Identification of a public CDR3 motif and a biased utilization of T-cell receptor V beta and J beta chains in HLA-A2/Melan-A-specific T-cell clonotypes of melanoma patients

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Abstract

Background: Assessment of T-cell diversity, besides giving insights about the molecular basis of tumor antigen recognition, has clinical implications since it provides criteria for evaluating antigen-specific T cells clinically relevant for spontaneous and vaccine-induced anti-tumor activity. Melan-A is one of the melanoma antigens most frequently recognized by peripheral and tumor-infiltrating lymphocytes in HLA-A2+ melanoma patients. Many clinical trials involving anti-tumor vaccination have been conducted using modified versions of this peptide.

Methods: We conducted an in-depth characterization of 210 T-cell receptor beta chain (TRB) clonotypes derived from T cells of HLA-A2+ melanoma patients displaying cytotoxic activity against natural and A27L-modified Melan-A peptides. One hundred and thirteen Melan-A-specific clonotypes from melanoma-free subjects, 199 clonotypes from T-cell clones from melanoma patients specific for melanoma antigens other than Melan-A, and 305 clonotypes derived from T cells of HLA-A2+ individuals showing unrelated specificities, were used as control. After sequence analysis, performed according to the IMGT definitions, TRBV and TRBJ usage, CDR3 length and amino acid composition were compared in the four groups of clonotypes.

Results: TRB sequences of Melan-A-specific clonotypes obtained from melanoma patients were highly heterogeneous, but displayed a preferential usage of few TRBV and TRBJ segments. Furthermore, they included a recurrent "public" amino acid motif (Glycine-Leucine-Glycine at positions 110-112-113 of the CDR3) rearranged with dominant TRBV and TRBJ segments and, in one case, associated with a full conservation of the entire TRB sequence.

Conclusion: Contrary to what observed for public anti-Melan-A T-cell receptor alpha motifs, which had been identified in several clonotypes of both melanoma patients and healthy controls, the unexpectedly high contribution of a public TRB motif in the recognition of a dominant melanoma epitope in melanoma patients may provide important information about the biology of anti-tumor T-cell responses and improve monitoring strategies of anti-tumor vaccines.
**Background**

T-cell receptor (TR) plays a central role in the immune response, interacting with peptide antigens (Ags) and with major histocompatibility complex (MHC) molecules. TR alpha (TRA) and beta subunits are comprised of a variable (V) and a constant (C) amino acidic region. The TRBV region, referred according to the IMGT database [1], is encoded by V, diversity (D), and joining (J) gene segments. The juxtaposition of these segments [2], the lack of precision during V(D)J gene rearrangement and the removal and/or addition of non-template encoded nucleotides at V(D)J junctions [3], create a region of hypervariability known as complementarity-determining region 3 (CDR3).

Despite the potentially vast T-cell repertoire, restrictions of TR composition, known as TR bias, are commonly observed [4]. These TR constraints include the preferential usage of one TRV or TRJ region without conserved CDR3, the selection of conserved amino acids (up to five) or ‘motifs’ at the same CDR3 specific positions, and the selection of clonal TR sequences with identical CDR3 [4]. The different individual responses to discrete Ags are manifested in terms of personal, or “private”, and shared, or “public”, motifs in the TR sequences [4]. A private TR repertoire describes a situation in which T cells of distinct subjects responding to the same peptide-MHC complex have no significant overlaps in their TR sequences. In contrast, TR repertoires are defined public when Ag-specific T cells in several individuals use the same TR motifs, either in the TRA or TRB chains. To date, TRA and TRB public motifs have been described in human T-cell responses directed against viral peptides [4], while, in the anti-melanoma Ag response, only public TRA motifs have been reported [5-7]. However, TRA constraints, in particular within TRAV12-1 (previously defined Vα2 or TCRAV2.1) T cells, were observed not only in melanoma patients [5-7], but also in cord blood, thymocytes and PBL of non-tumor-bearing controls [5], as well as in several subjects with vitiligo [8,9]. On the contrary, no public TRB motifs were identified in the sequences of Melan-A-specific T cells of melanoma patients and controls [5-8,10-19]. The unreported identification of public TRB in anti-melanoma Ag response may be related to the use of different methodological approaches employed to obtain T-cell lines or clones and to analyze CTL activity, as well as to prepare, characterize and analyze TR sequences. Another explanation can be the low number of patients analyzed in different studies. To bypass these limitations we took advantage, in the present study, of the availability of several published and unpublished TR sequences obtained from a number of melanoma patients in order to study different aspects of TRB chain structural constraints imposed by the melanoma Ag MART1/Melan-A (hereafter reported as Melan-A). This differentiation Ag is a membrane-embedded protein of 118 amino acids expressed both by melanocytes and melanoma cells. Among the melanoma-associated Ags identified so far, Melan-A has received particular attention because of its immune dominance in HLA-A2+ patients. A large number of T-cell clones generated from HLA-A2+ patients are cross-reactive against either the natural nonamer/decamer Melan-A peptide (26/27–38) or the Alanine-to-Leucine substituted heteroclitic Melan-A A27L peptide [20,21]. Here, we identified several melanoma/HLA-A2-restricted TRB clonotypes (sequences showing different CDR3 in a given individual), and, after the definition of a common TR nomenclature, numbering and CDR3 designation, we studied in details their molecular features.

**Methods**

The TRB sequences analyzed in this study were obtained either from previously reported or still unpublished studies. The rationale underlying selection of the 4 groups of TR sequences was to take into account three characteristics of the TR clonotypes which may generate biases in the selection of CDR3 region, i.e. Melan-A specificity, HLA-restriction and categories of individuals analyzed. Two hundred and ten Melan-A-specific clonotypes ([5,7,10-18] and manuscript in preparation), sequenced starting from T-cell lines or clones obtained from PBL and/or tumor-infiltrating lymphocytes (TIL) of melanoma patients (“Mel/M-A* group; Table 1), were compared with 113 Melan-A-specific clonotypes (“Ctrl/M-A* group) from healthy controls and from a subject with vitiligo [5,8,19], 199 clonotypes specific either for melanoma Ags other than Melan-A peptide or with undetermined specificity (“Mel/no-M-A* group) obtained from T cells of melanoma patients [22-41], and 305 clonotypes prepared from HLA-A2+ melanoma-free patients (“Ctrl/HLA-A2+/+ group) selected because sequenced from T-cell lines and clones displaying CTL activity against unrelated Ags [42-54]. One hundred and seventy clonotypes of the Mel/M-A group and 85 from the Ctrl/M-A group were specific for the HLA-A2-restricted A27L-modified Melan-A peptide and their CTL activity was evaluated using a multimer-based approach [[5,6,8,12-14,17-19], and manuscript in preparation], by competition assay [15], or by analyzing the production of IL-2 in response to HLA-A2 Melan-A-expressing melanoma cell lines [7]. The remaining 40 clonotypes derived from cells of melanoma patients displayed CTL activity against natural Melan-A peptide, as demonstrated by 51Cr release assay [10,11,16]. Twenty-eight clonotypes of the Ctrl/M-A group, although specific for Melan-A peptide, were obtained from HLA-A2-negative healthy controls. Details on type of treatment, including vaccination, the starting material (peripheral blood or TIL), the experimental procedures used to obtain T-cell lines and clones or to analyze CTL activity, as well as the methodologies for TR sequencing are specified in the references included in Table 1. Before analysis, sequences available
Table 1: Characteristics of the TR clonotypes analyzed in this study

| Clono type | seq | subjects/patients (patients ID) | HLA | vaccination | source | type of sequenced cells | specificity" | TRBV selection | references |
|------------|-----|---------------------------------|-----|-------------|--------|-------------------------|--------------|-----------------|------------|
| Mel/M-A    | 47  | 90 5 (8.22, 15, 30, 38)         | A2  | modified Melan-A | pre/post | PBL | T-cell clones | Melan-A*    | no         | in preparation |
|            | 6   | 6 1 (VER)                       | A2  | no          | PBL    | T-cell clones | Melan-A*    | no         | 5          |
|            | 26  | 27 3 (M199, M180, M138)         | A2  | no          | TIL    | T-cell clones | Melan-A*    | no         | 6          |
|            | 11  | 11 10 (Mela01, 02, 03, 04, 05, 06, 10, 13, 15, 16) | A2  | modified Melan-A CTL clones | pre | PBL | T-cell clones | Melan-A***  | no         | 7          |
|            | 2   | 17 2 (-)^a                      | A2  | no          | TIL    | CTL lines | Melan-A***  | 4, 28      | 10         |
|            | 30  | 119 3 (1, 2, 3)                 | A2  | no          | PBL/TIL | CTL lines | Melan-A***  | 7, 20, 29, 12, 5 | 11        |
|            | 18  | 54 2 (LAU 181,203)              | A2  | no          | TIL    | CD8-sorted cells | Melan-A*  | 27, 30     | 12         |
|            | 7   | 7 3 (NW28, 29, 30)              | A2  | Melan-A, Tyrosinase, gp100 | pre/post | PBL | CD8-sorted cells | Melan-A*  | no         | 13         |
|            | 27  | 50 1 (-)                        | A2  | no          | PBL/TIL | T-cell clones | Melan-A*    | no         | 14         |
|            | 9   | 10 3 (SK9-AV, M77, LB373)       | A2  | no          | PBL/TIL | T-cell clones | Melan-A*    | no         | 15         |
|            | 8   | 9 5 (8959, LB39, AV, 501, 9742) | A2  | no          | PBL/TIL | T-cell clones | Melan-A***  | no         | 16         |
|            | 12  | 27 1 (LAU444)                   | A2  | modified Melan-A | pre/post | TIL/PBL | CD8-sorted cells | Melan-A*  | 6, 28     | 17         |
|            | 7   | 17 1 (LAUI337)                  | A2  | Melan-A     | post   | PBL | T-cell clones | Melan-A*    | no         | 18         |
|            | 210 | 444                             | A2  | NA          | PBL/Thymocytes | T-cell clones | Melan-A*    | no         | 5          |
|            | 32  | 37 1 (PSA)                      | A2  | NA          | PBL    | T-cell clones | Melan-A*    | no         | 8          |
|            | 28  | 28 4 (HD001, HD002, HD010, CB886) | Various A2- |                | PBL    | CD8-sorted cells | Melan-A* | no         | 19         |
| Ctrl/M-A   | 113 | 118                             | A2, A24 & DC′ peptide-pulsed IL-7+ autologous melanoma cells | post | PBL/TIL | - | - | no | 22 |
| Mel/noM-A  | 1   | - Patient 1                     | A11, A32 | pre/post | PBL/TIL/DTH | - | - | 27 | 23 |
|            | 2   | 2 1 (FON)                       | A2, A29 | no | TIL | T-cell clones | autologous melanoma | no | 24 |
### Table 1: Characteristics of the TR clonotypes analyzed in this study (Continued)

|   |   |   | Cw16 | MNNG-treated melanoma cells | pre/post | PBL | T-cell clones | BAGE MAGE1 |   |   |
|---|---|---|------|-------------------------------|----------|-----|--------------|-----------|---|---|
| 4 | 9 | 1 (MZ2) & Cw16 | - | no | TIL/PBL/Skin | - | - | 14, 29, 23 | 26 |
| 9 | 40 | 1 (-) | B14 | no | TIL/Tissue | T-cell clones/lines/TIL | - | - | 6 | 27 |
| 52 | 87 | 4 (1, 2, 5, 6) & A2 | no | TIL, PBL, normal skin | - | - | 27, 9, 20, 29 | 28, 7 | 31 |
| 11 | 42 | 1 (2) | autologous stem cells after CTX | pre/post | PBL | - | - | 2, 3 | 32 |
| 3 | 3 | 2 (BON, MAR) & A2, A25 | no | TIL | - | - | 28, 2, 24 | 33 |
| 3 | 3 | 1 (MZ2) & A1 | autologous melanoma cells | PBL | T-cell clones | MAGE1 | no | 34 |
| 5 | 10 | 1 (9742) & A2 | no | PBL/TIL | T-cell clones, PBL-PHA | autologous melanoma cells | no | 35 |
| 19 | 38 | 1 (JB) & A1, A28 | DNP-modified melanoma cells | post | TIL | - | - | 27, 9, 20 | 36 |
| 4 | 15 | 2 (1200, 501) & A1, A2 | no | TIL | bulk/CTL microcultures | A1/A2+ melanoma cells | no | 37 |
| 22 | 172 | 3 (1622, 1464, 1214) & A24, 26; A3, A11, A24 | no | Tissue | - | - | 6, 27, 28, 24, 30 |
| 1 | 1 | 1 (0831) & A2 | no | TIL | - | - | 8 | 38 |
| 16 | 100 | 5 (1, 2, 3, 4, 6) & A2 | no | Tissue | - | - | 4, 28, 25, 29 | 39 |
| 4 | 192 | 1 (1803) & A1 | no | PBL | T-cell clones | gp100 | no | 40 |
| 199 | 957 | Ctrl/HLA-A2+ & A2 | no | TIL | bulk + cultures | - | 20 | 41 |
| 41 | 46 | 15 (BD, CL, DD, DP, HL, JE, JM, JN, JW, KD, KE, MO, MP, NM, SW) & A2 | NA | PBL | T-cell clones | M58-66 (flu) | 19 | 42 |
| 56 | 606 | 12 (PB1, PB2, PB3, PB4, RA1, RA2, RA3, RA4, RA5, RA11, RA14, RA15) & A2 | NA | PBL/SFL | T-cell clones/CD8+sorted lines | GLC/A2 (EBV) | 2, 20, 29, 9 | 43 |
Table 1: Characteristics of the TR clonotypes analyzed in this study (Continued)

| 9 | 9 | 2 (FM, JI) | PBL | T-cell lines | A2 | NA | PBL | T-cell lines | A2 | NA | PBL | T-cell lines | A2 | NA |
|---|---|------------|-----|----------------|---|----|-----|----------------|---|----|-----|----------------|---|----|-----|----------------|---|----|-----|----------------|---|----|-----|----------------|---|----|-----|----------------|
| 42 | - | 5 (B, F, M, P, T) | A2 | Sample No. | no | 44 | 42 | 5 | no | 44 | 42 | 5 | no | 44 |
| 79 | - | 9 (D, F, H, K, M, N, P, R, S) | A2 | Sample No. | no | 45 | 79 | 9 | no | 45 | 79 | 9 | no | 45 |
| 33 | 43 | 4 (BMT, HD, RA, KT) | A2 | Sample No. | no | 46 | 33 | 43 | no | 46 | 33 | 43 | no | 46 |
| 5 | 92 | 3 (003, 065, 868) | A2 | Sample No. | no | 47 | 5 | 92 | no | 47 |
| 1 | 7 | 1 (HEU) | TIL | T-cell clones | lung cancer antigen alpha-actinin-4 | no | 48 | 1 | 7 | 1 (HEU) | TIL | T-cell clones | lung cancer antigen alpha-actinin-4 | no | 48 |
| 3 | 31 | 1 (HEU) | TIL/PBL | T-cell clones | mHag HA-2 | no | 49 | 3 | 31 | 1 (HEU) | TIL/PBL | T-cell clones | mHag HA-2 | no | 49 |
| 14 | 28 | 2 (-, 5H13) | PBL | T-cell clones | 19-kDa M. tuberculosis | no | 50 | 14 | 28 | 2 (-, 5H13) | PBL | T-cell clones | 19-kDa M. tuberculosis | no | 50 |
| 9 | 15 | 1 (2) | PBL | T-cell clones | MAGE-10 | no | 51 | 9 | 15 | 1 (2) | PBL | T-cell clones | MAGE-10 | no | 51 |
| 6 | 9 | 1 (-) | TIL | T-cell clones | Mutated malic enzyme | no | 52 | 6 | 9 | 1 (-) | TIL | T-cell clones | Mutated malic enzyme | no | 52 |
| 2 | 29 | 1 (LB37) | PBL | CD8-sorted cells | TALpep | no | 53 | 2 | 29 | 1 (LB37) | PBL | CD8-sorted cells | TALpep | no | 53 |
| 5 | 24 | 2 (MS2, MS7) | PBL | T-cell culture | MS7-88 (flu) GL9 (EBV) | no | 54 | 5 | 24 | 2 (MS2, MS7) | PBL | T-cell culture | MS7-88 (flu) GL9 (EBV) | no | 54 |

* Number of sequences from which the clonotypes have been selected.

Abbreviations: CTL: Cytotoxic T Lymphocytes; CTX: Chemotherapy; DNP: Dinitrophenyl; DTH: delayed-type hypersensitivity site; ID: Identification number; MNNG: N-methyl-N'-nitro-N'-nitrosoguanidine; NA: not applicable; Seq: sequences; SFL: synovial fluid lymphocytes; TIL: Tissue infiltrating lymphocytes.

* CTL specificity against modified Melan-A analyzed by *multimers; ** competition assay; *** production of IL-2 in response to HLA-A2 Melan-A-expressing melanoma cell lines **** or CTL specificity against natural Melan-A analyzed by 51Cr release assay.

* Clonotypes identified either in pre or in post vaccination.

* : data not available.

* MAGE-4, MAGE-10, GmTV, gp100, Melan-A, FluMP, FluBNP-pulsed dendritic cells.

* Identical clonotypes are included if found in different patients.

** Bold: total number of clonotypes and of sequenced TRBV chains in each group.
only in nucleotide form were translated into their amino acid counterparts. All sequences analyzed in this study are supplied in the supplemental tables (additional file 1, 2, 3 and 4) showing, respectively, the clonotypes from Mel/M-A, Ctrl/M-A, Mel/noM-A and Ctrl/HLA-A2+ groups. In order to obtain uniformed information, TRBV gene family and CDR3 amino acid positions were named and numbered according to the IMGT indications http://imgt.cines.fr[55].

**Statistical analysis**

To analyze TRBV or TRBJ segment usage, the 95% confidence intervals of the respective proportions were calculated. "Preferentially used" were defined those segments whose lower limit of the respective 95% confidence interval was higher than the mean percentage of TRBV or TRBJ transcripts usage, obtained by arbitrarily hypothesizing a uniform distribution of all segments. When proportions were compared, Fisher’s exact test was employed, while the differences between the means of CDR3 length distributions in the four groups of clonotypes were evaluated by Kruskal-Wallis test and Dunn’s post-hoc test. Results were considered significant for p < 0.05.

**Results**

**Preferential TRBV and TRBJ usage in HLA-A2/Melan-A restricted response in melanoma patients**

We first investigated whether clonotypes identified in HLA-A2+ melanoma patients with CTL specificity against Melan-A (Mel/M-A group) had a preferential usage of par-
ticular TRBV chains and whether these preferential TRBV were also predominantly utilized in the control (Ctrl/M-A, Mel/noM-A and Ctrl/HLA-A2+ groups) clonotypes. As shown in Figure 1A, multiple transcripts covering the majority of the TRBV families were observed in the 4 groups of clonotypes, although some TRBV segments were preferentially used. In particular, while TRBV6 and TRBV27 were highly represented in all groups of clonotypes, TRBV4 was overrepresented in response to melanoma Ags but not to unrelated Ags, TRBV19 was preferentially used in clones of HLA-A2+ control individuals, and TRBV28 appeared to be preferentially selected only by Melan-A-specific CTL. TRBV usage comparison among the 4 groups suggested that the proportion of clonotypes

Figure 2
Amino acid frequency. Amino acid frequency in the entire IMGT-defined CDR3 (A) and in the position 110, 112 and 113 of the CDR3 (B) in the indicated groups of sequences.
using TRBV27 chains was higher in Mel/M-A, Ctrl/M-A and Mel/noM-A sequences compared to Ctrl/HLA-A2+ clonotypes (p = 0.03; p = 0.004; p < 0.001), while TRBV28 was significantly more frequent in Mel/M-A clonotypes than in Mel/noM-A and Ctrl/HLA-A2+ groups (p = 0.001 and p < 0.001).

Among Mel/M-A clonotypes there was a high number of clonotypes bearing the TRBJ2-1, TRBJ2-7 and TRBJ1-5 segments (Figure 1B). However, the first two TRBJ chains, however, were highly utilized also in other groups of clonotypes (Figure 1B), and had also been frequently observed among peripheral blood T-cells from healthy individuals [56].

The mean CDR3 length was highly similar (p = NS) in Mel/M-A, Ctrl/M-A and Mel/noM-A groups (mean ± SD: 12.37 ± 1.29, 12.32 ± 1.43 and 12.35 ± 1.71, respectively), but significantly lower in Ctrl/HLA-A2+ clonotypes (11.95 ± 1.50) in respect to Mel/M-A (p < 0.01) and Mel/noM-A sequences (p < 0.05). Furthermore, the majority of Mel/M-A and Ctrl/M-A CDR3 were 12 amino acid long (32.9% and 31.9% respectively), while most of CDR3 of Mel/noM-A and Ctrl/HLA-A2+ sequences were 13- and 11-amino acid-long, respectively (Figure 1C).

Collectively, the present analysis demonstrated that in melanoma patients there is a biased T-cell response to Melan-A, which is characterized by TR clonotypes using preferentially TRBV28 and TRBJ1-5 segments and containing a 12-amino acid-long CDR3.

Public motifs in Melan-A-specific clonotypes

The amino acid composition of TRB hypervariable regions of Melan-A-specific CTL from melanoma patients were subsequently analyzed in detail. Serine, Glycine, Alanine and Glutamine were by far the most frequently used residues in the IMGT-defined CDR3, and were almost equally represented in all groups of analyzed sequences (Figure 2A). However, while Alanine, Serine, and Glutamine were abundantly present because of their occurrence at the consensus sequences; in light gray: other preferentially used amino acids at the given position; in bold: amino acids belonging to N-D-N region; in the boxes: hydrophilic amino acids at position 109 and 114.
tions 105, 106, 107 and 114 in the majority of canonical TRBV and TRBJ chains. Glycine, as reported for murine [57] and human sequences [56], was clearly predominant in the region created by N-D-N recombination events. Furthermore, in the N-D-N region of Mel/M-A and Ctrl/M-A sequences there was an increased Leucine usage (Figure 2A), and Glycine and Leucine were overrepresented at CDR3 positions 110, 112 and 113 (Figure 2B). Moreover, the overall percentage of non-polar amino acids at these CDR3 positions in the clonotypes carrying 12-amino acid-long CDR3s, which were the most commonly represented among the Melan-A-specific T-cell clones, was significantly higher in the Mel/M-A group (75%) compared to Ctrl/M-A (62%, p = 0.017), Mel/M-A (52%, p < 0.001) and Ctrl/HLA-A2+ (38%, p < 0.001) groups. This indicates that non-polar amino acids may be important for Melan-A-peptide-TR interaction. Furthermore, we found a public clonotype identified in two laboratories from cells of two melanoma patients: one was sequenced in our laboratory starting from a T-cell clone (ID 16) obtained from patient 22 [manuscript in preparation], the other from a T-cell clone (ID 27) obtained in the laboratory of Trautmann et al [6] employing melanoma-infiltrating lymphocytes of patient M180 (Figure 3).

Table 2: Nucleotide composition of available N-D-N regions of public Melan-A-specific clonotypes of melanoma patients

| Clone ID   | 3' V region | N1 | P   | D region | N2 | 5' J region | TRBV | TRBJ | References |
|------------|-------------|----|-----|---------|----|-------------|------|------|------------|
| D/a        | GCCACAGTTTA... | . . . . | . . . . | . . . . | . . . . | . . . . | 28 | 1-5 | 14 |
| 30         | GCCCTGGAGTGT | . . . . | . . . . | . . . . | . . . . | . . . . | 30 | 1-2 | in preparation |
| 508.55     | GCCACAGCTT... | . . . . | . . . . | . . . . | . . . . | . . . . | 7  | 1-5 | 14 |
| 17         | GCCAGCA... | TCTGGG | . . . . | . . . . | . . . . | . . . . | 28 | 1-5 | in preparation |
| 814S1      | XXXXXXA...... | . . . . | . . . . | . . . . | . . . . | . . . . | 30 | 1-5 | 12 |
| 82899S32   | XXXXXXX..... | GGCATAT | . . . . | . . . . | . . . . | . . . . | 30 | 1-5 | 12 |
| A/5        | AGTGTAGTAT... | . . . . | . . . . | . . . . | . . . . | . . . . | 20 | 1-5 | 14 |
| 25         | GCCACGAG...... | CAAA | . . . . | . . . . | . . . . | . . . . | 28 | 1-6 | in preparation |
| 814S2      | XXXXXXXAG... | CCGAT | . . . . | . . . . | . . . . | . . . . | 30 | 1-5 | 12 |
| 82899S526  | XXXXXXXAGT... | G | . . . . | . . . . | . . . . | . . . . | 30 | 1-5 | 12 |
| 42         | GCCACAGT... | . . . . | . . . . | . . . . | . . . . | . . . . | 28 | 1-5 | in preparation |
| D/b        | GCCACAGTATAT... | . . . . | . . . . | . . . . | . . . . | . . . . | 19 | 1-5 | 14 |
| 6          | AGTGC... | GCGAT | . . . . | . . . . | . . . . | . . . . | 20 | 1-5 | in preparation |
| 4          | GCCACGAG... | ATACCA | GGGAC | . . . . | . . . . | . . . . | 28 | 1-5 | in preparation |
| 39         | GGTGGAGTGT | G | . . . . | . . . . | . . . . | . . . . | 30 | 1-5 | in preparation |
| NA         | XXXXXXXAGT... | GAT | . . . . | . . . . | . . . . | . . . . | 30 | 1-2 | 12 |
| 16         | GCCACGA... | CXTT | GACAGGG | . . . . | . . . . | . . . . | 28 | 1-5 | in preparation |
| 6E4        | GCCACAGTTT... | TCT | C | GGG | . . . . | TTGGG | 28 | 1-1 | 18 |
| 40         | GCCACAGTTTTA... | . . . . | . . . . | . . . . | . . . . | . . . . | 28 | 1-1 | in preparation |
| B/22       | GCCACGAGT... | G | . . . . | . . . . | . . . . | . . . . | 28 | 1-5 | 14 |
| 41         | GCCACGAG... | GGA | . . . . | . . . . | . . . . | . . . . | 28 | 1-5 | in preparation |
| B/9        | GCCACGAGTTT... | TCA | GGGAC | . . . . | . . . . | . . . . | 28 | 1-5 | 14 |

*Nucleotides not available
contained identical 12-amino acid-long CDR3s, created by the joining of TRBV28 and TRBJ1-5 segments and containing a Glycine-Leucine-Glycine stretch at positions 110-112-113 of the CDR3. This motif was recurrent among other sequences derived from several patients, since it was found in 27 additional clonotypes sequenced in different laboratories and obtained from 15 melanoma patients. This peculiar motif rearranged only with members of TRBJ1 cluster, because 19 out of 29 clonotypes were joined with TRBJ1-5 segments, 7 with TRBJ1-1, 2 with TRBJ1-2 and one with TRBJ1-6 (Figure 3). TRBV usage was also restricted in these clonotypes since 16 of them were TRBV28, 7 were TRBV30 and 2 were TRBV20. The recurrent motif was found in Melan-A-specific CTL isolated from PBL and from tumor sites of HLA-A2+ melanoma patients, independently of the stage of disease and of the methodological approaches used for T-cell cloning. The same motif was identified in two Melan-A-T-cell clones derived from cells of healthy donors [5,19], but not in the remaining 504 clonotypes sequenced from T-cell lines or clones with specificity for other Ags. Similarly, the Glycine-Leucine-Glycine motif at position 110-112-113 was absent in the 219 clonotypes identified analyzing 353 sequences randomly obtained from CD8+ lymphocytes of healthy subjects (data not shown). Furthermore, no common motifs were found when Melan-A-specific sequences of melanoma patients were compared using particular BV or BVBJ combinations. Of clinical relevance, the Glycine-Leucine-Glycine motif was detected in lymphocytes obtained from untreated patients, representing spontaneous anti-tumor responses, as well as from patients having undergone vaccination with the natural or modified peptides (Figure 3). Interestingly, one clonotype sequenced in our laboratory (ID 4) was detected both in samples prepared before and after the vaccination [58]. Furthermore, all but one clonotype containing the Glycine-Leucine-Glycine motif were sequenced from T-cell clones whose specificity was identified using modified Melan-A peptide-multimers. The specificity of the remaining clone for natural Melan-A peptide was established by the analysis of the ability of Melan-A-transfected COS-7 cells to stimulate IFN-γ release. This last clonotype (ID 1E2), identified by Cole et al [10], bore TRBV28 and TRBJ1-1 chains and differed only by the amino acid at position 109 (Figure 3) from ID 57, ID CTL01 and ID 6E4 clonotypes [6,7,18], which were sequenced starting from 3 melanoma patients. Furthermore, the same motif was present, at slightly different positions of the CDR3, in 7 other Melan-A-specific clonotypes [5,7,10,19], but never in non-Melan-A clonotypes. While the Glycine-Leucine-Glycine stretch is composed exclusively by non-polar or frankly hydrophobic amino acids, all the amino acids at position 114 and several of those at position 109 were hydrophilic (Figure 3). Finally, we looked for very similar sequences at the same CDR3 positions because it is conceivable that these sequences adopt equivalent structures in the recognition complex. We found a Glycine-Valine-Glycine stretch in 8 clonotypes, 5 of which were identified in melanoma patients [4,12,14,30] and manuscript in preparation] and 3 in controls [3,5].

Since previous studies focusing on the analysis of shared TR amino acid sequences in humans did not address the extent to which TRB nucleotides are shared among public amino acid stretches, we identified the N-D-N regions of the 22 available nucleotide sequences of clonotypes with Glycine-Leucine-Glycine at position 110, 112 and 113. As summarized in Table 2, all N-D-N regions were different, with the only exception of those of ID D/a and ID 30 sequences, in which, however, the Adenine at the extreme 3’V region must be ascribed to the TRBV segment in clone ID D/a and to the D region in clone ID 30. Finally, the alignment of the 22 nucleotide sequences with the TRBV, TRBJ and TRBD germine gene segments allowed us to calculate the germline contribution and the number of nucleotide deletions (the so-called “nibbling”) and additions during the VDJ recombination process. The exonucleolytic nibbling was highly heterogeneous: at 3’V end varied from 0 to 7 nucleotides, at 5’I end ranged from 4 to 9, at 3’D from 0 to 9 and at 5’D from 0 to 7. Similarly, N-addition was highly different at both sites ranging from 0 to 9 nucleotides at N1 and from 0 to 6 at N2 position. Finally, also TRBD region length is diverse since it varies from 3 to 8 nucleotides.

Discussion

T-cells recognize peptide Ags in the context of MHC molecules through their TR, and during chronic infections, autoimmunity and alloreactivity a preferential use of particular TRA or TRB regions has been observed [4]. Therefore much effort has been put into the characterization also of tumor Ag-specific TRs. Several data demonstrated a major role of TRAV than TRBV chains in TR-Ag recognition, due to the higher number of contacts of this chain with peptides [59], and, accordingly, a preferential usage of a TRAV chain has been observed in Melan-A-specific T cells from melanoma or vitiligo patients and healthy donors [5-9]. However, this has not been considered a result of TR repertoire narrowing due to affinity focusing during Ag-driven immune responses, but to reflect a structural constraint already present in the pre-immune TR repertoire [5,9]. Differently from TRAV, the TRBV repertoire of Melan-A-specific T lymphocytes appears to be large and diverse in terms of clonal composition and TRBV region usage, as multiple clonotypic transcripts, covering the majority of the TRBV families, have been identified in HLA-A2+ patients [5-7,14,17]. Conversely, other authors reported that the recognition of melanoma Ags involved the use of T lymphocytes bearing specific TRBV chains, such TRBV5, TRBV9, TRBV19, TRBV27, and TRBV28.
Indeed, Mandruzzato et al. [14, 16, 18, 23, 30, 35] have previously identified the Glycine-Leucine-Glycine stretch of the CDR3, which is surrounded by hydrophilic residues, can favor the interaction with the antigenic Melan-A peptide, which has a similar central Glycine-Isoleucine-Glycine motif, with the large non-polar side chain of the Isoleucine protruding extensively from the molecular surface [63]. The relevance of this and of other structural affinities in the two sequences, such as the potential interactions between the hydrophilic residues flanking their central positions, might be assessed with more confidence when further data on the recently crystallized TR-Melan-A-MHC complex [64] will be available, and the spatial relationships between Melan-A and CDR3 amino acids will become clearer.

Looking at the biochemical structure of the public motif identified, one may speculate that the Glycine-Leucine-Glycine stretch positioned in the central region of the CDR3, which is surrounded by hydrophilic residues, can favor the interaction with the antigenic Melan-A peptide, which has a similar central Glycine-Isoleucine-Glycine motif, with the large non-polar side chain of the Isoleucine protruding extensively from the molecular surface [63]. The relevance of this and of other structural affinities in the two sequences, such as the potential interactions between the hydrophilic residues flanking their central positions, might be assessed with more confidence when further data on the recently crystallized TR-Melan-A-MHC complex [64] will be available, and the spatial relationships between Melan-A and CDR3 amino acids will become clearer.

**Conclusion**

The finding of a conserved amino acid motif in the CDR3, together with the selective use of certain TRBJ and TRBV segments, indicates an important role of the TRB chain in fine-tuning TR affinity of Melan-A-specific T cells of melanoma patients and argues against the hypothesis that high affinity TRs against self-Ags, like Melan-A, are removed during selection in the thymus or, alternatively, by tumor-induced deletion of dominant TR clonotypes [65].

Further studies are needed to elucidate the clinical relevance of these melanoma-associated clones, which were found not only in T-cell clones isolated from PBL but also from tumor sites, thus suggesting some lymph-node homing properties of the T cells bearing the public motif. However, whatever the function of these clonotypes is, the occurrence of this public CDR3 sequence may have implications for the tracking of tumor Ag-specific T cells in different clinical settings. In particular, sensitive molecular approaches targeting TRBV28+TRBJ1-5+ cells bearing Gly-
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Additional material

Additional file 1
Supplemental table 1. Table of TRB sequences of 210 clonotypes from Melan-A-specific T-cell lines or clones obtained from HLA-A2+ melanoma patients.
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Additional file 2
Supplemental table 2. Table of TRB sequences of 113 clonotypes from Melan-A-specific T-cell clones of subjects without melanoma
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Additional file 3
Supplemental table 3. Table of TRB sequences of 199 clonotypes from T-cell lines or clones obtained from melanoma patients with variable Ag-specificity and no known Melan-A restriction.
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Additional file 4
Supplemental table 4. Table of TRB sequences of 305 clonotypes from HLA-A2+ T-cell lines or clones with specificities unrelated to melanoma.
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Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
FS, and AS have made substantial contribution in the acquisition and alignment of sequences, in the analysis and interpretation of data, and helped to draft the manuscript. LC, BP, PGN, and PN, have been involved in drafting and critically revising the manuscript. LI conceived and interpreted of data, and helped to draft the manuscript. All authors read and approved the final version of the manuscript.
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