Distinct sets of $\alpha\beta$ TCRs confer similar recognition of tumor antigen NY-ESO-1$_{157}$–165 by interacting with its central Met/Trp residues

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Naturally acquired immune responses against human cancers often include $\mathrm{CD}^8^+$ T cells specific for the cancer testis antigen NY-ESO-1. Here, we studied T cell receptor (TCR) primary structure and function of 605 HLA-A*0201/NY-ESO-1$_{157}$–165-specific CD8$^+$ T cell clones derived from five melanoma patients. We show that an important proportion of tumor-reactive T cells preferentially use TCR AV3S1/BV8S2 chains, with remarkably conserved CDR3 amino acid motifs and lengths in both chains. All remaining T cell clones belong to two additional sets expressing BV1 or BV13 TCRs, associated with $\alpha$-chains with highly diverse VJ usage, CDR3 amino acid sequence, and length. Yet, all T cell clonotypes recognize tumor antigen with similar functional avidity. Two residues, Met-160 and Trp-161, located in the middle region of the NY-ESO-1$_{157}$–165 peptide, are critical for recognition by most of the T cell clonotypes. Collectively, our data show that a large number of $\alpha\beta$ TCRs, belonging to three distinct sets (AVx/BV1, AV3/BV8, AVx/BV13) bind pMHC with equal antigen sensitivity and recognize the same peptide motif. Finally, this in-depth study of recognition of a self-antigen suggests that in part similar biophysical mechanisms shape TCR repertoires toward foreign and self-antigens.

The specificity of CD8$^+$ cytolytic T lymphocyte (CTL) responses relies on the interaction of clonotypically distributed antigen receptors (TCR) on the surface of the effector cell with small immunogenic peptide fragments displayed at the surface of a target cell by self-MHC class I molecules (pMHC) (1). Binding of the heterodimeric $\alpha\beta$-chains of the TCR to the pMHC complex is a key step leading to T cell activation and cell killing. The $\alpha\beta$ TCRs bind agonist pMHCs with relatively low affinity ($K_d$ =1–100 $\mu$M) through complementary determining regions (CDR) present on their variable domains (2). The mature TCR repertoire is shaped by both positive and negative intrathymic selection, leading to an estimated 2.5 $\times$ 10$^8$ different TCR clonotypes in the human peripheral T cell pool (3). A relatively small number of CTL precursor cells is normally selected in response to the antigenic stimulus and comprises cells bearing several TCRs ($\approx$10 to $\approx$50 clonotypes) differing from each other yet having the ability to recognize the same pMHC complex. The recent development and availability of multimerized MHC–peptide complexes combined to TCR spectratyping with high-throughput DNA sequencing allowed the study of the development of antigen-driven CD8$^+$ T lymphocyte response to chronic antigen exposure, e.g., in viral infection (CMV, EBV, HIV) or in cancer patients. The impact of TCR diversity on recognition of single antigenic pMHC complexes has been extensively investigated, and the relative contributions of each TCR $\beta$-chain have been addressed in several models. In a few systems, strong biases in the TCR repertoire selection of antigen-specific T cells, resulting in the preferential usage of particular $\alpha\beta$ TCR combinations, have been reported (4, 5) [supporting information (SI) Text].

Over the last 10 years, 24 class I and class II TCR–pMHC complexes have been crystallized and identified a substantial degree of structural variability in TCR–pMHC recognition (reviewed in ref. 6). In many cases, the TCR V$\alpha$ interactions with the pMHC seem to predominate and, thus provide some basis for a conserved diagonal orientation of the TCR on the pMHC. Importantly, all aspects of TCR binding to pMHC have direct implications for TCR function. For instance, there is a direct link between peptide–MHC affinity and the efficiency of recognition by CD8$^+$ T lymphocytes (7), as similarly, there is a close relationship between the $K_d$ of the TCR binding and the activity of the T cell in cytolytic assays (8, 9). Nevertheless, despite all of these efforts, the structural bases of TCR repertoire selection for pMHC complexes remain elusive.

The molecular recognition events taking place at the TCR–peptide–MHC interface are of great interest for medical applications. In particular, studies underlying pMHC recognition have important implications for the design of therapeutic antigen-based vaccines or of engineered TCRs. Therefore, the analysis of the primary structure of both $\alpha$- and $\beta$-chains of the TCR, especially when combined with the functional analysis of the corresponding clonal populations of T cells, can provide important insights in TCR repertoire selection in response to a given pMHC ligand (10). Here, we report a detailed and combined molecular and functional characterization of the TCR repertoire specific for the cancer testis (CT) antigen NY-ESO-1$_{157}$–165 bound to HLA-A*0201, by the analysis of large numbers of T cell clones and TCRs derived from melanoma patients with naturally occurring CD8$^+$ T cell responses.

Results

Naturally Occurring NY-ESO-1$_{157}$–165-Specific CD8$^+$ T Cell Responses Display a Preferential Usage of BV1, BV8, and BV13 TCRs. Previous studies have focused much attention on tumor antigens encoded by cancer germ-line genes, whose expression can be found in various types of tumors but not in normal adult tissues, with the exception of testis. In particular, because of its frequent expression in tumors and immunogenicity in advanced cancer patients, NY-ESO-1 is currently viewed as an ideal candidate for thera-

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peutic tumor antigen-based vaccines. Recently, we reported an unusually strong natural tumor-specific immune response against NY-ESO-1157–165 in patient LAU 50 with advanced melanoma (11), characterized by expansions of codominant T cell clonotypes bearing distinct BV1, BV8, or BV13 TCRs (12). These data prompted us to examine the proportion of BV1, BV8, and BV13 TCRs present within NY-ESO-1-specific T cells of peptide-stimulated PBMCs from four additional melanoma patients with naturally occurring NY-ESO-1-specific CTL responses (13). By using HLA-A2/NY-ESO-1157–165 multimers in combination with mAbs directed against the variable region of TCR BV1, BV8, and BV13, we found that the majority of multimer+ T cells expressed TCRs with these BV elements (Fig. S1), in line with a recent report by Le Gal and colleagues (10).

Restricted CDR3β Diversity and High Sequence Homology Among NY-ESO-1-Specific TCRs Bearing the TCR BV8 Gene Segment. Using an approach that combines flow cytometry based-cell sorting, TCR spectratyping, and sequencing at the single cell level, we recently identified, in a single patient (LAU 50), nine codominant NY-ESO-1-specific T cell clonotypes characterized by distinct BV1, BV8, or BV13 TCR sequences (12). Extensive studies of their clonal composition revealed a high degree of sequence homology of the NY-ESO-1-specific TCRs bearing the TCR BV8 gene segment (Fig. S2). These data prompted us to examine in great detail 605 NY-ESO-1-specific T cell clones generated in vitro from patients LAU 50, LAU 97, LAU 155, LAU 156, and LAU 198 (Fig. S1). Complete TCR analysis by sequencing allowed the identification of a total of 49 NY-ESO-1-specific clonotypes, classified into BV1 (12 times), BV8 (21 times), or BV13 (16 times) TCR usage (Fig. 1), including the nine characterized clonotypes from patient LAU 50 (12). A striking feature was that, with two exceptions, all BV8-expressing NY-ESO-1-specific clonotypes shared the same relatively short length of the β-chain CDR3 region (7 aa). Moreover, there was a preferential usage of either the BJ1S1 or the BJ2S1 gene segments and a marked conservation of the CDR3 amino acid composition, particularly at positions 3, 6, and 7 with prominent usage of a glycine, a glutamate, and a glutamine residue, respectively. Interestingly, the TCR analysis of BV1- and BV13-specific T cell clones yielded different results. Indeed, despite conserved BV usage, most of these clonotypes used different BJs and different CDR3β loop sequences of highly variable lengths ranging from 6 to 13 aa (Fig. 1).

BV8 T Cell Clonotypes Pair with a Highly Restricted Set of Vβ-Chains in Contrast to the BV1 and BV13 NY-ESO-1-Specific T Cell Clonotypes. To assess the full extent of conservation of the primary TCR structure of the BV8 T cell clonotypes, we next examined the TCR α-chain clonal diversity and junctional features of the 20 NY-ESO-1-specific T cell clones defined as dominant clonotypes based on their frequencies, implying that at least three individual T cell clones share the identical TCR β-chain sequence (Fig. 1). Strikingly, sequencing of the complementary TCR α-chains demonstrated the highly recurrent usage of the AV3S1 gene segment by all BV8S2 T cell clonotypes (Fig. 2). Moreover, we observed a marked conservation of the V(D)J junctional sequences and a unique CDR3α length (6 aa) within clonotypes derived from the same and distinct patients. Aside the preferential usage of the AJ31 gene segment, the dominant NY-ESO-1-specific T cell clonotypes were also characterized by their preferred selection of aspartate/leucine at CDR3α positions 1/6, respectively. In sharp contrast, the Vα repertoires associated with both BV1 and BV13 TCRs were highly heterogeneous and comprised various AJs and various CDR3α lengths ranging from 7 to 14 aa (Fig. 2).

Fig. 1. Sequence analysis of TCR junctional transcripts derived from BV1-, BV8-, and BV13 NY-ESO-1157–165 multimer+ T cell clones from five melanoma patients. Transcripts were reverse-transcribed and amplified from multimer+ T cell clones by using either pairs of BV1/BC, BV8/BC, or BV13/BC primers. PCR products were directly purified and sequenced. Conserved residues forming the CDR3 loop and preferential BJ gene element usages are highlighted in gray. The number of in vitro generated T cell clones displaying a given sequence is depicted. Of note, the NY-ESO-1-specific CD8+ T cell response seen in patients LAU 156 and LAU 198 was composed of, respectively, a single dominant BV8 and BV13 T cell clonotype, confirming the results obtained by using anti-BV antibodies and flow cytometry (Fig. S1).

Similar Efficient Tumor Cell Recognition and Killing by NY-ESO-1-Specific T Cell Clonotypes with Distinct Primary TCR Structures. To evaluate the relationship between the NY-ESO-1-specific T cell clonotypes and functional avidity for antigen recognition, 82 T cell clones generated from patient LAU 50, distributed according to their TCR BV1, BV8, or BV13 clonotypic expression, were assessed in chromium release assays for their ability to recognize graded concentrations of the native NY-ESO-1157–165 peptide pulsed to T2 cells (Fig. 3). Remarkably,
most of the BV1-, BV8-, and BV13-derived clonotypes showed similar functional avidity because 50% maximal lysis of T2 cells was found at similar peptide doses (10^{-9}–10^{-10} M). One exception was BV13 clonotype 2 that exhibited a slightly but statistically significant inferior functional avidity of antigen recognition compared with the other tested clonotypes. Lysis of targets sensitized with an analog peptide with a C to A substitution at peptide position 9 was achieved at 10^{-9} M, which corresponds to 1 log lower peptide concentrations (10^{-10}/10^{-11} M), which corresponds to the increased pMHC stability (14). The ability of the BV1, BV8, and BV13 clonotypes to specifically lyse NY-ESO-1-expressing tumor cell lines revealed that all distinct T cell clonotypes efficiently and similarly recognized the NY-ESO-1-expressing melanoma cell lines Me 275 and Me 290 (Figs. S3 and S4). Altogether, our results demonstrate that T cells bearing three distinct sets of αβ TCRs (AVx/BV18, AV3S1/ BV8S2, or AVx/BV13) shared similar avidity for antigen recognition and tumor reactivity, irrespective of their BV usage.

Both Met and Trp Residues Located in the Middle Region of the Antigenic Peptide Are Critical TCR Contact Residues Regardless of TCR Gene Segment Usage. To gain information about the specific role of each single amino acid of the NY-ESO-1 peptide in the interaction with well defined TCRs, we analyzed the cytotoxicity of 11 BV1, BV8, and BV13 molecularly defined T cell clonotypes against T2 cells loaded with a set of NY-ESO-1 peptide analogs single alanine-substituted peptide variants. The recognition patterns of each codominant clonotype are depicted in Fig. 4.4. The relative antigenic activity was calculated by using the native peptide NY-ESO-1 as reference. As described (13, 14), the peptide with the C165A replacement appeared to be the most efficient antigenic peptide analog, improving HLA-A2 binding and resulting in a marked increase in the recognition by every clonotype. All four BV8 clonotypes exhibited a more homogeneous pattern of fine specificity of recognition compared with the recognition patterns found for BV1 and BV13 T cell clones. Strikingly, several amino acid residues located in the middle region of the antigenic peptide (P3–P8) appeared critical for the recognition by BV1, BV8, or

![Fig. 2.](image_url) Functional amino acid sequence of TCR α-chains associated with each TCR β transcript derived from dominant NY-ESO-1-specific T cell clonotypes. Conserved residues forming the CDR3 loops and preferential A/J/B gene element usages are highlighted in gray. * Clonotypes recently described in ref. 10.

![Fig. 3.](image_url) Fine specificity of NY-ESO-1 antigen recognition of tumor-reactive T cell clones derived from patient LAU 50. The relative TCR functional avidity was compared by using T2 target cells (HLA-A2*/TAP-/-) pulsed with graded concentrations of either the native NY-ESO-1 peptide (SLLMWITQC) or the analog NY-ESO-1 peptide (SLLMWITQC). Complete collection of data (n = 82 clones) representing the peptide concentration (either native or analog) used to achieve 50% of maximum lysis is shown. Each data point represents the result of an individual clone. Mean ± SD values (nanomolar) are shown. *, P ≤ 0.03 with the Welch two-sample t test.

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<th>Clonotype ID</th>
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<td>5 LAU 155-clono2 BV1S1 S L A T G G D T G T Q 283 AV2S31 N K A S G N T P L 29</td>
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| 6 LAU 50-clono2 BV8S2 S L G G T E A 1S1 AV3S31 D A E A R L 31 |
| 7 LAU 50-clono3 BV8S2 S L G G N E Q 2S1 AV3S31 D G E S R L 58 |
| 8 LAU 155-clono2 BV8S2 S L G G T E A 1S1 AV3S31 D G D A R L 31 |
| 9 *LAU 50-clono1 BV8S2 Q G G T E A 2S1 AV3S31 D K N A R L 31 |
| 10 LAU 50-clono4 BV8S2 K Q G G N E Q 2S1 AV3S31 D G V G K L 23 |
| 11 *LAU 156-clono1 BV8S2 K G G G N E Q 2S1 AV3S31 D S A R L 31 |
| 12 LAU 155-clono2 BV8S2 S L G G T E A 1S1 AV3S31 D G E S R L 58 |
| 13 *LAU 155-clono1 BV8S2 N G G G N E Q 2S1 AV3S31 D G E S R L 58 |
| 14 LAU 155-clono2 BV13S1 L K G L T Q 2S5 AV3S31 D A S Y G Q N F 26 |
| 15 *LAU 50-clono1 BV1S1 S V T G T G G G 2S1 AV14S1 S D S N Y Q L 33 |
| 16 LAU 155-clono1 BV1S1 S V T G T G G D 2S2 AV4S1 R K T S Y D K V 50 |
| 17 *LAU 50-clono2 BV1S1 S V T G T G G D 2S2 AV4S1 R K T S Y D K V 50 |
| 18 LAU 198-clono1 BV1S1 S Y V G G A E L 2S2 AV6S2 R E G P S G G Y S Y G K L 52 |
| 19 LAU 50-clono3 BV13S1 S Y V G G A E L 2S2 AV6S2 R E G P S G G Y S Y G K L 52 |
| 20 LAU 155-clono1 BV13S1 S V T G T G G G 2S1 AV14S1 S D S N Y Q L 33 |
Prevalent Contribution of the TCR β-Chain for the Binding Free Energy Toward the NY-ESO-1\textsubscript{157–165}/HLA-A*0201 Complex. The \textit{in silico} modeling of the TCR–pMHC was performed for the 20 dominant NY-ESO-1-specific T cell clonotypes. For each model and for the 2BNR structure (15), we calculated side chains and backbone contributions to the binding free energy made by the TCR residues (\textit{SI Text}). Fig. 4C shows a histogram count of the \( \Delta G_{\text{bind}} \) as a function of the \( \Delta G_{\alpha \text{bind}} - \Delta G_{\beta \text{bind}} \) difference. Except for BV1-clono2 (LAU 155) TCR, a large majority of models showed a preferential β-chain contribution (i.e., a negative \( \Delta G_{\beta \text{bind}} - \Delta G_{\alpha \text{bind}} \) difference), revealing its greater importance compared with the α-chain, in the binding with NY-ESO peptide and MHC complex.

Discussion

In the present work, we performed a detailed analysis of primary structure and function of the TCR αβ repertoire toward the immunodominant HLA-A2/NY-ESO-1\textsubscript{157–165} epitope. The analysis of a large number of T cell clones (605 clones) showed that an important proportion of tumor-reactive CD8\textsuperscript{+} T lymphocytes preferentially expressed the TCR AV3S1/BV8S2 chains with a remarkably high degree of conservation of CDR3α and β domains, in terms of both length and amino acid composition at certain positions. These data are further emphasized by the comparable recognition patterns of fine specificity exhibited by distinct AV3/BV8 T cell clonotypes, as revealed by the experiments using a set of single alanine-substituted NY-ESO-1 peptides. Collectively, this report demonstrates that similarly to the CD8\textsuperscript{+} T cell responses observed against viral influenza A matrix protein and EBV EBNA3 epitopes (4, 5), highly restricted and conserved TCR αβ usage can also be observed in CTL responses toward self/tumor antigens such as NY-ESO-1.

Another major finding is that besides the selected AV3S1/BV8S2 set of T lymphocytes, NY-ESO-1-specific TCR repertoires were biased toward the usage of BV1 and BV13 gene segments. However, in contrast to the AV3S1/BV8S2 set, the BV1 and BV13 TCR sets revealed corresponding V\( \alpha \) repertoire toward the high diversity. Overall, these TCR α-chains were characterized by different V\( \alpha \) and J\( \alpha \) usage of highly variable CDR3 length and amino acid composition. Similar to our data, a recent study showed that CTL specific for the protective immunodominant nucleoprotein epitope (NP366–374) of influenza A viruses in B6 mice used a highly restricted VB repertoire but no conserved V\( \alpha \) of the binding free energy contributions made by the TCR V\( \beta \) residues and the TCR V\( \alpha \) residues toward the HLA-A*0201/NY-ESO-1\textsubscript{157–165} complex. Histogram count of 19 of the 20 TCR models was generated \textit{in silico}, as a function of the \( \Delta G_{\text{bind}} \) difference. The \( \Delta G_{\alpha \text{bind}} - \Delta G_{\beta \text{bind}} \) difference is in kilocalories per mole. Of note, the 2BNR (15) \( \Delta G_{\beta \text{bind}} - \Delta G_{\alpha \text{bind}} \) difference was −6.4 kcal/mol, conferring again a preponderant role to the β-chain in the binding process.

**Fig. 4.** Contribution of individual NY-ESO-1\textsubscript{157–165} amino acid residues to functional antigen recognition by specific T cell clonotypes. (A) The relative antigenicity of alanine-substituted NY-ESO-1 peptide analogs was assessed on T2 target cells in the presence of graded peptide concentration. We used five and six distinct T cell clonotypes (BV1, BV8, and BV13) isolated from patients LAU 155 and LAU 50, respectively. The molar concentration required for 50% maximal lysis by each T cell clone and for each peptide was calculated from peptide titration experiments. The relative antigenic activity of each peptide analog was determined by using the native peptide NY-ESO-1 as a reference (\textit{SI Text}). The recognition patterns of BV1-, BV8-, and BV13-derived clonotypes are depicted in separate panels of graphs. (B) Estimation of the binding free energy contributions of each residue of the NY-ESO-1\textsubscript{157–165} Peptide for the TCR BV13 clonotype 1 isolated from patient LAU 155. Of note, the side chains of the central residues (P4–P8) showed important favorable contributions in the binding between peptide and TCR, whereas the contributions of the outer side chains (P1, P2, P3, and P9) were not significant. (C) Comparison of the sum BV13 T cell clonotypes. Both methionine (P4) and tryptophan (P5) residues were particularly crucial for their interaction with the TCR, as demonstrated by the low recognition by all of the BV1, BV8, and BV13 clonotypes for the peptides containing M160A and W161A substitutions (Fig. 4A). Finally, other residues (Leu-P3, Ile-P6, Thr-P7, and Gln-P8) were highly critical for only some but not other clonotypes (either BV1, BV8, or BV13). The contributions of the side chains of the NY-ESO-1\textsubscript{157–165} peptide to the binding free energy of the BV13 clonotype 1 from patient LAU 155 were calculated according to the Molecular Mechanics–Generalized Born Surface Area (MM-GBSA) approach (Fig. 4B and \textit{SI Text}) and are in good agreement with the functional recognition pattern found for the same clonotype (Fig. 4A). This particular clonotype was chosen because of the availability of a very closely related TCR-pMHC x-ray structure, designated as the 2BNR complex (15).
repertoire (16). Despite the limited set of human T cell clones analyzed, similar conclusions were reached by another study based on HIV-specific CTL responses from long-term survivors (17). Altogether, these observations reveal an unsuspected diversification of the TCR αβ repertoire, which would have been qualified as "highly restricted" had such analyses remained limited to the TCR β-chain repertoire. Therefore, TCR repertoire studies need to include the characterization of both TCR α- and β-chains and not only the latter as done in the majority of previous studies.

Because NY-ESO-1-specific T cells expressed biased TCR BV1 and BV13 chains but can use a variety of TCR α chains, our data suggest that recognition of the NY-ESO-1,157–165/HLA-A*0201 complex may predominantly depend on the TCR β-chain. In this regard, the in silico modeling of the NY-ESO-1-specific T cell repertoire combined with the MM-GBSA decomposition of the binding free energy into TCR α and β contributions revealed that a large majority of specific T cell clonotypes have a preferential β-chain contribution to the TCR–pMHC binding compared with their corresponding α-chain. Collectively, our findings emphasize the prevalent role of the β-chain in contributing to more favorable interactions with the pMHC ligand than the α-chain. Curiously, the results described here and in the above-mentioned studies do not exactly recapitulate those found by others that rather suggest a predominant role of TCR α-chain in determining the primary repertoire of antigen-specific T cells (18–20). In line with this view, Yokosuka et al. (18) have investigated the potential spectrum of TCR αβ-chains to exhibit antigen specificity by systematically analyzing hundreds of individual TCR αβ pairs for antigen-specific recognition. Their findings show that to recognize the HIVgp160 peptide/H-2Dd complex, CTLs have to possess a single TCR α-chain but can use a variety of TCR β-chains. Restricted α-chain usage (i.e., Vα2.1) was also found among HLA-A2/Melan-A specific T cells (19, 20). Consistent with these results, Miles et al. (21) have recently shown that MHC restriction in the CD8+ T cell response to an epitope from EBV, which is naturally immunogenic across two human MHC class I alleles, can be controlled exclusively by TCR α-chain usage.

Many self-antigens are expressed in the thymus (22), associated with negative selection of T cells expressing specific TCRs. However, in some instances explaining why self/tumor antigen-specific TCRs are of lower avidity as opposed to pathogen-specific TCRs (23). Because our observations were made on an extended pool of dominant T cell clonotypes isolated from two different patients (LAU 50 and LAU 155), they indicate that this represents a general feature, i.e., that distinct TCRs (AVx/BV1, AVx/BV8S2, AVx/BV13) share comparable peptide recognition patterns, suggesting conserved binding modes of distinct TCR-specific TCRs. In particular, preliminary observations on in silico models suggest that some conformational motifs within the CDR3β loop may be critical for the binding to the NY-ESO-1,157–165 peptide (data not shown). The issue of which residues within the CDR1, CDR2, and CDR3 loops present dominant contributions to (i) antigen recognition and (ii) MHC recognition deserves additional studies combining detailed structural and functional investigations. A better understanding of structural principles that govern TCR recognition is essential to promote research and clinical applications. The quality of TCRs recruited during disease, or by vaccination, dominantly influences the potency of immune responses (27). To improve therapeutic immune interventions, TCRs need to be fully characterized, and the mechanisms for their recruitment and function must be elucidated. Results from such studies impact, e.g., on the design of antigens for vaccination and on the choice of optimal TCRs for adoptive T cell therapy, with or without TCR gene transfer. Here, we show that the TCR β-chain, comparable with the TCR α-chain in other models, may play an important role in the recognition of pMHC complexes and that depending on the pMHC model, each chain may present dominant contributions to antigen recognition. In addition, similar mechanisms may control TCR repertoire shaping toward foreign or self-antigens because the predominant contribution of the TCR α- or β-chain can be observed under various physiopathological contexts, e.g., acute or chronic in vivo triggering by either viral or tumor antigens. Finally, our data demonstrate that three distinct sets of TCRs (AVx/BV1, AV3/ BV8, and AVx/BV13) are able to recognize the same NY-ESO-1/MHC complex with comparable and high functional avidity, conferring successful tumor recognition.
Materials and Methods

Patients, Cell Preparation, and Flow Cytometry. Five HLA-A*0201 melanoma patients (LAU 50, LAU 97, LAU 155, LAU 156, and LAU 198) provided written informed consent and thus participated in this study of the Ludwig Institute for Cancer Research (LICR) and of the Multidisciplinary Oncology Center, which was approved by Institutional Review Boards and the LICR Protocol Review Committee. Samples were collected and processed for peptide stimulation experiments and for HLA-A2/peptide multimer staining as described in the SI Text.

Generation of T Cell Clones and Functional Cytolytic Assays. Multimerpos CD8pos T cells were sorted in a FACS Vantage SE machine (Becton Dickinson) and cloned by limiting dilution and expanded as described in the SI Text. The functional avidity of antigen recognition was analyzed for in vitro generated T cell clones, isolated from patients LAU 50 and LAU 155, and bearing molecularly defined mIC TCR clonotypes as detailed in the SI Text.

TCR-BV and -AV Repertoire Analysis. TCR-BV and -AV repertoire analysis was performed after spectratyping, sequencing, and clonotyping as detailed in the SI Text.

Binding Free Energy Calculations. The estimation of the binding free energy contributions of each residue of the NY-ESO-1 peptide to the TCR–pMHC association was performed on the TCR BV13 clonotype 1 from LAU 155, which is highly related to the TCR AV23-BV13 (1G4) bound to NY-ESO-1 by HLA-A*0201 (15), as described in the SI Text.

Computational TCR Modeling. To calculate the binding free energy contributions made by the TCR VÎµ and VÎ¼ residues, homology models of the variable domain of the TCR bound to the SLLMWITQC peptide presented by HLA-A*0201 were built using Modeller 6v2 (28), based on the 2BGR complex (15) and other crystal structures of TCR from the Protein Data Bank (SI Text).

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Supporting Information

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SI Text

In a few systems, strong biases in the T cell receptor (TCR) repertoire selection of antigen-specific T cells, resulting in the preferential usage of particular αβ TCR combinations, have been reported (1–3). The human CTL response to influenza A matrix in individuals with HLA-A*0201 is characterized by prominent usage of the TCR-AV10.2-BV17 containing a conserved Arg–Ser–Ser sequence within the CDR3β regions (1, 3). Another example involves the T cell response to the EBNA3 antigen of Epstein–Barr virus (EBV) seen in HLA-BS-positive individuals whereby nearly all specific CD8+ T cells use TCR-AV4.1-BV6.2 and share conserved CDR3 sequences (2). More recently, Gillespie et al. (4) described the preferential usage of AV15.1 and BV17 TCR, with striking sequence conservation throughout the hypervariable region, in a HLA-B*57-restricted immune response in HIV-1 infection.

Results. Naturally occurring NY-ESO-157–165-specific CD8+ T cell responses display a preferential usage of BV1, BV8, and BV13TCRs. By using HLA-A2/NY-ESO-157–165 multimers in combination with mAbs directed against the variable region of TCR BV1, BV8, and BV13, we found that the majority of multimer+ T cells expressed TCRs with these BV elements (Fig. S1), in line with a recent report by Le Gal et al. (5). In patients LAU 50, LAU 155, LAU 156, and LAU 198, the proportion of NY-ESO-1-specific T cells bearing TCR BV1 was lower compared with cells expressing TCR BV8 and/or BV13 segments. BV8-positive cells were found predominantly in patient LAU 156, whereas most of the tumor-specific T cells of patient LAU 198 displayed the BV13 segment.

Analysis of distinct TCR BV1, BV8, and BV13 NY-ESO-1-reactive T cells by using clonotypic primers. The TCR BV and AV nomenclature used was according to Arden et al. (6) and Genevee et al. (7). To study the clonal composition of NY-ESO-1-specific T cell clones generated from patient LAU 50 (8), two distinct sets of primers specific for each clonotypic CDR3 sequence of these characterized T cell clones were designed: forward (Fwd) and reverse (Rev) (Fig. S2A). The sets designed for BV1 and BV13 T cell clonotypes were highly specific and allowed efficient clonotypic discrimination of each respective clone (Fig. S2 B and D). In contrast, all designed BV8 clonotypic primers displayed some degree of cross-reactivity among clonotypes 1–4 (Fig. S2C).

In addition, BV8 clonotype-specific PCRs were clearly less efficient in identifying the different clonotypes because a large fraction of the obtained PCRs (44%) gave a single positive product (+/+ instead of +/−), and full clonotypic discrimination required extensive sequencing (Fig. S2E). Consistent with these results, sequence alignment and comparison of the four BV8 NY-ESO-1-specific T cell clonotypes revealed a high degree of homology in both the length and sequence of the CDR3β region (Fig. S2B and ref. 8).

Assessment of tumor cell killing by NY-ESO-1-specific T cell clones from patient LAU 50. Overall, all distinct T cell clonotypes efficiently recognized the NY-ESO-1 expressing melanoma cell lines Me 275 and Me 290, the former of which was autologous (i.e., derived from a metastasis of patient LAU 50). Consistent with the data obtained from peptide-pulsed T2 cells (see Fig. 3 of the main text), BV13-clono2 cells recognized tumor cells slightly less efficiently (Fig. S3).

Distinct NY-ESO-1-specific T cell clonotypes from patient LAU 155 exhibit similar functional avidity of antigen recognition. We compared the efficiency of peptide recognition by NY-ESO-1-specific T cell clonotypes isolated from another melanoma patient, LAU 155, by peptide titration killing assays on 17 representative T cell clones of defined TCR clonotypic sequences (Fig. S4). Most BV1-, BV8-, and BV13-derived clones killed T2 cells with an IC50 ranging widely between 10−10 and 10−3 M for the native and 2 × 10−11 and 10−9 M for the analog peptide, respectively. BV13 clonotypes displayed slightly superior peptide recognition compared with BV1 and BV8 clones, in line with their increased ability to recognize NY-ESO-1-expressing tumor cell lines (Me 275, Me 290) (data not shown). Altogether, these data further support the notion that T cells bearing three distinct sets of TCRs (either AVx/BV1, AV3/BV8, or AVx/BV13) shared similar avidity for antigen recognition, irrespectively of their BV usage.

Discussion. An important aspect is the functional antigen recognition and its relation to TCR sequence and structure. Our work was focused on dominant T cell clonotypes, which had expanded successfully in vivo. Therefore, it was particularly interesting to investigate whether their TCRs conferred efficient antigen recognition also in vitro, justifying the effort to generate a very large number of functional T cell clones. Indeed, they recognized and killed NY-ESO-1+ tumor cells and peptide-pulsed T2 cells efficiently and with similar functional avidity, despite the usage of largely different TCRs. These data are consistent with our recent report (8) showing that in patient LAU 50, both BV8 and BV13 tumor-reactive clonotypes efficiently secreted effector mediators such as granzyme B, perforin, and IFN-γ, and killed tumor cells when tested directly ex vivo in a LiveCount assay (9).

Altogether, these observations revealed that T cell clonotypes bearing molecularly defined distinct TCRs not only displayed full effector function but also killed target cells with similar antigen sensitivity, indicating that different TCR αβ usage was not associated with different functional TCR avidity.

Another finding was that despite the relative diversification of the NY-ESO-1-specific TCR repertoire with the characterization of up to 12, 21, and 16 distinct BV1, BV8, and BV13 V(D)J junctional sequences, respectively (see Fig. 1 in the main text), the number of dominant NY-ESO-1-specific T cell clones found among individual melanoma patients was in fact rather limited. In patients LAU 50 and LAU 155, we, respectively, identified 10 and 6 distinct and codominant T cell clonotypes bearing BV1, BV8, and BV13 TCRs. Strikingly, we recently reported that the great majority of NY-ESO-1-specific T lymphocytes, in patient LAU 50, were composed of BV8 clonotypes 1 and 2 and of BV13 clonotypes 1 and 2 (8). Finally, NY-ESO-1/A*0201-specific T cell responses were dominated by a single dominant clone in patients LAU 50 and LAU 198, respectively. Collectively, our data indicate that similarly to our study on T cell responses against the immunodominant Melan-A/MART1 antigen (10), responses to NY-ESO-1 are also caused by selection and amplification of particular T cell clonotypes.

Materials and Methods. Patients, cell preparation, and flow cytometry. Peripheral blood mononuclear cells (PBMCs) were obtained by density centrifugation using Ficoll–Hypaque (Amersham Pharmacia) and cryopreserved in RPMI medium 1640 supplemented with 40% FCS and 10% DMSO (1 × 107 to 2 × 107 cells per vial). For peptide stimulation experiments, CD8+ T lymphocytes were positively selected from PBMCs using anti-CD8-coated magnetic microbeads (Miltenyi Biotech), resulting in more than 90% CD3+CD8+ lymphocytes. Cells from the CD8neg fraction were irradiated (3,000 rad) and used as autologous antigen-presenting cells (APCs). CD8pos-enriched lymphocytes (1 × 106 per well)
were stimulated with the NY-ESO-1 analog peptide (1 μM) and irradiated autologous APCs in 2 ml of RPMI medium 1640 containing 8% of human serum, 10 units/ml human recombinant IL-2 (hrIL-2; a gift from GlaxoSmithKline), and hrIL-7 (10 ng/ml; R&D Systems). A second round of peptide-specific stimulation was performed after 15 days, and cells were cultured for 15 additional days before HLA-A2/NY-ESO-1 multimer analysis. Synthesis of phycoerythrin (PE)- or allophycocyanin-labeled HLA-A*0201/peptide multimers was performed as described in ref. 11 with NY-ESO-1 analog peptide 157–165 C165A (SLLMWITQC) and the analog peptide 157–165 C165A (SLLMWITQC) and the analog peptide.咱们对CD8pos T cells were sorted in a FACSVantage SE machine for 30 min at 4°C in PBS, 0.2% BSA, 50 M EDTA, and then with appropriate antibodies (30 min, 4°C). Cells were washed once in the same buffer and immediately analyzed on a BD Vantage flow cytometer by using CellQuest software (Becton Dickinson).

Generation of T cell clones and functional cytolytic assays. Multimerpos CD8pos T cells were sorted in a FACSVantage SE machine (Becton Dickinson), cloned by limiting dilution, and expanded in RPMI medium 1640 supplemented with 8% human serum, 10 units/ml human recombinant IL-2 (hrIL-2; a gift from GlaxoSmithKline), 1 mM TCEP, and CDR3 clonotype reverse as follows: BV1 clonotype 1, GCTCATTGCCCCCCA-3 and BV13 clonotype 2, 5'-GGAGGGGTG AATTATAGT-3'. Two distinct sets of primers (Metabion) specific for the CDR3 region of each identified BV1, BV8, and BV13 T cell clonotype from patient LAU50 were validated and used for clonotyping PCR as described in ref. 8, (i) CDR3 clonotype forward and Cβ reverse, and (ii) BV subfamilies (BV1, BV8, or BV13) forward and CDR3 clonotype reverse as follows: BV1 clonotype 1, 5'-AGGCCAAGGACGGGAGG-3' and rev-5'-GCCCTTGTCTGCAGGCTAG-3'; BV1 clonotype 2, 5'-GTGATGAGAACATCAGGCC-3' and rev-5'-AAATAGTCGGGGCTGATTG-3'; BV8 clonotype 1, 5'-ACTCTTGTGGCGACCCAC-3' and rev-5'-AAAGCTCTAGTACCCCCCCTG-3'; BV8 clonotype 2, 5'-ACTCTTGTGGCCAGCATCT-3' and rev-5'-AAGCTTCTAGTCCCGGAGA-3'; BV8 clonotype 3, 5'-GTTTGGGGGGCGAATGGCGAC-3' and rev-5'-GAACCTGTCATGCCCCCA-3'; BV13 clonotype 1, 5'-GAAAAGGGGGGGACCT-3' and rev-5'-GGAGGGGTG AATTATAGT-3'.

Binding free energy calculations. To calculate the binding free energy contributions of each residue of the NY-ESO-1 peptide to the TCR–pMHC association, we chose the TCR BV13 clonotype 1 from LAU 155 and used the atomic coordinates of the highly related TCR AV23 BV13 (1G4) bound to NY-ESO-157165/HLA-A*0201, retrieved from the Protein Data Bank (PDB ID code 2BNR) (14). Four TCR residues were mutated first, to generate a 3D model of the TCR BV13 clonotype 1 from LAU 155 (see Fig. 4 of the main text); Thr-95 and Ser-96 of the CDR3α were replaced by Gln and Thr, respectively, and the Asn-97 and Thr-98 of the CDR3β were replaced by Ala. The in silico amino acid substitutions were introduced into the reference TCR atomic coordinates by means of the Molecular Mechanics–Generalized Born Surface Area (15, 16) (MM–GBSA) approach for binding free energy estimation and decomposition. This method was used previously on a TCR-pMHC system and successfully reproduced the results of an experimental alanine scanning (16) as well as allowed a straightforward decomposition of the binding free energy into backbone and side-chain contributions for each residue as detailed in ref. 16. The averaged binding free energy contributions were calculated for all residues. All calculations were performed by using the CHARMm package (version c31b1) (17) and the CHARMm22 all-atoms force field (18). Contributions were averaged over a state of the art 1-ns MD simulation in explicit solvent, by using periodic boundary conditions and at constant pressure (1 atm) and temperature (300 K).

Computational TCR modeling. Homology models of the variable domain of the TCR bound to the SLLMWITQC peptide presented by HLA-A*0201 were built by using Modeller 6v2 (19), based on the 2BNR complex and other crystal structures of TCR from the Protein Data Bank. The 2BNR complex (14) was used as a structural template for TCR and pMHC homology modeling. From the available experimental structures of TCR or TCR–pMHC class I complexes, we selected the variable domains of 11 TCR (Vα and Vβ) as additional templates for the homology modeling: 1ao7, 1bd2, 1f60, 1lgf, 1kb5, 1kj2, 1lp9, 1mi5, 1ndf, and 2ckb. Lower-resolution redundant structures were excluded. Three unbound TCR Vα (1bb8, 1i9e, and 1h5b) and two unbound TCR Vβ (1bec and 1ltk) structures were added to the templates list. The TCRVα structure 1934.4 was also used (20). Because the only complete TCR–pMHC template used was 2BNR, it was expected that the resulting models will have
a similar overall TCR orientation over pMHC, although small adjustments are possible. Once a homology model was obtained, each CDR loop was refined individually, while the rest of the complex, including the other CDR loops, remained unchanged. The loop modeling algorithm consists of a cycle of molecular dynamics simulations, combined with simulated annealing, optimizing the Modeler pseudoenergy function. Whenever possible, this function was improved by adding restraints specific to the nature of the CDR loop, derived from the rules given by Al-Lazikani et al. (21), where the identification of key amino acids defines limitations to the conformation space accessible to a CDR loop. The loops were refined one by one, in the following order, CDR2β, CDR1β, CDR2α, CDR1α, then both CDR3α and β were modeled at the same time. From each model that was obtained, we used CHARMm to calculate and decompose the binding free energy according to the MM-GBSA approach (15, 16), except that the quantities were not averaged along an MD simulation trajectory but calculated from the single structural model because of computing limitations. A 4 kcal/mol cutoff was applied to all residue contributions. Thus, all contributions that were larger than 4 kcal/mol or lower than −4 kcal/mol were set to 4 kcal/mol and −4 kcal/mol, respectively. This was done because values were calculated from a single structure and not averaged along a molecular dynamics simulation trajectory, leading to possible overestimation of favorable or unfavorable contributions. This cutoff was based on the maximum values that are usually observed by experimental alanine scanning (22). To assess the impact of the cutoff on the results, different values ranging from ±3 kcal/mol to ±6 kcal/mol were tested. No significant change in the calculated ΔGαbind and ΔGβbind contributions were found, illustrating the robustness of the results (data not shown). Of note, because of its particularly long CDR3α and β, the BV13 clone1 from patient LAU 198 can be considered as an outlier and was removed from the calculations. In particular, the last step of the loop-modeling algorithm needs to estimate theoretically the conformation of a 23-aa loop, which is out of reach for the method.

Fig. S1.  TCR-BV usage of naturally occurring HLA-A*0201/NY-ESO-1_{157-165} multimer⁺ T cells in peptide-stimulated PBMCs from melanoma patients. Flow cytometry analysis of CD8⁺ multimer⁺ T lymphocytes (R1) by using TCR BV1-, BV8-, and BV13-specific mAbs was performed. Data are shown as the percentage of BVx-expressing cells among CD8⁺ multimer⁺ T cells. Of note, only patients exhibiting the highest frequencies of NY-ESO-1-specific T cells are depicted (LAU 50, LAU 155, LAU 156, and LAU 198).
Fig. S2. Characterization of dominant T cell clones by clonotypic PCR and sequencing. (A) Unique primers corresponding to the CDR3 gene segment of each identified TCR clonotype were designed and validated (see SI Materials and Methods). Two distinct sets of primers were used: (i) BVx forward–clonotype reverse (BVclono-Rev) and (ii) clonotype forward (BVclono-Fwd)–C\(_\gamma\)/H9252 Reverse. (B–D) Clonotypic BV1 (B), BV8 (C), and BV13 (D) PCRs were performed on \textit{in vitro} generated NY-ESO-1-specific T cell clones from patient LAU 50. The size for each amplified BV-CDR3-BC product is indicated (in base pairs). PCRs performed on distinct T cell clones bearing the same TCR clonotype are also depicted. (i) refers to low PCR expression levels. (E) The proportion (in percentage) of PCRs generating +/+ signals (positive PCR products with both BVclono-Fwd and BVclono-Rev sets of primers), +/- (positive PCR products with BVclono-Fwd only), and -/+ signals (positive PCR products with BVclono-Rev only). The percentage of BV1, BV8, and BV13 clonotypes confirmed by sequencing is depicted within each column and highlighted as gray bars. Of note, 64% of “true” BV8 T cell clonotypes gave a +/- PCR signal (false negatives). Conversely, 25% of +/+ PCR signals were not specific for the correct BV8 clonotype sequence (false positives). n represents the number of single T cell clones analyzed.
A. Tumor reactivity for the autologous melanoma cell lines Me 275 (A2+/NY-ESO-1+) and the allogeneic lines Me 290 (A2+/NY-ESO-1+) and NA8 (A2+/NY-ESO-1+) in the absence or presence of NY-ESO-1\textsubscript{157-165} analog peptide (1 μM) at an effector:target ratio of 30:1. Representative examples of percentage of specific lysis by BV1, BV8, and BV13 clonotypes 1 and 2 are depicted. Representative examples of percentage of lysis by BV1, BV8, and BV13 clonotypes 1 and 2 are depicted.

B. Complete set of data (n = 82 clones) representing the specific lysis of HLA-A2+ tumor cells expressing NY-ESO-1 in the absence of peptide (30:1; effector:target ratio). Each data point represents the result of an individual clone. Mean ± SD values (in percentage) are shown.
Fig. S4. Assessment of NY-ESO-1-peptide recognition of NY-ESO-1-specific T cell clones derived from patient LAU 155. (A) Representative examples of percentage of specific lysis by BV1, BV8, and BV13 clonotypes are depicted. The data show lysis of T2 cells loaded with varying concentrations of either native NY-ESO-1 peptide (open symbols) or its C165A substituted peptide analog (filled symbols). (B) Complete set of data (n = 17) representing the peptide concentration (either native or analog) required for 50% of maximum lysis. Each point represents the result of an individual clone. Mean ± SD values (nanomolar) are shown.