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Single Amino Acid Replacements in an Antigenic Peptide Are Sufficient to Alter the TCR Vβ Repertoire of the Responding CD8+ Cytotoxic Lymphocyte Population[1]

Alexis M. Kalergis,* Toshiro Ono,[2]* Fuming Wang,[3]† Teresa P. DiLorenzo,* Shinichiro Honda,* and Stanley G. Nathenson[4]*†

Cytotoxic CD8+ T lymphocytes are activated upon the engagement of their Ag-specific receptors by MHC class I molecules loaded with peptides 8–11 amino acids long. T cell responses triggered by certain antigenic peptides are restricted to a limited number of TCR Vβ elements. The precise role of the peptide in causing this restricted TCR Vβ expansion in vivo remains unclear. To address this issue, we immunized C57BL/6 mice with the immunodominant peptide of the vesicular stomatitis virus (VSV) and several peptide variants carrying single substitutions at TCR-contact residues. We observed the expansion of a limited set of TCR Vβ elements responding to each peptide variant. To focus our analysis solely on the TCR β-chain, we created a transgenic mouse expressing exclusively the TCR α-chain from a VSV peptide-specific CD8+ T cell clone. These mice showed an even more restricted TCR Vβ usage consequent to peptide immunization. However, in both C57BL/6 and TCRα transgenic mice, single amino acid replacements in TCR-contact residues of the VSV peptide could alter the TCR Vβ usage of the responding CD8+ T lymphocytes. These results provide in vivo evidence for an interaction between the antigenic peptide and the germline-encoded complementarity-determining region-β loops that can influence the selection of the responding TCR repertoire. Furthermore, only replacements at residues near the C terminus of the peptide were able to alter the TCR Vβ usage, which is consistent with the notion that the TCR β-chain interacts in vivo preferentially with this region of the MHC/peptide complex. ***The Journal of Immunology,*** **1999, 162: 7263–7270.**

Cytotoxic CD8+ T lymphocytes (CTL) play an important role in the cellular immune response to virus infections (1, 2). These T lymphocytes recognize Ags as peptides of 8–11 amino acids bound to MHC class I molecules on the surface of infected cells (3). The MHC class I/peptide complex is the ligand for the TCR, a heterodimeric protein consisting of covalently bound α- and β-chains (4, 5). The variable regions of the TCR α- and β-chains are encoded by V/J and V/D/J transcriptional elements, respectively (4, 5). These genetic elements are rearranged as a result of somatic recombination in the thymus during early steps of T cell ontogeny (5).

As is found for Abs, the portions of the TCR variable region predicted to interact with the Ag have been called complementarity-determining regions (CDRs)[5] (5, 6). While the CDR1 and CDR2 are germline encoded by each variable gene segment, the CDR3 results from the V(D)J junction. Since the CDR3 shows the highest diversity within the TCR structure, it was initially proposed that this portion of the TCR would interact with the antigenic peptide, while the less diverse CDR1 and CDR2 would contact the MHC α-helices (7). However, the recently solved crystal structures of three MHC class I-restricted TCRs complexed with their cognate ligands have revealed that in addition to the CDR3, the CDR1 could also interact with the peptide (8–11). In these structures, the CDR3 of the α- and β-chains come in close apposition, interfacing the center of the peptide, while the CDR1 of the α- and β-chains contact residues of the antigenic peptide near the N and C termini, respectively (8–11). It is interesting that all the structures show the same overall orientation, in which the TCR interfaces the MHC class I/peptide complex in a diagonal orientation parallel to the β sheets of the MHC Ag binding groove, with the α- and β-chains flanking the amino and carboxyl termini of the peptide, respectively (8–12). This diagonal orientation revealed by the crystal structures agrees with that previously predicted by in vivo studies performed with CTL clones and transgenic mice (13, 14).

However, despite structural and biochemical data addressing the role that the different regions of the TCR play in the interaction with the MHC/peptide complex, still largely unknown are the structural features that determine which members of the highly diverse TCR repertoire are expanded during the onset of a T cell response triggered by a particular Ag. In this regard, it has been observed in a variety of systems that the T cell response to an Ag is biased toward the usage of a limited set of TCR variable gene families (15–18). These observations have revealed that the T cell responses are preferentially oligoclonal, involving the activation of a restricted set of T cell clones (17). This oligoclonality is presumably determined by the structure of the antigenic peptide and its interaction with the responding TCR molecules.

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[5] Abbreviations used in this paper: CDR, complementarity-determining region; VSV, vesicular stomatitis virus; B6, C57BL/6; β_2m, β_2-microglobulin.

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To study the role that the structure of the antigenic peptide plays in the expansion of the responding T cells during an immune response, we analyzed the effect that single amino acid replacements in the sequence of an antigenic peptide have on the repertoire of activated T cells. As a system, we have used an eight-amino acid-long peptide corresponding to residues 52–59 from the nucleocapsid of the vesicular stomatitis virus (VSV) (19). The VSV peptide binds to H-2K^b, triggering a strong immune response mediated by cytotoxic CD8$^+$ T cells. Previous analysis of VSV-specific CTL clones revealed the preferential usage of the Vβ13 and Vβ8 elements (20). Functional studies and analysis of the crystal structure of the H-2K^b/VSV complex have allowed the potential TCR-contact residues of the VSV peptide to be identified (21–23). In the present work, we asked how single amino acid replacements in TCR-exposed positions of the VSV peptide would affect the TCR repertoire of the responding CD8$^+$ T cell population.

When C57BL/6 (B6) mice were immunized with the VSV peptide, the predominant TCR Vβ element used by the responding CD8$^+$ T cell population was Vβ13. Immunization of these mice with certain singly substituted VSV-derived peptide variants caused a change in the predominant TCR Vβ usage. To focus our study on changes in the TCR β-chain resulting from alterations in the sequence of the VSV peptide independently of the TCR α-chain, we created a transgenic mouse that expressed exclusively the TCR α-chain of a VSV-specific, H-2K^b-restricted T cell clone (N30.7, Vα2/Vβ13) (20, 24). When the transgenic mice were immunized with the VSV peptide, Vβ13 was again the predominant Vβ element present in VSV-specific CTL populations. However, the TCR Vβ usage was profoundly altered when these transgenic mice were immunized with peptides carrying single replacements near the C terminus of VSV. Peptides with replacements at the N terminus of VSV were able to induce a strong cytotoxic response but did not alter the TCR Vβ usage. Our results are discussed with regard to the orientation of the TCR/ligand interaction in vivo and the effects that point mutations in the epitope sequence can have on the nature of responding cytotoxic T cells.

Materials and Methods

Peptide synthesis and purification

The wild-type VSV peptide (RGYVYQGL) and singly substituted variants were synthesized by a solid-phase method using Fmoc chemistry on an automated 433A peptide synthesizer (Applied Biosystems, Foster City, CA) at the Peptide Synthesis Facility of the Albert Einstein College of Medicine. All peptides were purified to >98% homogeneity by reversed-phase HPLC on a Vydac C-18 column (2.1 or 4.6 mm × 25 cm, 300 Å) using HP-1090M HPLC (Hewlett Packard). The identity of the purified peptides was determined by a tandem quadrupole mass spectrometer (TSQ700, Finnigan MAT, San Jose, CA).

Animals and immunizations

B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The N30.7 Vα2 transgenic mice were designed as described previously by Wang et al. (24). These mice carry the TCR α-chain of the H-2K^b/VSV-specific CTL clone N30.7 (20) as a transgene in a TCRα$^{-/-}$ background. All animals were maintained under approved institutional guidelines in the Animal Institute of the Albert Einstein College of Medicine.

Mice were immunized by injecting 15 μg of peptide emulsified in CFA in the hind footpads. Seven days later, animals were boosted with 15 μg of peptide emulsified in IFA. One week after the booster, animals were sacrificed and spleens removed for culture and generation of peptide-specific CTL populations.

Culture of peptide-specific CTL populations and cytotoxicity assays

Splenic cells (5 × 10^7) were cultured for 7 days in 10 ml of IMDM, supplemented with 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μM 2-ME, and 10% heat-inactivated FCS (HyClone, Logan, UT), in the presence of 1 μM of the peptide used for immunization. At day 7, cells were restimulated with the same peptide (1 μM) presented by mitomycin C-treated syngeneic B6 splenic cells. Cultures were supplemented with 50 U/ml rhIL-2. At day 14 of culture, cells were harvested for cytotoxic assays and Vβ family usage analyses. This protocol was followed for generation of peptide-specific CTL populations from both wild-type B6 and N30.7 Vα2 transgenic mice.

Peptide-specific cytotoxicity mediated by CTL populations was determined by ^{51}Cr release assay. RMA/s cells were incubated for 1 h at 37°C with 1.85 MBq of $Na_2{}^{51}CrO_4$ and 1 μM of specific peptide. These target cells (3 × 10^3) were mixed with CTL effector cells in E:T ratios ranging from 30 to 50. After a 4-h incubation at 37°C and 8% CO_2 in air, supernatants were collected and the amount of ^{51}Cr released was measured in a gamma counter. The percentage of specific lysis was determined as 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Experimental release is the radioactivity in the supernatant of target cells mixed with CTLs, spontaneous release is that in the supernatant of target cells incubated alone, and maximum release is that in the supernatant after complete lysis of target cells with 1% Triton X-100 in PBS.

Preparation of the H-2K^b/VSV tetramers and binding to VSV-specific CD8$^+$ CTL populations

The detailed procedure for preparation and characterization of H-2K^b/VSV tetramers will be described elsewhere (A. M. Kalergis, E. Palmieri, E. C. Goyarts, S. Honda, and S. G. Nathenson, manuscript in preparation). Briefly, using a form of the H-2K^b molecule carrying a single unpaired cysteine residue at the C terminus, H-2K^b/β_2-microglobulin (β_2m)/VSV complexes were obtained from the folding of rβ_2m and H-2K^{b}cys in the presence of VSV peptide as previously described (21). After purification by gel filtration (G-75, Pharmacia), H-2K^b/β_2m/VSV complexes were treated with 1-biotinamido-4- (4′-(maleimidomethyl)-cyclohexanecarboxamido)-butane (Pierce, Rockford, IL), a biotinylating reagent specific for free –SH groups. This procedure ensures the incorporation of biotin only at the free cysteine residue engineered at the C terminus of H-2K^b. Tetramerization of the biotinylated H-2K^b/VSV complexes was obtained by incubation with streptavidin-PE at a molar ratio equal to 8:1, ensuring the saturation of the four biotin-binding sites of streptavidin. H-2K^b/VSV tetramers were purified from the excess of H-2K^b/VSV monomers by gel filtration in a G-200 column (Pharmacia).

VSV-specific CD8$^+$ CTL populations from B6 and N30.7 Vα2 transgenic mice were double stained with 0.5 μg of H-2K^b/VSV tetramers-PE and 0.5 μg of anti-CD8α-FITC mAb (PharMingen, San Diego, CA) for 1 h in the presence of 0.1% BSA (fraction V, Sigma, St. Louis, MO). Cells were washed three times with 0.1% PBS-BSA and immediately analyzed using FACScan (Becton Dickinson, Mountain View, CA). Splenocytes from naive B6 and N30.7 Vα2 transgenic mice were included as controls in all of these experiments.

Flow cytometric analysis of TCR Vβ usage in peptide-specific CD8$^+$ CTL populations

At day 14 of culture, 1 × 10^5 cells from the peptide-specific CTL populations were double stained for CD8α and for TCR Vβ with a panel of FITC-conjugated Abs specific for different TCR Vβ families. Those Abs included: anti-Vβ2 (B20.6), anti-Vβ4 (KT4), anti-Vβ5 (MR9-4), anti-Vβ6 (RR4-7), anti-Vβ7 (TR310), anti-Vβ8.1/8.2 (MR5-2), anti-Vβ8.3 (1B3.3), anti-Vβ9 (MR10-2), anti-Vβ10 (B21.5), anti-Vβ11 (RR3-15), anti-Vβ12 (MR11-1), anti-Vβ13 (MR12-3), and anti-Vβ14 (14-2). All of the Abs

Peptide name	TCR 1	2	3	TCR 4	5	TCR 6	7	8
VSV	R	G	Y	V	Y	Q	G	L
K1	K	-	-	-	-	-	-	-
I1	I	-	-	-	-	-	-	-
K6	-	-	-	-	-	K	-	-
R6	-	-	-	-	-	R	-	-
E6	-	-	-	-	-	E	-	-
D6	-	-	-	-	-	D	-	-
I6	-	-	-	-	-	I	-	-
M6	-	-	-	-	-	M	-	-

FIGURE 1. Sequence of VSV and peptide variants used for immunization. For peptide variants, only residues different from those of the VSV peptide are shown. "TCR" indicates the TCR-contact residues of the VSV peptide.

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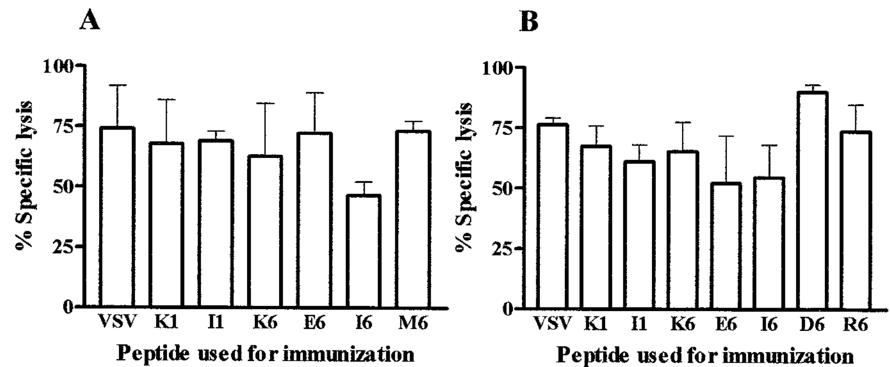

FIGURE 2. All of the peptide variants trigger an equivalent cytotoxicity response in immunized B6 (*A*) and N30.7 Vα2 transgenic mice (*B*). Mice were immunized by injection of 15 μg peptide emulsified in CFA in the hind footpads and were boosted 7 days later with 15 μg peptide emulsified in IFA. One week after the booster, splenocytes were cultured in the presence of IL-2 and the same peptide used for immunization. After 14 days of culture, cytotoxicity was determined by ^{51}Cr release from peptide-loaded RMA/s cells using 1 μM peptide and an E:T ratio of 40.

were purchased from PharMingen. Cells were incubated for 30 min on ice with 0.1 μg of anti-CD8α-PE and 0.1 μg of the specific anti-Vβ mAb in the presence of 0.1% BSA (fraction V, Sigma). After incubations, cells were washed three times with 0.1% PBS-BSA and immediately analyzed using FACScan (Becton Dickinson). Data are reported as the fraction of total CD8$^+$ cells expressing a particular Vβ family. Statistical analyses were performed using the Student *t* test.

Results

Immunization of B6 mice with the VSV peptide triggers a CD8$^+$ T cell-mediated cytotoxic response biased to the relative contribution of a few TCR Vβ elements

It has been previously observed that mice mount a cytotoxic immune response after immunization with peptides of the proper length and sequence for MHC class I binding (25, 26). In this study, immunization of B6 mice with the VSV peptide (Fig. 1), which binds to H-2K^b, triggered a strong peptide-specific cytotoxic response (Fig. 2*A*). The responding CD8$^+$ CTL populations were strongly biased to the participation of T cells that showed the preferential usage of the Vβ13 and Vβ8.1/8.2 (8.1 or 8.2) elements (Table I). These Vβ families represented up to 50% of the total CD8$^+$ CTL population, with the Vβ13 gene element being always predominant and significantly expanded as compared with unimmunized mice ($p < 0.001$) (Table I). No significant expansion of any of the other Vβ families tested was consistently observed after immunization with VSV peptide (Table I).

To evaluate the Ag specificity of the entire CD8$^+$ T cell population responding to the VSV peptide, tetramers of the H-2K^b/VSV complex were utilized for binding to CD8$^+$ T cells. MHC/peptide tetramers are extremely sensitive reagents for the detection of peptide-specific T cells (27). As shown in Fig. 3*B*, the majority of CD8$^+$ T cells responding to VSV peptide bound to H-2K^b/VSV tetramers, indicating that this MHC/peptide complex is the specific ligand for those CD8$^+$ lymphocytes. As control, splenocytes obtained from naive B6 mice were also stained with H-2K^b/VSV tetramers and anti-CD8 mAb; however, CD8$^+$ T cells from these naive animals did not bind H-2K^b/VSV tetramers (Fig. 3*A*).

Single amino acid replacements toward the C terminus of the VSV peptide cause an alteration in the TCR Vβ repertoire of the responding peptide-specific CD8$^+$ CTL populations in B6 mice

The H-2K^b/VSV crystal structure shows that positions 1 (N terminus) and 6 (toward the C terminus) of the VSV peptide are solvent exposed and available to contact the TCR (21, 22). Functional studies from our laboratory have suggested proximity between the TCR β-chain and position 6 of the VSV peptide (24, 28). We therefore evaluated whether single amino acid replacements at position 6 of the VSV peptide could alter the TCR Vβ repertoire of the responding CD8$^+$ CTL populations.

Like the VSV peptide, VSV-derived peptide variants carrying single substitutions at position 6 (Fig. 1) were able to elicit cytotoxic responses in B6 mice (Fig. 2*A*). However, the TCR Vβ usage of the CD8$^+$ T cells expanded by immunization with position 6 peptide variants was significantly altered compared with the Vβ usage of anti-VSV CTL populations. For example, in the case of the M6 peptide, in which the glutamine residue at position 6 was replaced by methionine, CD8$^+$ CTL populations were induced that showed a usage of Vβ13 similar to those induced by VSV, but M6 also induced abundant Vβ11$^+$ CD8$^+$ T cells (Fig. 4*A*). The Vβ11 element was expressed by 20% of the M6-induced CTLs, a frequency significantly higher than the 6% of Vβ11$^+$ cells present in the VSV-induced CTL populations ($p < 0.0001$).

Furthermore, a peptide variant carrying lysine in place of glutamine at position 6 (K6) induced CD8$^+$ CTL populations that preferentially used Vβ8.1/8.2 (Fig. 4*A*). The Vβ8.1/8.2 usage of these cells (24%) was significantly higher than that observed in VSV-specific CD8$^+$ T cells ($p < 0.0086$). The Vβ13 element was

Table I. *Immunization with VSV peptide induces the expansion of CD8$^+$ T cells that express a restricted set of TCR Vβ elements in B6 and N30.7 Vα2 transgenic mice*[a]

	Vβ2	Vβ4	Vβ5	Vβ6	Vβ7	Vβ8.1/2	Vβ8.3	Vβ9	Vβ10	Vβ11	Vβ12	Vβ13	Vβ14
B6 naive	2.2	10.4	3.5	6.0	3.6	14.3	8.8	7.1	9.5	6.6	6.8	4.6	0.7
B6 VSV immune	1.8	5.9	7.9	2.6	8.5	20.1**	6.7	4.2	4.9	5.9	1.6	29.1*	6.6
N30.7 Vα2 Tg naive	2.0	9.1	5.7	6.1	6.5	13.5	6.9	3.7	3.6	5.3	5.2	3.3	3.3
N30.7 Vα2 Tg VSV immune	1.7	8.2	5.9	3.1	5.2	2.9	2.1	2.0	1.5	1.8	1.3	68.7***	4.5

[a] In B6 mice, immunization with VSV peptide expanded preferentially CD8$^+$ T cells expressing Vβ13, followed by Vβ8.1/2 (*$p < 0.001$ and **$p < 0.05$, when compared with nonimmunized mice). On the other hand, in VSV-immunized N30.7 Vα2 transgenic mice, Vβ13 was the only element significantly expanded in CD8$^+$ T cells (***$p < 0.005$ when compared with nonimmunized littermates). Mice were immunized by injecting 15 μg of VSV peptide emulsified in CFA in the hind footpads and boosted 7 days later with 15 μg of VSV peptide in IFA. At 1 wk after the booster, splenocytes were cultured in the presence of IL-2 and the same peptide used for immunization. After 14 days of culture, TCR Vβ usage was analyzed by flow cytometry using a panel of TCR Vβ-specific Abs. Data are expressed as percentage of CD8$^+$ T cells that express a particular Vβ element. Each number represents the average of three independent experiments.

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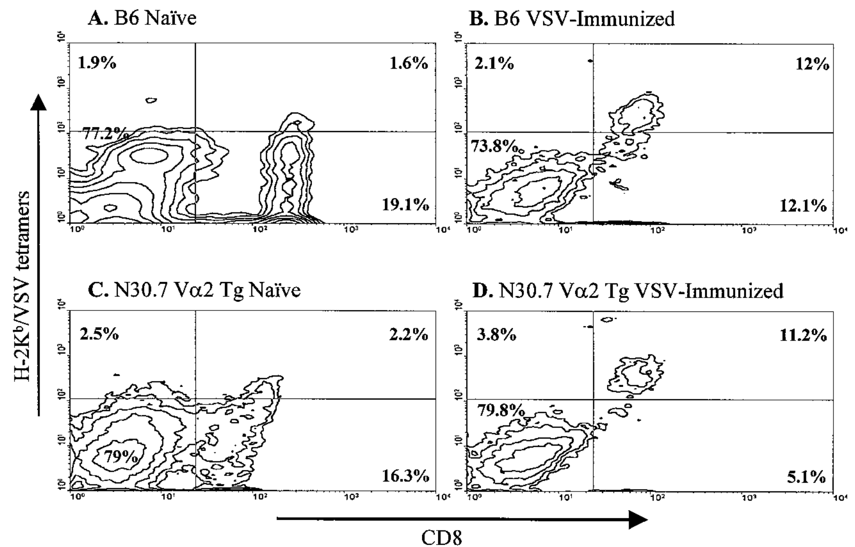

FIGURE 3. CD8$^+$ T cells from VSV peptide-specific CTL populations bind specifically to H-2K^b/VSV tetramers. CTL populations from B6 (*B*) and N30.7 Vα2 transgenic (*D*) mice immunized with VSV peptide were double stained with H-2K^b/VSV tetramers-PE and anti-CD8α-FITC and analyzed using FACS. Splenocytes from naive B6 (*A*) and N30.7 Vα2 transgenic (*C*) mice were included as controls.

practically absent in K6-specific CTL populations from these mice (5.7% compared with the 25.2% observed in VSV-specific CTLs, $p < 0.0002$).

Two other peptides carrying replacements at position 6 of VSV were used for immunization of B6 mice; these were E6 and I6, in which glutamine 6 was replaced by glutamic acid or isoleucine, respectively. The preferential TCR Vβ usage observed in E6-specific CD8$^+$ CTL populations was Vβ8.1/8.2 (14%, Fig. 4*A*). The Vβ13 element (7.6%) was significantly reduced when compared with VSV-specific CD8$^+$ CTLs (25.2%, $p < 0.005$). In the case of I6 peptide-specific CD8$^+$ CTL populations from B6 mice, the frequency of the Vβ13 gene element was also decreased (4.3%, $p < 0.01$) (Fig. 4*A*).

Single amino acid replacements at the N terminus of the VSV peptide do not alter the TCR Vβ repertoire of the responding peptide-specific CD8$^+$ CTL populations in B6 mice

Two replacements were made at position 1 of VSV, where the wild-type arginine residue was either changed to lysine (a conservative change) or isoleucine (a nonconservative change). These K1 and I1 peptide variants (Fig. 1) were able to trigger peptide-specific cytotoxic responses in B6 mice similar to that observed for the VSV wild-type peptide (Fig. 2*A*). When the TCR Vβ usage was analyzed, the CD8$^+$ CTL populations specific for these two peptides showed a pattern similar to that observed in VSV-induced CD8$^+$ CTL populations (Fig. 4*B*). Thus, the predominant Vβ elements used by K1- or I1-induced CD8$^+$ CTL populations were Vβ13 and Vβ8.1/8.2. In the case of K1-induced CTL populations, there appeared to be a decrease in Vβ13 usage when compared with VSV-induced CTL populations, but this difference was not statistically significant.

Immunization of N30.7 Vα2 transgenic mice with VSV peptide triggers a CD8$^+$ T cell-mediated cytotoxic response biased to the contribution of a single TCR Vβ element

To focus our study only on the repertoire of TCR β-chains independently of variations in the TCR α-chain, we created a transgenic mouse expressing the TCR α-chain of the H-2K^b/VSV-specific CTL clone N30.7, in an endogenous TCRα-deficient background. T cells obtained from these mice express only the transgenic TCR α-chain (Vα2, Fig. 5), since they carry a mutation in the endogenous Cα gene (24, 29). When N30.7 TCRα transgenic mice were immunized with the VSV peptide, a strong, specific cytotoxic response was obtained

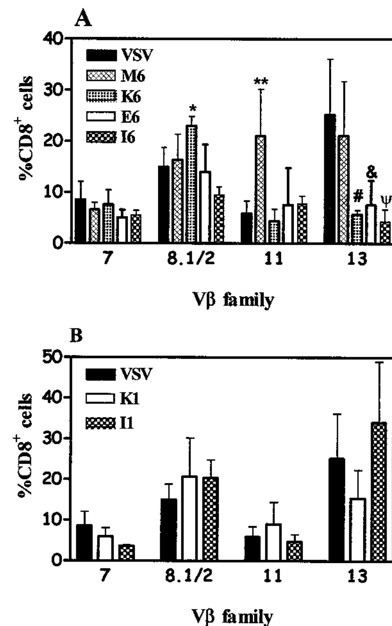

FIGURE 4. Only single replacements near the C terminus of VSV peptide alter the TCR Vβ usage in peptide-specific CTL populations derived from B6 mice. Mice were immunized by injection of 15 μg peptide emulsified in CFA in the hind footpads and were boosted 7 days later with 15 μg peptide emulsified in IFA. One week after the booster, splenocytes were cultured in the presence of IL-2 and the same peptide used for immunization. After 14 days of culture, TCR Vβ usage was determined by flow cytometry using a panel of TCR Vβ-specific Abs. The results are expressed as percentage of CD8$^+$ T cells that express a particular Vβ gene element. Only the Vβ elements that were expanded upon immunization with each peptide are shown. *A*, M6 peptide induces the expansion of Vβ13$^+$ CD8$^+$ T cells but also expands Vβ11$^+$ CD8$^+$ T cells (**, $p < 0.0001$ when compared with VSV peptide). K6, E6, and I6 peptides abolished the predominance of Vβ13 in peptide-specific CTLs (#, $p < 0.0002$; &, $p < 0.005$; and ψ, $p < 0.001$ when compared with VSV peptide). In K6-specific CTL populations, Vβ8.1/8.2 was increased as compared with VSV peptide (*, $p < 0.0086$). *B*, Peptide variants carrying mutations at P1 (N terminus of VSV) do not significantly alter the TCR Vβ usage of peptide-specific CTL populations. Groups of five mice were immunized with each peptide. Each bar represents the average of three to five independent experiments.

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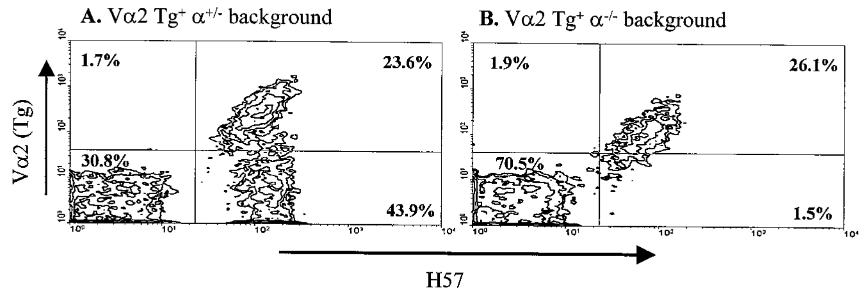

FIGURE 5. $\alpha\beta$ T cells from N30.7 Vα2 TCRα transgenic mice carrying a homozygous mutation in their endogenous TCRα locus express only the transgenic TCR α-chain. Splenocytes obtained from N30.7 Vα2 TCRα transgenic mice heterozygous (*A*) or homozygous (*B*) for a mutation in the TCRα locus were double stained with the anti-TCR β-chain (H57-FITC) and the anti-Vα2 (B20.1-PE) Abs and analyzed by FACS.

(Fig. 2*B*). In these mice, Vβ13$^+$ CD8$^+$ cytotoxic T cells represented up to 70% of the VSV peptide-specific CTL population (Table I and Fig. 6*A*). Neither Vβ8.1/8.2 nor any other of the tested Vβ elements were observed to predominate in VSV-specific CTL populations from these mice (Table I).

Similarly to what was observed with the B6 mice immunized with the VSV peptide, the CD8$^+$ T cells responding to the VSV peptide from N30.7 Vα2 transgenic mice bound H-2K^b/VSV tetramers (Fig. 3*D*). Thus, those CD8$^+$ lymphocytes are specific for the H-2K^b/VSV complex. Naive CD8$^+$ T cells from spleens of unimmunized N30.7 Vα2 transgenic mice did not bind H-2K^b/VSV tetramers (Fig. 3*C*).

Single amino acid replacements toward the C terminus of VSV peptide cause an alteration in the TCR Vβ repertoire of the responding peptide-specific CD8$^+$ CTL populations in N30.7 Vα2 transgenic mice

As was observed in B6 mice, immunization of N30.7 Vα2 transgenic mice with peptide variants carrying certain replacements at position 6 of the VSV peptide also resulted in significant alterations of the TCR Vβ usage. R6-specific CD8$^+$ CTL populations induced in N30.7 Vα2 transgenic mice show two major TCR β-chain families (Fig. 6*A*): Vβ11 (48.7%) and Vβ13 (38.2%). These frequencies differ significantly from those observed in VSV-specific CTL populations (Vβ11, 1.8%, and Vβ13, 69%; $p < 0.001$ in each case), indicating that this single amino acid replacement in the peptide favors the expansion of Vβ11$^+$ T cells. In contrast, the K6 and I6 peptides maintained Vβ13 as the major Vβ element present in the responding CD8$^+$ CTL populations (Fig. 6*A*). This result contrasts with the observed reduction in Vβ13 usage in B6 mice immunized with those peptides (Fig. 4*A*; see *Discussion*).

As seen in Fig. 6*A*, a negative charge in position 6 of the VSV peptide induced the expansion of Vβ7$^+$ CD8$^+$ T cells. Thus, D6 peptide-specific CTL populations show Vβ7 (27.5%) and Vβ13 (24.6%) as the two major Vβ elements involved in D6 recognition. These values differ significantly from VSV-induced CTL populations, in which Vβ7 is expressed in only 5% of the CD8$^+$ T cells ($p < 0.001$). Furthermore, E6 peptide-induced CD8$^+$ CTL populations show Vβ7 as the only significant Vβ element (73%), contrasting the low frequency of this Vβ family in VSV-induced CTL populations (5%, $p < 0.0001$).

Single amino acid replacements at the N terminus of VSV peptide do not alter the TCR Vβ repertoire of the responding peptide-specific CD8$^+$ CTL populations in N30.7 Vα2 transgenic mice

N30.7 TCRα transgenic mice mounted a strong cytotoxic response to the K1 and I1 peptides (Fig. 2*B*) and showed Vβ13 as the predominant Vβ element present in the peptide-specific CD8$^+$ CTL populations (Fig. 6*B*). None of the other Vβ elements tested were significantly abundant in these peptide-specific CTL populations. Thus, while peptides carrying single substitutions at position 1 of the VSV peptide were able to trigger a cytotoxic immune response in both wild-type B6 and N30.7 Vα2 transgenic mice, they did not significantly alter the TCR Vβ usage in CD8$^+$ CTL populations expanded after peptide immunization.

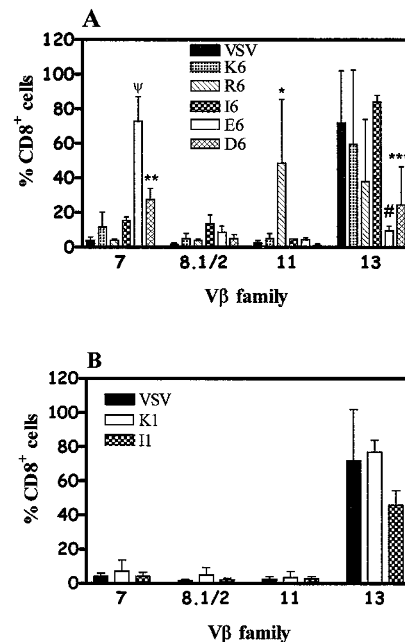

FIGURE 6. Only single replacements near the C terminus of VSV peptide alter the TCR Vβ usage in peptide-specific CTL populations derived from N30.7 Vα2 transgenic mice. Mice were immunized by injection of 15 μg peptide emulsified in CFA in the hind footpads and were boosted 7 days later with 15 μg peptide emulsified in IFA. One week after the booster, splenocytes were cultured in the presence of IL-2 and the same peptide used for immunization. After 14 days of culture, TCR Vβ usage was analyzed by flow cytometry using a panel of TCR Vβ-specific Abs. The results are expressed as percentage of CD8$^+$ T cells that express a particular Vβ gene element. Only the Vβ elements that were expanded upon immunization with each peptide are shown. *A*, Similarly to VSV, peptide variants K6, R6, and I6 induce the expansion of T cells expressing Vβ13. In addition, R6 peptide expanded T cells expressing Vβ11 TCRs (*, $p < 0.001$ when compared with VSV peptide). Peptide variants carrying negative residues at position 6 (E6 and D6) reduced the predominance of Vβ13 and increased Vβ7 in peptide-specific CTLs (ψ, $p < 0.0005$; **, $p < 0.002$; #, $p < 0.0021$; and ***, $p < 0.005$, when compared with VSV peptide). *B*, Peptide variants carrying mutations at P1 (N terminus of VSV) do not significantly alter the TCR Vβ usage of peptide-specific CTL populations. Groups of three mice were immunized with each peptide. Each bar represents the average of two or three independent experiments.

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VSV peptide variants that alter the TCR Vβ repertoire are not recognized by VSV-specific CTL populations

Since certain VSV peptide variants carrying mutations at position 6 of the VSV peptide abolished the predominance of Vβ13 in the responding $CD8^+$ T cell populations, it might be expected that those peptide variants would not be recognized by VSV-specific CTL populations that are enriched in Vβ13 TCRs. Indeed, VSV-specific CTL populations from B6 mice cross-reacted only with those peptide variants that did not alter the Vβ usage (Table II), namely, K1, I1, and M6 peptides. These three peptides induced an equivalent expansion of $V\beta13^+$ $CD8^+$ T lymphocytes upon immunization as did the wild-type VSV peptide (Fig. 4, *A* and *B*). These results support a correlation between TCR Vβ usage and the Ag specificity of CTL populations.

Discussion

The recently solved TCR/MHC-peptide structures have provided key information about the interactions taking place during Ag recognition, revealing that not only the TCR CDR3 loops, as was initially proposed, but also the germline-encoded CDR1 loops can contribute to the interaction with the antigenic peptide bound to the MHC groove (8–11). Evidence for the occurrence of CDR1-peptide interactions in vivo has not yet been shown. Here we provide in vivo evidence for an active role of the antigenic peptide in the expansion of T cells carrying a restricted set of Vβ elements during a cytotoxic immune response. We observe that single amino acid substitutions in the antigenic peptide can profoundly alter the distribution of Vβ elements on a responding peptide-specific CTL population. Such findings are consistent with the notion that there is an interaction between the germline-encoded CDRβ loops and the peptide.

It has been reported previously that T cell responses to specific Ags can be biased, as shown by the expansion of a limited number of TCR Vα and Vβ elements (15–18). We report here that the $H\text{-}2K^b$-restricted cytotoxic T cell response to the VSV peptide in B6 mice is mediated mainly by $CD8^+$ T cells expressing Vβ13 or Vβ8.1/8.2 TCR molecules (Table I, Fig. 4*A*). That $H\text{-}2K^b$/VSV is the specific ligand for those $CD8^+$ T cells is demonstrated by the fact that they bind to $H\text{-}2K^b$/VSV tetramers (Fig. 3). We have also found that single amino acid substitutions at TCR-contact residues of the VSV peptide can alter the TCR Vβ usage of a peptide-specific CTL population. Furthermore, only substitutions near the C terminus of the peptide altered the Vβ usage, a finding consistent with the notion of an interaction between this region of the peptide and the TCR Vβ domain (Fig. 4*A*). However, it is possible that the point mutation in the peptide could affect the Vα usage in the B6 mice and that the observed alteration in the Vβ usage could be a secondary effect due to VαVβ pairing restrictions. This could be a feasible alternative, especially in view of previously reported restrictions on the pairing of specific TCR Vα and Vβ elements (30–33).

To evaluate whether the effect of the peptide mutation on the TCR Vβ usage is a direct or indirect effect on the β-chain, we fixed the TCR α-chain as a transgene, allowing the analysis of TCR β-chains independently of the TCR Vα usage. With this aim, a transgenic mouse was engineered expressing the TCR α-chain from a VSV-specific clone (N30.7) in an endogenous Cα-deficient background (24, 29). Although these mice express only the transgenic TCR α-chain and no endogenous α-chains (Fig. 5), they potentially can express any of the endogenous TCR Vβ elements (Table I). When these mice were immunized with the VSV peptide, only Vβ13 TCRs were significantly expanded. In agreement with what was observed in the B6 mice, only amino acid replacements near the C terminus of the VSV peptide altered the Vβ usage in peptide-immunized N30.7 Vα2 transgenic mice (Fig. 6*A*). Since these mice express the transgenic TCR α-chain in the absence of any endogenous α-chain, the alterations of the TCR Vβ usage resulting from replacements near the C terminus of VSV peptide are not an indirect effect due to alterations of the TCR α-chain, but are a direct effect on the TCR β-chain. Such data are consistent with an orientation in which the TCR β-chain is interacting in vivo with the C-terminal region of the VSV peptide; furthermore, since the observed alterations in the TCR Vβ usage affected entire CTL populations, it is highly probable that this TCR/MHC-peptide orientation is common for most of the T cell clones involved in the recognition of K^b/VSV complex.

Despite the similarities between B6 and TCRα transgenic mice in the response to K^b/VSV, some important differences were observed after peptide immunization. It is noteworthy that Vβ8.1/8.2, Vβ elements that were abundant in VSV-specific CTL populations derived from B6 mice, were significantly reduced in VSV-specific CTL populations derived from N30.7 Vα2 transgenic mice. Moreover, in these transgenic mice there was no significant Vβ8.1/8.2 usage after immunization with any of the peptides tested. In contrast, these TCR Vβ elements were abundant in most of the peptide-specific CTL populations derived from B6 mice and even predominant in the case of the K6 peptide. Furthermore, TCRα transgenic mice responded to K6 and I6 peptides by expanding Vβ13 TCRs, while B6 mice responded to the same peptides by preferentially expanding Vβ8.1/8.2 TCRs, with no significant Vβ13 expansion. These observations could be the result of structural restrictions imposed by the transgenic TCR α-chain on the universe of Vβ elements with which it can pair to produce a functional TCRαβ heterodimer able to recognize $H\text{-}2K^b$/VSV. VαVβ pairing restrictions have been observed previously in other systems in which structural constraints on TCR formation could limit the association between different TCR Vα and Vβ elements (30–33).

The molecular basis for the changes of the TCR Vβ usage in response to small alterations in the antigenic peptide sequence described here is currently unknown. However, one or more of the following three explanations could account for this phenomenon: 1) a direct contact between the CDR1 of the TCR β-chain and position 6 of the VSV peptide, 2) the requirement for particular CDR1 and/or CDR2 structures to position a given CDR3 sequence during recognition of the VSV peptide, or 3) a conformational change in the structure of the TCR binding domain of the MHC due to the single mutation in the peptide.

Table II. *VSV-specific CTL populations derived from B6 mice recognize peptide variants that do not alter TCR Vβ13 usage*[a]

Peptide[b]	Specific Lysis[c]	% Vβ13[d]
VSV	100.0	25.2
K1	96.0	15.3
I1	98.0	34.0
M6	95.0	21.1
E6	47.0	7.6
K6	22.0	5.7
I6	11.0	4.3

[a] The ability of a VSV-specific CTL population to recognize peptide variants carrying single amino acid replacements at either position 1 or 6 was evaluated. Recognition of each peptide variant was standardized with regard to recognition of VSV peptide (100%). Cytotoxicity was determined by ^{51}Cr release from peptide-loaded RMA/s cells at 1 μM peptide and E:T ratio equal to 40.

[b] Target peptide.

[c] Specific lysis: standardized considering cytotoxicity obtained with VSV peptide as 100%.

[d] Percentage of $CD8^+$ T cells expressing Vβ13 in CTL population induced by the target peptide.

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The first explanation implies a direct contact between the CDR1 of the TCR β-chain and position 6 of the VSV peptide. Thus, single replacements at that position of the peptide could impose restrictions on the universe of TCR Vβ elements that would be expanded upon peptide immunization. This hypothesis suggests a role for CDR1β in recognition of the peptide bound to the MHC, a notion supported by the recently reported high-resolution crystal structures of TCRs complexed with their cognate ligands. In both human and mouse systems, the CDR1 of the TCR β-chain interacts with residues toward the C terminus of the antigenic peptide bound to the MHC groove (8–10). In fact, in the 2C/H-2K^b-dEV8 structure reported by Garcia et al., 2C-CDR1β makes 6 contacts with position 6 of the dEV8 peptide as compared with CDR3β, which makes only one (10). In addition, site-directed mutagenesis studies have provided evidence for a role of CDR1β loops in recognition of MHC class I-peptide complexes (34, 35), although those experiments could not determine whether the CDR1β loop was interacting with residues of the peptide or of the MHC. Thus, a direct interaction between the CDR1β loop and the antigenic peptide is a feasible explanation for the changes in Vβ usage reported here.

Alternatively, the second possibility is that particular CDR1 and CDR2 structures would be required to accommodate or position a CDR3 loop of a specific sequence during Ag recognition. We have recently reported that single replacements in P6 of the VSV peptide induced charge-compensatory changes at position 98 of the TCR CDR3β loop (CDR3β98) (24). While negatively charged residues at P6 expanded TCRs with a positive charge at CDR3β98, the opposite finding was observed with positively charged residues at P6 of VSV (24). This requirement for certain CDR3 sequences could limit the germline-encoded CDR1 and CDR2 loop structures that would allow Ag recognition. Further support for this view comes from the observation that in hybridomas recognizing MHC class II/peptide complexes, the use of a particular Vβ gene segment correlated with the presence of a given CDR3β sequence motif (36).

Finally, the third explanation suggests that a single change in the peptide sequence could induce a conformational change in the MHC structure that would alter its binding domain for TCRs. Such an alteration could then be responsible for the expansion of CTLs carrying TCRs with different variable gene elements. To explore this possibility for the E6 peptide, which most profoundly alters Vβ usage in the TCR α-chain transgenic mice (Fig. 6*A*), we have recently solved the structure for the H-2K^b/E6-peptide complex and compared it with the H-2K^b/VSV structure (C. T. Thomson, A. M. Kalergis, S. G. Nathenson, and J. C. Sacchettini, manuscript in preparation). The only conformational changes observed between these two MHC/peptide complexes were localized to the peptide. Therefore, in the case of the E6 peptide, the alteration in Vβ usage is not due to conformational changes in MHC residues. However, in two previous crystallographic studies, peptide variants carrying single substitutions at TCR-contact residues were observed to induce changes in the conformation of at least one MHC residue (37, 38). These two studies raise the possibility that for peptides other than E6, conformational changes in MHC residues could be involved in causing the changes in the TCR Vβ gene usage observed as a result of single substitutions at position 6 of the VSV peptide.

When considered together with previous observations made in MHC class II-restricted responses (36, 39, 40), our results reveal that alterations in the TCR repertoire of the responding T cell population due to minor changes in the Ag are a general and biologically significant phenomenon affecting both CD4$^+$ and CD8$^+$ compartments. Previous studies performed in CD4$^+$ T cell-mediated responses specific for antigenic peptides bound to MHC class II molecules have shown that replacements at positions toward the C terminus of the peptide can alter the TCR Vβ usage of the responding CD4$^+$ T cells (36, 39, 40). By studying entire peptide-specific CD8$^+$ cytotoxic T cell populations, we have observed that replacements at TCR-contact residues of the antigenic peptide can alter the TCR repertoire, supporting the notion that this is a common feature shared by both MHC class I- and II-restricted immune responses.

We demonstrate here that cytotoxic immune responses are particularly sensitive to minor epitope alterations. It is striking that only a single replacement in a TCR-contact residue of the VSV peptide can completely modify the TCR repertoire of the responding CTL population. In addition, those peptide variants that altered the TCR repertoire are not recognized by VSV-wild-type peptide-specific CTL populations. Such a phenomenon could be relevant for understanding the strategies used by pathogens to evade the immune system. It is well known that antigenic variation is a common strategy employed by a number of different microorganisms, including viruses, bacteria, and protozoans to escape recognition by immune cells (41). Our findings show that a change in a single amino acid residue of an immunodominant T cell epitope would require the host organism to generate an entirely new immune T cell response.

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