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T-cell receptor usage by melanoma-specific clonal and highly oligoclonal tumor-infiltrating lymphocyte lines

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ABSTRACT Tumor-infiltrating lymphocytes (TIL) obtained from human melanomas can specifically lyse autologous tumor *in vitro* and mediate tumor regression *in vivo*. To develop more effective therapeutic reagents and to further understand the T-cell response to tumors, the diversity of T-cell receptors (TCRs) involved in melanoma antigen recognition has been studied. The TCR variable (V) genes, joining (J) segments, and N diversity regions used by five clonal lines and one highly oligoclonal, melanoma-specific, CD8$^+$ TIL line were examined utilizing PCR amplification with V gene subfamily-specific primers and anchor PCR. The TIL lysed multiple allogeneic melanomas expressing matched surface major histocompatibility complex class I molecules. TCR analysis confirmed the clonal nature of the TIL lines; however, the TCR repertoire was diverse. Even among the three HLA-A2 restricted TIL (TIL 1200, TIL F2-2, and TIL 5), no common V gene usage was found. Comparison of the third complementarity-determining regions of the TCRs from the HLA-A2 restricted TIL revealed no homology. Results presented here identify T-cell clonotypes that recognize epitopes on highly prevalent, shared melanoma tumor-associated antigens presented in the context of HLA-B55, HLA-A1, and HLA-A2. These T cells and the antigens they recognize represent important components for the design of new immunotherapies for patients with advanced melanoma.

Tumor-infiltrating lymphocytes (TIL) isolated from human breast tumors (1), melanomas (2), renal cell cancers (3), pancreatic cancers (4), lymphomas (5), hepatomas (6), ovarian malignancies (7), and colon cancers (8) can be expanded *in vitro* in the presence of exogenous interleukin 2 (IL-2). TIL obtained from melanoma patients may specifically lyse autologous targets and mediate tumor regression in clinical trials (2, 9). Although TIL may contain both CD4$^+$ and CD8$^+$ T cells, clinical response appears to correlate with the presence of tumor-specific, CD8$^+$ cytolytic T cells (10, 11). TIL reactivity against tumor is mediated via T-cell receptors (TCRs), which are heterodimers consisting of α and β chains. Each chain is composed of a variable (V), a joining (J), and a constant (C) region, as well as a diversity (D) region in the β chain. TCR rearrangement, resulting in the random assembly of different V, D, and J segments into a single gene, generates a highly diverse TCR repertoire. However, the greatest TCR diversity is produced by random insertion or deletion of nucleotides at the junctions between the V and J segments for the α chain and between the V and D and the D and J segments for the β chain. The V–J and V–D–J junctions of the α and β chains, respectively, code for the putative third complementarity-determining region (CDR3), which is thought to play an important role in antigen recognition (12, 13).

The antigens recognized by CD8$^+$ T cells are short peptides, usually 8–10 amino acids long, derived by processing endogenous proteins and presented in the context of major histocompatibility complex (MHC) class I molecules (14, 15). TIL derived from melanomas may lyse autologous tumors and MHC matched allogeneic tumors, suggesting that some tumor-associated antigens (TAAs) are commonly expressed by tumors of the same histology obtained from different patients (16). The suggestion of shared TAAs has led to the speculation that T cells specifically reactive against such TAAs may express a limited TCR repertoire. Examples of restricted TCR usage have been reported in autoimmunity (17–19), allograft rejection (20), and peptide immunization (21, 22) and in the presence of parasitic (23) or viral (24, 25) infections.

Restricted usage of TCR V genes by T cells that recognize tumor antigens is controversial. In some studies, limited TCR V gene usage was found (26–28), while other reports demonstrated diverse TCR usage by TIL (29, 30). We have previously examined the TCR V gene usage in 15 melanoma-specific human TIL lines (unpublished data). Although several TCR V genes were commonly used by these TIL lines, their presence did not correlate with HLA haplotype or clinical response. The analysis of polyclonal TIL lines could not establish which TCR clonotypes mediated tumor recognition *in vitro* or *in vivo*. Therefore, the current study was undertaken to examine TCR usage in melanoma-specific clonal and highly oligoclonal TIL lines. These TIL specifically lysed multiple melanoma lines expressing matched MHC restriction elements, suggesting that shared melanoma-associated epitopes were recognized. The TCR diversity observed in the melanoma-specific TIL clones used in this study suggests that there is no restricted TCR V gene usage to a single immunodominant epitope. Alternatively, each TCR clonotype identified in this study recognizes a distinct TAA epitope.

METHODS

Generation of TIL Lines and Clones. TIL were generated from tumor biopsies of patients with metastatic melanoma treated at the Surgery Branch of the National Cancer Institute, as described (2, 31). Briefly, tissue from surgical specimens was dissociated into single cell suspensions and cultured in complete medium (CM) consisting of either RPMI-1640 medium (Biofluids, Rockville, MD) with 10% human AB

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Abbreviations: TIL, tumor-infiltrating lymphocyte(s); CTL, cytotoxic T lymphocyte; TCR, T-cell receptor; TAA, tumor-associated antigen; NK, natural killer; LAK, lymphokine-activated killer; EBV, Epstein–Barr virus; MHC, major histocompatibility complex; C, constant; D, diversity; V, variable; J, joining; CDR, complementarity-determining region; IL, interleukin; MHC, major histocompatibility complex.

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serum (BioWhittaker) or AIM V serum-free medium (GIBCO), supplemented with 10 μg of gentamicin sulfate per ml (BioWhittaker), 50 units of penicillin per ml, 146 μg of L-glutamine per ml (GIBCO), and 6000 international units of recombinant human IL-2 (rhIL-2) per ml (provided by Cetus). Growing cultures were supplemented with fresh CM every 2–3 days and cell density was maintained below 5×10^5 cells per ml. TIL 1200 was a 45-day-old bulk TIL culture used for the treatment of patient 1200 (HLA-A1, A2; B8, B44). TIL C10-1 and TIL F2-2 were isolated from microcultures (1000 T cells per well) of a tumor digest from patient 1200. TIL 5 was isolated from a microculture (4000 lymphocytes per well) of a tumor digest from patient 501 (HLA-A2, A24; B18, B35). TIL F11-21 was isolated from a microculture (1 cell per well) of bulk TIL obtained from patient 1102 (HLA-A2, A24; B55, B62). TIL A10 was isolated from a microculture (0.3 cell per well) of bulk TIL obtained from patient 537 (HLA-A1, A26; B44, B70). Tumor specificity and MHC restriction of each TIL were examined by lysis of a panel of HLA matched and mismatched melanoma lines, Epstein–Barr virus (EBV) transformed B-cell lines, Daudi, and K562 in standard 4-hr ^{51}Cr release assays (8).

RNA Isolation and cDNA Synthesis. Total cellular RNA was isolated using the guanidine isothiocyanate/acid phenol method (32) from $1–5 \times 10^6$ TIL. For PCR, first strand cDNA was synthesized from 1–5 μg of total RNA using $(dT)_{22}$ and Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) as described (33).

Generation and Screening of cDNA Libraries. cDNA libraries were generated for the TCR analysis of TIL 5 and TIL A10. First and second strand cDNA was synthesized from 2 μg of poly(A)$^+$ RNA as described (33). Double-stranded cDNA was cloned into *Eco*RI sites of λgt10, packaged *in vitro*, and plated (Packagene Lambda DNA packaging system, Promega, Madison, WI). Recombinant λ phage were screened by plaque hybridization with ^{32}P-labeled TCR C_α or C_β region probes. λ Clones containing TCR cDNAs were plaque purified three times and full-length clones were identified by PCR using λgt10 primers that flank the cloning site (Clonetech, Palo Alto, CA).

PCR Primers. V gene subfamily-specific PCR primer sequences were designed based upon alignments of all known TCR V_α and V_β gene sequences. All oligonucleotides were synthesized using an ABI 392 DNA/RNA synthesizer (Applied Biosystems). The C_α and C_β primers used for anchor PCR and the V_α and V_β sequences and specificity controls for PCR analysis are described elsewhere (refs. 34 and 35; unpublished data).§

PCR Conditions. TCR DNA fragments were amplified from cDNA using the PCR as described (36) with the following modifications. Briefly, 1% of the first strand cDNA synthesized from each TIL was subjected to amplification in a 50-μl reaction containing 1 unit of Ampli-Taq (Perkin–Elmer), 200 μM dNTP (Pharmacia), 1 μM V_α or V_β subfamily-specific primer, and 1 μM of the corresponding C_α or C_β region primer. Amplifications were performed in a Perkin–Elmer 9600 DNA thermocycler (Perkin–Elmer) using the following cycle profile: 30 cycles of 92°C denaturation for 1 min, 60°C annealing for 1 min, and 72°C extension for 2 min. PCR products were separated on 2% agarose gels along with molecular size standards. Visualization of a band of the appropriate size on an ethidium bromide-stained gel indicated the presence of that TCR subfamily.

Anchor PCR. Amplification and cloning of TCR genes were performed by anchor PCR as described (37) with a few modifications. In brief, first strand cDNA was treated with RNase H and purified over a GlassMax column (GIBCO/BRL). One-tenth of the purified cDNA was dC tailed using terminal deoxynucleotide transferase (GIBCO/BRL). The amplification reaction was performed in a 50-μl final reaction volume using 25 ng of tailed cDNA, 4 pmol of anchor primer (GIBCO/BRL), 2 pmol of either TCR C_α (34) or C_β (35) specific primers, and 0.5 unit of *Taq* DNA polymerase (Perkin–Elmer/Cetus). Amplification was performed for 35 cycles at 92°C for 60 sec, 54°C for 60 sec, and 72°C for 120 sec followed by a 15-min extension period at 72°C.

Cloning and Sequencing. The PCR products were separated on a low-melt agarose gel (GIBCO/BRL); DNA fragments were purified using the PCR DNA purification system (Promega) and cloned into the T/A vector PCR II (Invitrogen). Cloned anchor PCR products were sequenced using the dideoxynucleotide chain-termination method with T7 DNA polymerase (Sequenase 2.0, United States Biochemical) as described (38). The resultant sequences were analyzed using the Genetics Computer Group, Inc., software package (39).

RESULTS

Reactivity and Specificity of TIL Lines. Six CD8$^+$ TIL lines were generated from four patients with metastatic melanoma. Tumor specificity was determined by assaying *in vitro* lysis of a panel of melanoma cell lines (Table 1). TIL 1200, TIL 5, and TIL F2-2 were derived from HLA-A2$^+$ patients and lysed HLA-A2$^+$, but not HLA-A2$^-$, melanomas. TIL C10-1 and TIL F11-21 lysed only HLA-A1$^+$ and HLA-B55$^+$ melanoma targets, respectively. TIL A10 lysed autologous tumor and did not lyse HLA-A1$^+$ targets. Since it was not tested against allogeneic HLA-A26$^+$, HLA-B44$^+$, and HLA-B70$^+$ targets, the restriction of TIL A10 could not be defined. None of the TIL exhibited nonspecific lysis due to lymphokine-activated killer (LAK) or natural killer (NK) activity as demonstrated by lack of lysis of Daudi and K562.

Analysis of V Gene Usage by TIL Lines. TCR repertoire was examined by PCR with V gene subfamily-specific primers and by sequence analysis of cloned anchor PCR products (TIL F11-21, TIL F2-2, TIL C10-1, TIL 1200) or sequence analysis of clones from cDNA libraries (TIL A10, TIL 5). TCR V gene usage by the six TIL is shown in Table 2. TIL A10 (V_α2.2, V_β4), TIL 5 (V_α1.1, V_β7), TIL F11-21 (V_α15, V_β15), and TIL F2-2 (V_α17, V_β6) each expressed a single V_α and a single V_β indicating clonality. TIL 1200 expressed two V_α (V_α2, V_α9) and as many as six V_β (V_β4, V_β5, V_β6, V_β13, V_β14, V_β22) chains when analyzed by PCR with V gene-specific primers (data not shown). However, analysis of 25 consecutive TCR α anchor PCR clones and 13 consecutive TCR β anchor PCR clones identified only V_α9 and V_β22. Similar analyses have shown that the frequency of TCR anchor PCR products is proportional to the frequency of each clonotype in a T-cell population (30). Therefore, TIL 1200 consisted predominantly of a single T-cell clone expressing V_α9 and V_β22. Sequence analysis of anchor PCR clones from TIL C10-1 revealed that all 15 TCR β cloned anchor PCR products were V_β13. However, sequence analysis of 20 consecutive TCR α anchor PCR clones revealed two V_α genes, V_α8.2 (11 of 20 cloned anchor PCR products) and V_α14.1 (9 of 20 cloned anchor PCR products).

Analysis of D, J, and N Diversity Segment Usage by TIL Lines. The V–J and V–D–J junctional sequences are unique to each T-cell clonotype and contribute to TCR diversity. Occasionally, TCR rearrangements result in nonfunctional gene products. To determine which TCR α gene contributes a functional gene product in TIL C10-1 and to define its clonality, the V–J or the V–D–J regions of the cloned TCR genes from TIL C10-1 were sequenced (Fig. 1*A*). All 15 TIL C10-1 TCR β cloned products were comprised of V_β13/D_β1.1/J_β1.5. Both TCR α chains found in TIL C10-1 were

§Sequences of PCR primers described in this paper are available upon request.

Table 1. Specificity and reactivity of clonal and oligoclonal TIL lines

Tumor target	HLA-A locus	HLA-B locus	% lysis by TIL line[a]					
			1200[b]	F2−2[b]	5[c]	C10-1[b]	F11-21[d]	A10[e]
501	A2, A24	B18, B35	ND	ND	**52**	ND	9	1
526	A2, A3	B50, B62	**60**	**46**	**16**	0	ND	ND
624	A2, A3	B7, B14	**68**	ND	**36**	−2	14	ND
1102	A2, A24	B55, B62	**20**	**42**	ND	2	**61**	ND
1143	A2, A11	B7, B60	**67**	**25**	ND	3	ND	ND
888	A1, A24	B52, B55	2	1	−2	**43**	**57**	ND
938	A1, A24	B7, B8	8	ND	ND	**55**	10	ND
397	A1, A10	B8, B62	4	ND	−2	**42**	13	3
537	A1, A26	B44, B70	ND	ND	ND	ND	6	**21**
586	A29, A31	B8, B44	1	1	0	−2	ND	ND
K562	ND	ND	3	ND	ND	10	ND	ND
Daudi	ND	ND	11	−6	−1	−1	1	0.2
EBV 888	A1, A24	B52, B55	2	2	ND	2	7	ND
EBV 501	A2, A24	B18, B35	6	12	ND	10	7	ND

Melanoma-specific reactivity was determined by standard 4-hr ^{51}Cr release assays. Percent lysis identified in boldface type was significantly different from background lysis. Melanoma lines were derived and HLA typed as described (27). Specific lysis for each TIL line was performed twice and representative experiments are shown. ND, not done.

[a]Percent lysis is shown for an effector-to-target ratio of 40:1 except for TIL 5, which is for an effector-to-target ratio of 10:1.

[b]Derived from patient 1200 (HLA-A1, A2; B8, B44).

[c]Derived from patient 501 (HLA-A2, A24; B18, B35).

[d]Derived from patient 1102 (HLA-A2, A24; B55, B62).

[e]Derived from patient 537 (HLA-A1, A26; B44, B70).

productively rearranged and used $V_\alpha 8.2/J_\alpha 49$ (11/20) and $V_\alpha 14.1/J_\alpha 32$ (9/20). However, the amino acid translation of these TCR cDNAs indicated that only the $V_\alpha 8.2/J_\alpha 49$ transcripts can produce functional TCR α chains. While the $V_\alpha 14.1/J_\alpha 32$ cDNA can produce a full-length TCR α protein, the J region lacks the correct amino acid sequence reported for $J_\alpha 32$ and the highly conserved FGXG motif (40). This motif is highly conserved both among the human and the murine J_α segments (40). It was also found in previously described functional TCRs containing $J_\alpha 32$ and in the other TCR α gene ($V_\alpha 8.2/J_\alpha 49$) expressed by TIL C10-1, suggesting that it is essential for the structural integrity of TCR α gene products (41). Therefore the $V_\alpha 14.1/J_\alpha 32/C_\alpha$ TCR transcript in TIL C10-1 likely encoded a nonfunctional TCR α chain and the TCR α chain encoded by $V_\alpha 8.2/J_\alpha 49/C_\alpha$ was responsible for tumor recognition.

Since the CDR3 region encoded by the V–D–J and V–J junctions of the TCRs is thought to be involved in antigen recognition, the junctions from the three HLA-A2 restricted TIL (TIL F2-2, TIL 1200, and TIL 5) were compared (Fig. 1*B*). TCR V_α and J_α genes utilized by TIL F2-2, TIL 1200, and TIL 5 were $V_\alpha 17/J_\alpha 42$, $V_\alpha 9/J_\alpha 16$, and $V_\alpha 1.1/J_\alpha 49$, respectively. The TCR V_β, D_β, and J_β genes utilized by the HLA-A2 restricted TIL were V_β 6/D_β 1.1/J_β 1.5 (TIL F2-2), $V_\beta 22/D_\beta 2.1/J_\beta 2.1$ (TIL 1200), and $V_\beta 7/D_\beta 2.1/J_\beta 2.1$ (TIL 5). No restricted V gene usage or sequence homology at the N diversity regions was detected in the TCRs from the three HLA-A2 restricted TIL.

Table 2. TCR V gene usage by melanoma-specific TIL clones and oligoclonal lines

TIL line	TCR α usage	TCR β usage	HLA restriction
A10	$V_\alpha 2.2$[a]	$V_\beta 4$	ND[b]
5	$V_\alpha 1.1$[c]	$V_\beta 7$[c]	A2
F2-2	$V_\alpha 17$[d]	$V_\beta 6$[e]	A2
C10-1	$V_\alpha 8.2$,[f] $V_\alpha 14.1$[f]	$V_\beta 13$[g]	A1
1200[h]	$V_\alpha 9$[i]	$V_\beta 22$[j]	A2
F11-21	$V_\alpha 15$[k]	$V_\beta 15$[l]	B55

[a]Five of 5 TCR α genes cloned into λgt10 phage and sequenced corresponded to $V_\alpha 2.2$.

[b]Not determined. A10 lysed only autologous tumor when tested against a small panel of melanomas.

[c]Six of 6 TCR α and TCR β genes cloned into λ phage and sequenced corresponded to $V_\alpha 1.1$ and $V_\beta 7$, respectively.

[d]Nine of 9 cloned anchor PCR products analyzed corresponded to $V_\alpha 17$.

[e]Ten of 10 cloned anchor PCR products analyzed corresponded to $V_\beta 6$.

[f]Analysis of 20 consecutive, cloned anchor PCR products revealed that 11 corresponded to $V_\alpha 8.2$ and 9 corresponded to $V_\alpha 14.1$.

[g]Fifteen of 15 cloned anchor PCR products analyzed corresponded to $V_\beta 13$.

[h]PCR with family-specific primers identified two V_α genes ($V_\alpha 2$ and $V_\alpha 9$) and six V_β genes ($V_\beta 4$, $V_\beta 5$, $V_\beta 6$, $V_\beta 13$, $V_\beta 14$, and $V_\beta 22$) genes, but analysis of anchor PCR products revealed only $V_\alpha 9$ and $V_\beta 22$.

[i]Twenty-five of 25 cloned anchor PCR products analyzed by PCR and confirmed by sequencing expressed $V_\alpha 9$.

[j]Thirteen of 13 cloned anchor PCR products analyzed corresponded to $V_\beta 22$.

[k]Sixteen of 16 cloned anchor PCR products analyzed corresponded to $V_\alpha 15$.

[l]Thirteen of 13 cloned anchor PCR products analyzed corresponded to $V_\beta 15$.

DISCUSSION

In most prior studies, TCR V gene usage was determined in T cells isolated from tumor biopsies or from IL-2 expanded bulk TIL cultures (26, 27, 29, 30). Increases in the frequency of TCR V gene subfamilies were seen but the antitumor reactivity of the T cells bearing these receptors was unknown. Furthermore, analysis by PCR, Southern blotting, or immunofluorescence alone cannot distinguish between productively and nonproductively rearranged TCRs. Thus, this type of analysis is unable to determine which TCR α/β pair mediates melanoma antigen recognition. The TIL lines reported here are clonal in nature and specifically recognize human melanoma cells, indicating the TCR clonotypes identified in these clones are responsible for *in vitro* lysis of melanoma targets.

In contrast to other studies that describe restricted TCR V gene usage in melanoma TIL (26–28), evidence accumulated in this study and others (29, 30) indicates that multiple TCR V gene segments are capable of recognizing melanoma TAA. Among the HLA-A2 restricted, melanoma-specific, CTL clones that we have examined, three different clonotypes were identified ($V_\alpha 1.1/V_\beta 7$, $V_\alpha 9/V_\beta 22$, and $V_\alpha 17/V_\beta 6$).

A

	V gene	J region	C region
TIL C10-1 Vα8.2/Jα49/Cα	TACTTTTGTGCA Y F C A	GAGAATATGATGAACACCGGTAACCAGTTCTATTTTGGGACAGGGACAAGTTTGACGGTCATTCCAA E N M M N T G N Q F Y F G T G T S L T V I P N	ATATCCAGAACCCTGAC I Q N P D
TIL C10-1 Vα14.1/Jα32/Cα	ATGTATTTCTGTGCT M Y F C A	TATAGGGGCCTTGGGGTGGTGCTACAAACAAGCTCATCTTTGGAACTGGCACTCTGCTTGCTGTCCAGCCAAGTAC Y R G L G V V L Q T S S S L E L A L C L L S S Q V H	ATATCCAGAACCCTGAC I Q N P D
TIL C10-1 Vβ13/Dβ1.1/Jβ1.5/Cβ1	TACTTCTGTGCCAGC Y F C A S	CGACCTACTATAACGGTCCCGTATAGCAATCAGCCCCAGCATTTTGGTGATGGGACTCGACTCTCCATCCTAG R P T I T V P Y S N Q P Q H F G D G T R L S I L E	AGGACCTGAACAAGGTG D L N K V

B

	V gene	J region	C region
TIL F2-2 Vα17/Jα42/Cα	ACCTACTTCTGTGCA T Y F C A	GCAAGCAAGGGAGGAAGCCAAGGAAATCTCATCTTTGGAAAAGGCACTAAACTCTCTGTTAAACCAA A S K G G S Q G N L I F G K G T K L S V K P N	ATATCCAGAACCCTGAC I Q N P D
TIL 1200 Vα9/Jα16/Cα	ATGTATTACTGTGCT M Y Y C A	CTAATCCCAGGAGGCCAGAAGCTGCTCTTTGCAAGGGGGACCATGTTAAAGGTGGATCTTA L I P G G Q K L L F A R G T M L K V D L N	ATATCCAGAACCCTGAC I Q N P D
TIL 5 Vα1/Jα49/Cα	GAGTACTTCTGTGCT E Y F C A	GTGGGTGCCACCGGTAACCAGTTCTATTTTGGGACAGGGACAAGTTTGACGGTCATTCCAA V G A T G N Q F Y F G T G T S L T V I P N	ATATCCAGAACCCTGAC I Q N P D
TIL F2-2 Vβ6/Dβ1.1/Jβ1.5/Cβ1	ATGTATCTCTGTGCCAGCAGC M Y L C A S S	TTAGTAGTCTGGGACAGGGGTGGTAATCAGCCCCAGCATTTTGGTGATGGGACTCGACTCTCCATCCTAG L V V W D R G G N Q P Q H F G D G T R L S I L E	AGGACCTGAACAAGGTG D L N K V
TIL 1200 Vβ22/Dβ2.1/Jβ2.1/Cβ2	ATGTACTTCTGTGCC M Y F C A	GCTGGGGAGACTAGCGGGGTGTCGTACAATGAGCAGTTCTTCGGGCCAGGGACACGGCTCACCGTGCTAG A G E T S G V S Y N E Q F F G P G T R L T V L E	AGGACCTGAAAAACGTG D L K N V
TIL 5 Vβ7/Dβ2.1/Jβ2.1/Cβ2	CTGTATCTCTGTGCCAGCAGC L Y L C A S S	CAAGATCTCCTGAGTTGGGATGAGCAGTTCTTCGGGCCAGGGACACGGCTCACCGTGCTAG Q D L L S W D E Q F F G P G T R L T V L E	AGGACCTGAAAAACGTG D L K N V

FIG. 1. TCR α and TCR β junctional sequences from melanoma-specific cytotoxic T lymphocyte (CTL) clones. (*A*) Clone C10-1 contains two in frame TCR α transcripts and one in frame TCR β transcript. Although the $V_\alpha14.1/J_\alpha32/C_\alpha$ transcript was in frame, the N region sequence produced a frame shift in $J_\alpha32$ resulting in the loss of the highly conserved FGXG structural motif. Splicing at an alternative site at the 3′ end of the $J_\alpha32$ segment resulted in the restoration of the reading frame in the C region. The TCR α gene using $V_\alpha8.2/J_\alpha49/C$ and the TCR β gene were productively rearranged. Boxes indicate the 3′ ends of the V genes and the 5′ ends of the C regions. The genomic part of the J regions is underlined. The N regions are unmarked. (*B*) Alignment of the TCR α and TCR β junctional sequences from three HLA-A2 restricted TIL. The amino acid sequence for each J region matches the sequence reported for other transcripts using the same J region. No DNA or amino acid sequence homology was observed in the N regions.

Alignment of junctional TCR gene sequences and polypeptide sequences from these HLA-A2 restricted clonotypes revealed no sequence homology or common structural motifs within the CDR3. A comparison of TCR clonotypes from our five CTL clones and on oligoclonal line to four other clones that have been described finds no common TCR V gene usage and no homology within the CDR3 region (ref. 28; P. Shamamian, personal communication). Therefore, we find no evidence for restricted TCR V gene usage in melanoma-specific CTL clones.

Three of the TIL analyzed in this study (TIL F2-2, TIL C10-1, TIL 1200) were isolated from a single patient. TIL F2-2 and TIL 1200 were HLA-A2 restricted, while TIL C10-1 was HLA-A1 restricted. It follows that at least two different tumor epitopes were recognized by lymphocytes within the tumor bed of this patient, one presented in the context of HLA-A1 and the other presented in the context of HLA-A2. This result is consistent with the findings of others where two CTL clones from a single patient are believed to recognize different T-cell epitopes (ref. 28; unpublished data). In addition, multiple CTL clonotypes may be derived from a single patient that recognize one or more tumor-associated epitopes presented in the context of the same restriction element. It has been postulated that at least six distinct melanoma peptides can be presented by HLA-A2 (42). Given the potential for a large number of T-cell epitopes presented on melanoma cells, it is not surprising that the TCR V gene usage in tumor-reactive CTLs would also be diverse.

Analysis of patient 1200 has provided information relevant to the development of immunotherapy-based cancer treatment. First, expansion of individual T-cell clonotypes is dependent on the culture conditions since two independent expansions of TIL from the same tumor biopsy yielded different clonotypes ($V_\alpha9/V_\beta22$ T cells in TIL 1200 and $V_\alpha8.2/V_\beta15$ and $V_\alpha17/V_\beta6$ T cells in TIL C10-1 and TIL F2-2). This result suggests that the culture conditions may influence the expansion of therapeutically relevant cells. Second, patient 1200 had a partial tumor regression following treatment with TIL 1200. Therefore, it is possible that a clonal or highly oligoclonal anti-tumor CTL population can successfully treat patients with advanced cancer. Third, the antigen recognized by TIL 1200 is expressed on most melanomas since all HLA-A2$^+$ melanomas established in the Surgery Branch, National Cancer Institute, are lysed by this TIL (data not shown). Given the broad distribution of the nominal antigen and the clinical efficacy of TIL 1200, cloning of the antigen recognized by this TIL and the selective expansion of the $V_\alpha9/V_\beta22$ clonotype may lead to more effective treatments for HLA-A2$^+$ patients with melanoma. Insertion of the genes coding for the $V_\alpha9.V_\beta22$ clonotype into alternate effector cells may be a mechanism for possible use in the treatment of patients with melanoma.

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