TCR-α CDR3 Loop Audition Regulates Positive Selection

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How positive selection molds the T cell repertoire has been difficult to examine. In this study, we use TCR-β-transgenic mice in which MHC shapes TCR-α use. Differential AV segment use is directly related to the constraints placed on the composition of the CDR3 loops. Where these constraints are low, efficient selection of αβ pairs follows. This mode of selection preferentially uses favored AV-AJ rearrangements and promotes diversity. Increased constraint on the α CDR3 loops leads to inefficient selection associated with uncommon recombination events and limited diversity. Further, the two modes of selection favor alternate sets of AJ segments. We discuss the relevance of these findings to the imprint of self-MHC restriction and peripheral T cell activation. The Journal of Immunology, 2006, 177: 2477–2485.

The intrinsic peptide-MHC reactivity within the preselection repertoire (1, 2) forms the basis for self-peptide MHC-driven customization to select the most useful self-restricted TCRs (3, 4). Structural studies have revealed conserved features of the engagement of TCR with peptide-MHC mediating T cell activation including the extent of the buried interface and the docking orientation (reviewed in Refs. 5–8). Germline-encoded variable domain CDR1 and CDR2 loops predominately interact with MHC while the hypervariable CDR3 loops generally make more contact with peptide. Within this framework, the roles of the CDR loops vary considerably. In contrast, much less is known about how TCR interacts with the peptide-MHC to direct positive selection.

Positive selection imprints self-MHC restriction promoting peptide recognition in the context of the selecting MHC haplotype. The benefit of self-MHC restriction derives from the focus of TCR reactivity being placed on MHC and, by implication, limiting the participation of self-peptide. Increased contact with antigenic peptide can provide the increased affinity required for activation of peripheral T cells. Defining the role of peptide in positive selection has proved elusive, although two features of selecting peptides have emerged. First, selecting peptide-MHC complexes bind to TCR with lower affinity in comparison with antigenic peptide-MHC (9); low density antigenic peptide-MHC complexes can also elicit positive selection (10, 11). Second, the peptide requirement is relaxed, with multiple peptides mediating selection of particular TCRs, and individual peptides competent to select diverse repertoires (12). The view that contacts between TCR and MHC alone can mediate positive selection has also been proposed (13).

An attractive approach to circumvent the problem of combinatorial complexity within the repertoire is to express a transgenic β-chain and focus on selection of the α repertoire (reviewed in Ref. 14). Here, we extend this approach to investigate how reactivity to self-peptide and MHC is balanced during selection using the HYD^Smcy system (15) which offers several advantages. First, the response exhibits self-restriction as the presenting MHC molecule, H2-D^b, also mediates thymic selection (16). Second, the TCR-β/α rearrangement of the prototypic D^Smcy-specific receptor, B6.2.16, is representative of the wild-type (WT) response. Third, the TCR-α variable segment AV9 is strongly selected in B6.2.16β mice skewing the repertoire to D^Smcy reactivity (17). We show AV9 selection is highly dependent on MHC haplotype; non-H2^d haplotypes select AV9 poorly and generate repertoires with low D^Smcy reactivity. Selection of AV9 by H2^d and the corresponding reactivity to D^Smcy thus represent the imprint of MHC restriction within the repertoire and its translation into a peripheral T cell response, respectively.

The mechanism driving selection of AV9 becomes apparent when AV9 CDR3 libraries from different MHC haplotypes are analyzed. A novel approach was applied in which postselection TCR-α joining (AJ) segment use is compared with the preselection recombination blueprint (18) to indicate selective pressure. Two modes of selection were discerned reflecting a structural division of germline AJ segments. The first mode predominates in the context of H2^d and preferentially selects rearrangements using longer, flexible AJ segments (type I) and is consistent with a reduced role for AV9 CDR3 loops in contacting self-peptide. Consistent with this, we show that the AV9-D^β interaction has a key role within the B6.2.16β/AV9 TCR/D^Smcy complex. The second mode of AV9 selection, favored in the presence of non-H2^d haplotypes, is consistent with increased involvement of peptide-MHC during selection and uses TCRs with shorter, more rigid CDR3 loops (type II).

A striking parallel is seen among B6.2.16β/AV9 rearrangements which recognize the D^Smcy complex. Increased affinity is achieved by AV9 CDR3 loop contact with the peptide and is strongly associated with a shift from type I to type II rearrangements. These data lead to a general model for positive selection in which α-chain selection can play a key role in imprinting self-restriction and in so doing maintains high TCR-CDR3 loop diversity. Implications of these findings are discussed in the contexts of T cell selection and immunity.
Materials and Methods

Mice

B6.2.16 TCR-β (C57BL/6 background) transgenic mice have been described (19). H2b, H2a, and F1H2a/b. B6.2.16 TCR-β-transgenic mice were made by appropriate crosses with B10.BR (H2b) and DBA/1 (H2a) mice obtained from Harlan Olac. Bone marrow chimera were made by irradiating (900 rad) which had been depleted of NK cells by i.p. injection of 100 μg of PK1.36 anti-NK1.1 Ab. A total of 1×10^7 T cell-depleted bone marrow cells from H2b B6.2.16 TCR-β-transgenic donors were injected i.v. T cell depletion was done using anti-Thy1.2 Dynabeads following the manufacturer’s instructions (Dynal Biotech). All mouse procedures were approved by a local ethical review process and under Home Office authority.

Peptides

WT and variant peptides (substituted residues are in bold type) (KCSRN QYLY (WT), ACSRNRQYL (A1), KASRNQYL (A2), KCSNARQYL (A4), KCSRRNQYL (A6), KCRSNRQYL (A7), KCSENRQY (A8), AAS NRRQYL (D′/AA), and ISSRNQYLD′/SSmyc) were synthesized by the Central Research Resources Unit of the Clinical Sciences Centre (London, U.K.). Peptide binding to H2d was tested using stabilization of D′ on the TAP-deficient cell line RMA-S (20) as previously described (15).

Cell culture

D′Smcy-specific T cell clones were isolated and maintained as previously described (15). Briefly, splenocyte suspensions were cultured with 100 nM D′ Smcy peptide and limiting dilution was performed after 10 days. Clones S1FP and SSFP were derived from immunized (syngeneic male spleen) C57BL/6 females. Clones B61 and BR3 originate from nonimmunized bone marrow chimeras. B61 derives from a B6.2.16 background into B10.BR (H2b) female recipient and BR3 from a B6.2.16 background into B10.BR (H2a) female recipient.

Flow cytometry

PE-labeled D′Smcy tetramer was obtained from ProImmune. Allophycocyanin-conjugated anti-CD4, PerCP-conjugated anti-CD8, H2D′-Ig DimerX and PE-conjugated anti-mouse IgG1 were purchased from BD Pharmingen. For tetramer staining, cells were incubated with the tetramer for 15 min at room temperature, washed, and subsequently incubated with the indicated Abs. DimerX was loaded with peptide and used for staining according to the manufacturer’s instructions. Samples were run on a FACScalibur or FACScan instrument and data was analyzed using CellQuest software (BD Biosciences).

Cell isolation

Splenic CD8+ T cells were selected using anti-CD8 Dynabeads following the manufacturer’s instructions (Dynal Biotech). For cell sorting, lymph node (in-FACSCalibur or FACScan instrument and data was analyzed using according to the manufacturer’s instructions. Samples were run on a FACScalibur or FACScan instrument and data was analyzed using CellQuest software (BD Biosciences).

Primers

Most AV primers and the ACa primer have been described previously (21, 22). Sequences of primers AV7, 12, 14, 15, 19, ACR, and ACseq are: AV7, TCTGTAGTCTTCACAGAAATC; AV12, CCTCTGTATCTCTCGT CGACC; AV14, TCCTGTTGGACCAAAAAAGAC; AV15, GAGGCAA GACCTATAGGTG; AV19, CACCTTTCAGGCGCGTCGAA; ACR, GAAACCCAGACCTGCTGTG; ACseq, CATAGCTTTCATGTC CAGC. The BV5, BV6, and BV13 primers are respectively: TTGACTTCT GTGGCCAGCGTGTG, GAAGAGCCTCTGGCCAAGCACA and CAG GAAACAGCTGATG.

RNA preparation and cDNA synthesis

RNA was extracted with the RNAzol B reagent (Biogenesis). CDNA was synthesized with Superscript II RNase H- Reverse Transcriptase (Invitrogen Life Technologies) and random hexamers (Amersham Biosciences) using 50–250 ng of RNA in a final volume of 60 μl.

TCR analysis of D′Smcy-specific clones

BV usage was determined by flow cytometry with a panel of specific Abs. AV usage was determined by PCR using the 20 AV-specific primers and the ACA primer. For J segment usage and CDR3 analysis, PCR was performed using the BV8 and BC primers (TCR-β) and the AV9 and ACA primers (TCR-α) and PCR products were cloned using the TA Cloning kit (Invitrogen Life Technologies) and sequenced using the M13R primer.

Semiquantitative analysis of AV usage

Exponential PCRs were generated by subjecting 1 μl of cDNA sample to 25 PCR cycles using an AV-specific primer and the ACA primer. The same procedure was followed for all AV-AC combinations. Reaction products were run on a 1% agarose gel and analyzed by Southern blot. A probe specific for the C region of the TCR-α chain was synthesized with the ACAs and ACR primers and labeled with 32P using the Ready To Go DNA Labeling kit (Pharmacia Biotech). Signal quantification and band intensity analysis were performed using the Storm 820 system and ImageQuant software (Amersham Biosciences). Nomenclature for TCRAV has been previously described (23).

AV9 TCR sequencing

cDNA was amplified by PCR with the AV9 and ACA primers and PCR products were cloned into the pCR2.1 vector using the TA Cloning kit (Invitrogen Life Technologies). PCR was conducted on individual colonies again using the AV9 and ACA primers and sequenced using the ACseq primer. Nomenclature for TCRAJ has been published previously (24).

Results

Invariant BV8.2 in association with variable AV9 dominates the D′Smcy response

The prototype D′Smcy-specific TCR, B6.2.16, is composed of BV8.2-BJ2.3 and AV9 (19, 25). β-chain use in this response is highly restricted as further D′Smcy-specific CD8+ T cell clones used identical β-chain rearrangements (Table I). TCR-α use also resembles the prototype in being constrained to AV9.

In B6.2.16β-transgenic mice, selection of AV9 into the CD8+ T cell repertoire is strongly favored (17). The resulting repertoire is highly skewed to D′Smcy recognition because the vast majority of AV9 rearrangements facilitate binding to D′Smcy tetramer (17). The association of the B6.2.16β chain with AV9 in positive selection and peripheral Ag recognition of the Smcy peptide indicates a mechanistic link between these two peptide-MHC-driven processes.

MHC haplotype directs α-chain selection in B6.2.16β-transgenic mice

To understand the molecular mechanism underlying skewed AV selection, we established the role of MHC haplotype in directing AV selection. AV use was determined within splenic CD8+ T cells by semiquantitative AV transcript analysis. Fig. 1a confirms the previously described skew to AV9 among H2b-selected B6.2.16β CD8+ T cells (17).

Table I. D′Smcy CD8+ T cell clones have restricted TCR use

| Clone  | BV   | CDR3β | BJ  | AV   | CDR3α | AJ   |
|-------|------|-------|-----|------|-------|------|
| B6.2.16 | 9.2  | GDNSEETL | 2.3 | 9    | EGDQCGGAIK | 57   |
| S1FP  | 8.2  | GDNSEETL | 2.3 | 9    | EGDQCGGAIK | 33   |
| SSFP  | 8.2  | GDNSEETL | 2.3 | 9    | EGDQCGGAIK | 56   |

Two D′Smcy CD8+ T cell clones were isolated from different female B6 mice immunized with syngeneic male cells. TCR AV and BV use was determined. The expressed TCR gene segments and sequence of the CDR3 junctional regions are indicated. Both clones use a BV rearrangement identical to that of B6.2.16. The clones use different AV9 rearrangements.
H2b and H2b haplotypes select markedly different profiles with alternative dominant AV segments (AV8 and AV10 in H2b and H2b, respectively) and limited use of AV9 (Fig. 1, b and c). To determine whether decreased AV9 expression in the absence of H2b was due to negative selection, the F1 H2b× H2b B6.2.16β AV spectrum was determined. Like H2b, the F1 TCR repertoire was dominated by AV9 (Fig. 1d) supporting positive selection as the driving force behind haplotype-specific AV9 selection.

H2b selects AV9-AJ rearrangements resembling the recombination blueprint

Having established that the favored selection of AV9 with the transgenic B6.2.16β chain is dependent on MHC haplotype, we next determined the underlying mechanism. We hypothesized that this pairing makes more optimal interactions with H2b than with H2b/H2b class I-peptide complexes. Because the TCR-β chain is fixed, the only variable component within selected B6.2.16β/AV9 TCRs is the α CDR3 loop. In the absence of H2b, positive selection of the AV9/B6.2.16β combination may require a greater contribution from the AV9 CDR3 loop to compensate for suboptimal interactions of the other CDR loops. To explore this hypothesis, AV9 CDR3 loop composition among the AV9/B6.2.16β population was analyzed.

To deduce the relative selection pressure placed on TCR hypervariable loops during selection, a novel analysis was used relating the dynamics of preselection TCR-α locus recombination to the postselection repertoire. AV-AJ recombination is a directed process, with preferential joins of 3′ AV segments to 5′ AJ segments (18). As AV9 is one of the most 3′ segments, its pattern of recombination is particularly predictable, being strongly biased to 5′ AJ segments. Comparison of AV-AJ joins used in the postselection repertoire with the preselection recombination blueprint will be indicative of the pressure falling on α CDR3 loops during selection. As previously described (17), H2b-selected AV9 sequences use mainly AJ56 and 57 (Fig. 2a), located at the 5′ end of the AJ cluster and which reflect the recombination blueprint (18). H2b-selected haplotypes have a very different picture with AV9 rearrangements using AJ segments located in the central part of the locus, despite ensuing from infrequent recombining events (Fig. 2, b and c). Selection of such receptors must result from a more rigorous selective process that rejects the more common AV-AJ joins and leads to the reduced selection seen in non-H2b haplotypes. The H2b× F1 strain shows a distribution intermediate between that of the parental strains including the strong bias to 5′ AJ segment use (Fig. 2d).

H2b selects a structurally distinct class of AV9 CDR3 loops

Having ascertained that the efficiency of AV9 selection correlates with the use of commonly recombined AJ segments, we next analyzed the characteristics of the CDR3 loops. An immediate observation was that the length of the CDR3 loop is dependent on the AJ segment used rather than the selecting haplotype. For instance, CDR3 loops using AJ56 are generally longer than those using AJ33 independently of the selecting MHC haplotype (Fig. 3). A more detailed examination of AJ segment amino acid composition revealed two structurally distinct types. First, glycine content upstream from the conserved F-G-X-G motif increases with increasing germline AJ segment length reaching 20% for the longest segments (Fig. 4a). Second, in the longer J segments, glycines are often paired and/or are positioned adjacent to alanine and serine residues which also promote peptide flexibility (26). Based on length and the flexible motifs, this subset of J segments (type I) is likely to endow TCR-α CDR3 loops with properties distinct from those using the remaining shorter and less flexible AJ segments (type II). The AJ classification is shown in Fig. 4b and this nomenclature will be used to classify TCR rearrangements and CDR3 loops based on their AJ segment.

Fig. 3 shows the haplotype-selected AV9 libraries plotted according to AJ segment use, AJ segment type (I/II), and CDR3 length. Several features emerge from this analysis as summarized in Fig. 5a. First, the germline length differential of type I and II AJ segments is exaggerated following positive selection into functional CDR3 loops. Second, in all haplotypes, type I AJ segment use is focused at the 5′ end of the complex in the region of favored recombination (Fig. 3). The overall coincidence of the recombination blueprint with type I segment use is shown more clearly in Fig. 5b. Type I rearrangements located within the region of favored recombination dominate AV9 selection in the presence of H2b constituting 70% (Figs. 3, a and d, and 5a). Third, type II rearrangements which dominate non-H2b-mediated AV9 selection (Figs. 3, b and c, and 5a) bear no relationship to the recombination blueprint with type I segment use.
blueprint (Fig. 5b) with particular AJ segments being favored by each haplotype despite occurring infrequently.

AV9 CDR3 loops selected by H2b but not H2k or H2q are highly variable

If limited selection of AV9 by H2k/H2q reflects increased engagement of the CDR3 loop with peptide-MHC, AV9 CDR3 loop variability should be correspondingly lower in these haplotypes in comparison with H2b. To address this, we analyzed the most common AJ segment/length combination(s) for each haplotype (Fig. 6). In H2b, the 11-aa AJ56 type I CDR3 set (26% of sequences) showed high variation with 7 sequences among the 24 isolated. Variation in side-chain character of P3 insertions was extreme and included proline despite its frozen N-C bond which imposes a turn in the polypeptide chain. The high variation within this small sample suggests a broad range of CDR3 loops are compatible with selection by H2b. This is indeed the case as a further 8 loops of this AJ segment/length were found in the analysis described below (see Fig. 8) and a further 7 were described by Bouneaud et al. (17).

This degree of variability was not seen in the H2k-o r H2q-selected repertoires. Instead, variability was limited and common features were shared. For H2k, the most common rearrangement was 8-aa, type II AJ33 CDR3 loops (27%). A single CDR3 sequence dominated (26 of 27 isolated). The remaining rearrangements occurred with similar frequency. The 15 type II, 7-aa AJ34 set (16%) were represented by just two sequences. Again, these shared common features both had deleted the final AV9 germline-encoded residue (glutamic acid) and either retained the germline AJ34 sequence or replaced the first 2-aa (SS) with WG through deletion and insertion of ttgggg. The 17 type I, 11-aa AJ53 set (16%) was composed of three structurally similar CDR3s with conservative substitution of aromatic side chains (Y/W).

Peptide-independent interaction of AV9 with D

The majority of the B6.2.16/AV9 TCRs selected by H2b also recognize the DβSmy complex (17). This close overlap is most easily explained by both processes involving similar formats of TCR interaction with peptide-MHC. In both cases, the AV9 variable segment is crucial for recognition but the CDR3 loop is not tightly constrained consistent with it playing a limited role in peptide-MHC interaction.

This hypothesis was directly assessed in two ways using DβSmy multimers. Because the common docking orientation adopted in all MHC class I/TCR structures positions the CDR3 loop in the vicinity of the amino portion of the bound peptide, the role of this region of the Smyc peptide (KCSRNRQYL) was analyzed by residue substitution (shown in bold type): 1) I and S substitutions at p1, 2 (ISSRNRQYL) to alter the N-terminal hydrophobicity profile and 2) minimizing N-terminal side-chain size (AAISRNRQYL). Dimeric Dβ was complexed with the WT and variant Smyc peptide and used to stain the H2b-selected B6.2.16β CD8+/H11001 T cell repertoire (Fig. 7a). Binding was tolerant of both types of substitution, showing that the stable interaction of AV9/ B6.2.16β TCRs with DβSmyc is largely independent of the amino region of the peptide.

Second, we tested whether the DβSmyc multimer can be recognized by AV9 CDR3 loops selected by H2k and H2l haplotypes (Fig. 1)
despite having very different composition. This was confirmed by staining the thymic and peripheral CD8<sup>+</sup>/H<sub>11001</sub> repertoires with WT Db<sup>Smcy</sup> tetramer (Fig. 7b). To ensure recognition was not due to an alloreactive population, the analysis was done by using H<sub>2b</sub>B6.2.16<sup>+</sup>/H9252 into H<sub>2k</sub> and H<sub>2b</sub>B6.2.16<sup>+</sup>/H9252 into H<sub>2q</sub> irradiation bone marrow chimeras to direct central tolerance to H<sub>2b</sub>. Maintenance of the proportion of tetramer binding from the thymic to the peripheral repertoire shows the CD8 repertoire is stably maintained.

These data show that the strong bias to AV9 for Db<sup>Smcy</sup> recognition does not involve the CDR3 loop, rather the interaction of the AV9 CDR1/2 loops must contribute to the observed stable binding to Db<sup>Smcy</sup>.

**High-affinity D<sup>β</sup>Smcy binding selects for type II AV9 rearrangements which contact peptide**

The strong association between type II CDR3 loops and selection by H<sub>2k</sub> and H<sub>2q</sub>, but not H<sub>2b</sub>, indicates an increased contribution from the CDR3 region through specific molecular interactions with self-peptide-MHC. Although this mode of selection can be achieved by type I (AJ53 in H<sub>2k</sub>) it is more commonly associated with type II segments (AJ 23, AJ 33 in H<sub>2k</sub> and AJ 34 in H<sub>2q</sub>).

To directly determine whether type II AV9 CDR3 loops are preferentially suited for peptide-MHC engagement, we made use of the above observation that binding of the D<sup>β</sup>Smcy tetramer is largely independent of TCR-α-peptide interaction. We reasoned, however, that TCR-α-peptide interaction may become relevant among high-affinity TCRs. D<sup>β</sup>Smcy-specific populations were sorted using WT D<sup>β</sup>Smcy and D<sup>β</sup>(D227K)Smcy tetramer which does not interact with CD8 (27, 28) and binds a small, high-affinity subpopulation (Fig. 8, a and b). The WT tetramer sorted cohort had a very skewed AV9 profile with type I rearrangements representing 87.5%. This is higher than the proportion (70%) seen within the whole H<sub>2b</sub>CD8<sup>+</sup> T cell population (Fig. 5a) and is likely to reflect the ability of type I CDR3 loops to avoid steric clash with the peptide-MHC complex. The high-affinity subpopulation (Fig. 8b), however, has a very different AV profile with a 3.9-fold enrichment of type II rearrangements (12.5–49.1%) focused on AJ37 and AJ33. A further sort of the highest affinity cells bound by the mutant tetramer was further enriched to 71% type II loops (Fig. 8c). The switch from type I to type II loops involves the use of rare recombination events and produces a distribution of CDR3 size, AJ segment use, and type I/II use reminiscent of the non-H<sub>2b</sub>-selected AV9 repertoires (Fig. 3). The most common AV9/AJ33 rearrangement (CDR3: EGMDSNYQL) found in both high-affinity sorts is used by the T cell clone S1FP (Table I) allowing TCR-α-peptide interaction to be evaluated using dimeric Db folded with an alanine scan series of Smcy peptide variants at positions with potential for TCR interaction (29). S1FP was compared with clones B61 which has a typical type I TCR using the type I AJ56 segment and BR3, which, although using a type II AJ segment (AJ33) actually has a type I TCR due to n-nucleotide additions that introduce a GSS motif. For the type I clones, binding of the mutant peptide complexes was enhanced or maintained. Conversely, the type II clone lost affinity for the complex with the p1A mutation demonstrating its CDR3 loop makes a productive interaction with the N-terminal lysine of the Smcy peptide. These data support the hypothesis that type II loops are particularly suited for peptide interaction. All clones behaved identically with respect to the alanine p4-, 6-, 7-, and 8-substituted D<sup>β</sup>Smcy dimers which completely fail to bind (Fig. 8d). These data demonstrate that the B6.2.16β chain, shared by the three clones, makes multiple critical contacts with the C region of the peptide.
Discussion

A key finding of this work is a division among TCR AJ segments. Type I AJ segments are longer and characterized by consecutive, small amino acids, especially glycine, which endow flexibility to polypeptide chains (26). Within the residues contributing to the CDR3 loop, glycine content increases sharply with J segment length reaching over 20%. These motifs are positioned at or close to the apex of the loops and have the potential for structural accommodation to peptide-MHC. Further, the small side chains of the amino acids within the motifs offer fewer opportunities for noncovalent interactions with peptide-MHC.

TCR structures with different peptide-MHC ligands are available for two type I AV chains and directly show the flexibility of the motif regions (30–34). The remaining, type II AJ segments are shorter and likely to be less flexible. Use of this class correlates with greater selective pressure on the CDR3 loops during T cell selection.

We present direct evidence that the classification represents an important functional division whereby type I rearrangements are more peptide tolerant while type II loops are more suited for peptide engagement. Almost 90% of DβSmcy-specific T cells selected by H2b.B6.2.16-transgenic mice use type I AJ segments and directly show the flexibility of the motif regions (30–34). The remaining, type II AJ segments are shorter and likely to be less flexible. Use of this class correlates with greater selective pressure on the α CDR3 loops during T cell selection.

A second striking feature reflecting the functional division in AJ segment use is seen when the recombination process is considered. Postselection type I, but not type II, AJ segment use resemble the preselection recombination blueprint indicating a less rigorous selection process. This correlation applies both to the CD8+ T cell AV9 libraries derived from the four haplotypes analyzed (Fig. 3), and the WT and mutant Smcy tetramer selected libraries (Fig. 8). The requirement for Smcy peptide interaction demonstrated among the high-affinity DβSmcy population imposes a stringent filter on CDR3 composition explaining the use of rarely recombining type II AJ segments. Where such a stringent filter does not apply, such as for the lower affinity DβSmcy specific population, type I loops arising from common rearrangements can often be used.

Thus, use of common V-J rearrangements using type I segments is a strong indicator of limited peptide interaction, while preferential use of type II AJ segments arising from uncommon V-J joins is indicative of more extensive peptide engagement. By these criteria, the CD8+ T cell AV9 libraries selected by H2b and H2bSmcy reflect more limited CDR3 engagement than those derived from H2b and H2a. Favored selection of the AV9 gene segment can be traced to the lower constraints placed on AV9 TCRs to mediate positive selection. It is likely that, for H2b, favorable interactions established through the BV chain and the AV CDR1/CDR2 loops with self-peptide-MHC provide the majority of contacts necessary to reach “selecting” affinity and reduce the requirement for AV CDR3 involvement. This idea is supported by work from Sandberg et al. (35) showing that TCR affinity for MHC and peptide can be interchangeable, and by our own analysis of DβSmcy recognition which suggests a robust Dβ/AV9 CDR1/2 interaction (Fig. 7a). Analysis of CDR3 loop composition provides further support for this interpretation as the 11-aa type I AJ56 CDR3s selected commonly by H2b tolerate insertions of a broad range of structurally diverse amino acids including proline (Fig. 6). The character of the insertions thus appears less important than the length and flexibility of the loop.

FIGURE 4. AJ segment classification. a, Glycine content increases with AJ segment length. Percent glycine is calculated within the 7- to 10-aa block upstream from the conserved F-G-X-G. b, Type I and II J segments are listed. The clusters of small, flexible amino acids are indicated in bold.
In contrast, the repertoire selected by H2k is very biased to type II AV9 CDR3s. The most likely explanation is that the B6.2.16/H9252/AV9 combination does not provide sufficient contacts with either Kk or Dk molecules to dispense with a strong AV9 CDR3 contribution. Instead of adopting a more passive role, this domain is now required to participate in the selection process by establishing more extensive interaction with self-peptide-MHC. In contrast with H2b-mediated selection, in which loop flexibility is important and can be obtained with all type I AJ segments within the recombination hotspot, for H2k-mediated selection, AJ amino acid composition becomes critical. As well as the preferred use of type II loops, the formation of specific non-covalent peptide-MHC contacts would be expected to limit the diversity of CDR3 composition, resulting in the selection of very specific, almost invariant, type II AJ rearrangements (Fig. 6) positioned outside the recombination hotspot (Fig. 3). A potential link between extensive peptide contact and rigid CDR3 loops has been suggested previously (36).

The third haplotype analyzed, H2q, again generates a B6.2.16b/H9252/AV9 CD8+ T cell population skewed to type II AJ segment use (Fig. 5a). The type II TCRs mainly use the AJ34 segment and, again, show low CDR3 variability. H2q also selects type I receptors (AJ53), which although fitting into this classification, have constrained composition (Fig. 6). In this case, flexibility appears to be a secondary characteristic and, as in type II receptors, the emphasis is set on amino acid composition of the CDR3 loop.

Interestingly, evidence for the existence of two types of TCR with stricter or more relaxed peptide requirements for selection has been reported. Positive selection of the OT-I TCR is promoted by rare self-peptides with high homology to the cognate peptide (37). Conversely, selection of the B6.2.16, F5, N15, and P14 TCRs can be mediated by multiple self-peptides with minimal homology to the cognate peptide (38–40). This dichotomy mirrors the results obtained here in which two very different types of TCR emerge from the thymus depending on the selecting MHC. Thus, like most B6.2.16b/A9 TCRs selected by H2k, OT-I has short, relatively inflexible CDR3 loops (type II). Such type II TCRs may require a higher degree of shape complementarity with peptide-MHC to be selected, because their CDR3 domains may undergo minimal conformational change. It is not, therefore, surprising that only a few self-peptides with specific structural characteristics can select type II TCRs. In contrast, TCRs containing a flexible CDR3 region (type I receptors) will be better equipped to scan and adapt to different peptide-MHC complexes. This conformational plasticity may be particularly advantageous during positive selection when low-affinity TCR interactions are sufficient to rescue immature thymocytes from death. If a good “fit” between the CDR1/2 regions and the MHC molecule is attained, these contacts may contribute most of the affinity required for positive selection. In such cases, a mobile CDR3 loop will have the advantage of accommodating to the peptide-MHC surface and avoiding steric clashes. B6.2.16, F5, N5, and P14 are all type I receptors containing one, and in the case of F5 two, flexible CDR3s, hence it is likely that the range of peptides able to select these TCRs is wider.

**FIGURE 6.** High variability among AV9 CDR3 loops selected by H2b. Predicted amino acid sequences of AV9 rearrangements selected by H2b (a), H2k (b), and H2q (c). Non-germline-encoded residues are indicated in bold. The frequency of each rearrangement is indicated.

![FIGURE 6](image)

**FIGURE 7.** D^8Smcy tetramer binding does not require TCR-α-peptide contact. a, Staining of splenic H2bB6.2.16b/CD8+ T cells with PE-labeled dimeric D^8 loaded with D^8Smcy and variant peptides (substituted residues in bold type) (KCSRNRQYL), D^8AASmcy (AASRNRQYL), and D^8ISSmcy (ISSRNRQYL) peptides. Percentages of positive cells within the CD4+CD8+ gate are indicated. b, Identification of D^8Smcy-specific CD8+T cells in H2b B6.2.16b→H2b: H2b B6.2.16b→H2k, and H2k B6.2.16b→H2k bone marrow chimeras. Percentage of D^8Smcy-specific T cells within the gated CD4+CD8+ population in spleen and thymus is indicated. In H2k and H2q chimeras, cells were further gated on H2b expression to exclude cells of host origin. Analysis was performed 9–10 wk after reconstitution. The first row shows staining control performed on WT B6 CD8+ single-positive thymocytes.
Overall, our results suggest a general mechanism for repertoire selection. The model predicts that rearrangements which interact more extensively with peptide-MHC may facilitate selection of a broader CDR3 repertoire. Where a rearranged β-chain, in combination with a particular AV CDR1/2 contribution (B6.2.16/AV9 in the experimental system used here) makes sufficient contact with peptide-MHC, the required contribution of the AV CDR3 loop becomes negligible. Combinations of this type have a selective advantage because a higher proportion of rearrangements will mediate selection, increasing the representation of both the rearrangement and the AV family and ensuring TCR diversity. The model is consistent with analyses of the WT TCR repertoire which show substantially less diversity than was predicted on theoretical grounds (41), suggesting that selection narrows the repertoire, leading to the repeated use of particular rearrangements both in selection and immune responses (this study, Ref. 42). This model suggests that the structures of the invariant modules of T cell recognition MHC class I, II, and the TCR variable segments are finely calibrated to allow positive selection of a repertoire with the optimal balance of interaction placed onto MHC and self-peptide. This balance may be disturbed in autoimmune repertoires and the findings we describe offer an experimental approach into this complex issue.

An alternative interpretation for the conserved TCR pairing seen in the D<sup>8</sup>Smcy response is suggested by recent MHC-peptide-TCR structures in which AV CDR1/2 loops are responsible for positioning the CDR3 loops for optimal contact with peptide-MHC (43, 44). As this mechanism is highly peptide specific, in contrast with the selection and peripheral recognition events studied here which involve different peptides, it is very unlikely to apply to this system. Second, Kb knockout (KO) mice select a much higher proportion of AV9 into the CD8<sup>+</sup> T cell repertoire than D<sup>b</sup> KO mice, consistent with a favorable AV9/D<sup>b</sup> interaction which is maintained in the context of a diverse self-peptide repertoire (45). Finally, a recent model suggests that in addition to the cognate interaction, engagement of self-peptide-MHC complexes with TCR is required for T cell activation (46, 47), suggesting the same self-peptide-MHC complexes might be involved in both T cell selection and immune responses. Thus, the functional differences of type I and II AJ loops that came to light in the thymus are also likely to have important implications for peripheral immunity. TCRs with type I loops are likely to recruit such self-peptide-MHC complexes more easily due to their reduced peptide requirement. Indeed, flexibility of TCR loops has been suggested to contribute to cross-reactivity (34, 48), and use of type I loops may identify
more promiscuous T cells. Cross-reactivity and ease of activation may make T cells bearing type I TCRs particularly useful in the early stages of the response. Type II TCRs which are likely to achieve higher affinity (36) may become more relevant as clonal expansion increases their number. Relevant to this notion is the enrichment of type II AV9 sequences within the high affinity set of D1SmCys-specific TCRs (Fig. 8, b and c).

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Disclosures

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