Genetic Basis for T Cell Recognition of a Major Histocompatibility Complex Class II-restricted Neo-Self Peptide

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Summary

We have analyzed the genetic basis for T cell recognition of an endogenous major histocompatibility complex class II-restricted self peptide. Transgenic mice expressing the influenza virus PR8 hemagglutinin I-E\(^d\)-restricted determinant S1 (HA Tg mice) mediate negative selection of PR8 S1-specific T cells, but respond to immunization with a virus containing a closely related analogue, S1(K113). Sequence analysis of S1(K113)-specific T cell receptors (TCR) from nontransgenic mice revealed a dominant TCR clonotype that cross-reacts with PR8 S1. This clonotype is eliminated by negative selection in HA Tg mice; nonetheless, modified versions of this TCR that used altered junctional sequences and a novel V\(\alpha\)/V\(\beta\) pairing to evade negative selection by the S1 self peptide were identified. The remaining S1(K113)-specific TCRs from HA Tg mice were highly diverse; 13 of 15 S1(K113)-specific TCRs from HA Tg mice used unique V\(\alpha\)/V\(\beta\) pairings. Thus, tolerance to PR8 S1 as a self peptide does not limit the diversity of the T cell response to S1(K113).

The specificity with which T cells recognize antigenic peptide fragments bound to MHC class I or class II molecules is determined by the \(\alpha/\beta\) heterodimer of the TCR, which is generated during thymic maturation of developing T cells through the rearrangement of V, J, and, for \(\beta\) chains, D gene segments (1-3). The availability of large numbers of germline gene segments, random processing of junctional sequences during rearrangement, and pairwise assembly of different \(\alpha/\beta\) chain combinations provide the potential to generate a vast diversity of TCRs with unique sequences and distinct specificities (4). However, the peripheral T cell repertoire is restricted by selective processes that act on developing thymocytes. Positive selection limits the repertoire to those TCRs capable of recognizing self MHC molecules (5-9), while negative selection eliminates TCRs with high avidities for self peptide–MHC complexes (10, 11). Although recent studies suggest that endogenous self peptides bound to thymic MHC molecules may participate in both of these processes (12-15), little is known about the genetic basis by which TCRs recognize self peptide–MHC complexes during T cell repertoire formation.

Current understanding of the genetic basis for T cell recognition of peptide–MHC complexes has mostly been derived from structure/function analyses of peripheral T cells. In early studies, TCRs with the same specificity were often found to use one of a few V gene segment combinations containing similar V-(D)-J junctional sequences, suggesting an important role for junctional sequences in establishing the specificity of the TCR (16-18). Additionally, simple compensatory changes in junctional sequences were shown to modify the specificity of TCRs for analogues of a peptide antigen (18-21), indicating that the V-(D)-J junctional sequences can determine the specificity of the TCR for the peptide moiety of the peptide–MHC complex. In other cases, however, TCRs directed to the same peptide–MHC complex can use diverse combinations of V gene segments (22-26), and different V gene segments can be used to establish specificity of TCRs for analogues of a peptide antigen (21, 27). It is not understood why T cell responses to some peptide–MHC complexes contain limited numbers of closely related V gene segments while others are more diverse. One possibility is that structurally restricted T cell responses arise when the peripheral T cell repertoire contains V\(\alpha\)/V\(\beta\) pairs that are uniquely able to recognize particular peptide–MHC complex(es), providing a framework by which V-(D)-J junctional sequences can determine specificity for the peptide antigen (28). An alternative model suggests that negative selection of T cells by endogenous self peptides might influence the diversity of responses to antigens that are close homologues of self peptides (29). This possibility arose from studies of T cell responses to
peptides derived from influenza virus hemagglutinin (HA)\(^1\) (22) and Plasmodium berghei (23), which appeared to be more diverse than responses to homologues of self antigens such as myelin basic protein (30) and pigeon cytochrome \(c\) (16, 31, 17). To date, however, these models have been difficult to distinguish, because the endogenous self peptides that are present during T cell repertoire formation and the genetic basis by which TCRs recognize these peptides during repertoire formation remain poorly defined.

To examine the genetic basis for T cell recognition of a defined class II-restricted self peptide, we have analyzed transgenic mice that express the influenza virus PR8 HA I-E\(^d\)-restricted determinant S1 as a neo-self peptide (HA Tg mice). S1 is expressed in many tissues (including the thymus) in HA Tg mice and mediates negative selection of PR8 S1-specific T cells (32). Nonetheless, T cells that react with a closely related analogue of S1, designated S1(K113), can be isolated from HA Tg mice (32). By comparing the sequences of PR8 S1- and S1(K113)-specific TCRs from non-Tg mice, we have identified a TCR clonotype that dominates both responses and cross-reacts with each analogue. This TCR clonotype is eliminated in HA Tg mice. Interestingly, modified versions of the clonotype were identified among S1(K113)-specific TCRs from HA Tg mice. These results allow evaluation of the genetic basis by which a group of closely related TCRs recognize the neo-self PR8 S1 peptide. In addition, because most of the S1(K113)-specific TCRs from HA Tg mice use unique V\(\alpha\)/V\(\beta\) pairings, the results demonstrate that negative selection of PR8 S1-reactive T cells does not limit the diversity of S1(K113)-specific TCRs in HA Tg mice.

### Materials and Methods

**Mice.** HA Tg mice, which contain the PR8 HA gene coupled to the SV40 early region promoter/enhancer, have previously been described (32). These HA Tg mice were initially generated by injection of BALB/cxC57Bl6 zygotes, and had been back-crossed to BALB/c mice (obtained from The Jackson Laboratory, Bar Harbor, ME, or Charles River Laboratories, Wilmington, MA) for 8-13 generations before use in the experiments described here. Non-Tg mice were in most cases equivalently back-crossed transgenic-negative littermates; in a few cases, back-crossed transgene-negative littermates; in a few cases, non-Tg donor mouse, and the numerical suffix indicates the individual hybridoma clone; for example, hybridoma S520-8 was obtained from mouse number 520. B indicates a BALB/c donor mouse. All S1(K113)-specific hybridomas were found to be I-E\(^d\)-restricted, based on their ability to react with S1(K113) presented by I-E\(^d\)- but not I-Ad-transfected L cells (32). The majority of the PR8 S1-specific hybridomas have previously been described (22).

**Sequence Analysis.** Sequence analysis of TCR-\(\alpha\) and -\(\beta\) chains was performed using V region family- and C region-specific primers to amplify V regions in the PCR (23, 36). Roughly 10\(^6\) hybridoma cells were washed with 1 ml of NTE (0.1 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) at 4°C and then lysed for 5 min on ice in 200 \(\mu\)l of cold NTE plus 0.5% NP-40. Nuclei were pelleted, and supernatants were added to 200 \(\mu\)l of 0.3 M NaCl, 0.2 M Tris-HCl, pH 8.0, 25 mM EDTA, 2% SDS, and 400 \(\mu\)g/ml protease K, and samples were incubated at 37°C for 1 h. RNA samples were extracted with phenol/chloroform, were ethanol precipitated, and were redissolved in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA. Region-specific primers were used to prime cDNA synthesis from aliquots (roughly 1/10 of the total sample) using reverse transcriptase in 100-\(\mu\)l volumes under standard conditions (37). After incubation, reaction mixtures were boiled, and aliquots (2 \(\mu\)l) were subjected to 35 cycles of amplification in the PCR under standard conditions (36) using either C\(\alpha\)1 (5'-ATCTTTTAACTGGATGGCTC-3') and C\(\beta\)1 (5'-dCTCTGCTTTTGAGCGGCACG-3') or C\(\beta\)3 (5'-dCAAGGAGACCTTGGGTGG-3') as region-specific primers to amplify V regions in the PCR (23, 36). Roughly 10\(^6\) hybridoma cells were washed with 1 ml of NTE (0.1 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) at 4°C and then lysed for 5 min on ice in 200 \(\mu\)l of cold NTE plus 0.5% NP-40. Nuclei were pelleted, and supernatants were added to 200 \(\mu\)l of 0.3 M NaCl, 0.2 M Tris-HCl, pH 8.0, 25 mM EDTA, 2% SDS, and 400 \(\mu\)g/ml protease K, and samples were incubated at 37°C for 1 h. RNA samples were extracted with phenol/chloroform, were ethanol precipitated, and were redissolved in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA. Region-specific primers were used to prime cDNA synthesis from aliquots (roughly 1/10 of the total sample) using reverse transcriptase in 100-\(\mu\)l volumes under standard conditions (37). After incubation, reaction mixtures were boiled, and aliquots (2 \(\mu\)l) were subjected to 35 cycles of amplification in the PCR under standard conditions (36) using either C\(\alpha\)1 (5'-ATCTTTTAACTGGATGGCTC-3') and C\(\beta\)1 (5'-dCTCTGCTTTTGAGCGGCACG-3') or C\(\beta\)3 (5'-dCAAGGAGACCTTGGGTGG-3') as region-specific primers to amplify V regions in the PCR (23, 36). Amplified products were fractionated by electrophoresis on a 1.5% agarose gel, purified using GeneClean II (BIO-101, Inc., Vista, CA), and directly sequenced using C\(\alpha\)1 or C\(\beta\)1 as described (22). Nonproductive TCR-\(\alpha\) and -\(\beta\) transcripts of BW5147 fusion partner origin (an aberrant \(\alpha\) chain transcript [34] and an out-of-frame \(\beta\)5 rearrangement) were routinely detected. In hybridoma SB3-5, the two V\(\alpha\)10 transcripts were separated by size fractionation of full-length cDNA copies of the V\(\alpha\) regions on a 6% polyacrylamide gel. These products were eluted from gel slices, reamplified for 10 additional PCR cycles, and then sequenced separately. Most of the hybridomas containing in-frame V\(\alpha\)4, -8, or -10 gene segments were subsequently sequenced using V\(\alpha\) family-specific 5'-proximal primers—V\(\alpha\)4L-V (5'-d-CACAA[T/C]TC[A/C]GTGACTCA-3'), V\(\alpha\)8L-V (5'-d-CACCAAGGAGCATTGG-3'), and V\(\alpha\)10L-V (5'-d-TGAGAGG[A/G]ACAGAAGCAGG-3')—to differentiate between individual family members.

\(^{1}\text{Abbreviations used in this paper: GST, glutathione S-transferase; HA, hemagglutinin; HA Tg, transgenic mice that express PR8 HA I-E\(^d\)-restricted determinant S1 as a neo-self peptide; LNC, LN cell; SW, A/Swine/31.}\)
Analysis of Vβ8.3/Gly-Ala-Gly/Jβ1.3 Rearrangements in LNC. Non-Tg and HA Tg mice were immunized at the base of the tail with 2,000 hemagglutinating U of PR8, RV6, or SW virus in 50% CFA. After 7 d, total LNC RNA was isolated from the inguinal and lumbar LNs using guanidinium isothiocyanate/NaCl centrifugation (37). The RNA was used as a template for reverse transcription primed by the Cβ2 primer, and the resulting cDNA was amplified in the PCR for 35 cycles under standard conditions (36). Amplification was directed by the primer Vβ8.3-PCR (5′-d'-ACGCAAAGACCTTCTTCTCCTGC-3′) (23) and by a 5′-32P-end-labeled primer Jβ1.3/Gly-Ala-Gly (5′-d-TAGAGC- GTATTTCCCGCCCC-3′), which is complementary to the β chain junctional sequences of hybridomas SB3-5 and SB5-334, and which shows good homology to most of the Vβ8.3/Jβ1.3 rearrangements (see Fig. 2 A). cDNA from hybridoma SB3-5 was also amplified using the same primers to identify the length of the characteristic Vβ8.3/Gly-Ala-Gly/Jβ1.3 rearrangements. Amplified products were separated on a 6% polyacrylamide gel and quantified by autoradiography. Samples containing roughly equivalent amounts of 32P-labeled reaction products were again fractionated, and these products were separated on a 6% polyacrylamide gel. These samples were analyzed using a Phosphorlmager SF and ImageQuant software (version 3.3; Molecular Dynamics, Inc., Sunnyvale, CA), to quantitate the amount of radioactivity present in Vβ8.3/Gly-Ala-Gly/Jβ1.3 rearrangements of different lengths.

Results and Discussion

Specificity of S1-specific TCRs from Non-Tg and HA Tg Mice. S1 is the major I-E4-restricted CD4+ T cell determinant recognized by BALB/c mice in response to the PR8 HA (39). All PR8 S1-specific T cells isolated to date can react with a synthetic PR8 S1 peptide (SFERFEIF-PKE) corresponding to residues 110–120 of the PR8 HA polypeptide; the majority can also react with a synthetic peptide comprising amino acids 111–119 (40–43). In previous studies analyzing the reactivity of S1-specific TCRs with analogue peptides, two-thirds of PR8 S1-specific TCRs from BALB/c mice were found to cross-react with an analogue peptide S1(K113) containing an Arg to Lys substitution at residue 113 (SFKEKFEPKE); the remaining third of the PR8 S1-specific TCRs displayed undetectable reactivity with S1(K113) (43). We recently generated corresponding panels of S1(K113)-specific T cell hybridomas in non-Tg mice and in HA Tg mice expressing an HA polypeptide under the control of the SV40 early region promoter/enhancer (32). When analyzed for their reactivity with PR8 S1, 11/16 of the S1(K113)-specific TCRs from non-Tg mice were found to cross-react with the PR8 S1 determinant, one reacted with roughly 1,000-fold more PR8 S1 than S1(K113), and the remaining 4/16 displayed no detectable reactivity with PR8 S1. Thus, the majority (roughly two-thirds) of PR8 S1- and S1(K113)-specific TCRs from BALB/c background mice cross-react with both determinants, and in each case a minority (roughly one-third) reacts only with the immunizing determinant. By contrast, none of the 15 S1(K113)-specific TCRs from HA Tg mice displayed substantial cross-reactivity with PR8 S1, which is a neo-self peptide in HA Tg mice. Only two of the S1(K113)-specific T cell hybridomas displayed any detectable reactivity with PR8 S1, delivering partial signals in response to high concentrations of PR8 virus (32). Thus, the specificity of the T cell response to S1(K113) is altered by negative selection of T cells that can cross-react with PR8 S1, a finding which is consistent with the expression of HA in many tissues (including the thymus) in HA Tg mice.

A Dominant TCR Gene Segment Combination Is Induced in Non-Tg Mice in Response to S1(K113) and PR8 S1. To examine the genetic basis for the generation of S1(K113)-specific TCRs, the hybridomas were analyzed for the sequences of their TCR-α and -β chain V and J regions. Two productive α chain sequences were isolated from 4 of the 31 S1(K113)-specific hybridomas, because of incomplete allelic exclusion at the TCR-α locus (23, 44, 45). In all cases, only a single productive β chain rearrangement was isolated. The Vα and Vβ gene segments were assigned to families based on homology with previously published sequences (2). In the case of members of the Vα1, Vα4, Vα8, and Vα10 gene families (which comprise the majority of the Vα gene segments used by the hybridomas), the Vα gene segments were further divided and given arbitrary subfamily designations (e.g., Vα4.1, Vα4.2, etc.) based on amino acid differences that are likely to represent distinct germline gene segments. Within these families, individual gene segments could differ by as few as two amino acid differences (such as Vα4.1 and Vα4.3) or as many as 28 amino acid differences (such as Vα4.1 and Vα4.6) (data not shown).

The TCR V and J gene segments and the α and β chain junctional amino acid sequences of the 16 S1(K113)-specific T cell hybridomas from non-Tg mice are shown in Table 1, along with 13 PR8 S1-specific TCRs from BALB/c mice (22). The antigen used to induce each hybridoma is shown, and the hybridomas are grouped according to their ability to react with the PR8 S1 and S1(K113) determinants. Many different Vα and Vβ gene segments were found to be used in unique combinations among these S1-specific TCRs. 6 Vβ and 14 Vα gene segments (derived from six different Vα gene families) are represented. Likewise, 17 Jα and 9 Jβ gene segments were used, and the amino acid sequences of the associated α and β chain J regions were correspondingly diverse.

Despite this overall diversity, more than half (11/21) of the TCRs that cross-react with both PR8 S1 and S1(K113) expressed either Vα10.1 or 10.2 (which differ from each other by only two amino acid substitutions at residues α43 and α82 in the numbering system of Chothia et al.; 46) in association with Jα47, Vβ8.3, and Jβ1.3. This set includes one TCR (MB1-1) that used a Vα10/Jα47-Vβ8.3/Jβ2.4 combination, the β chain of which was processed during recombination to encode the same junctional amino acids as the Jβ1.3 gene segment. Two other TCRs used a Vα10.1-Vβ8.3/Jβ1.3 combination but used different Jα gene segments. The dominance of the Vα10/Jα47-Vβ8.3/Jβ1.3 combination in the response of non-Tg mice to the S1 analogues is emphasized in the case of hybridomas generated from mice S533 and S629, in which two different
Table 1.  TCRs Used by S1-specific Hybridomas from Non-Tg Mice

| Hybridoma Induced by Vα | α Juncions | Jα | Vβ | β Juncions |
|-------------------------|------------|----|-----|------------|
| Cross-reactive hybridomas* |            |    |     |            |
| SB3-5 S1 (K113)         | CAMDGAGSKL...IF | 10.1 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| SB5-534 S1 (K113)       | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| S520-8 S1 (K113)        | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| S533-1 S1 (K113)        | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| S533-6 S1 (K113)        | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| S629-17 S1 (K113)       | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| S629-52 S1 (K113)       | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| P1D3A6 PR8 S1           | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| LD1 PR8 S1              | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| 1E10 PR8 S1             | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| MBI-1 PR8 S1            | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| 7/6AH1 PR8 S1           | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| S533-30 S1 (K113)       | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| SB2-28 S1 (K113)        | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| MT1-14 PR8 S1           | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| MT1-27 PR8 S1           | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| S520-2 S1 (K113)        | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| S533-10 S1 (K113)       | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| MT1-33 PR8 S1           | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| MT1-7 PR8 S1            | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| 2B11 PR8 S1             | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| S1 (K113) specific*     |            |    |     |            |
| SS19-12 S1 (K113)       | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| SB1-2 S1 (K113)         | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| SS19-5 S1 (K113)        | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| SS19-6 S1 (K113)        | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| SB1-18 S1 (K113)        | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| PR8 S1 specific*        |            |    |     |            |
| LD3 PR8 S1              | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| MT1-6 PR8 S1            | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |
| V2.1 PR8 S1             | CAMDGAGSKL...IF | 10.2 | 8.3 | CASSVGAGNTL...YF | 1.3 |

V and J gene segments were assigned to families based on homology with previously described sequences (2, 38, 53, 54), with members of the Va1, -4, -8, and -10 gene families assigned arbitrary subfamily designations (e.g., Va10.1 and Va10.2) based on differences in their deduced amino acid sequences. In the case of a chains, between 20 and 85 amino acid residues NH2 terminal to the conserved Cys at a90 were determined; for b chains, 20-40 amino acid residues NH2 terminal to the conserved Cys at b92 were determined. The analogue used for the generation of each hybridoma (either SI[K113] or PR8 S1) is indicated. Although two productive a chain rearrangements were detected in hybridomas SB3-3 and LD1, the similarity of their Va10/Ja47 rearrangements to those of other SI-specific hybridomas suggests that these a chains were used to generate specificity for SI. The TCR sequences of the majority of PR8 S1-induced hybridomas have been described (22). The b chain junctions of hybridomas 7/6AH1 and MT1-7 contain corrections of the previously published sequences, and the LD1 Va10/Ja47 a chain is a previously unreported second a chain rearrangement from this hybridoma. The nucleotide and amino acid sequences of these hybridoma TCRs, along with those presented in Table 2, are available from EMBL/GenBank/DDBJ under accession numbers U21393-U21456, U21477-U21481, and M34194-M34219.

*Hybridomas were designated as cross-reactive if the concentrations of SV and PR8 virus and/or SI[K113] and PR8 SI synthetic peptides required to induce half-maximal responses differed by <50-fold. If the half-maximal concentrations differed by >50-fold, hybridomas were designated as either SI[K113]- or PR8 S1-specific (22, 43).

In addition to the reported a chain, a nonproductive a chain rearrangement was also detected from each of these hybridomas (data not shown), allowing unambiguous assignment of the a chain used to generate antigen specificity.
examples of Vo10/Ja47-VB8.3/JB1.3 TCRs were isolated from each mouse.

The α and β chain junctional sequences of the Vo10/Ja47-VB8.3/JB1.3 TCRs were all of identical length and contained similar sequences (Fig. 1 A). The majority of the α chains varied at a single amino acid residue (amino acid α94), which was occupied by six different amino acids (Ala, Arg, His, Leu, Phe, and Pro) among the 11 examples of Vo10/Ja47 α chains. Likewise, the β chain junctional sequences were all of identical length and contained a Gly-Ala-Gly motif at residues αβ97–99 that was generated by a variety of unique recombination events and nontemplated nucleotide additions involving the VB8.3, Dβ1, and JB1.3 gene segments (Fig. 1 A). Two TCR-β chains (from hybridomas LD1 and E10) expressed the sequence Gly-Ser-Gly at residues αβ97–99, where the Ser at αβ98 can be encoded by the germline JB1.3 gene segment. As noted above, in one instance a different gene segment (Jβ2.4) was processed to replace a germline-encoded Gln residue with the Gly residue at αβ99 that is contained in the germline sequence of the JB1.3 gene segment.

The selection of Vo10/Ja47-VB8.3/JB1.3 TCRs containing a conserved junctional sequence strongly suggests that this Gly-Ala-Gly motif is important in generating specificity for the SI(K113)-I-Eα and PR8 SI-I-Eα complexes. To examine this directly, non-Tg mice were immunized with $1 analogue viruses, and LNC were analyzed according to size (Fig. 2 B); in each case multiple reaction products were detected, and in-frame β chain rearrangements were detected at a three-base interval (47, 48). These major reaction products were amplified in roughly equal proportions from LNC of a naive BALB/c mouse. By contrast, LNC from a BALB/c mouse immunized with PR8 virus gave rise to two- to threefold higher levels of the reaction product corresponding to the characteristic VB8.3/Gly-Ala-Gly/JB1.3 rearrangement (peak 3) (Fig. 2 C). A similar induction of peak 3 reaction products was observed after immunization with SW virus, containing the SI(K113) substitution (data not shown). As a negative control, additional mice were immunized with the virus RV6, which is a variant of PR8 that contains a Glu to Lys substitution in SI at position 115 (SFERF_KIFPKE). The RV6 S1 determinant is presented by I-Eα but induces T cells that are non-cross-reactive with PR8 S1 (49). Non-Tg mice immunized with RV6 displayed no induction of peak 3 reaction products, resembling naive mice (Fig. 2 D). Thus,

Figure 1. TCR-α and β junctional sequences of structurally related TCRs. The nucleotide and deduced amino acid sequences of the V/J junctions of selected hybridomas from non-Tg (A) and HA Tg mice (B) are shown. Nucleotides likely to have been contributed by the V and J gene segments, and nucleotides likely to have been contributed by the Dβ1 gene segment are underlined. The β chain junction of hybridoma MB1-1 is likely to have used the Dβ2 gene segment rather than Dβ1. Vo, VB, Ja, and JB gene segment assignments were made as described in Tables 1 and 2. Amino acid residues are numbered according to Chothia et al. (46), with the conserved Cys residues present near the 3' end of the V segments assigned as residue 90 or 92 for α and β chains, respectively. The reactivity of hybridomas for SW and PR8 viruses was previously determined (32) and is indicated as the ratio of the doses of SW and PR8 virus that induce half-maximal stimulation. A value of 1.00 indicates that a hybridoma reacts equally with both viruses; a value of >1.00 indicates that a hybridoma is more reactive to PR8 virus, and a value of <1.00 indicates that a hybridoma is more reactive to SW virus. *These hybridomas also contained a second in-frame α chain rearrangement. $In addition to the reported α chain, a nonproductive α chain rearrangement was also detected in each of these hybridomas. $Not determined.
TCR-β chains that use the characteristic Vβ8.3/Gly-Ala-Gly/Jβ1.3 rearrangement were induced in LNC from non-Tg mice in response to immunization with PR8 S1 and S1(K113).

The predominant expression of TCRs using the Vα10/Jα47-Vβ8.3/Jβ1.3 combination in non-Tg mice is striking since all other TCRs, either cross-reactive or PR8 S1 or S1(K113) specific, used unique combinations of TCR V and/or J gene segments. The bias toward Vα10/Jα47-Vβ8.3/Jβ1.3 expression could reflect overexpression of this gene segment combination in the preimmune repertoire. For example, preferential recombination of these gene segments or prior expansion in response to a cross-reactive foreign (or perhaps self) antigen could increase the frequency of T cells expressing these TCRs. However, analysis of LNC from naive mice did not provide evidence that TCR-β chains corresponding in length to Vβ8.3/Gly-Ala-Gly/Jβ1.3 rearrangement were already expanded relative to other TCR-β chains (Fig. 2 B). Alternatively, the Vα10/Jα47-Vβ8.3/Jβ1.3 combination might be particularly well suited to generating PR8 S1- and S1(K113)-specific TCRs. Although TCRs that can react only with PR8 S1 or S1(K113) are present in the T cell repertoire of non-Tg mice, Vα10/Jα47-Vβ8.3/Jβ1.3 TCRs that cross-react with both analogues were repetitively isolated in each response and contained similar junctional sequences that included the non-germline-encoded Gly-Ala-Gly motif in their β chain junction. These TCRs contained variation at individual amino acid residues in the α chain (residue α94) and β chain (residue β96) junctions, which could modify the relative reactivity of these TCRs for PR8 S1 and S1(K113) up to tenfold (e.g., hybridomas S533-1 and S533-6; Fig. 1 A). In most cases, however, differences in the α94 and β96 junctional sequences (as well as the use of Vα10.1 versus Vα10.2 gene segments) had little effect on the relative reactivity of these TCRs with the different S1 analogues.

The overall homology between these TCRs suggests that they share an intrinsic reactivity toward both S1 analogues and are likely to adopt a common orientation that allows similar interactions with both PR8 S1-I-E^d and S1(K113)-I-E^d. By this model, the remaining TCRs, which use diverse combinations of V gene segments, might adopt unique orientations and confer distinct specificities (either cross-reactive or non-cross-reactive) for the S1 analogues.

**Negative Selection by Neo--self S1 Eliminates the Dominant TCR Gene Segment Combination from HA Tg Mice.** The α and β chain V and J gene segments and junctional sequences used by S1(K113)-specific TCRs from HA Tg mice are summarized in Table 2. A diverse array of gene segments was again used in many unique combinations to generate S1(K113)-specific TCRs; 12 different Vα genes from 6 Vα gene families, 12 Jαs, 7 Vβs, and 9 Jβs were represented among the 15 TCRs. None of the S1(K113)-specific TCRs from HA Tg mice used the Vα10/Jα47-Vβ8.3/Jβ1.3 combination that dominated the cross-reactive TCRs from non-Tg mice, a finding which is consistent with the negative selection of TCRs that can react with the PR8 S1 determinant (32). Interestingly, two TCRs (from hybridomas S598-20 and S322-427) used β chains encoded by Vβ8.3 and Jβ1.3 that included the Gly-Ala-Gly (or Gly-Ser-Gly) sequence (Fig. 1 B) identified as being critical in establishing the specificity of S1-specific TCRs from non-Tg mice. The Vβ8.3/Gly-Ala-Gly/Jβ1.3 TCR-β chains from HA Tg mice, however, were one amino acid longer than those from non-Tg mice, containing two Gly residues in place of the single amino acid residue found at the variable position β96 in TCRs from non-Tg mice. In addition, in place of the Vα10/Jα47 pairing repetitively identified among TCRs from non-Tg mice, the Vβ8.3/Gly-Ala-Gly/Jβ1.3 β chains of S598-20 and S322-427 were found to be associated with α chains using the same Vα8.3/Jα40 gene segment combination, differing from.
Table 2. TCRs Used by S1(K113)-specific Hybridomas from HA Tg Mice

| Hybridoma | Induced by | Va | α Junc | Jα | VB | β Junc | Jβ |
|-----------|------------|----|--------|----|----|--------|----|
| S598-20*  | S1(K113)   | 8.3| CALRYGNEKI... TF| 40 | 8.3| CASSGGGAGNTL... YF| 1.3|
| S322-427* | S1(K113)   | 8.3| CALRYGNEKI... TF| 40 | 8.3| CASSGGGGAGNTL... YF| 1.3|
| S532-28*  | S1(K113)   | 8.3| CALRAYGNEKI... TF| 40 | 10 | CASSQAGGAEQ... FF| 2.1|
| S106-1*   | S1(K113)   | 8.3| CALTHOSSGNKL... IF| 25 | 1  | CASSPPDRGIEQ.YF| 2.7|
| S518-6*   | S1(K113)   | 8.2| CAMRQQGSAKL... IF| 48 | 8.3| CASSRRDKNTL... YF| 2.4|
| S322-507  | S1(K113)   | <1.2| CAMRQQGSAKL... IF| 48 | 8.1| CALRTGGGNNKL... TF| 47 |
| S630-62   | S1(K113)   | <1.2| CALRTGGGNNKL... TF| 47 | 8.3| CASSDRGGERL... FF| 1.4|
| S518-7*   | S1(K113)   | 4.7| CALARGVNNRI... FF| 24 | 1  | CASSQEGGGRQNTLYF| 2.4|
| S238-17   | S1(K113)   | 4.3| CALDHTGGADRL... TF| 37 | 1  | CASSQRLGSEYQ... YF| 2.7|
| S324-228  | S1(K113)   | <3  | CAAYNAGAKL... TF| 32 | 8.2| CASGLTANDY... TF| 1.2|
| S518-12   | S1(K113)   | 4.2| CVLATSQGKQ... YF| 13 | 10 | CASSFRQPNTEV... FF| 1.1|
| S102-26   | S1(K113)   | 4.5| CALTANSQGTQ... RF| 11 | 10 | CASSTRGGGARNTQ... YF| 2.5|
| S532-8*   | S1(K113)   | 1.1| CAARINSGTYQ... RF| 11 | 2  | CASSRQVSNPL... YF| 1.6|
| S518-5    | S1(K113)   | 10.3| CAXKDRGSLAGRL.HF| 15 | 6  | CASSQGAEV... FF| 1.1|
| S101-1*   | S1(K113)   | 15 | CAAASRTQYQGF... TF| 41 | 2  | CSAWGAQQNTL... YF| 2.4|

V and J gene segments were sequenced and assigned to gene families as described in Table 1. Two functional α chain transcripts were detected in hybridomas S322-507, S630-62, and S324-228; the similarity of the Va8/J147 rearrangements from hybridomas S322-507 and S630-62 suggests that these α chains are used to generate specificity for S1(K113). The nucleotide and amino acid sequences of these hybridoma TCRs, along with those presented in Table 1, are available from EMBL/GenBank/DDBJ under accession numbers U21393-U21456, U21477-U21481, and M34194-M34219.

*In addition to the reported α chain, a nonproductive α chain rearrangement was also detected from each of these hybridomas (data not shown), allowing unambiguous assignment of the α chain used to generate specificity for S1(K113).

Consistent with the failure to isolate hybridomas expressing the Va10/Va47-VP8.3/Jβ1.3 clonotype that was isolated from non-Tg mice was not isolated among hybridomas from HA Tg mice. Since the S1(K113)-specific TCRs from HA Tg mice are at least 1,000-fold more reactive with S1(K113) than with PR8 S1 (Fig. 1 B), these changes modified the specificity of TCRs expressing the VP8.3/Gly-Ala-Gly/Jβ1.3 β chain and allowed T cells expressing these TCRs to evade negative selection by the neo-self PR8 S1 peptide.

Consistent with the failure to isolate hybridomas expressing the Va10/Va47-VP8.3/Jβ1.3 clonotype from HA Tg mice, TCR-β chains corresponding in length to the VP8.3/Gly-Ala-Gly/Jβ1.3 rearrangement (i.e., peak 3 rearrangements) were not induced in LNC from HA Tg mice that had been immunized with PR8 (Fig. 2 E) or SW virus (data not shown). In addition, we have not observed induction of the peak 2 rearrangement that would correspond to the longer VP8.3/Gly-Ala-Gly/Jβ1.3 rearrangement in LNC from SW virus–immunized HA Tg mice (data not shown). As indicated by the relative frequencies of hybridomas from HA Tg and non-Tg mice, however, S1(K113)-specific TCRs containing VP8.3/Gly-Ala-Gly/Jβ1.3 rearrangements are relatively rare in HA Tg mice. In this regard, it is noteworthy that coordinate changes, i.e., both longer β chain junctional sequences and pairwise association with a Va8.3/Jα40 combination, appeared to be necessary to alter this TCR sufficiently to allow it to evade negative selection. For example, TCRs using the longer VP8.3/Jβ1.3 β chain in association with Va10/Va47 were not isolated, despite the high frequency of TCRs using this gene segment combination among S1(K113)- and PR8 S1-specific TCRs from non-Tg mice. Simple changes in the α and β chain junctional sequences might not be able to sufficiently modify the specificity of these TCRs away from reactivity with PR8 S1, even though changes in junctional amino acid sequences can substantially alter the specificity with which peripheral TCRs react with peptide analogues (18, 20, 21). This may be because the Va10/Va47-VP8.3/Jβ1.3 combination has limited adaptability and cannot be modified by junctional variation to generate a TCR that reacts with S1(K113) but does not cross-react with PR8 S1. Alternatively, because the signaling threshold that directs negative selection of TCRs by self peptides may differ
from that necessary to activate peripheral T cells (50, 51), substantial structural changes may be necessary to allow a TCR to evade negative selection by a self peptide. By this model, junctional variation might be able to modify the specificity with which peripheral T cells recognize PR8 S1 and S1(KI13). However, greater modifications may be necessary to alter reactivity with PR8 S1 sufficiently to prevent negative selection of developing thymocytes in HA Tg mice.

With respect to the genetic basis for the negative selection of self-reactive TCRs by self peptides, the other significant finding lies in the diversity of the TCRs used by HA Tg mice in response to S1(KI13): 14 out of 15 of these TCRs expressed unique combinations of Vα, Jα, Vβ, and Jβ gene segments. We had previously postulated that the degree of similarity with self antigens might limit the structural diversity of T cell responses to antigens that are close homologues of self peptides, based on the finding that TCRs induced in response to PR8 S1 used a much more diverse array of gene segments than had been reported for TCRs induced in response to homologues of self antigens (22). Similar conclusions have been reached by others (29), based on the highly diverse repertoire of TCRs specific for an H-2Kd-restricted foreign determinant from Plasmodium berghei (23) and the limited TCR repertoire in response to an HLA-Cw3 determinant, which is a close homologue of a putative self determinant and is restricted by the same MHC molecule (52). In the current study, the diversity of the S1(KI13)-specific TCRs in HA Tg mice, despite negative selection of TCRs that can cross-react with the neo-self PR8 S1, clearly indicates that homology with self peptides need not limit the structural diversity of T cell responses.

Indeed, because of the dominance of the Vα10/Jα47-Vβ8.3/Jβ1.3 combination among S1-specific TCRs from non-Tg mice, the S1(KI13)-specific TCRs from HA Tg mice were actually more diverse with respect to V and J gene segment usage than those from non-Tg mice. Instead of tolerance limiting the diversity of some T cell responses, the results here suggest that structurally restricted T cell responses arise when the peripheral T cell repertoire contains V gene combinations that are well suited to recognize certain peptide-MHC complex(es), perhaps because of an ability to adopt orientations that favor interactions with a particular complex(es). If these germline gene combinations are not available (as occurred in HA Tg mice due to negative selection of Vα10/Jα47-Vβ8.3/Jβ1.3 T cells), a more diverse repertoire of TCRs can be induced.

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