvale, CA). Cells of the CD8$^-$ fraction were irradiated (30 Gy) and used as autologous APC. CD8$^+$ highly enriched lymphocytes (1×10^6/condition) were stimulated with peptide (1 μM) and irradiated autologous APC in 2 ml of CTL medium (14) containing human rIL-2 (100 U/ml; Glaxo, Geneva, Switzerland; provided by Dr. M. Nabholz, Institut Suisse de Recherches Experimentales sur le Cancer, Epalinges, Switzerland) and human rIL-7 (10 ng/ml; R&D Systems Europe, Oxon, U.K.). Cells were cultured for 2 wk before A2/Melan-A tetramer-guided β-chain variable (BV) chain usage analysis.

Tetramers

Complexes were synthesized as previously described (12, 15). Briefly, purified recombinant HLA heavy chain and β_2-microglobulin were obtained by means of a prokaryotic expression system (pET, R&D Systems, Minneapolis, MN). The heavy chain was modified by deletion of the transmembrane-cytosolic tail and COOH-terminal addition of a peptide sequence containing the BirA enzymatic biotinylation site. Heavy chain, β_2-microglobulin, and peptide were refolded by dilution. The 45-kDa refolded product was isolated by fast protein liquid chromatography and then biotinylated by recombinant BirA (Avidity, Denver, CO) in the presence of biotin, ATP, and Mg^{2+} (all from Sigma, St. Louis, MO). Tetramers were prepared by addition of PE-streptavidin and then concentrated to 1 mg/ml. As the antigenic peptide, the Melan-A_{26-35} E26A/A27L analogue (ALA GIGILTV) was used, which has a higher binding stability to HLA-A*0201, and T cell antigenicity and immunogenicity higher than those of the natural Melan-A decapeptide EAAGIGILTV or the nonapeptide AAGIGILTV (16).

Monoclonal Abs and flow cytometric analysis

A panel of 21 anti-BV Abs was used in this study (Immunotech, Beckman-Coulter, Marseilles, France). Anti-BV1, -2, -3, -5.1, -5.2, -7, -8, -11, -12, -13.1, -13.6, -14, -16, -17, -20, -21.3, and -22 mAbs were FITC conjugated, whereas anti-BV5.3, -9, -18, and -23 were used as purified mAbs. Anti-CD8 PercP was purchased from Becton Dickinson (San Jose, CA). Staining and washing were performed in PBS, 5% FCS, and 0.2% sodium azide. For indirect fluorescence labeling, cells were incubated 1) with tetramers (0.2 μg/sample in 20 μl) for 20 min at room temperature and washed, 2) with purified anti-BV mAbs for 30 min at 4°C and washed, 3) with sheep anti-mouse FITC-labeled Ab for 30 min at 4°C and washed, 4) with IgG1 and IgG2a Abs for 10 min at 4°C, and 5) with anti-CD8 PercP-labeled mAb for 30 min at 4°C. Staining with directly labeled anti-BV mAbs was similarly performed for steps 1, 2, and 5. After completing the staining scheme, the cells were washed once and analyzed immediately in a FACSCalibur (Becton Dickinson). Data analysis was performed using CellQuest software.

Results

Short term cultured TIL(N) from A2$^+$ melanoma patients frequently contain high proportions of A2/Melan-A tetramer$^+$ CD8$^+$ lymphocytes

Melan-A has been previously shown to be one of the most prevalent melanoma-associated Ags, as it is recognized by the large majority of TIL cultures (17). In a previous report we observed that short term cultured TILN from A2 melanoma patients often contain high percentages of A2/Melan-A tetramer$^+$ cells that may allow TCR repertoire analysis by flow cytometry (12). Thus, samples from a total of six TILN and six TIL were first analyzed by staining with an anti-CD8 mAb together with A2/Melan-A tetramers. We have previously established that staining of LN cell suspensions with A2/Melan-A tetramers allows the identification of Melan-A-specific cells at frequencies as low as 0.2% of the CD8$^+$ subpopulation of LN cells. This detection limit was calculated from background staining observed in a series of seven HLA-A2$^-$ LNs (12). Based on these data, percentages of CD8$^+$ A2/Melan-A tetramer$^+$ cells largely over the tetramer detection limit were found in four of six TILN and four of six TIL (Table I). These samples were selected for repertoire analysis.

The functional Ag specificity of the A2/Melan-A tetramer$^+$ population was confirmed by flow cytometry sorting, as illustrated in Fig. 1 for TILN LAU 233. Reanalysis of the CD8$^+$ A2/Melan-A tetramer$^+$ and CD8$^+$ tetramer$^-$ populations showed a high degree of purity (>96%). After overnight culture, the sorted populations were assayed for Ag-specific cytolytic activity. As shown in Fig.

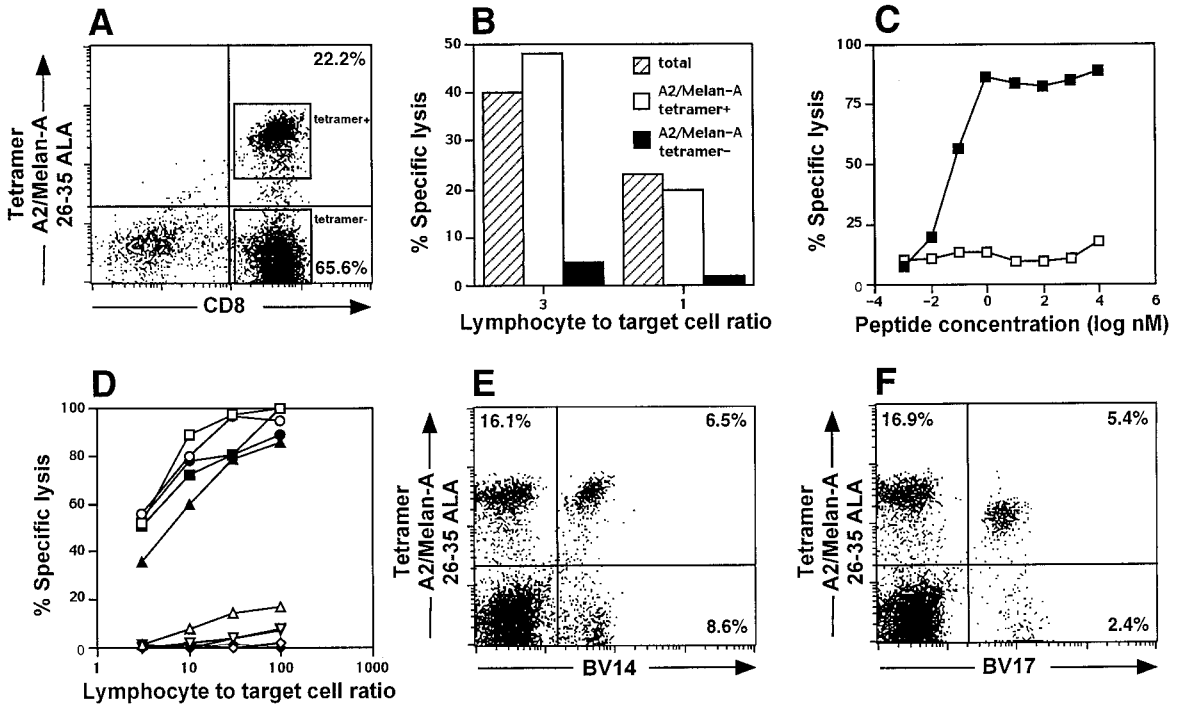

FIGURE 1. Assessment of functional reactivity and BV usage by A2/Melan-A tetramer-positive and -negative TILN from an A2$^+$ melanoma patient. Short term cultured TILN from HLA-A2$^+$ melanoma patient LAU 233 were triple stained with anti-CD8 mAbs together with anti-BV mAbs and A2/Melan-A tetramers as detailed in *Materials and Methods*. Frequencies of cells in the quadrants were calculated with CellQuest software. Results are shown for gated CD8^{bright} cells. The A2/Melan-A tetramer vs CD8 dot plot is shown for gated live lymphocytes (*A*). Selected dot plots for BV-14 (*E*) and BV-17 (*F*) are shown. CD8^{bright} lymphocytes from TILN were sterile sorted into A2/Melan-A tetramer$^+$ and tetramer$^-$ populations. After overnight culture, sorted populations were tested for their cytolytic activity against ^{51}Cr-labeled T2 cells (HLA-A2$^+$). *B*, Bars represent the difference in specific lysis obtained in the presence of peptide Melan-A$_{26-35}$ (EAAGIGILTV; 1 μM) minus the percent lysis on T2 cells without peptide. *C*, Specific cytolytic activity of tetramer$^+$ lymphocytes in response to graded concentrations of the peptide Ag (■; Melan-A$_{26-35}$; EAAGIGILTV) or to an irrelevant antigenic peptide (□; NY-ESO-1$_{157-165}$; SLLMWITQC) (29). *D*, Specific cytolytic activity of tetramer$^+$ lymphocytes on the autologous melanoma line Me 305 (□), the HLA-A2$^+$ Melan-A-expressing melanoma cell line Me 290 (○), the HLA-A2$^+$ Melan-A-negative melanoma cell line NA-8 MEL (△), or the HLA-A2$^-$ melanoma cell lines, Me 242 B1 (▼) and Me 329 M2 (◆), in the absence (open symbols) or the presence (filled symbols) of the antigenic peptide Melan-A$_{26-35}$ (1 μM).

1*B*, sorted CD8$^+$ A2/Melan-A tetramer$^+$ T cells specifically lysed peptide-loaded T2 cells even at a low E:T cell ratio, whereas no significant specific lysis was observed for the CD8$^+$ Melan-A tetramer$^-$ population. The former population displayed a potent specific lytic activity in the presence of parental Melan-A peptide concentrations as low as 100 pM, suggesting that they expressed relatively high avidity TCRs (Fig. 1*C*). In addition, CD8$^+$ A2/Melan-A tetramer$^+$ cells specifically lysed autologous tumor cells (Fig. 1*D*). Similar results were obtained for other TIL(N) populations (LAU 203 and LAU 156; data not shown). Finally, lysis of nonautologous melanoma tumor cell lines by CD8$^+$ A2/Melan-A tetramer$^+$ cells was strictly dependent on coexpression of both HLA-A2 and Melan-A (Fig. 1*D*).

A2/Melan-A tetramer-guided TCR BV region analysis of TIL(N)

The TCR BV usage of A2/Melan-A tetramer$^+$ cells present within TIL(N) samples was analyzed using a panel of 21 mAbs directed against the variable region of the TCR β-chain together with anti-CD8 mAb and A2/Melan-A tetramers. An illustration of the results obtained is shown in Fig. 1 for patient LAU 233 (BV14, Fig. 1*E*; BV17, Fig. 1*F*). It is of note that in some cases (Fig. 1*F*) the intensity of the tetramer signal detected in the tetramer$^+$ BV$^+$ fraction was lower than that of the tetramer$^+$ BV$^-$ signal, most likely as the result of steric hindrance between tetramers and some anti-BV Abs competing for neighboring binding sites. Indeed, inversion in the order of addition of reagents (i.e., staining with the tetramer before staining with the anti-BV mAb) resulted in the reciprocal pattern; that is, a lower intensity of BV signal in the tetramer$^+$ BV$^+$ fraction, but a comparable intensity of the tetramer signal in both tetramer$^+$ BV$^+$ and tetramer$^+$ BV$^-$ fractions (not shown). This phenomenon was observed for certain anti-BV mAbs, but not for others (Fig. 1*E*), probably dependent on the topology of their TCR binding sites.

A compilation of the results of BV usage analysis for TILN from four melanoma patients is illustrated in Fig. 2 for CD8$^+$ A2/Melan-A tetramer$^+$ T cells (Fig. 2*A*) and CD8$^+$ A2/Melan-A tetramer$^-$ T cells (Fig. 2*B*). The fraction of BV repertoire covered within CD8$^+$ A2/Melan-A tetramer$^+$ TILN populations by our panel of anti-BV mAb was similar in different TILN (70% for LAU 253, 74% for LAU 50, and 78% for LAU 233), with the exception of TILN LAU 203, where only 41% of the available BV repertoire was covered. Relatively small proportions of CD8$^+$ A2/Melan-A tetramer$^+$ T cells were detected that express many of the BV tested. However, high proportions of CD8$^+$ A2/Melan-A tetramer$^+$ T cells were found to express only certain BV. In particular, CD8$^+$ A2/Melan-A tetramer$^+$ T cells expressing BV14 were present in a relatively high percentage (from 9.4–33.7%) in all the samples tested. In contrast, over-representation of CD8$^+$ A2/Melan-A tetramer$^+$ T cells expressing some other BV was characteristic of single samples (i.e., BV2 and BV13.1 for patient LAU 50, BV17 for patient LAU 233, BV20 for patient LAU 203). The increased proportion of CD8$^+$ A2/Melan-A tetramer$^+$ T cells expressing a certain BV could not simply be explained by selective

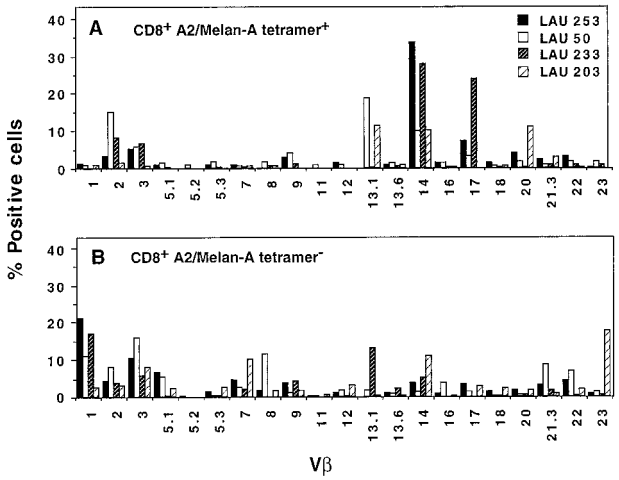

FIGURE 2. A2/Melan-A tetramer-guided TCR BV region analysis of TILN. TILN from HLA-A2$^+$ melanoma patients were stained A2/Melan-A tetramers together with anti-BV and anti-CD8 mAbs as detailed in *Materials and Methods*. Results were analyzed with CellQuest software. After gating on CD8^{bright} living lymphocytes A2/Melan-A tetramer vs BV dot plots were analyzed. Dot plots were divided into four quadrants as illustrated in Fig. 1, *E* and *F*, and the percentages of CD8$^+$ A2/Melan-A tetramer$^+$ or tetramer$^-$ cells expressing a given BV were calculated. For example in Fig. 1*E* the *upper right* quadrant contains tetramer$^+$ cells that express BV14. These cells represent 6.5% of the total CD8$^+$ population. Considering that total tetramer$^+$ cells account for 22.5% of CD8$^+$ cells in the sample, it follows that the tetramer$^+$ BV14$^+$ fraction represents 28.8% of total tetramer$^+$ cells (*A*). A similar calculation was performed for tetramer$^-$ cells (*B*).

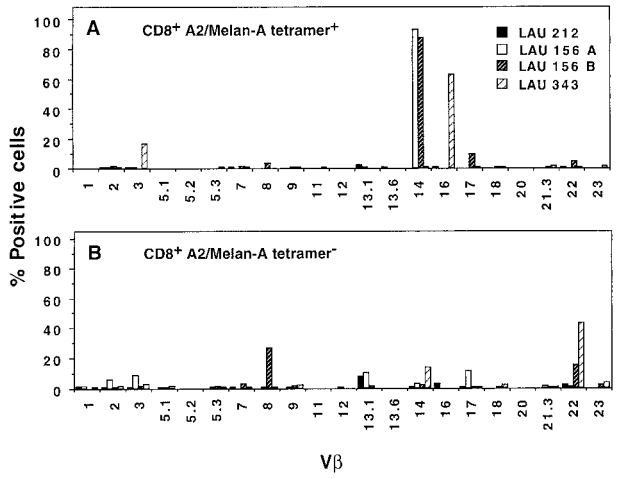

FIGURE 3. A2/Melan-A tetramer-guided TCR BV region analysis of TIL. TIL from HLA-A2$^+$ melanoma patients were stained as described in Fig. 2. Percentages of BV$^+$ A2/Melan-A tetramer$^+$ (*A*) or BV$^+$ A2/Melan-A tetramer$^-$ (*B*) cells were calculated with CellQuest software as detailed in Fig. 2. Results are shown for gated CD8^{bright} cells.

growth of cells expressing that particular BV in the culture, because a parallel expansion was often not detected in the corresponding CD8$^+$ A2/Melan-A tetramer$^-$ T cell fraction (i.e., for patient LAU 253, BV14 represent 34% of CD8$^+$ A2/Melan-A tetramer$^+$ T cells but only 3.7% of CD8$^+$ A2/Melan-A tetramer$^-$ T cells). Over-representation of T cells expressing certain BV up to 21.1% (BV1 patient LAU 253) was also detected among CD8$^+$ A2/Melan-A tetramer$^-$ cells, possibly reflecting T cell responses driven by Melan-A-unrelated tumor Ags. This hypothesis may be supported by the fact that BV usage analysis of short term cultured normal lymph node cells from two A2$^+$ melanoma patients failed to reveal expansion of CD8$^+$ T cells expressing a certain BV at levels >6% (data not shown).

A2/Melan-A tetramer-guided TCR BV region analysis of TIL

A similar analysis of BV usage by CD8$^+$ A2/Melan-A tetramer$^+$ cells was performed on four TIL from three melanoma patients (Fig. 3). In general, the BV usage by CD8$^+$ A2/Melan-A tetramer$^+$ cells present in the TIL appeared to be much more restricted than that in TILN. Remarkably, BV14$^+$ cells represented 93 and 87% of CD8$^+$ A2/Melan-A tetramer$^+$ cells in TIL LAU 156A and LAU 156B, respectively. Because these two TIL were obtained from two independent resections of the same metastatic lesion at an 8-mo interval, this could indicate the presence (and prevalence) of a unique Melan-A specific clonotype within the lesion. For TIL 343, two BVs (BV3, 17%; BV16, 63%) were over-represented. From this patient the corresponding TILN (obtained in the course of the same surgical intervention) were also available, although in very limited numbers. TILN LAU 343 contained 90% of CD8$^+$ cells, but only 0.25% of those were A2/Melan-A tetramer$^+$. However, and interestingly, 71% of the CD8$^+$ A2/Melan-A tetramer$^+$ cells were BV3 positive (not shown). Thus, the tetramer-guided analysis of BV usage in TIL allowed us to determine the BV segment used by the large majority of Melan-A-specific T cells. In striking contrast, for TIL LAU 212, we failed to detect any significant proportion of CD8$^+$ A2/Melan-A tetramer$^+$ cells expressing any of the anti-BV mAb included in our panel, thus suggesting over-representation of alternative BV segments. Over-representation of T cells expressing certain BV were also detected among CD8$^+$ A2/Melan-A tetramer$^-$ cells (i.e., BV8 for TIL LAU 156B, BV22 for TIL LAU 343), possibly in relation to T cell responses to other tumor Ags.

Analysis of BV usage of CD8$^+$ A2/Melan-A tetramer$^+$ T lymphocytes in peptide-stimulated PBMC of a melanoma patient

Several attempts to analyze the TCR repertoire selected in vitro by stimulation with Melan-A natural peptides or analogues have recently been reported (9, 18). To verify the feasibility of this approach and to directly compare the BV usage by Melan-A-specific CTL present in TILN and in peptide-stimulated PBMC from the same patient, we performed the experiment illustrated in Fig. 4. CD8$^+$ highly enriched PBMC from melanoma patient LAU 203 were stimulated with either the nonapeptide Melan-A$_{27–35}$ or the decapeptide Melan-A$_{26–35}$ in the presence of autologous APC. Two parallel cultures were set for each peptide used. The analysis of BV repertoire usage of CD8$^+$ A2/Melan-A tetramer$^+$ cells detected in the cultures at the end of a 2-wk stimulation period revealed significant expansion of CD8$^+$ A2/Melan-A tetramer$^+$ cells expressing many different BV, including the ones previously identified in the autologous TILN (BV13.1, BV14, and BV20; Fig. 2). However, although the percentages of CD8$^+$ A2/Melan-A tetramer$^+$ cells present in the different cultures were roughly constant (3.5 and 4.6% for the AAGIGILTV-stimulated cell cultures, and 4 and 4.4% for the EAAGIGILTV-stimulated cell cultures), the expansion of CD8$^+$ A2/Melan-A tetramer$^+$ cells expressing a certain BV varied considerably between parallel cultures. Similar results were obtained with PBMC from another melanoma patient (not shown).

Thus, although the tetramer-guided analysis of the BV repertoire usage of in vitro peptide-stimulated PBMC can provide useful information about the diversity of BV usage by T cells of defined Ag specificity, it does not allow quantitative evaluation of the relative contributions of single BV to the overall repertoire or the comparison between T cell repertoires elicited by stimulation with peptide

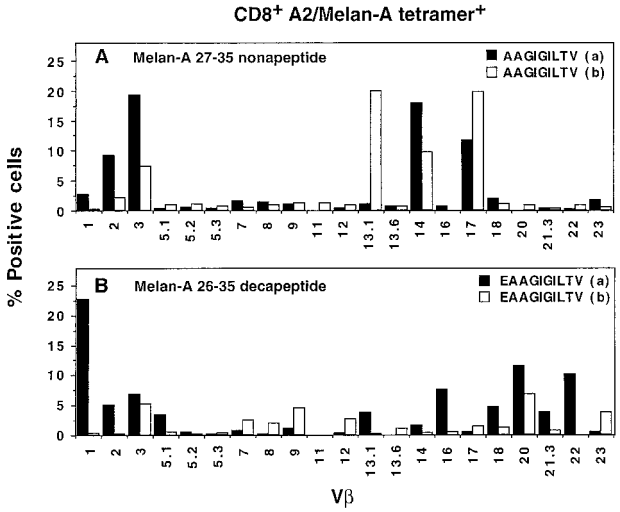

FIGURE 4. BV usage analysis of $CD8^+$ A2/Melan-A $tetramer^+$ peptide stimulated PBMC from melanoma patient LAU 203. $CD8^+$ highly enriched cells from patient LAU 203 were stimulated with peptides and autologous APC as detailed in *Materials and Methods*. Two parallel cultures (*a* and *b*) were simultaneously set for either Melan-$A_{27–35}$ nonapeptide (*A*) or the Melan-$A_{26–35}$ decapeptide (*B*). After 2 wk of culture, cells were triple stained with anti-CD8 mAb, A2/Melan-A tetramers and the listed anti-BV mAb. Numbers represent the percentage of BV^+ A2/Melan-A^+ cells on gated $CD8^{bright}$ cells calculated with CellQuest software as detailed in Fig. 2. The percentages of $CD8^+$ A2/Melan-A $tetramer^+$ cells contained in the cultures at the moment of the analysis were 3.5% (AAGIGILTV; *a*), 4.6% (AAGIGILTV; *b*), 4.0% (EAAGIGILTV; *a*), and 4.4% (EAAGIGILTV; *b*), respectively.

variants. A parallel analysis of PBMC stimulated with the A2-restricted immunodominant peptide from influenza matrix (Flu-$MA_{58–66}$) was similarly performed for three melanoma patients using tetramers containing the Flu-MA peptide. In agreement with previous studies (19, 20) reporting highly restricted BV usage by Flu-MA-specific $CD8^+$ T lymphocytes, the large majority of A2/Flu-MA $tetramer^+$ T cells expressed BV17 (81.3% for patient LAU 97, 98.9% for patient LAU 156, and 97.8% for patient LAU 241). These results further underline the large diversity of BV usage by Melan-A-specific $CD8^+$ T cells when directly compared with highly restricted BV usage by $CD8^+$ T cells specific for virally derived Ags (19–22).

Discussion

This study represents the first direct assessment of TCR BV usage by antitumor human T cells of defined Ag specificity present within melanoma lesions. Indeed, analysis of the T cell repertoire displayed by human tumor-reactive T cell populations has to date relied on the use of PCR analysis of total populations with a panel of TCR BV-specific primers (23). However, the use of this technique does not allow targeting of the analysis to T cell populations of defined Ag specificity and has led to conflicting results (6, 7, 24, 25). The recent development of A2/peptide fluorescent tetramers (12, 15) allowed us to perform a multiparameter flow cytometric analysis of CD8, anti-BV, and A2/Melan-A tetramer triple-stained populations, which enables the direct and quantitative assessment of BV usage by Melan-A-specific $CD8^+$ T cells.

Using A2/Melan-A tetramers we have recently shown that ex vivo stained metastatic LN of A2 melanoma patients can contain significant numbers of Ag-experienced Melan-A-specific T cells (12). We have also observed that the proportion of such T cells can significantly increase during the first weeks of in vitro culture in the presence of exogenously added cytokines only. In this study we found that 8 of 12 short term cultured TIL and TIL(N) samples from A2 melanoma patients contained increased proportions (from 4 to 24% of total $CD8^+$ cells; see Table I) of Melan-A-specific T cells. These results further underline the immunodominant character of anti-Melan-A-specific responses in the context of HLA-A*0201. However, it is not clear why enrichment of Melan-A-specific T cells is not always found. Interestingly, in the case of patient LAU 198 (for whom no significant amount of $CD8^+$ A2/Melan-A $tetramer^+$ T cells was observed), a melanoma cell line derived from the analyzed lesion showed loss of HLA-A2 surface expression, as assessed by staining with HLA-A2-specific Ab BB7.1 (data not shown). In addition, whether the presence of increased numbers of A2/Melan-A $tetramer^+$ cells in ex vivo or short term cultured TIL(N) samples of A2 melanoma patients may represent an independent prognosis indicator for overall survival remains to be established.

In the present study we have used A2/Melan-A tetramers containing the Melan-$A_{26–35}$-enhanced analogue E26A/A27L (ALAGIGILTV). In a previous study we showed the interchangeability of A2/Melan-A tetramers containing both the parental Melan-$A_{26–35}$ (EAAGIGILTV) peptide or the A27L (ELAGIGILTV) modified analogue for the staining of CTL-specific monoclonal populations (12). We have also extensively shown that A2/Melan-A A27L $tetramer^+$ sorted populations from both TILN and TIL and peptide-stimulated PBL were functionally extremely active in killing both peptide-pulsed cells and autologous tumor cells. In contrast, no specific lysis was detected for A2/Melan-A A27L $tetramer^-$ populations (12–14). Similar results were obtained for the E26A/A27L peptide analogue (not shown). In addition, in our experience both polyclonal and monoclonal Melan-A-specific populations obtained from TILN or TIL or from PBL stimulated with parental Melan-$A_{26–35}$ peptide, cross-recognized the A27L analogue with a similar or high avidity compared with the parental sequence (16). Overall, these data support the idea that the large majority of Melan-A-specific $CD8^+$ T cells efficiently cross-recognize peptide A27L and E26A/A27L and are efficiently stained by A2/Melan-A A27L or E26A/A27L tetramers. However, the possibility that a very minor population of Melan-A-specific cells that do not cross-react with the analogue or exhibit very low avidity recognition could be missed by this approach cannot be formally excluded.

Numerous studies have focused on the complexity of TCR usage by T cells of defined Ag specificity (26). Responses to T cell Ags were sometimes rather diverse or, in other cases, strongly constrained (27). The human TCR repertoire expressed by CTL specific for MHC class I-restricted viral peptides has been shown to be very limited. Indeed, similar or even identical TCR α/β primary structures were found in different individuals sharing the presenting HLA allele (19–22, 28). In contrast to these observations, the analysis of CTL clones derived from a single melanoma patient and specific for an HLA-A1-restricted epitope derived from MAGE-1 protein indicated that the TCR repertoire directed against this tumor Ag was rather diverse (10). The presence of multiple shared tumor-associated Ags has led to conflicting studies of T cell variable usage in TIL cultures (6, 7, 24, 25). Overall, the knowledge concerning the repertoire of tumor-specific T cells remains very limited.

Several attempts to analyze the TCR repertoire specific for Melan-A have been made. Some studies initially reported restricted TCR V gene usage (AV2, BV7, and BV14) by Melan-A-specific T cell clones (7). However, subsequent studies failed to confirm this trend (6, 8, 9, 11). The results of the present study show that a large repertoire of Melan-A-specific $CD8^+$ T cells is available for each patient. In agreement with these structural data we have recently reported a large functional heterogeneity of Ag

recognition by Melan-A-specific CTL clones (16). Whether a correlation can be found between Melan-A-specific T cell fine specificity and BV usage remains to be established. Indeed, it would be of great interest to use the approach described here to assess the functional properties and, in particular, the tumoricidal potential of Melan-A-specific populations expressing single BV and isolated by tetramer-guided cell sorting. However, the feasibility of these studies is limited by the large number of cells required for the assessment of Ag-specific cytolytic activity. The development of new techniques based on flow cytometric analysis of single-cell cytolytic functions may allow new means of addressing this issue in the near future.

The BV repertoire was larger in PBMC and TILN compared with TIL. This could be explained if, despite the relatively high frequencies at which A2/Melan-A tetramer-positive precursors can be detected in both normal LN and PBMC of A2 melanoma patients (12, 14), only few specific circulating precursors can infiltrate tumor metastases. A2/Melan-A tetramer-positive cells expressing BV14 were over-represented in most samples analyzed, whereas over-representation of other BV was characteristic of individual samples. These results may at least partially explain the BV14 restriction previously reported for Melan-A-specific CTL (7) and may reflect a relatively high frequency of BV14-bearing cells in circulating Melan-A-specific CTL precursors. Alternatively, it may be related to the high affinity/avidity of Ag recognition by BV14-bearing CTL. These issues are currently under investigation.

In the present study the analysis of the T cell repertoire of Melan-A-specific CTL was based on the use of a panel of anti-BV Abs. This approach constitutes only a first level of analysis of the complexity of Ag-specific T cell responses represented by the differential use of the BV, and does not reveal whether the BV-restricted response analyzed is, for example, of monoclonal or polyclonal nature or whether Ag-specific clonotypes present in the population exhibit any other common features. To analyze this second level of complexity we are currently performing molecular TCR analysis (26) of Melan-A-specific T cells isolated by tetramer-guided cell sorting. The combination of these powerful techniques will be instrumental to dissect changes in the repertoire of tumor-specific CTL responses during the course of the disease as well as in vaccinated patients.

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