The Journal of Immunology

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J Immunol 2002; 169:6253-6260; ;
doi: 10.4049/jimmunol.169.11.6253
http://www.jimmunol.org/content/169/11/6253

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Dominant TCR-α Requirements for a Self Antigen Recognition in Humans

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TCR-α and -β chains are composed of somatically rearranged V, D, and J germline-encoded gene segments that confer Ag specificity. Recent crystallographic analyses revealed that TCR-α has more contacts with peptide than TCR-β, suggesting the possibility that peptide recognition predominantly relies on TCR-α. T cells specific for the self Ag Melan-A/MART-1 possess an exceptionally high precursor frequency in human histocompatibility leukocyte Ag-A2 individuals. This provided a unique situation for assessment of the structural relationship between TCR and peptide/MHC ligand at both the pre- and postimmune levels. Molecular and phenotypic analysis of many different Melan-A-specific T cell populations revealed that a structural constraint is imposed on the TCR for engagement with Melan-A peptides presented by HLA-A2, namely the highly preferential use of a particular TCRAV segment, AV2. Examination of CD8 single-positive thymocytes indicated that this preferential use in forming the Melan-A-specific TCR is mainly imposed by intrathymic positive selection. Our data demonstrate a dominant function of TCRAV2 segment in forming the TCR repertoire specific for the human self Ag Melan-A/MART-1 and support the view that Ag recognition is mediated predominantly by TCR-α. *The Journal of Immunology*, 2002, 169: 6253–6260.

Antigen-specific immune responses are both initiated and maintained by TCR recognition of Ag peptide assembled within the MHC groove. Selection of the TCR repertoire during thymic development begins with positive selection of T cells expressing receptors with low affinity for self-MHC, and negative selection of the high-affinity cells (1). Persistence in the peripheral repertoire requires homeostatic division, and this also relies on TCR recognition of self-epitopes (1). TCR-α and -β chains are composed of somatically rearranged V, D, and J germline-encoded gene segments (2), and contain three highly variable complementarity-determining regions (CDRs)[2] that confer Ag specificity. CDR1 and 2 are encoded by germline V segments, whereas CDR3 is created by somatic rearrangement and makes a greater contribution to TCR diversity (2). Crystallographic analysis of several class-I-restricted and one class-II-restricted TCRs has shown that TCR-α has more contacts with peptide than TCR-β and is presumably of greater importance in peptide recognition (3–8). Specifically, dominance of the TCRAV domain in peptide recognition has been established (4, 6). Besides, TCR-α is also important in making contacts with the α-helices of the MHC (9). In addition to structural analyses, studies of single TCR-transgenic mice and clonal TCR reconstitution systems suggest that recognition of foreign Ags is predominantly dependent on TCR-α and that multiple TCR-β-chains can create the same Ag specificity when paired with a single TCR-α during generation of the T cell repertoire (10–13).

The melanocyte differentiation Ag Melan-A/MART-1 is a self protein of unknown function expressed by melanocytes and most malignant melanoma cells, but not by other tissues (14, 15). Among the several melanoma-associated Ags so far identified, it has received particular attention because of its "immunodominance" in patients carrying the HLA-A2 allele: it is recognized by 90% of tumor-infiltrating lymphocytes from HLA-A*0201 patients. This immunodominance is subject to extremely stringent requirements (16): only two largely overlapping peptides, Melan-A 26–35 and 27–35, of intermediate affinity for HLA-A*0201 (17, 18). HLA-A2 tetramers synthesized around the Melan-A A27L peptide analog have been used to identify Melan-A-specific T cells ex vivo among circulating lymphocytes from both melanoma (19–21) and vitiligo patients (22, 23). A surprising finding was a large Melan-A-specific naive T cell pool: up to 70% of HLA-A*0201 healthy donors possess discrete amounts of tetramer-positive cells, free from signs of previous Ag encounter (23–27). The size of the circulating Melan-A T cell pool in adults is at least 100-fold larger than current estimates for naive Ag-specific lymphocyte precursors, and approaches that of epitope-specific memory CD8 T cells. Sequence homology between the Melan-A peptide and a broad range of self-derived proteins (28, 29) suggests that cross-recognition of many ligands may play an important role in positive thymic selection and generation of a large naive T cell pool. High frequencies of Melan-A-specific T cells among CD8 single-positive (SP) thymocytes from HLA-A2 individuals (27) indicate that a high thymic output makes a major contribution to this precursor frequency.

It is thus of interest to determine whether specific TCR sequence motifs are involved in Melan-A recognition and in positive thymic selection of Melan-A-specific T cells. Previous experiments, confined to TCR-β, indicated that generation of the large Melan-A-specific T cell pool was not due to selection of a particular TCR

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Received for publication August 7, 2002. Accepted for publication September 27, 2002.

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[2] Abbreviations used in this paper: CDR, complementarity-determining region; SP, single positive.

0022-1767/02/$02.00

(27, 30–33). We now demonstrate that TCR-α is predominant in recognition of this self Ag and suggest that stringent allele/epitope/TCR-α requirements constitute the molecular basis for Melan-A/MART-1 responses in humans.

Materials and Methods

Cells and tissues

Peripheral blood from seven healthy donors, seven melanoma patients, and eight vitiligo patients was collected after signed informed consent. All subjects were selected on the basis of HLA-A2 expression. Molecular typing for HLA-A genes was performed by PCR-sequence-specific primers technique. PBMC were isolated by Ficoll centrifugation (Ficoll-Paque; Amersham Pharmacia Biotech, Uppsala, Sweden). Pediatric thymus tissue from 10 children undergoing corrective cardiac surgery was obtained at Ospedale Infantile Regina Margherita (Turin, Italy) in accordance with the guidelines of the local Ethical Committee. Three specimens were HLA-A2 positive.

T cell sorting and cloning

PBMC were sorted into defined populations on a FACSVantage SE using CellQuest software (BD Biosciences, Mountain View, CA). To maximize purification efficiency, the flow speed used during cell sorting corresponded to ($\sim 2 \times 10^3$) cells per second. Purification of 1–2 $\times$ 10^3 A2/Melan-A$^+$ cells required 3–4 h sorting. Immediate reanalysis of the sorted populations revealed >98% purification efficiency. For the cloning experiments, part of sorted cells were plated at 0.3 cells/well in complete RPMI medium in the presence of irradiated PBMC (5×10^5 cells/ml), 2 μg/ml PHA, and 250 U/ml rIL-2. Proliferating clones were expanded in complete RPMI medium supplemented with 5% human serum (Euroclone, Wetherby, U.K.) and 500 U/ml rIL-2. For the functional tests, part of ex vivo tetramer-sorted T lymphocytes were expanded for 2 wk with 1 μg/ml PHA, 250 U/ml rIL-2, and 0.5×10^6/ml allogenic-irradiated PBMC to obtain a sufficient number of cells. For some experiments, CD8 cells were enriched from PBMC using CD8 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and either directly stained, or when necessary, expanded for 8 days with 1 μg/ml PHA. CD8 SP thymocytes were negatively enriched with CD4 microbeads.

T cell lines

For the generation of peptide-specific T cell lines, PBMC were monocyte-depleted by exclusion of plastic adherent cells. PBMC (10^5/well) were cultured in complete RPMI medium in the presence of 10^5 peptide-pulsed T2 cells (34) using 1–2 μg/ml of Melan-A A27L peptide. Low-dose (25 U/ml) IL-2 was added on day 5. Cells were stimulated at 3-wk intervals.

Peptides and tetramers

The following peptides were purchased from Primm (Milan, Italy): Melan-A$_{26-35}$ (EAAGIGILTV), Melan-A A27L (an analog of the Melan-A$_{26-35}$ epitope carrying a substitution of Ala for Leu at position 2 from the NH2 terminus, ELAGIGILTV) (35), influenza matrix Flu-MA$_{58-66}$ (GILGFVFTL), and MAGE-3$_{271-279}$ (FLWGPRALV). All peptides were >90% pure as indicated by analytical HPLC. Lyophilized peptides were diluted in DMSO and stored at −20°C. MHC/peptide tetramers built around the first three peptides were purchased from Beckman-Coulter (iTAg MHC tetramers; San Diego, CA). For some experiments, a mutated A2/Melan-A tetramer, containing the substitution Val for Ala at position 245 known to alter the interaction between class I and CD8 (36, 37), was used (38).

Abs and flow cytometry

PBMC were stained with tetramers-PE (0.5 μg/10^6 cells) for 15 min at 37°C; after two washes, indirect double-staining was performed on ice using anti-human CD8 mAb (OKT8, IgG2a) and FITC-labeled goat anti-mouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) as second mAb. The specificity of each tetramer was confirmed by staining CTL lines or clones specific for HLA-A2 in association with the peptide of interest (data not shown). To minimize background staining, tetramers were titrated and used at the lowest concentration that still gave a discernible population. Triple stainings with tetramer-PE, anti-CD45RA-CYC (BD Biosciences), and either anti-CD45RO-FITC (Caltag Laboratories, Burlingame, CA) or anti-CD56-FITC (Southern Biotechnology Associates), were performed to assess the naive/effector/memory phenotype of tetramer$^+$ cells. For the TCR repertoire studies, the following Abs (mAbs) were used: anti-AV2 (Beckman-Coulter), anti-AV24 (C15, IgG1), anti-AV12-FITC (Endogen, Woburn, MA), and anti-BV1, -3, -4, -5, -6, -8, -12, -13, -14, -16, -20, -21-FITC (Beckman-Coulter). Cells were analyzed on a FACSCalibur (BD Biosciences).

TCR down-regulation assay

T2 target cells were pulsed for 1.5 h with various concentrations of Melan-A A27L peptide in serum-free medium (X-VIVO 15; BioWhittaker, Walkersville, MD) at 37°C, washed four times, and then added to effector cells to give an E:T ratio of 1:5. After 3 h at 37°C, TCR internalization was measured by flow cytometry using HLA-A2 tetramers synthesized around the Melan-A A27L peptide. The use of the same reagent (A2/Melan-A tetramer) for identification of Ag-specific cells and evaluation of surface TCR expression poses a problem when data are analyzed, since tetramer staining intensity decreases due to TCR down-regulation and a cell may escape detection if its fluorescence intensity drops below the gate established to identify specific cells. Therefore, when Ag concentration is increased, a large proportion of the sample population is "lost" because of its undetectability. For this reason, the use of the percentage decrease of tetramer fluorescence intensity as the sole parameter is inappropriate. Therefore, two parameters where used to calculate the percentage of surface TCR within the tetramer$^+$ population: the percentage decrease of tetramer fluorescence intensity and the percentage of tetramer$^+$ cells still present within the gate.

Cytotoxicity assay

Sorted tetramer$^+$ cells and peptide-specific T cell lines were assayed for specific lysis against an HLA-matched melanoma cell line (501 mel; a kind gift of L. Rivoltini, Cancer Immunotherapy Unit, Istituto Nazionale Tumori, Milan, Italy) or peptide-pulsed T2 cells, respectively, in a standard 4-h chromium release assay. Briefly, 10^6 target cells were labeled with 200 μCi ^{51}Cr for either 1.5 h (melanoma cells) or 15 h (T2 cells). T2 cells were pulsed with 3 μg/ml Melan-A A27L peptide for 30 min at room temperature. T2 cells pulsed with an HLA-A*0201-restricted, irrelevant peptide (MAGE-3$_{271-279}$) were used as a negative control at the same concentration as the relevant peptide. A total of 10^3 target cells/well were mixed with appropriate amounts of effector cells to give final E:T ratios ranging between 30:1 and 2:1. After 4 h at 37°C, 25 μl supernatant was collected, seeded in Lumaplate 96 solid scintillation plates (Packard Instruments Company, Meriden, CT), and counted in a beta counter. The percentage of specific lysis was calculated as: 100 $\times$ (experimental release − spontaneous release)/(maximum release − spontaneous release). Spontaneous release was assessed by incubating target cells in the absence of effectors and maximum release was determined in the presence of 1% Nonidet P-40 detergent (BDH Biochemicals, Poole, U.K.).

Amplification and sequencing of TCR transcripts

Total RNA from 10^6 cells from each clone was extracted using EUROZOL (Euroclone). First-strand cDNA was synthesized using oligo(dT) and Moloney murine leukemia virus M-MLV Reverse Transcriptase (Invitrogen, Paisley, U.K.) in 20 μl final volume. Aliquots of the cDNA were PCR amplified in parallel with a panel of 32 AV- and 24 BV-specific oligonucleotides in conjunction with one AC- (5′-GTTGCTCCAGGCCGCG GCACTGTT-3′) and one BC-specific (5′-TGCTGACCCCACTGTCGAC CTCCTTCCCATT-3′) oligonucleotide. The AV-specific primers were taken from the literature (39, 40).

The BV-specific primers were as follows: BV1 5′-GCACAACAGTTC CCTGACTTGCAC-3′, BV2 5′-TCATCAACCATGCAAGCCTGACCT-3′, BV3 5′-GTCTCTAGAGAGAAGAAGGAGCGC-3′, BV4 5′-TTCCCA TCAGCCGCCCAAACCTAA-3′, BV5 5′-ATACTTCAGTGAGACACAG AGAAA-3′, BV6 5′-TCTCAGGTGTGATCCAAATTCGGG-3′, BV7 5′-CACCTGAATGCCCCAACAGCTCTC-3′, BV8 5′-ATTTACTTTAACA ACAACGTTCCG-3′, BV9 5′-TTCCCTGGAGCTTGGTGACTCTGC-3′, BV10 5′-CTCCAAAAACTCATCCTGTACCTT-3′, BV11 5′-TCAACA GTCTCCAGAATAAGGACG-3′, BV12 5′-TGTCACCAGACTGGGAA CCACCAC-3′, BV13 5′-CACTGCGGTGTACCCAGGATATGA-3′, BV14 5′-GTCTCTCGAAAAGAGAAGAGGAAT-3′, BV15 5′-CAGGCACAG GCTAAATTCTCCCTG-3′, BV16 5′-TGAAAGAGTCTAAACAGGAT GAGT-3′, BV17 5′-TCCTCTCACTGTGACATCGGCCCA-3′, BV18 5′-GATGAGTCAGGAATGCCAAAGGAA-3′, BV19 5′-TCAATGCCCCA AGAACGCACCCTG-3′, BV20 5′-ATCAGCTCTGAGGTGCCCCAGA AT-3′, BV21 5′-ATTCACAGTTGCCTAAGGATCGAT-3′, BV22 5′-GGGCAGAAAGTCGAGTTTCTGGTT-3′, BV23 5′-TTTTATGAAAAG ATGCAGAGCGAT-3′, and BV24 5′-AAGTCAAGTCAGGCCCCAAA GCTG-3′.

The TCRAV-PCR profile was 2 min at 94°C, 30 cycles at 94°C for 1 min, 60°C for 30 s, 72°C for 30 s followed by a 7-min extension. The TCRBV-PCR profile was 2 min at 94°C, 35 cycles at 94°C for 30 s, 55°C for 20 s, 72°C for 1 min followed by a 7-min extension. PCR amplification

products were purified using JetSorb gel extraction kit (Genomed, Bad Oeynhausen, Germany) and directly sequenced with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) using an internal AC- (5′-GTCACTGGATTTAGAGTC-3′) and BC-specific (5′-TGCTTCTGATGGCTCAA-3′) oligonucleotide according to the manufacturer's instructions. Sequences were run on an ABI 377 DNA sequencer (Applied Biosystems). The TCR nomenclature proposed by Arden et al. (41) was adopted.

Results

Strong TCR-α chain conservation in Melan-A-specific cell clones

Freshly isolated PBMC from one HLA-A2 vitiligo patient (PSA) were stained with HLA-A2 tetramers synthesized around the Melan-A A27L analog peptide (35) together with an anti-CD8 mAb and analyzed by flow cytometry. As shown in Fig. 1*A*, the A2/Melan-A tetramer$^+$ T cells constituted 0.16% of the CD8$^+$ cells. The CD8$^+$ tetramer$^+$ population was isolated by flow cytometry cell sorting. Sorted cells were too few to allow directly Ag specificity testing. They were split into two parts: one was shortly expanded in the presence of PHA for functional studies, the other immediately cloned by limiting dilution. Specificity of the sorted cells was demonstrated through peptide-specific TCR down-regulation experiments and cytotoxicity assays against an HLA-matched melanoma cell line (Fig. 1, *B* and *C*). To analyze the TCR repertoire displayed by the CD8$^+$ A2/Melan-A tetramer$^+$ T cells, RNA was extracted from 30 clones and cDNA subjected to RT-PCR using a panel of AV and BV oligonucleotides covering virtually 100% of the TCR-$\alpha\beta$ repertoire. In line with previously published data concerning the heterogeneity of the Melan-A-specific TCR-β repertoire (27, 30–33), Melan-A-specific CD8$^+$ T cells from this vitiligo patient were found to rearrange many distinct BV gene segments (Fig. 1*D*). In contrast, all clones expressed the same rearranged AV gene segment (AV2, 28 of 28 clones; the AV rearrangement of two clones could not be determined) (Fig. 1*E*). Direct sequencing with an internal AC-specific primer was performed to determine the complete TCR-α CDR3 region of the Melan-A-specific clones (Fig. 2). The AV2 segment was always productively rearranged and the CDR3 regions were all different, indicative of a large degree of polyclonality in the specific TCR repertoire. Twenty-two of 28 clones (78%) used the AV2.1 segment, while the remaining six used either AV2.2 (five cases) or AV2.3 (one case); these last six clones also used the same AJ segment (AJ48) and contained an identical CDR3α size (16 aa). Two clones (PSA.S.6 and PSA.S.26) possessed only two nucleotide differences in their CDR3 and their amino acids were identical. A total of 11 clones expressed a second rearranged AV segment; this also was productive in four cases, in line with figures reported by others (42, 43).

Circulating Melan-A-specific cells from a second HLA-A2 patient (SCO) affected by melanoma were similarly analyzed. The percentage of A2/Melan-A tetramer$^+$ T cells in this patient and the functional analysis of sorted cells are illustrated in Fig. 3. The molecular characterization of 21 clones obtained after sorting and cloning of the tetramer$^+$ cells confirmed a highly selective usage of AV2 segment in this patient, which contributed to form the expressed Melan-A-specific TCR in 20 of 21 clones (Figs. 3*D* and 4). Eight clones expressed a second rearranged AV segment and this was also productive in one case.

Dominant usage of TCRAV2 is widely conserved in the Melan-A-specific peripheral repertoire

To assess whether the selective use of this AV segment is a generalized feature of Melan-A-specific cells, PBMC from 14

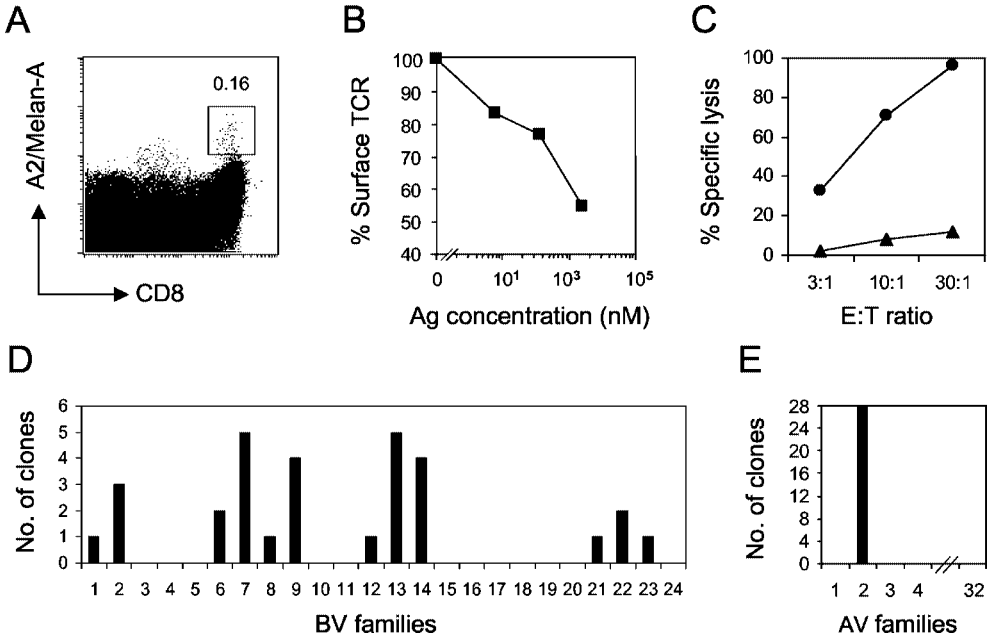

FIGURE 1. Ex vivo sorted Melan-A-specific T cells from one HLA-A2 vitiligo patient display a highly restricted TCRAV usage. *A*, Freshly isolated PBMC from one HLA-A2 vitiligo patient (PSA) were stained with A2/A27L tetramers and anti-CD8 mAb and analyzed by flow cytometry. The percentage of tetramer$^+$ cells within the CD8$^+$ population is shown. Cells within the gate were FACS sorted using a FACSVantage SE. *B*, Sorted Melan-A-specific CD8$^+$ T cells that had been shortly expanded in vitro were tested for their peptide specificity through TCR down-regulation experiments. HLA-A2 TAP-deficient T2 cells were pulsed with various concentrations of Melan-A A27L peptide and added to effector cells at a final E:T ratio of 1:5. After 3 h at 37°C, TCR internalization was measured by flow cytometry using A2/Melan-A tetramers. The percentage of surface TCR was calculated with a modified method (see *Materials and Methods*). *C*, Sorted tetramer$^+$ cells that had been expanded in vitro were assayed for specific lysis against a Melan-A-expressing, HLA-matched melanoma cell line (501 mel) in a standard 4-h chromium release assay. T2 cells pulsed with a HLA-A2 restricted, irrelevant peptide (MAGE-3$_{271–279}$) were used as negative control. ●, 501 mel; ▲, T2 + irrelevant peptide. *D* and *E*, Part of the cells were cloned by limiting dilution immediately after sorting and 30 independent clones were analyzed for their TCRBV (*D*) and TCRAV (*E*) usage by RT-PCR; the TCRAV rearrangement of two clones could not be determined.

PSA clones	AV		N	AJ	
34	2.1	CAV	KD	TPLVFG	29.1
60		CA	AP	SGNTPLVFG	29.1
33		CA	TP	GNTPLVFG	29.1
13		CAV	GY	NNDMRFG	43.1
14		CAVN		NNDMRFG	43.1
29		CAVN		GYALNFG	41.1
53		CAV	PPP	GYALNFG	41.1
27		CAV	E	GYSTLTFG	11.1
54		CAVN		SGYSTLTFG	11.1
21		CAV	SR	GFGNVLHCG	35.1
59		CAV	T	IGFGNVLHCG	35.1
55		CAV	GA	GKSTFG	27.1
38		CAVN	KG	YGNKLVFG	47.1
41		CAV	S	GNNRLAFG	7.1
28		CAV	GV	DSWGKLQFG	24.2
43		CAV	G	GSARQLTFG	22.1
45		CA	E	DSSYKLIFG	12.1
56		CA	S	NAGNMLTFG	39.1
20		CAV	T	SGGYNKLIFG	4.1
2		CAVN	G	DNYGQNFVFG	26.1
36		CA	M	GGGADGLTFG	45.1
8		CA	AGEG	NYGGSQGNLIFG	42.1
6	2.2	CAMS	L	SNFGNEKLTFG	48.1
26		CAMS	L	SNFGNEKLTFG	48.1
48		CAM	TG	SNFGNEKLTFG	48.1
50		CAM	AI	SNFGNEKLTFG	48.1
49		CAMS	V	SNFGNEKLTFG	48.1
31	2.3	CVV	SP	SNFGNEKLTFG	48.1

FIGURE 2. TCR-α chain amino acid sequences of Melan-A-specific cell clones from one HLA-A2 vitiligo patient. CDR3α junctional amino acid sequences of 28 ex vivo sorted Melan-A-specific T cell clones from this patient are shown. Amino acids either partially or completely formed by nontemplate added nucleotides (N nucleotides) are underlined. Two clones (PSA.S.6 and PSA.S.26) possessed only two nucleotide differences in their CDR3, and their amino acids were identical. These sequence data are available from GenBank/European Molecular Biology Laboratory (EMBL)/DNA Data Base in Japan (DDBJ) under accession no. AY135832-AY135859.

HLA-A2 patients (seven with vitiligo and seven with melanoma) were analyzed. PBMC were enriched in $CD8^+$ cells with magnetic beads and stained with A2/Melan-A tetramers in association with an anti-AV2 mAb either directly or, when the number of $CD8^+$ cells was too low, following short expansion in the presence of PHA. It is important to note that the mAb we used is specific for the AV2.1 segment and does not cross-react with AV2.2 or AV2.3 (data not shown). Thus, the number of AV2-expressing cells is underestimated when this reagent is used. The high percentages of AV2.1-expressing cells in the $tetramer^+$ population of all our patients (range 50–87%; mean ± SD, 67 ± 10.9%) was consistent with a highly preferential use of AV2 in forming the Melan-A-specific TCR (Fig. 5), whereas AV12- and AV24-expressing cells were always undetectable (Fig. 5*B*). No other AV families could be tested due to lack of other commercialized AV-specific Abs. We repeated the same stainings using HLA-A2 tetramers built around the natural 26–35 peptide, as well as tetramers containing a mutation known to reduce the nonspecific binding between HLA-A2 and CD8 (38). In both cases, we obtained comparable percentages of $tetramer^+$ $AV2^+$ cells (data not shown). HLA-A2 tetramers built around the Flu-MA peptide were also used in conjunction with the AV2.1 mAb to stain these samples. None of the A2/Flu-MA $tetramer^+$ cells turned out to be $AV2.1^+$, indicating that preferential usage of this AV segment is specifically relevant for recognition of the self Ag Melan-A (Fig. 5).

Consistent with previous data indicating that Melan-A-specific CTL from both melanoma and vitiligo patients frequently show signs of an in vivo Ag encounter (23, 25, 44), all patients analyzed in this part of the work contained variable proportions of A2/Melan-A $tetramer^+$ lymphocytes displaying an effector/memory

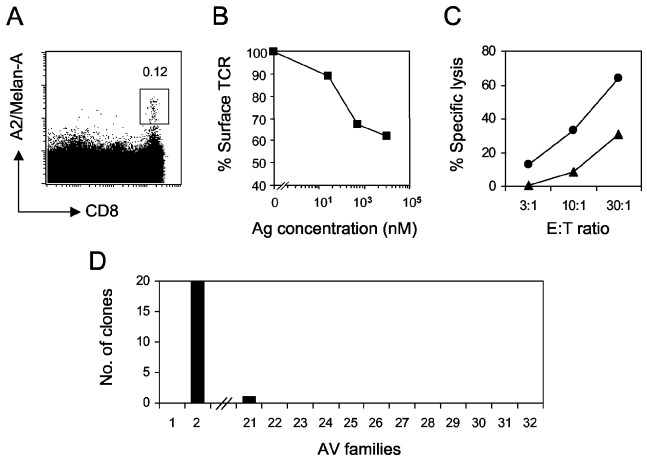

FIGURE 3. Restriction of TCRAV usage in ex vivo-sorted Melan-A-specific T cells from one HLA-A2 melanoma patient. *A*, Freshly isolated PBMC from this patient (SCO) were stained with A2/A27L tetramers and anti-CD8 mAb and analyzed by flow cytometry. The percentage of $tetramer^+$ cells within the $CD8^+$ population is shown. $Tetramer^+$ cells were FACS sorted using a FACSVantage SE. *B*, Sorted Melan-A-specific $CD8^+$ T cells that had been shortly expanded in vitro were assayed for their TCR down-regulation upon peptide encounter, as described in *Materials and Methods*. *C*, Tumor reactivity of the sorted cells that had been expanded in vitro was assessed by chromium release assay against a HLA-matched melanoma cell line (501 mel). T2 cells pulsed with a HLA-A2 restricted, irrelevant peptide were used as negative control. ●, 501 mel; ▲, T2 + irrelevant peptide. *D*, Part of the sorted cells were immediately cloned by limiting dilution after sorting and 21 independent clones were analyzed for their TCRAV usage by RT-PCR.

phenotype (between 6 and 59%; mean ± SD, 31.3 ± 17.6%, data not shown). To define whether the predominant use of AV2 by the Melan-A-specific repertoire is defined at the preimmune level or reflects a postimmune selection, we analyzed the Melan-A-specific repertoire by tetramer staining PBMC in seven HLA-A2 healthy subjects. PBMC were enriched in $CD8^+$ cells with magnetic beads

SCO clones	AV		N	AJ	
106	2.1	CAV	KH	QFYFG	49.1
64		CAV	GSA	GNQFYFG	49.1
80		CAVN	SH	TGNQFYFG	49.1
1		CAVN		TGNQFYFG	49.1
5		CAV		SGGYQKVTFG	13.1
73		CA	LLGG	GYQKVTFG	13.1
43		CA	SGG	GADGLTFG	45.1
41		CAV	GG	GGADGLTFG	45.1
74		CAV	GG	TSYGKLTFG	52.1
3		CAVN	LFG	GTSYGKLTFG	52.1
57		CAV	GSG	MRFG	43.1
25		CAVN	QG	YQLIWG	33.1
81		CAVN	Q	GGKLIFG	23.1
18		CA	AD	NARLM FG	31.1
46		CAVN	L	DGQKLLFA	16.1
95		CAV	TL	GFGNVLHCG	35.1
26		CAVN		QAGTALIFG	15.1
31		CA	DG	GATNKLIFG	32.1
38		CAVN	EQ	EYGNKLVFG	47.1
23		CAV	PDQ	GAGSYQLTFG	28.1
126	21.1	CAA	I	NYGGSQGNLIFG	42.1

FIGURE 4. TCR-α chain amino acid sequences of Melan-A-specific cell clones from one HLA-A2 melanoma patient. CDR3α junctional amino acid sequences of 21 ex vivo-sorted Melan-A-specific T cell clones from this patient are shown. Amino acids either partially or completely formed by nontemplate added nucleotides (N nucleotides) are underlined. These sequence data are available from GenBank/EMBL/DDBJ under accession no. AF532837-AF532857.

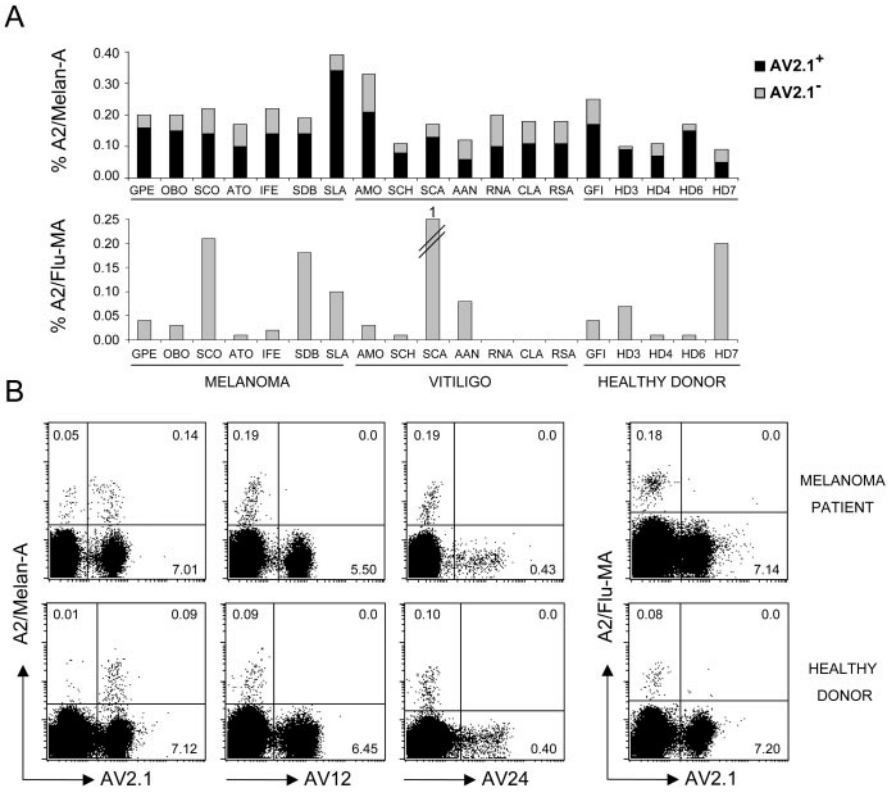

FIGURE 5. Dominant usage of TCRAV2 is a conserved feature of the Melan-A-specific peripheral repertoire. *A*, PBMC from seven HLA-A2 vitiligo patients, seven HLA-A2 melanoma patients, and seven HLA-A2 healthy donors were enriched for $CD8^+$ cells with magnetic beads and analyzed by double staining with A2/Melan-A tetramers and anti-AV2.1 mAb. Only the cases containing detectable A2/Melan-A positive populations are shown. Bars represent the total percentages of $tetramer^+$ cells within the $CD8^+$ population; ■ represent the percentages of $AV2.1^+$ cells and ▨ the percentages of $AV2.1^-$ cells within the $tetramer^+$ $CD8^+$ population. *Lower panel*, The same samples analyzed by double staining with A2/Flu-MA tetramers and anti-AV2.1 mAb. *B*, Representative double stainings of $CD8^+$ cells from one patient (SDB) and one healthy donor (HD3) with different combinations of tetramer/anti-AV mAbs.

and stained with A2/Melan-A tetramers. A detectable population was found in five cases (71%) (Fig. 5). Virtually all $tetramer^+$ cells exhibited a $CD45RA^+CD56^-$ phenotype, indicative of a naive repertoire (data not shown). These data are in agreement with previous reports of high frequencies of circulating Melan-A-specific CD8 T cells with a bona fide naive phenotype ($CD45RA^+CD45RO^-CCR7^+CD27^+CD28^+CD56^-CD57^-$) in healthy HLA-A2 individuals (23–27). Once again (Fig. 5) a large fraction of $tetramer^+$ cells, comparable to that found in the patients, expressed the AV2 segment (range 55–90%; mean ± SD, 73 ± 15.4% of the $CD8^+$ cells).

Requirement of single TCRAV and heterogeneous TCRBV segments for Melan-A recognition during expansion of Ag-specific cell lines

Next, peptide-specific cell lines were established from the PBMC of two vitiligo, two melanoma, and one healthy subjects. In all cases, enrichment of $tetramer^+$ cells upon two rounds of stimulation correlated with similar expansion rates of AV2.1 cells (Fig. 6*A* for one representative case) as well as with enhanced TCR down-regulation upon peptide engagement (Fig. 6*B*) and enhanced peptide-specific cytotoxicity (Fig. 6*C*). The percentage of AV12 cells increased only slightly or not at all during stimulation (Fig. 6*A*). At the highest expansion levels, virtually all $tetramer^+$ cells in the peptide-specific lines were $AV2.1^+$ (Fig. 6*A*), whereas their BV repertoire as assessed by the use of mAbs specific for 12 BV segments was still highly heterogeneous (Fig. 6*D*). These experiments demonstrate that AV2 has a predominant role in determining Melan-A-recognition and that the same Ag specificity can be created by pairing of a conserved TCR-α chain with multiple TCR-β chains. In addition, they rule out the possibility that A2/Melan-A tetramers cross-react with AV2-containing TCR, irrespective of their Ag specificity.

TCR-α dominates in shaping the intrathymic Melan-A-specific repertoire

The origin of the $AV2^+$, Melan-A-specific T cells, was directly investigated by tetramer staining T cells from the human thymus. High percentages of A2/Melan-A-, but not influenza-specific, cells are detected in the pool of CD8 SP, but not double-positive thymocytes (27). SP thymocytes constituted 1–5% of thymocytes that were rescued by TCR engagement and matured to express either CD4 or CD8. CD8 SP cells were enriched from the thymus of three $HLA\text{-}A2^+$ and three $HLA\text{-}A2^-$ individuals through magnetic depletion of CD4-expressing cells. Staining detected significant numbers of A2/Melan-A $tetramer^+$ cells in all three $HLA\text{-}A2^+$ cases (mean ± SD, 0.05 ± 0.006%; Fig. 7*A*), but in none of the $HLA\text{-}A2^-$ samples (data not shown). A2/Flu-MA $tetramer^+$ cells were never detected (Fig. 7*B*). Interestingly, most of the A2/Melan-A $tetramer^+$ thymocytes expressed AV2.1 (mean ± SD, 78 ± 20.2%) (Fig. 7, *A* and *C*), but neither $AV12^+$ nor $AV24^+$ (Fig. 7*C*). Altogether, these results indicate that the preferential usage of AV2 in forming the Melan-A-specific TCR is mostly imposed by intrathymic-positive selection.

Discussion

The data reported in this study show that TCR-α is dominant in formation of the TCR repertoire specific for the human self Ag Melan-A/MART-1 and support the view that Ag recognition is mediated predominantly by TCR-α. The most substantial preservation of TCR-α structure was the strongly preferential use of a particular AV segment, whereas the CDR3 regions were less conserved. In fact, crystallography of the TCR-$\alpha\beta$/peptide-MHC complex has revealed that the CDR1, CDR2, and CDR3 $\alpha\beta$ regions generally contribute to the buried surface area in the interface. However, dominance of the AV-encoded CDR1 and CDR2 domains in peptide recognition has since been established. This is

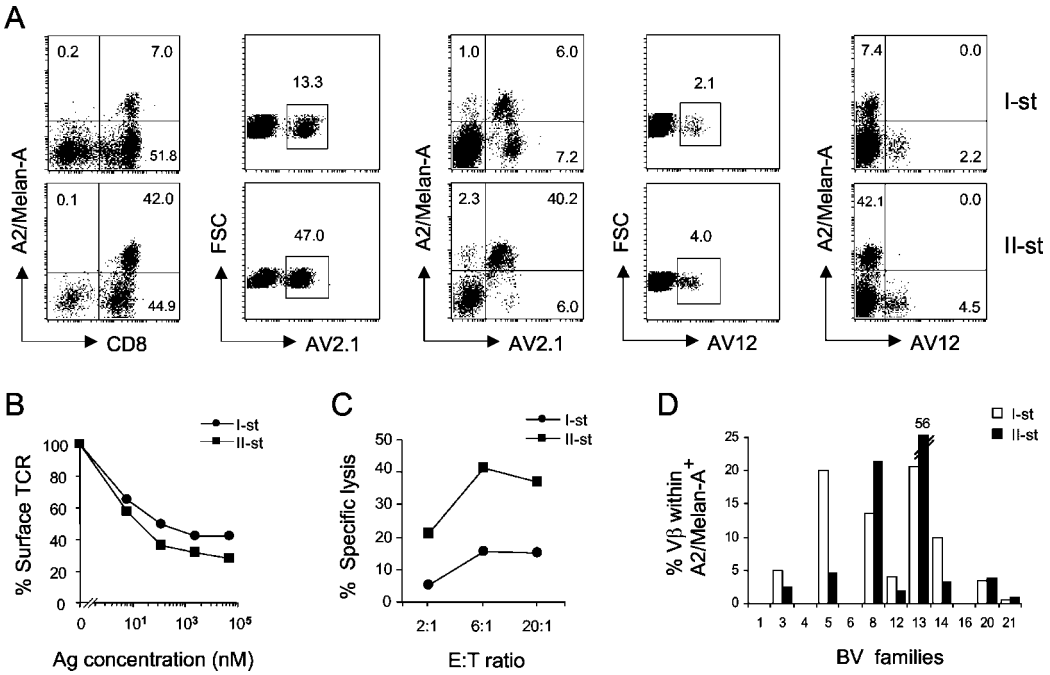

FIGURE 6. CD8 T lymphocytes selected during expansion of Melan-A-specific cell lines express TCRAV2 paired to heterogeneous TCRBV segments. *A*, PBMC from two vitiligo, two melanoma, and one healthy donors were in vitro expanded by two rounds of peptide-specific stimulations and stained with A2/Melan-A tetramers, anti-AV2.1, and anti-AV12 mAbs at each time point through either single or double stainings. The results for patient SDB are shown. I-st, first stimulation; II-st, second stimulation. *B*, Peptide specificity of the cell lines was assessed by TCR down-regulation experiments 2 wk after each stimulation, as described in *Materials and Methods*. *C*, Functional activity of the cell lines was assessed by chromium release assay against T2 cells pulsed with Melan-A A27L peptide. T2 cells pulsed with a HLA-A2 restricted, irrelevant peptide were used as negative control and nonspecific lyses were subtracted from each point. *D*, The TCRBV repertoire expressed by the Melan-A-specific cell lines was assessed with mAbs specific for 12 BV in conjunction with A2/Melan-A tetramers.

exemplified by the D10 TCR, where 23 of 27 atomic contacts with the peptide involved AV and only four involved BV (6). This dominance had not been appreciated previously, although a generalized complex orientation mediated by the AV domain had been proposed (2). This is consistent with our results concerning recognition of a human self Ag and those of Yokosuka et al. (13) regarding recognition of foreign Ags. In single TCR transgenic mice models, those authors observed that a single TCR-α chain generates the same functional Ag specificity when paired to different TCR-β chains. Analogously, when we used Melan-A peptides to in vitro stimulate PBMC from various HLA-A2 patients, we observed that AV2-expressing cells were selectively enriched, whereas TCR-β chains remained highly polyclonal.

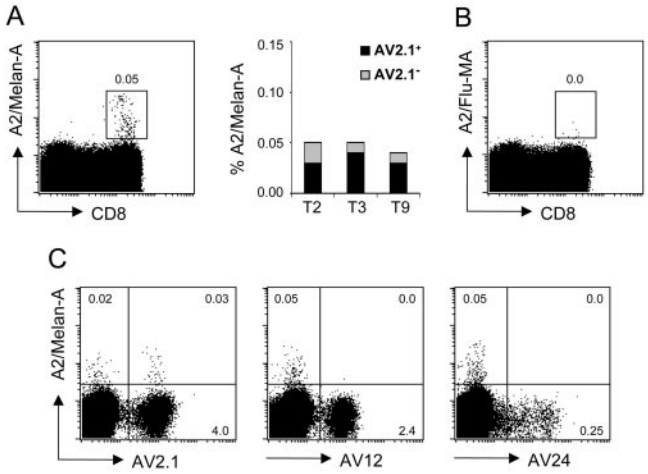

FIGURE 7. Preferential use of TCRAV2 by the Melan-A-specific CD8 T cells in thymus. *A* and *B*, CD4-depleted thymocytes from three HLA-A2 thymus specimens were stained with anti-CD8, anti-AV2.1 mAbs, and A2/Melan-A (*A*) or A2/Flu-MA (*B*) tetramers. Bars represent the total percentages of tetramer$^+$ cells; ■ represent the percentages of AV2.1$^+$ cells and ▨ the percentages of AV2.1$^-$ cells within the tetramer$^+$ population. *C*, Representative double stainings of CD8 SP thymocytes from one HLA-A2 donor with anti-AV2.1, anti-AV12, anti-AV24 mAbs, and A2/Melan-A tetramers.

The high precursor frequency of naive Melan-A-specific cells provided a unique situation for assessment of the structural relationship between TCR and peptide/MHC ligand at the preimmune level. The number of Melan-A-specific cells entering the peripheral repertoire is exceptionally high, although many self mimics can contribute to this large precursor population predominantly determined by a high thymic output (27). The composition of self-peptide ligands in the thymus is critical in shaping the preimmune T cell repertoire, but is Melan-A expressed and processed intrathymically? There are divergent opinions regarding this question. It was demonstrated that the immunoproteosome does not process the Melan-A 26–35 peptide (45), but the standard proteosome should also be expressed by thymic dendritic cells. If Melan-A peptides are processed in the thymus, as negatively selecting ligands they would narrow the structural diversity of self-specific TCR. Interestingly, the preferential use of a highly selected AV segment, as we observed for Melan-A-specific cells, has been reported as a feature of the "tolerant" repertoire (46). This raises the question of whether AV2 is one of the few segments capable of conferring Melan-A specificity to a TCR, or one of the few AV segments determining a low enough TCR affinity for Melan-A to allow positive selection. In any case, from our data the intrathymic generation of the large Melan-A-specific T cell pool appears to be favored by the selection of a particular TCR.

Preferential use of AV2 by Melan-A-specific T cells has been occasionally documented in HLA-A2 melanoma patients. Impressive dominance of this segment in forming the Melan-A-specific TCR was shown in one report where six of nine CTL clones obtained from five patients expressed TCRAV2.1 (47). However, TCRBV-restricted usage was also noted and practically all subsequent studies regarding the Melan-A-specific T cell repertoire have concentrated their attention on the TCR-β alone (30–33, 48, 49), presumably due to both technical reasons, i.e., lack of a large panel of AV-specific mAbs, and the persistent assumption that TCR-β is the main contributor to peptide recognition despite its recent rebuttal by crystallography. The only exception is one paper in which the clonally expanded Melan-A-specific TCR repertoire was monitored in a melanoma patient during immunization with melanoma-associated peptides (50); AV2-expressing cells were not detected among the clonally expanded populations. However, this patient already displayed a single monoclonal TCR-α transcript before immunization and all the repertoire studies were focused on clonally expanded cells, while in our case the AV2-expressing lymphocytes were mainly polyclonal even upon peptide-specific stimulation in vitro. A certain degree of heterogeneity among donors must also be taken in consideration. Our data suggest that AV2-containing TCRs are not the only ones involved in Melan-A recognition, and a few clones expressing other AV segments have been described (Ref. 51 and clone SCO126). Rather, it would seem more likely that AV2-containing TCRs have either a structural or a functional advantage during intrathymic-positive selection and peripheral expansion.

The immunodominance of Melan-A/MART-1 Ag in association with HLA-A2 has received particular attention from the immunotherapeutic standpoint, since this HLA allele is predominant in the North American Caucasian population and Melan-A reactivity is frequent in tumor-infiltrating lymphocytes from HLA-A2 melanoma patients. A specific allele/ligand combination, whose minimal terms are HLA-A*0201 and a single conserved glycine in position 5 shared by Melan-A peptides as well as by the large number of Melan-A epitope mimics so far described (29), has been proposed as the major contributor to this immunodominance (16). Our data now indicate that an additional structural constraint is imposed on the TCR for engagement with Melan-A peptides presented by HLA-A2. We would thus like to extend this previous observation by proposing that a unique allele/ligand/TCR-α combination, which includes TCRAV2, may be the molecular basis for Melan-A immunodominance.

In the light of our findings, the important role of TCR-α in determining Ag specificity, structurally suggested by crystallography, but functionally limited to "artificial" models, is now demonstrated for the first time in the case of a natural Ag response in humans and could represent the general molecular basis for recognition of both self and non-self Ags.

Acknowledgments

We thank Drs. Valeria Brazzelli and Giovanni Borroni (Instituto de Ricovero e Cura a Carattere Scientifico Policlinico San Matteo, Pavia, Italy); and Drs. Ausilia M. Manganoni and Graziella Carella (Spedali Civili, Brescia, Italy) for providing biological samples from melanoma and vitiligo patients; Divisione di Chirurgia Pediatrica (Ospedale Infantile Regina Margherita, Turin, Italy) for thymic biopsies; and Dr. Miriam Martinetti (IRCCS Policlinico San Matteo) for HLA-typing. We also thank Dr. Guido Forni (Department of Clinical and Biological Sciences, University of Turin, Orbassano, Italy) for careful critical reading.

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