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Immunobiological Analysis of TCR Single-Chain Transgenic Mice Reveals New Possibilities for Interaction between CDR3 α and an Antigenic Peptide Bound to MHC Class I¹

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The interaction between TCRs and peptides presented by MHC molecules determines the specificity of the T cell-mediated immune response. To elucidate the biologically important structural features of this interaction, we generated TCR β -chain transgenic mice using a TCR derived from a T cell clone specific for the immunodominant peptide of vesicular stomatitis virus (RGYVYQGL, VSV8) presented by H-2K^b. We immunized these mice with VSV8 or analogs substituted at TCR contact residues (positions 1, 4, and 6) and analyzed the CDR3 α sequences of the elicited T cells. In VSV8-specific CTLs, we observed a highly conserved residue at position 93 of CDR3 α and preferred J α usage, indicating that multiple residues of CDR3 α are critical for recognition of the peptide. Certain substitutions at peptide position 4 induced changes at position 93 and in J α usage, suggesting a potential interaction between CDR3 α and position 4. Cross-reactivity data revealed the foremost importance of the J α region in determining Ag specificity. Surprisingly, substitution at position 6 of VSV8 to a negatively charged residue induced a change at position 93 of CDR3 α to a positively charged residue, suggesting that CDR3 α may interact with position 6 in certain circumstances. Analogous interactions between the TCR α -chain and residues in the C-terminal half of the peptide have not yet been revealed by the limited number of TCR/peptide-MHC crystal structures reported to date. The transgenic mouse approach allows hundreds of TCR/peptide-MHC interactions to be examined comparatively easily, thus permitting a wide-ranging analysis of the possibilities for Ag recognition in vivo. *The Journal of Immunology*, 2001, 167: 4396–4404.

The specificity of the T cell-mediated immune response is dependent on the recognition by TCRs of antigenic peptides presented by MHC molecules. The $\alpha\beta$ TCR is a disulfide-linked heterodimer. The α - and β -chains each contain a constant region, assumed to be associated with CD3 accessory molecules that trigger downstream signaling, and a variable region involved in specific Ag recognition. The variable region of the α - and β -chains includes three Ag-binding loops or complementarity-determining regions (CDRs),⁷ referred to as CDR1, CDR2, and

CDR3, which are analogous to those found in Igs. CDR1 and CDR2 are encoded by V α or V β gene segments present in the germline. For the α -chain, the CDR3 loop is encoded following the joining of a V gene to one of a number of J genes, whereas for the β -chain, V-D-J joining encodes CDR3 (1, 2). This gene recombination is partly responsible for creating the diversity in CDR3 loop sequences, and further diversity is generated by deletion or addition of nucleotides at the junctions. It was recently demonstrated for Abs that diversity only in the H chain CDR3 is sufficient to develop responses to a variety of protein and hapten Ags (3).

Extensive structural and functional studies exploring the specificity of TCR recognition for peptide-MHC (pMHC) have been conducted. Recently reported crystal structures of TCR/pMHC complexes have revealed that TCRs bind to pMHC complexes with a generally diagonal footprint in which the α -chain is over the N-terminal half of the peptide and the β -chain over the C-terminal half (4–11). The crystal structures, along with functional analyses using TCR single-chain transgenic mice (12–14) or site-directed mutagenesis of CDR3 loops (15, 16), have all demonstrated the existence of interaction sites between the CDR3s and the antigenic peptide, thus identifying the CDR3 loops as important determinants for peptide recognition.

Although the overall diagonal orientation of the TCR over pMHC class I appears to be general, the particular contact sites vary for different TCRs bound to the same ligand (7), as well as for the same TCR complexed with different ligands (8, 10). For a fuller understanding of the general rules for TCR/pMHC interactions, a much more extensive functional and structural database is needed. Over the years, we have used a variety of structural and functional strategies to elucidate the interactions that occur between TCRs and the immunodominant peptide of vesicular stomatitis virus (RGYVYQGL, VSV8) presented by H-2K^b (17). In

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⁷ Abbreviations used in this paper: CDR, complementarity-determining region; pMHC, peptide-MHC; Tg α , TCR α -chain transgenic; Tg β , TCR β -chain transgenic; VSV, vesicular stomatitis virus.

early studies, we identified the residues at positions 1, 4, and 6 of VSV8 as potential TCR contacting sites by using x-ray crystallography (18) and a biological T cell recognition assay (19). Next we used a series of H-2K^b mutants to map the TCR binding surface on the MHC, work that allowed us to first propose the diagonal orientation of TCR over pMHC (20). Structural information regarding the interaction of TCRs with VSV8/H-2K^b is limited to a single low-resolution structure that is nonetheless sufficient to confirm the overall diagonal orientation observed in the TCR/pMHC structures that have been reported in other systems (21).

To better “visualize” the interactions that occur between TCRs and VSV8, and to observe a large number of such interactions comparatively easily, we have used the technique of variant peptide immunization of TCR single-chain (α or β) transgenic mice. In our previous efforts to identify the residues in CDR3 β that are important for the recognition of VSV8 and its variants (14), we introduced the V α 2 TCR α -chain of a VSV8-specific CTL clone (N30.7) into TCR α -deficient mice, so that all the T cells would express an identical α -chain. These TCR α -chain transgenic (Tg α) mice were immunized with VSV8 peptide analogs with a substitution at position 6, and the CDR3 β sequences of peptide-specific CTL were analyzed. We found that a substitution at position 6 of VSV8 can induce a compensatory change at position 98 of CDR3 β as well as a change in CDR3 β loop length. It was then concluded that position 98 of CDR3 β is a key residue determining the specificity of TCR recognition of position 6 of the peptide and that an optimal length of CDR3 β is required to facilitate interaction. Site-directed mutagenesis also confirmed the importance of position 98 in VSV8/H-2K^b recognition (22, 23).

Regarding the interactions between TCRs and VSV8, several questions remained to be answered, including: 1) Which positions of the peptide interact with CDR3 α ? 2) How does CDR3 α loop length and composition change to recognize different peptides? 3) Are these changes qualitatively different from those observed in CDR3 β ? and 4) Does position 4 of VSV8 contact CDR3 α and/or CDR3 β ? To approach these questions, we recently derived TCR β -chain transgenic (Tg β) mice using the V β 13 TCR β -chain from N30.7, the same VSV8-specific CTL clone whose α -chain was used to generate the Tg α mice used in our previous work (14). In these Tg β mice, the presence of the transgene inhibits the rearrangement and expression of endogenous TCR β -chain genes due to allelic exclusion. After immunization of the mice with VSV8 and variants bearing substitutions at potential TCR contact residues, the CDR3 α loop sequences of peptide-specific T cells were analyzed to see which, if any, amino acid substitutions could induce compensatory changes in CDR3 α composition and/or length. Here we show that this approach, in which hundreds of TCR/pMHC interactions could be examined, has revealed previously undescribed interactions between TCRs and VSV8 and has highlighted the general importance of the TCR J α region in determining Ag specificity.

Materials and Methods

N30.7 Tg α mice

Derivation of the Tg α mice has been described in detail previously (14). These mice are transgenic for the TCR α -chain of the VSV8-specific CTL clone N30.7 (24). They also carry a targeted disruption of both alleles of the endogenous TCR α locus and are therefore unable to express their endogenous TCR α -chain genes.

Generation of N30.7 Tg β mice

The VDJ segment of the TCR β -chain cDNA (V β 13) of the VSV8-specific CTL clone N30.7 was amplified by PCR (using primers containing appropriate intronic sequences, splice acceptor/donor sites, and restriction sites) and cloned into the *Clal/NotI* sites of the TCR β shuttle vector (Ref. 25;

provided by M. Davis, Stanford University, Stanford, CA). After removal of prokaryotic sequences by digestion with *PvuII/SalI*, the transgene construct was injected into (CBA \times C57BL/6)F₂ zygotes. Mice carrying the V β 13 transgene were identified by PCR analysis of tail DNA using the primer set 5'-CTTTGTCTCCTGGGTGCAGGT-3' and 5'-TTCACCACCCACCCAGTGCAT-3'. Transgene carriers were crossed for several generations with C57BL/6 mice to obtain transgenic mice homozygous for H-2^b. MHC haplotype was monitored by PCR using primer sets specific for H-2K^b and H-2K^k.

Generation of peptide-specific CTLs

Peptides (Table I) were synthesized and purified to >98% homogeneity by HPLC before being used for immunization. Tg α or Tg β mice were immunized in their hind footpads with 20 μ g of peptide emulsified in CFA and boosted 1 wk later with 20 μ g of peptide emulsified in IFA. One week after the boost, spleen cells were cultured at 5×10^6 /ml with 1 μ M immunizing peptide in IMDM supplemented with 10% heat-inactivated FBS (HyClone Laboratories, Logan, UT). Cells were restimulated once in vitro, and cell sorting was performed after 13 days of culture.

Cytolytic assay

CTL activity was measured in a standard 4-h ⁵¹Cr-release assay as described (31). The percentage of specific lysis was calculated as [(experimental release – spontaneous release)/(total release – spontaneous release)] \times 100%.

FACS analysis and cell sorting

To detect surface expression of the V β 13 transgene, dissociated lymph nodes of 6- to 8-wk-old mice were stained with an anti-V β 13 (MR12-3; BD Pharmingen, San Diego, CA) Ab. To sort Tg⁺ CTLs from in vitro cultures, cells were stained with anti-CD8 (53-6.7; BD Pharmingen) and anti-V β 13 Abs and CD8⁺V β 13⁺ cells were collected. FACS analysis and cell sorting of stained cells were performed on a FACScan and FACStar (BD Biosciences, Mountain View, CA), respectively.

Preparation of mRNA and cDNA and V α family usage typing

mRNA was extracted from 5×10^5 to 2×10^6 sorted Tg⁺ CTLs, and single-stranded cDNA was made as described (14). V α family usage was determined by PCR using a TCR C α primer paired with 1 of 20 V α primers, each specific for a particular V α gene family and designed to recognize all known members of that family (32). Each culture was found to express a limited number (i.e., 2–4) of V α families (data not shown).

Analysis of TCR CDR3 α sequences

Double-stranded TCR α -chain cDNA was prepared by PCR as described above from sorted CD8⁺V β 13⁺ cells. PCR products were cloned into

Table I. Peptides used for immunization of Tg α and/or Tg β mice^a

Peptide	Notation	Peptide Sequence
Wild type	VSV8	<u>R</u> <u>G</u> <u>Y</u> <u>V</u> <u>Y</u> <u>Q</u> <u>G</u> <u>L</u>
Position 1 variants	K1	K - - - - -
	E1	E - - - - -
	I1	I - - - - -
Position 4 variants	A4	- - - A - - -
	I4	- - - I - - -
	D4	- - - D - - -
	E4	- - - E - - -
	K4	- - - K - - -
R4	- - - R - - -	
Y4	- - - Y - - -	
Position 6 variants	K6	- - - - - K - -
	R6	- - - - - R - -
	D6	- - - - - D - -
	E6	- - - - - E - -

^a Positions 1, 4, and 6 of VSV8 are the potential TCR contacting residues (18, 26) and are underlined and bolded. These residues were substituted in the peptide variants as indicated, and the notation for each variant indicates the substituting residue and its position. In the variant peptide sequences, a dash indicates that the same residue as in VSV8 is present at that position. In general, single amino acid changes in the TCR contacting residues of VSV8 do not dramatically alter the affinity of the peptide for H-2K^b (Refs. 27–29, and data not shown) or its conformation within the peptide-binding groove (28, 30).

pCR2.1 (Invitrogen, San Diego, CA) and the TCR CDR3 α sequences in the resulting plasmids were determined. At least two Tg β mice were immunized with each peptide variant and the sequencing data pooled.

Results

TCR α -chains of VSV8-specific CTLs from Tg β mice exhibit conserved J α usage and a conserved residue at position 93 of the CDR3 loop

Mice transgenic for the TCR β -chain (V β 13) of the VSV8-specific CTL clone N30.7 were derived as described in *Materials and Methods*. FACS analysis revealed that virtually all peripheral CD8⁺ T cells in these Tg β mice expressed the V β 13 transgene on their surface, and V β family usage typing by RT-PCR (32) indicated that the presence of the rearranged V β 13 transgene eliminated the rearrangement and expression of endogenous TCR β -chain genes (data not shown). To investigate the features of CDR3 α that are important for interaction with VSV8/H-2K^b, Tg β mice were immunized with VSV8, and the CDR3 α sequences of VSV8-specific CTLs were determined.

Cytotoxicity assays showed that VSV8-specific CTLs could be induced in Tg β mice (Fig. 1), indicating that the transgenic TCR β -chain could pair with endogenous TCR α -chains to allow recognition of VSV8/H-2K^b. TCR V α family usage typing indicated that the V α 1, V α 2, V α 5, and V α 15 families were used in VSV8-specific CTLs. As the N30.7 TCR α -chain is a member of the V α 2 family (24), we first compared the CDR3 α loop sequences of the V α 2 TCRs with that of the N30.7 TCR (Table II). We found that the CDR3 α loop sequences from VSV8-specific CTLs elicited from Tg β mice showed striking similarity to that of the N30.7 TCR. Among the CDR3 α sequences from the V α 2 family, position 93 (Pro) and the J α 15 region (YQGGRALI) are highly conserved. (Note that position

93 occurs at the V-J junction and is generally not germline-encoded.) These data suggest that the residue at position 93 and the J α region of the CDR3 α loop are important elements for interaction with VSV8. We also found that the predominant CDR3 α length was 10-aa long (Table III), but that the CDR3 α length was less restricted as compared with that previously observed for CDR3 β (14).

To examine whether the conserved residues at position 93 and in J α were also associated with usage of a specific V α family, CDR3 α loop sequences from VSV8-specific CTLs expressing different V α families were examined and compared (Table II). We found that for three other V α families (V α 1, V α 5, or V α 15), the residue at position 93 and the J α region of CDR3 α were highly conserved. However, for these V α families, the residue at position 93 was Ser/Thr instead of Pro, which was found in V α 2 CTLs. Yet, J α 15 was again the predominant J α family among sequenced CDR3 α loops for all of the V α families examined. Our data suggest that in VSV8-specific CTLs, the specific J α usage is induced by the peptide independently of the V α family, whereas the identity of the conserved residue at position 93 is determined by both the antigenic peptide and the V α family.

In the crystal structure of the 2C TCR (4, 6), a portion of the J α region lies adjacent to the CDR3 β loop. This observation brought up a question: do J α 15 TCR α -chains preferentially pair with the transgenic TCR β -chain, resulting in predominant J α 15 usage even in naive Tg β mice? To address this issue, CDR3 α loops of TCRs from naive (i.e., unimmunized) Tg β mice were sequenced (data not shown). We found that in naive mice, there is no predominant J α usage and the occurrence of J α 15 among all CDR3 sequences is extremely low (1/22). Thus, the predominant J α 15 usage observed in VSV8-specific CTLs is

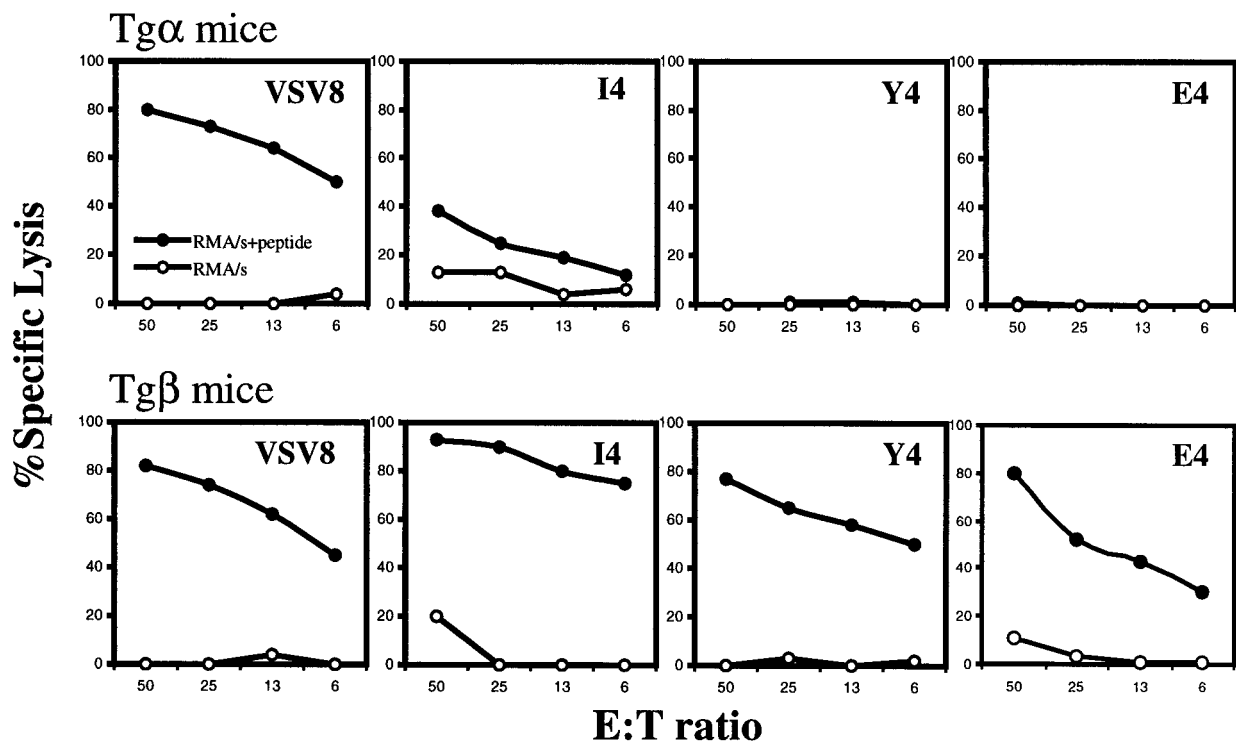


FIGURE 1. Induction of position 4 variant-specific CTLs in Tg α and Tg β mice. Tg α and Tg β mice were immunized with VSV8 or the indicated position 4 variants. After 1 wk in culture, cytotoxic activity was measured in a 4-h ⁵¹Cr-release assay using RMA/S cells incubated with (●) or without (○) the immunizing peptide (0.1 μ M) as targets.

Table II. CDR3 α sequences of N30.7 and VSV8-specific CTLs of the V α 2 family from a representative Tg β mouse^a

T cells	V α	CDR3 Sequence 93	CDR3 Length	J α Usage	Frequency
N30.7	2	PAYQGGRALI	10	15	
Tg β CTLs	2	PAYQGGRALI	10	15	1/12
		PYQGGRALI	9	15	2/12
		PSVYQGGRALI	11	15	1/12
		PHYQGGRALI	10	15	2/12
		PTYQGGRALI	10	15	2/12
		PGQGGRALI	9	15	1/12

^a Tg β mice were immunized with VSV8, and TCR CDR3 α sequences were determined after 2 wk of culture in the presence of VSV8. The single-letter amino acid code is used. CDR3 α is taken to be from position 93 to just before the J α -encoded "FG" (with the conserved C taken to be at position 90). Conserved CDR3 residues are shown in boldface and the J α region is underlined. Frequency, Number of each listed amino acid sequence obtained relative to the total number of sequences analyzed for that family. The N30.7 sequence was previously reported (24).

specifically induced by the immunizing peptide and is not caused by preferential pairing with the transgenic TCR β -chain.

Position 4 variants elicit CTLs in Tg β mice, but not in Tg α mice

Although immunization of N30.7 Tg α mice with VSV8 permits induction of peptide-specific CTLs (Ref. 14, and Fig. 1), immunization with the position 4 variants D4 (Val \rightarrow Asp), E4 (Val \rightarrow Glu), K4 (Val \rightarrow Lys), R4 (Val \rightarrow Arg), or Y4 (Val \rightarrow Tyr) was unable to elicit a CTL response, whereas I4 (Val \rightarrow Ile) induced only a weak response (Fig. 1 and data not shown). A possible explanation for these results is that position 4 of VSV8 interacts with the TCR α -chain, and that the fixed Tg α chain cannot tolerate any change at this position of the peptide. To determine whether position 4 might also interact with the β -chain, N30.7 Tg β mice were immunized with these same peptide variants. A cytotoxic response was obtained in all cases (Figs. 1 and 2), supporting the idea that position 4 of the peptide interacts principally with the TCR α -chain and not with the β -chain.

Certain substitutions at peptide position 4 induce changes in CDR3 α at position 93 and in J α usage

To map potential interaction sites between the TCR α -chain and position 4 of the peptide, CDR3 α sequence analysis was performed on CTLs from Tg β mice responding to VSV8 variants in which Val was substituted by a positively charged (Lys in K4 or Arg in R4) or bulky (Tyr in Y4) residue. Comparison of CDR3 α sequences within a specific V α family showed that nonconservative substitution of Val to Lys, Arg, or Tyr induced compensatory changes in the CDR3 α sequence (Table IV), suggesting a direct interaction between CDR3 α and position 4 of the peptide. Consistent with the results for VSV8-specific CTLs, TCRs of K4-, R4-, or Y4-specific CTLs had a highly conserved residue at position 93 and a conserved J α segment when a specific V α family was considered. When position 4 was changed to Lys, the residue at po-

sition 93 of CDR3 α was changed to Arg or Glu, the J α 15 gene segment used by VSV8-specific CTLs was replaced by J α 18 or J α 22, and changes in the CDR3 α length were also observed. For the Val \rightarrow Arg substitution, we observed changes in the residue at position 93 and J α usage, but not in CDR3 length, whereas the Val \rightarrow Tyr substitution induced compensatory changes in both the CDR3 α motif and length. These CDR3 α sequence data indicate that certain position 4 substitutions can induce changes both at position 93 and in the J α region, leading to alterations in the nature of the CDR3 α loop that have functional consequences, as revealed by the finding that K4-, R4-, and Y4-specific CTLs were unable to cross-react with VSV8 (Fig. 2).

VSV8 peptide variants with more conservative substitutions for Val at position 4 to similar hydrophobic residues (Ala in A4 or Ile in I4) could elicit strong CTL responses in Tg β mice (Fig. 1 and data not shown), and A4- or I4-specific CTLs could cross-react with VSV8 (Fig. 2 and data not shown). The predominant CDR3 α sequence used by V α 15 A4-specific CTLs (SXYQGGRALI; Table IV), with Ser at position 93 and usage of J α 15, was the same as that of VSV8-specific CTLs using the V α 15 family. For I4-specific CTLs using the V α 5 family, a substitution of Ile for Val also did not induce changes of the residue at position 93 or in the J α 15 usage, although the N-terminal residue (Y) of the J α 15 portion of CDR3 (YQGGRALI) was replaced with a random residue added at the VJ junction, thus maintaining a predominant CDR3 α length of 10 (Table IV). These findings suggest that both the CDR3 α sequence and the CDR3 α length are important in recognizing the antigenic peptide. Overall, VSV8 peptide variants with a position 4 substitution of Val to Ala or Ile induced a similar CDR3 α motif in the responding T cell population, which is consistent with the finding that the A4- and I4-specific CTLs were able to cross-react with VSV8.

Finally, when Tg β mice were immunized with VSV8 variants with a change at position 4 from Val to negatively charged residues (Glu in E4 or Asp in D4), CTL responses could be elicited

Table III. Summary of the CDR3 α sequences of VSV8-specific CTLs of different V α families^a

V α	CDR3 Sequence 93	CDR3 Length	J α Usage	Frequency
1	SXYQGGRALI	10	15	6/7
2	PXYQGGRALI	10	15	18/24
5	(S/T) X YQGGRALI	10	15	6/7
15	SXYQGGRALI	10	15	21/29

^a Tg β mice were immunized with VSV8, and TCR CDR3 α sequences were determined after 2 wk of culture in the presence of VSV8. The single-letter amino acid code is used. CDR3 α is taken to be from position 93 to just before the J α -encoded "FG" (with the conserved C taken to be at position 90). Conserved CDR3 residues are shown in boldface and the J α region is underlined. X, Nonconserved residues. The CDR3 length indicated is for the listed sequence only. Frequency, Number of each listed amino acid sequence obtained relative to the total number of sequences analyzed for that family.

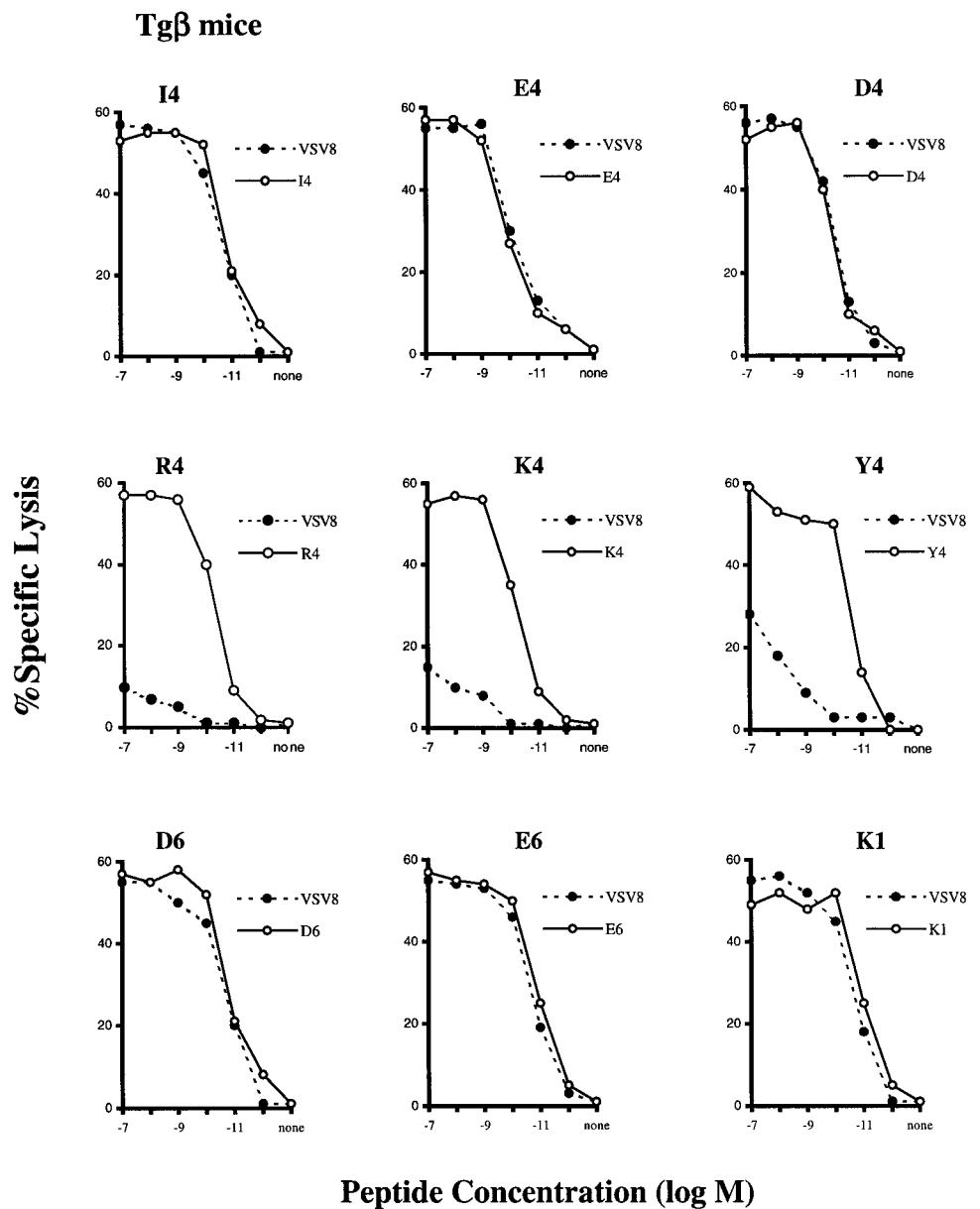


FIGURE 2. Cross-reactivity of peptide variant-induced CTLs with VSV8. CTLs were elicited from Tg β mice by immunization with the indicated peptide. After 2 wk in culture with the immunizing peptide, CTL recognition of varying concentrations of the immunizing peptide (○) or VSV8 (●) was measured in a 4-h ^{51}Cr -release assay using an E:T ratio of 2.

and, surprisingly, E4- or D4-specific CTLs showed strong cross-reactivity with VSV8 (Fig. 2). Although we expected that a Val \rightarrow Glu or Asp substitution at position 4 of VSV8 would change the CDR3 α motif (because Glu and Asp have very different electrostatic properties from Val), we found instead that TCRs of E4- or D4-specific CTLs had similar CDR3 α motifs to those of VSV8-specific CTLs (Table IV) in that J α 15 usage was maintained. However, although position 93 of CDR3 α was conserved for a specific V α family, for V α 5 E4-specific CTLs, Thr was found at position 93 rather than the Ser seen in V α 5 VSV8-specific CTLs. Also, although the J α 15 usage was maintained, two residues (YQ) at the N terminus of the J α region were not present, and the predominant CDR3 α loop length was 9 residues rather than 10. Similarly, when Val at position 4 of VSV8 was substituted with Asp, V α 1 D4-specific CTLs exhibited Leu at position 93 instead of the Ser seen there in V α 1 VSV8-specific CTLs, one residue (Y) was absent from the N terminus of J α 15, and the CDR3 α length was changed from 10 to 9 residues. Taken together, the data showed that the Val \rightarrow Asp/Glu substitution can induce CTLs having compensatory changes of the CDR3 α sequence at position 93

and deletions at the N terminus of the J α region, again suggesting that position 93 and the N-terminal region of J α may interact with position 4 of the peptide. Moreover, the CDR3 α length was altered, presumably to allow interaction between CDR3 α and the substituting residue at position 4 of the peptide.

Substitution at peptide position 6 to Asp or Glu induces a compensatory change at position 93 of CDR3 α

Previous results from Tg α mice showed that changes at position 6 of VSV8 could induce compensatory changes at position 98 of CDR3 β , suggesting that position 6 of the peptide specifically interacts with position 98 (14). From the crystal structures of TCR/pMHC class I complexes (4–8, 10, 11), residues close to the C terminus of the peptide interact with the TCR β -chain and not with the TCR α -chain. We would hypothesize that: 1) a nonconservative substitution for Gln at position 6 of VSV8 would not induce a CTL response in Tg β mice; and 2) even if substitutions of Gln to certain residues could induce a CTL response, these substitutions would not induce compensatory changes in the CDR3 α loop.

Table IV. Summary of the CDR3 α sequences of TCRs of position 4 variant-specific CTLs elicited from Tg β mice^a

Peptide	Recognition of VSV8	V α	CDR3 Sequence 93	CDR3 Length	J α Usage	Frequency
K4	No	2	RGSALGR LH	9	18	13/13
	No	4	EXSGSWQ L I	9	22	21/26
R4	No	2	EXSGSWQ L I	10	22	21/28
Y4	No	15	LSNYNV L Y	8	21	31/38
A4	Yes	15	SXYQGG R L I	10	15	8/10
I4	Yes	5	SXXQGG R L I	10	15	13/19
E4	Yes	5	TXQGG R L I	9	15	13/15
D4	Yes	1	LXQGG R L I	9	15	16/25

^a Mice were immunized with the indicated peptides, and CDR3 α sequences were determined after 2 wk of culture in the presence of the immunizing peptide. The single-letter amino acid code is used. CDR3 α is taken to be from position 93 to just before the J α -encoded "FG." Conserved CDR3 residues are shown in boldface, and the J α region is underlined. X, Nonconserved residues. The CDR3 length indicated is for the listed sequence only. "Recognition of VSV8" indicates whether the position 4 variant-specific CTLs can cross-react with VSV8. Frequency, Number of each listed amino acid sequence obtained relative to the total number of sequences analyzed for that family.

To test these hypotheses, Tg β mice were immunized with position 6 variants of VSV8. As expected, K6 (Gln→Lys) and R6 (Gln→Arg) peptide variants could not elicit CTL responses (data not shown). However, we found that D6 (Gln→Asp) or E6 (Gln→Glu) could induce very strong CTL responses in Tg β mice, and that D6- or E6-induced CTLs could cross-react with VSV8 (Fig. 2). Like position 4 variant-specific CTLs, we found that in the TCR α -chains of D6- or E6-specific CTLs, position 93 and J α usage were highly conserved (Table V). Surprisingly, we found that position 93 is compensatorily changed to a positively charged residue when Gln at position 6 is changed to the negatively charged Asp (D6) or Glu (E6). These data suggest the possibility of a direct interaction between CDR3 α and position 6 of the peptide. Such an interaction has not yet been revealed by the limited number of TCR/pMHC class I crystal structures reported to date (4–8, 10, 11).

Substitution at position 1 of VSV8 does not induce compensatory changes in CDR3 α

Our previous data from Tg α mice, as well as the crystal structures of TCR/pMHC complexes, suggested that position 1 of VSV8 might interact with the TCR α -chain (14, 33). To determine whether position 1 of VSV8 interacts with the CDR3 α loop, Tg β mice were immunized with VSV8 peptide variants bearing a substitution at position 1, and the CDR3 α loop sequences of these peptide variant-specific CTLs were examined (Table V). It was found that all position 1 peptide variants examined (i.e., K1, I1, and E1) elicited CTLs, all of which cross-reacted with VSV8 (Fig. 2, and data not shown). CDR3 α sequence analysis showed that position 1 substitution of Arg to Lys (K1), Ile (I1), or Glu (E1) did not induce changes at position 93 or in J α usage. Consistent with the reported crystal structures of TCR/pMHC class I complexes

(4–8, 10, 11), these data indicated that position 1 of the peptide is not specifically contacted by CDR3 α .

Discussion

The TCR α -chain, rather than the β -chain, interacts with position 4 of VSV8 in vivo

In our previous (14, 31, 33) and present studies, we used Tg α and Tg β mice as tools to define TCR interactions with VSV8/H-2K^b. Although VSV8 elicits strong CTL responses in both Tg α and Tg β mice, a wide variety of position 4 VSV8 variants elicit strong responses only in Tg β mice (Figs. 1 and 2), suggesting that position 4 of VSV8 interacts with the TCR α -chain. Alteration of position 4 of the peptide apparently disrupts interaction with the Tg α chain, rendering position 4 variants unable to induce CTL responses in Tg α mice. However, in Tg β mice, the TCR α -chain can vary, so a position 4 peptide variant can induce T cells expressing compatible α -chains. Our finding provides biological evidence that the central residue of the antigenic peptide primarily contacts the TCR α -chain rather than the β -chain. Crystal structures of TCR/pMHC class I complexes are consistent with this idea, despite the significant differences in details among the different structures (4–8, 10).

Position 93 and the J α region of CDR3 α are important elements for interaction with position 4 of the peptide

Analysis of CDR3 α sequences of TCRs of VSV8-specific CTLs from Tg β mice (Tables II and III) showed that the residue at position 93 and the J α region are highly conserved for one specific V α family. Even for different V α families, we discovered a predominant J α 15 usage. Taken together, we conclude that the residue at position 93 and the J α region of the CDR3 α loop are critical for recognition of VSV8. Because in VSV8-specific CTLs derived from Tg α mice, only two conserved residues were found in the

Table V. Summary of the CDR3 α sequences of TCRs of position 6 or position 1 variant-specific CTLs elicited from Tg β mice^a

Peptide	Recognition of VSV8	V α	CDR3 Sequence 93	CDR3 Length	J α Usage	Frequency
E6	Yes	13	(K/R)XYQGG R L I	10	15	25/27
D6	Yes	5	(K/R)XYQGG R L I	10	15	28/28
K1	Yes	15	SXYQGG R L I	10	15	16/19
E1	Yes	15	SXYQGG R L I	10	15	15/21
I1	Yes	15	(S/T)XYQGG R L I	10	15	11/16

^a Mice were immunized with the indicated peptides, and CDR3 α sequences were determined after 2 wk of culture in the presence of the immunizing peptide. The single-letter amino acid code is used. CDR3 α is taken to be from position 93 to just before the J α -encoded "FG." Conserved CDR3 residues are shown in boldface, and the J α region is underlined. X, Nonconserved residues. The CDR3 length indicated is for the listed sequence only. "Recognition of VSV8" indicates whether the variant-specific CTLs can cross-react with VSV8. Frequency, Number of each listed amino acid sequence obtained relative to the total number of sequences analyzed for that family.

CDR3 β loop (14), our current data from Tg β mice immunized with VSV8 suggest that a greater number of CDR3 α residues may make critical contacts with the peptide *in vivo*, a conclusion not inconsistent with the crystal structures of certain TCR/peptide-H-2K^b complexes (6).

Nonconservative substitution of Val at position 4 of VSV8 to Lys, Arg, or Tyr, which are distinct residues from Val in terms of size and other characteristics such as hydrophobicity and electropolarity, induces dramatic conserved changes at position 93 of CDR3 α and in J α usage (Table IV), indicating that these elements are important for interaction with position 4 of the peptide. The negatively charged residue Glu is present at position 93 in most K4- and R4-specific CTLs, suggesting that this Glu may interact through a salt bridge with the positively charged side chain at position 4. In addition, the change of J α usage indicates the importance of this region for interaction with position 4 of the peptide. Our data indicate that, unlike the CDR3 β loop in which position 98 is mostly involved in the interaction with position 6 of VSV8, residues at position 93 and in the J α segment of the CDR3 α loop may contact peptide position 4, providing strong biological evidence for the complexity of the interaction of the CDR3 α loop with position 4 of the peptide *in vivo*. Crystal structures of TCR/pMHC complexes (4–7) have revealed that position 93 and the J α region of the CDR3 α loop have extensive contacts with the central residue of the antigenic peptide. Based on the crystal structure of the 2C TCR complexed with its ligand (dEV8/H-2K^b), we have modeled the interaction between the VSV8-specific TCR N15 and the VSV8 peptide (Fig. 3). The model supports the likelihood of

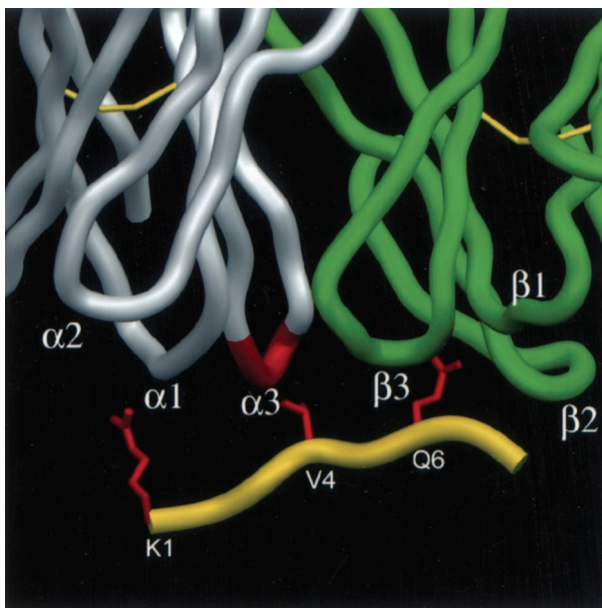


FIGURE 3. An interaction model between the VSV8-specific TCR N15 and the VSV8 peptide. This structural model was built, using the crystal structures of the unliganded N15 TCR (34) and VSV8/H-2K^b (18), to fit the overall structure of the 2C TCR complexed with dEV8/H-2K^b (6), and was presented using the program SETOR (35). TCR α , TCR β , and the VSV8 peptide (*bottom*) are colored gray, green, and yellow, respectively. CDR loops of the N15 α -chain are labeled $\alpha 1$, $\alpha 2$, and $\alpha 3$, whereas those of the β -chain are labeled $\beta 1$, $\beta 2$, and $\beta 3$. The J α region of CDR3 α is colored red. The side chains of the residues at positions 1, 4, and 6 of VSV8 are depicted as red sticks. The figure serves only to depict the likely overall placement of the N15 TCR relative to VSV8 based on the liganded 2C TCR structure. No attempt has been made to predict atomic contacts or the precise positioning of the CDRs after rearrangement upon ligand binding.

interaction between the J α region of CDR3 α and position 4 of VSV8.

Having determined that CDR3 α interacts with position 4 of VSV8 and its variants, we were initially surprised to find that immunization of Tg β mice with the position 4 variants A4 and I4 induces CDR3 α sequences that are quite similar to those elicited by VSV8 in terms of length, the residue at position 93, and the J α sequence (Tables II–IV). However, cytotoxicity assays then revealed that A4- and I4-induced CTLs could cross-react with VSV8 and that VSV8-induced CTLs could cross-react with A4 and I4 (data not shown). Because Val, Ala, and Ile all have hydrophobic side chains, we propose that the same types of interaction (possibly van der Waals) may be involved between these hydrophobic residues and the CDR3 α loop.

When the hydrophobic Val at position 4 of VSV8 was substituted with a negatively charged residue (Asp or Glu), we found that D4- or E4-induced CTLs also use the same J $\alpha 15$ segment as VSV8-specific CTLs (Table IV), indicating that Glu or Asp can apparently form similar contacts with the J α region as does VSV8. Thus, common structural features, rather than the charge, may be the determinant of the specificity of Glu or Asp interaction with the J α region of the CDR3 α loop. Consistent with this is our finding that D4- and E4-specific CTLs from Tg β mice can cross-react with VSV8. Although the change of Val to Asp or Glu at position 4 of VSV8 does not alter J α usage, it does induce changes at position 93. In previous studies with Tg α mice (14), a substitution of Gln to Glu or Asp at position 6 of VSV8 induced a change of Val or Thr at position 98 of CDR3 β to the positively charged residue Lys, suggesting that there is a salt bridge between position 98 of the CDR3 β loop and Glu at position 6 of VSV8. Here we observed that when Val at position 4 of VSV8 was changed to Glu or Asp, a positively charged residue was not induced at position 93, indicating the absence of a salt bridge with position 4 of the peptide variant. Instead, interaction between position 4 and Thr at position 93 might be mediated by a hydrogen bond.

The J α region is an important determinant of TCR specificity

Our current data reveal the importance of the J α region in recognizing a specific antigenic peptide *in vivo*. First, we found that VSV8 and its variants each induce a specific, predominant J α usage (Tables II–V). Second, for particular peptide-specific CTLs, J α usage is even conserved across different V α families, whereas position 93 tends to be conserved only within a particular family (Tables II and III). Third, substitutions for Val at P4 can trigger compensatory changes not only at position 93 but also at the J α region by deletion from the N terminus or replacement with a different J α segment (Table IV). Fourth, we found a correlation of J α usage with cross-reactivity of peptide variant-specific CTLs with VSV8: peptide variant-induced CTLs that could cross-react with VSV8 used J $\alpha 15$. No cross-reactivity was seen when a different J α was used (Tables IV and V). It is noteworthy that even among a panel of VSV8-specific CTL clones previously derived from standard C57BL/6 mice (24), a significant proportion (3 of 12) used J $\alpha 15$ (referred to as A10 in that earlier report), a striking finding considering the fact that the murine germline contains >60 different J α gene segments (36).

The crystal structures of TCR/pMHC class I complexes showed that the J region of CDR3 β does not contact the antigenic peptide (4–7). Our previous studies of CDR3 β loop sequences from position 6 variant-specific CTLs elicited from Tg α mice showed that J β usage is not highly conserved (14), suggesting that the J β region does not specifically interact with the peptide *in vivo* either. Instead, CDR3 β residues that contact peptide are encoded by the D segment and N addition. In contrast, structural studies (4–7)

reveal that the N terminus of J α is located at the apex of the CDR3 α loop, enabling the J α region to make multiple contacts with a central peptide residue. Our current data provide biological evidence for the importance of these J α interactions in determining TCR specificity *in vivo*. Given this difference between the TCR α - and β -chains, it is relevant to note that the murine germline contains >60 J α gene segments (36) but only 14 J β gene segments (37, 38).

Changes in CDR3 α loop length are induced by substitutions at position 4 of VSV8

Our previous studies in Tg α mice showed that nearly all sequenced CDR3 β loops for a particular position 6 variant-specific CTL population had an identical length, and that substitution at position 6 of VSV8 changed the CDR3 β loop length (14). These results suggest that the loop length could be very important for interactions between CDR3 β and position 6 of the peptide, and that loop length may facilitate the interaction between position 98 of the CDR3 β loop and position 6 of VSV8 or its variants.

Our data in Tg β mice now indicate that the length of the CDR3 α loop could also be important for TCR recognition of VSV8 and its variants. In Tg β mice, sequenced CDR3 α loops of TCRs of peptide-specific CTLs have a favored length (Tables II–V). A substitution of Val at position 4 to the large residues Tyr or Lys not only changed the CDR3 α loop sequence, but also shortened the CDR3 α loop length. Recently, we have found that trinitrophenyl-labeled-K4-specific CTLs from Tg β mice also have shorter CDR3 α loops than do VSV8-specific TCR α -chains (39). One possible explanation is that a CDR3 α loop of shorter length may create a bigger cavity at the TCR α and β interface, allowing bulky side chains or hapten groups to be accommodated.

The TCR α -chain may contact position 6 of VSV8 under certain circumstances

Using Tg α mice, we showed previously that interaction between position 98 of CDR3 β and peptide position 6 is critical for recognition of VSV8 and its variants (14). Thus, we predicted that K6 and R6 peptides would be unable to induce CTL responses in Tg β mice, as substitution of Gln at position 6 to Lys or Arg would likely abolish this critical contact with the Tg β chain. We found that this was indeed the case, a result compatible with the finding that VSV8-specific CTLs, including N30.7, are generally unable to recognize position 6 variants of VSV8 (22, 31, 33). However, we were surprised to find that both D6 and E6 could elicit strong CTL responses in Tg β mice, and that the residue at position 93 of CDR3 α was changed from a Ser to a positively charged residue (Table V), suggesting the possibility of an interaction between position 6 of the peptide and the TCR α -chain in the special situation where the β -chain may be somewhat incompatible with the residue at position 6. One possible explanation is the following: Because Gln, Asp, and Glu have similar structures, Asp/Glu can still interact with the Tg β chain CDR3, but with weaker affinity. Therefore, the residue at position 93 of CDR3 α is reciprocally changed to Lys/Arg to enhance the binding of position 6 of the peptide to the TCR. This hypothesis is supported by the crystallographic finding that the VSV8/H-2K^b and E6/H-2K^b complexes are very similar in structure (30). Further support for the idea that the TCR α -chain can interact with position 6 in certain circumstances comes from our finding that immunization of Tg β mice with a VSV8 variant in which Gln at position 6 is changed to trinitrophenyl-labeled-Lys can induce alterations both at position 93 and in J α usage (39).

An analogous interaction between the TCR α -chain and a residue toward the C terminus of the peptide has not yet been seen in

the limited number of TCR/pMHC class I crystal structures published to date (4–8, 10, 11). However, the extraordinary structure of the BM3.3 TCR complexed with pBM1/H-2K^b, in which the CDR3 α loop bends away from the MHC peptide-binding groove and makes absolutely no contacts with the peptide (11), is an indication that much remains to be discerned, using a combination of x-ray crystallography and various biological strategies, regarding the possibilities for interaction between the TCR and its ligand.

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