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Impact of Negative Selection on the T Cell Repertoire Reactive to a Self-Peptide: A Large Fraction of T Cell Clones Escapes Clonal Deletion

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Summary

How negative selection shapes a polyclonal population of self-reactive T cells has been difficult to address directly because of the lack of means to isolate T cells reactive to a particular self-peptide. Here, using mice transgenic for the TCR-β chain of a CTL clone directed against a male-specific peptide, we compared the preimmune repertoire reactive to this peptide in male and female animals. Surprisingly, in the presence of the deleting ligand, as many as 25%–40% of reactive T cells escaped clonal deletion. A correlation was found between T cell avidity, TCRα structures, and susceptibility to negative selection. These results suggest that numerous low-affinity self-specific T cells persist in the periphery and show that a deleting ligand can specifically narrow the structural diversity of the TCR repertoire.

Introduction

Clonal deletion in the thymus and/or in the periphery is a major mechanism for the establishment of tolerance to self-peptides (reviewed in Kruisbeek and Amsen, 1996; Stockinger, 1999). In the thymus, developing T cells reacting strongly to self-peptide:self-MHC complexes are eliminated by the process of negative selection. For example, thymocytes from mice bearing a TCR specific for an epitope of the male antigen H-Y are deleted in the thymus of male animals, whereas they develop normally in female mice (Kisielow et al., 1988). It has been estimated that the whole set of self-peptides is responsible for the elimination of 50%–65% of the positively selected thymocyte population (Ignatowicz et al., 1996; van Meerwijk et al., 1997). Studies using TCR transgenic mouse strains have established that susceptibility to negative selection is mostly imposed by a threshold of avidity. Modulation of thymocytes' avidity for ligand-expressing cells in the thymus by altering TCR affinity, TCR or coreceptor expression, or ligand density has been shown to influence the outcome of negative selection (Auphan et al., 1992; Robey et al., 1992; Homer et al., 1993; Ashton-Rickardt et al., 1994; Liu et al., 1995; Alam et al., 1996; Lyons et al., 1996). While these studies have shed light on the basic principles of negative selection, they are of limited value to estimate the efficiency of clonal deletion on a complex population of self-reactive T cells.

In nontransgenic animals, the preselection repertoire specific for a given self-peptide is likely to comprise a polyclonal population of T cells harboring TCRs of various affinities. To what extent negative selection narrows this polyclonal population has been difficult to quantify directly because self-specific T cells are present at very low frequency in the naive repertoire. To circumvent this difficulty, several groups have analyzed residual self-specific T cells following immunization with the self-antigen (Cibotti et al., 1994; Targoni and Lehmann, 1998; Peterson et al., 1999; de Visser et al., 2000; Sandberg et al., 2000). However, this strategy focused only on the self-specific T cells that were able to proliferate upon immunization and thus does not discriminate between anergy and clonal deletion. In addition, the fraction of self-specific T cells that escapes negative selection cannot be precisely quantified by this approach.

To answer these questions, it is essential to quantify and characterize peptide-specific T cells directly in the preimmune repertoire. To achieve this, we used mice transgenic for the TCR-β chain of an anti-H-Y T cell clone. The original B6.2.16 anti-H-Y, H-2D^b-restricted αβ-TCR recognizes the Smcy3 peptide derived from the protein encoded by the Y-linked gene *Smcy* (Markiewicz et al., 1998). We found that Smcy3-specific CD8 T cells were present at high frequency in the preimmune repertoire of female TCR-β Tg mice, as detected by MHC-peptide tetramer staining and functional assays. In males, a lower but significant proportion of CD8 T cells were also reactive to the Smcy3 peptide. Therefore, this system offers the unique opportunity to isolate and characterize a peptide-specific T cell repertoire in the presence or in the absence of the deleting ligand. Our results identify the imprint of negative selection mediated by one self-peptide on a peptide-specific T cell repertoire.

Results

Although the technology of MHC-peptide tetramers represents a major breakthrough in the detection of antigen-specific T cells, identification of peptide-specific T cell populations in nonimmunized animals remains difficult because of the extremely low frequency of these cells in the mature repertoire. In some instances, mice transgenic for a TCR-β chain isolated from a particular αβ-TCR were found to contain an elevated number of T cells displaying the same specificity as the original TCR (Dillon et al., 1994; Verdaguer et al., 1996; Gutgemann et al., 1998; Sant'Angelo et al., 1998; Baldwin et al., 1999). In this report, we have analyzed mice expressing the transgenic B6.2.16 TCR-β chain (hereafter referred to as TCR-β Tg) derived from the B6.2.16 D^b-restricted anti-H-Y αβ-TCR, which has been recently shown to recognize the male-specific peptide Smcy3 (Markiewicz et al., 1998; Uematsu et al., 1988).

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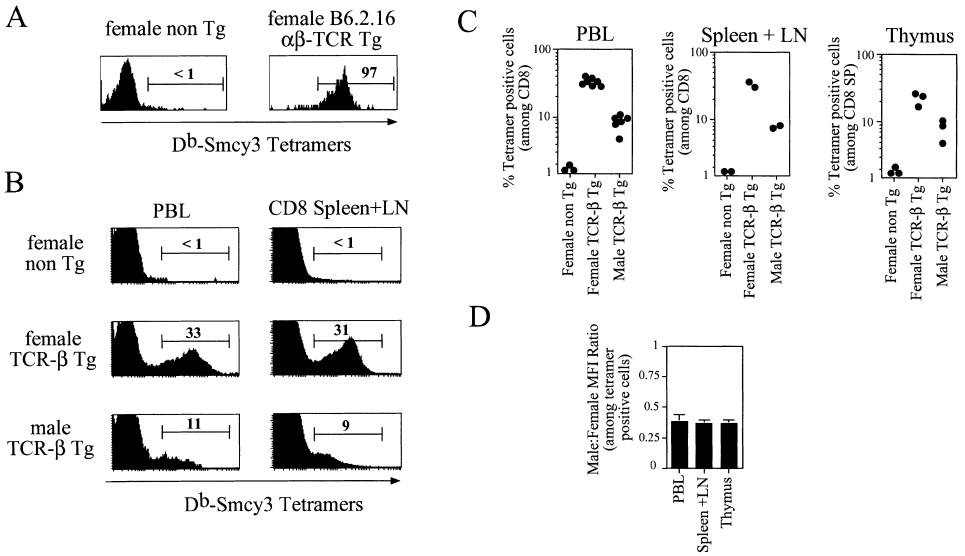

Figure 1. Detection of Smcy3-Specific T Cells Using MHC-Peptide Tetramers

(A) Specificity of D^b-Smcy3 tetramer staining. PBL from female B6 or $Rag^{-/-}$ anti-H-Y αβ-TCR Tg mice were stained with an anti-CD8 mAb and D^b-Smcy3 tetramers. Profiles are gated on $CD8^+$ cells.

(B) Comparison of D^b-Smcy3 tetramer staining of CD8 T cells from female or male TCR-β Tg mice. PBL (left column) or pooled lymph node cells and splenocytes (right column) from female non-Tg, female TCR-β Tg, or male TCR-β Tg mice were stained with anti-CD8 mAb and D^b-Smcy3 tetramers. Profiles are gated on $CD8^+$ cells.

(C) Quantitation of CD8 T cells stained with D^b-Smcy3 tetramers in the thymus or in the periphery of individual mice. For thymocytes, cells were stained with D^b-Smcy3 tetramers, anti-CD4 and anti-CD8 mAbs, and the frequency was calculated among CD8 SP cells.

(D) Reduced intensity of tetramer staining in male TCR-β Tg mice. The mean fluorescence intensity (MFI) of the tetramer-positive populations from male TCR-β Tg mice is compared to the corresponding value in female animals. Figure shows that the MFI of tetramer staining is reduced by ~3-fold in male as compared to female TCR-β Tg mice. Two to six sex-matched animals were analyzed for calculating each MFI ratio.

A Substantial Fraction of CD8 T Cells Reactive to D^b-Smcy3 Complexes Persists in Male TCR-β Tg Mice

To identify Smcy3-specific T cells, we prepared fluorescent tetramers of D^b-Smcy3 complexes. PBL and pooled lymph node cells and splenocytes from naive female non-Tg B6, TCR-αβ Tg, and TCR-β Tg mice and from male TCR-β Tg mice were stained with D^b-Smcy3 tetramers and an anti-CD8 antibody. As shown in Figure 1A, D^b-Smcy3 tetramers stained virtually all $CD8^+$ T cells from female TCR-αβ Tg mice but less than 1% of $CD8^+$ cells from female non-Tg B6 mice, demonstrating the specificity of this reagent. Interestingly, a large proportion (25%–33%) of $CD8^+$ cells from female TCR-β Tg mice were also stained with D^b-Smcy3 tetramers (Figure 1B). Thus, the presence of the TCR-β chain transgene causes the mature repertoire of female animals to contain an elevated number of tetramer-reactive cells. By contrast, no tetramer binding was observed in the CD4 population (data not shown). Because Scmy3 is a male-specific self-peptide, we then compared the tetramer staining pattern of peripheral T cells from female and male TCR-β Tg mice. As shown in Figure 1B, a significant fraction of male CD8 cells (7%–10%) were stained with the tetramers although both the frequency of stained cells and the intensity of tetramer staining was reduced in male as compared to female. In the periphery, the presence of the male-specific peptide was associated with a reduction of the number of tetramer binding cells by 60%–75% and with a 3-fold reduction of fluorescence intensity (Figures 1C and 1D). There was no significant difference in the thymus cellularity or in the CD4/CD8 ratio of male and female TCR-β Tg mice, and the vast majority of tetramer-stained peripheral T cells from female and male TCR-β Tg mice had a $CD44^{low}$ phenotype, characteristic of naive cells (data not shown). To evaluate whether the shaping of male Smcy3-specific T cell repertoire occurs in the thymus and/or in the periphery, we also stained CD8 simple-positive (SP) thymocytes with D^b-Smcy3 tetramers. Again, when compared to female CD8 SP thymocytes, male CD8 SP thymocytes exhibited reduced tetramer binding both in terms of frequency and staining intensity (Figures 1C and 1D). Thus, most differences between male and female anti-Smcy3 T cell populations are already apparent in the thymus.

As an independent approach to measure the frequency of Smcy3-reactive CD8 T cells, we stimulated splenocytes from male and female TCR-β Tg mice with various concentrations of Smcy3 peptide and took advantage of the early upregulation of CD69 expression upon TCR ligation (Testi et al., 1989). After 24 hr of culture, spleen cells were double stained with anti-CD8 and anti-CD69 antibodies. Figure 2 shows the number of CD69-positive cells as a function of Smcy3 peptide concentration. CD69 upregulation was clearly peptide specific since a D^b binding control peptide ($NP_{366-374}$) failed to stimulate male or female spleen cells (Figure 2A). The dose-response curves obtained with either male or female cells approximated a plateau that confirmed the high frequency of Smcy3-specific cells in female and to a lesser extent in male TCR-β Tg mice. Although TCR downregulation following T cell stimula-

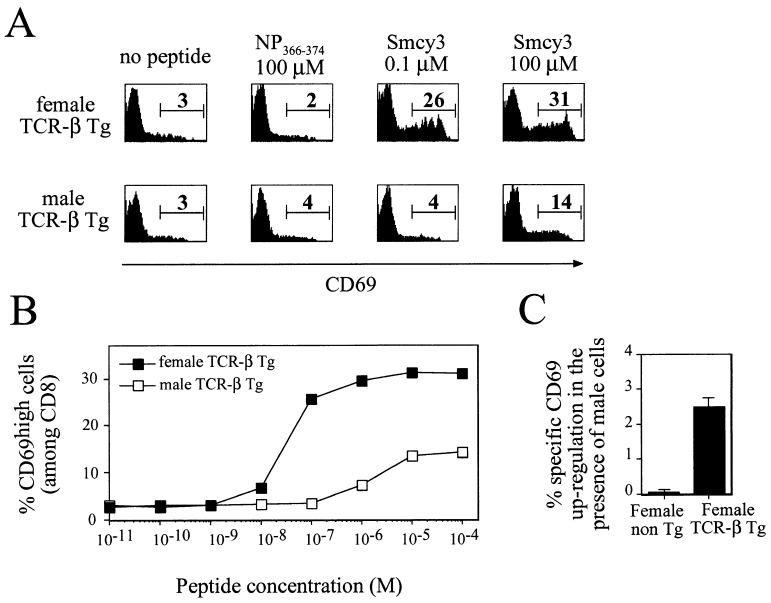

Figure 2. Detection of Smcy3-Reactive T Cells following Short Peptide Stimulation

(A) Splenocytes from female or male TCR-β Tg mice were incubated with the indicated concentration of the Smcy3 peptide. The D^b binding peptide $NP_{366-374}$ was used as a control. After 24 hr, cells were stained with anti-CD8 and anti-CD69 mAbs.

(B) Dose-response curves of CD69 upregulation following peptide stimulation. Note that male cells required 100-fold more peptide for maximal stimulation as compared to female cells. Similar results were obtained in three independent experiments.

(C) A fraction of Smcy3-specific T cells from female TCR-β Tg mice reacts with the endogenous level of D^b-Smcy3 complexes presented on male cells. Splenocytes from female Ly5.2 TCR-β Tg or non-Tg B6 mice were incubated with splenocytes from male Ly5.1 B6 mice for 24 hr and stained with antibodies against Ly5.2, CD8, and CD69. Control cultures were the same Ly5.2 cells incubated with splenocytes from female Ly5.1 B6 mice. The background frequency of Ly5.2 $CD69^+$ CD8 T cells in the control culture was subtracted to the frequency of Ly5.2 CD8 T cells that upregulated CD69 in the presence of male cells. Results are means of triplicate wells.

tion prevented costaining with MHC tetramers in this experiment, it should be noted that the frequencies of Smcy3-reactive T cells measured in this assay (11% and 28% of CD8 in male and female, respectively) were in close agreement with the tetramer staining profiles (Figure 1B). Importantly, female Smcy3-specific T cells were found to require 100-fold less peptide for stimulation as compared to male Smcy3-specific T cells. Finally, no CD69 upregulation was observed when male splenocytes were cultured in the absence of peptide, suggesting that male Smcy3-specific T cells are not reactive to the endogenous level of D^b-Smcy3 expression. In comparison, we also performed the CD69 assay with T cells from αβ TCR-Tg mice. Virtually all CD8 T cells (95%) from female $Rag^{-/-}$ TCRαβ Tg mice responded by upregulating CD69 when stimulated with a peptide concentration of 1.10^{-8} M, while 10- to 100-fold more peptide was required to achieve CD69 upregulation of T cells from male $Rag^{-/-}$ αβTCR Tg mice (data not shown). The latter observation is likely to reflect the reduced levels of CD8 at the surface of male αβ TCR Tg T cells that escaped negative selection (Teh et al., 1989).

Overall, we conclude from these results that in male TCR-β Tg mice, 25%–40% of T cells reactive to Smcy3 escape both central and peripheral deletion and that these T cells display a low-avidity phenotype.

To gain insight into the relationship between the avidity thresholds required for clonal deletion in the thymus and for activation in the periphery, we compared the level of D^b-Smcy3 complexes endogenously expressed on male cells to that displayed on the peptide-pulsed splenocytes used in the previous assay. To this end, we measured the number of CD8 T cells from female TCR-β Tg mice that specifically upregulated CD69 in the presence of male cells. Splenocytes from female Ly5.2 TCR-β Tg mice (or from female Ly5.2 non-Tg B6 mice) were incubated for 24 hr with spleen cells from male Ly5.1 B6 mice. The same Ly5.2 cells were also incubated with splenocytes from female Ly5.1 B6 mice to determine the background level of CD69 staining. As shown in Figure 2C, ~3% of CD8 T cells from female TCR-β Tg mice (corresponding to about 10% of the Smcy3-specific population) were specifically activated in the presence of male splenocytes (as compared to the control cultures). No specific CD69 upregulation was observed when splenocytes from female non-Tg B6 mice were incubated with male splenocytes (Figure 2C). From the results in Figure 2B, it can be seen that stimulation of female cells with a peptide concentration of 1.10^{-8} M also leads to an increase of $CD69^+$ cells of about 3% over the background. Thus, the level of activation induced by endogenous D^b-Smcy3 complexes was similar to that observed with a peptide concentration of 1.10^{-8} M. Interestingly, this concentration was still 10- to 100-fold lower than that required for activating male Smcy3-specific T cells (Figure 2B).

Male Smcy3-Specific T Cells Are Functional In Vitro

In several studies using αβ-TCR Tg mouse strains expressing the cognate antigen in the thymus, T cells that escaped negative selection were found to be functionally inert (reviewed in Stockinger, 1999). To further characterize the functional properties of male Smcy3-specific T cells, we analyzed the in vitro proliferation of splenocytes from male TCR-β Tg mice upon peptide stimulation in the absence of any exogenous cytokine. Spleen cells from male or female TCR-β Tg mice were labeled with the intracellular fluorescent dye CFSE and incubated with various concentrations of peptide for 3 days. As shown in Figure 3A, male TCR-β Tg CD8 T

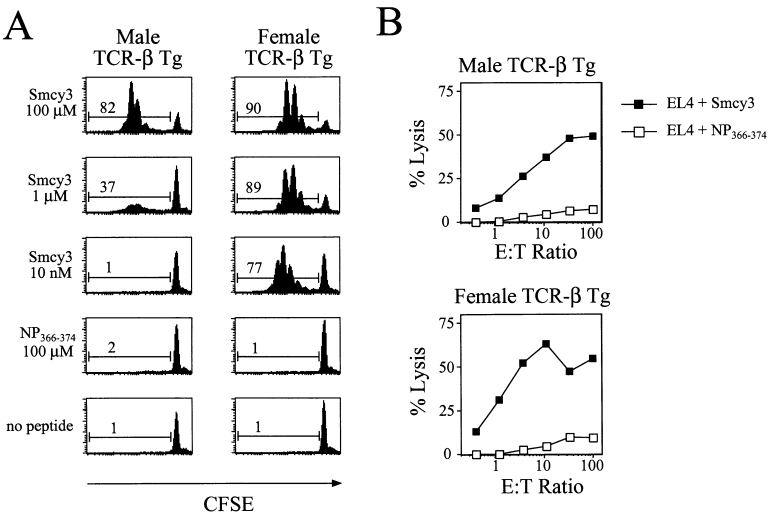

Figure 3. In Vitro Behavior of Female and Male Smcy3-Specific T Cells following Peptide Stimulation

(A) Male Smcy3-reactive T cells proliferate upon peptide stimulation. Splenocytes from female or male TCR-β Tg mice were labeled with CFSE and stimulated with the indicated concentration of Smcy3 peptide. The D^b binding peptide $NP_{366-374}$ was used as a control. After 3 days, cells were stained with an anti-CD8 mAb and analyzed by flow cytometry. Histograms are gated on $CD8^+$ cells.

(B) Male Smcy3-reactive T cells differentiate into cytotoxic effectors following peptide stimulation. Splenocytes from female or male TCR-β Tg mice were stimulated with 10 μM of Smcy3 peptide for 3 days and assayed for cytotoxicity on peptide-pulsed EL-4 cells. Results are means of triplicate wells. No cytoxocity was observed when the same cells were stimulated with the control peptide $NP_{366-374}$ (data not shown). Similar results were obtained in three independent experiments.

cells were able to proliferate vigorously upon Smcy3 peptide stimulation arguing against a state of profound anergy for these cells. No proliferation was observed with the control peptide (Figure 3A). In addition, this assay further confirmed the low avidity of male Smcy3-specific T cells, which, in contrast to female specific cells, were unable to proliferate when the peptide concentration was below 1 μM.

To determine whether male Smcy3-specific T cells could differentiate into cytotoxic effectors, we stimulated splenocytes from TCR-β Tg mice with a high peptide concentration for 3 days and assayed the cytolytic activity on peptide-pulsed EL-4 cells. Results shown in Figure 3B indicate that Smcy3-reactive CD8 T cells from male TCR-β Tg mice are perfectly capable of differentiating into cytotoxic effectors. Taken together, these in vitro assays suggest that the residual Smcy3-reactive T cells from male TCR-β Tg mice acquire normal functional capacities provided that sufficient peptide density is available for stimulation.

Origin of the Low-Avidity Phenotype of Smcy3-Specific T Cells in Male TCR-β Tg Mice

Studies using TCRαβ Tg mice have shown that several mechanisms could prevent T cells bearing autoreactive TCRs from being deleted, including downregulation of TCR and coreceptor as well as expression of dual TCRs (Teh et al., 1989; Schonrich et al., 1991; Zal et al., 1996; Girgis et al., 1999). To evaluate the role of these mechanisms in the context of a polyclonal TCR repertoire, we stained PBL of male and female TCR-β Tg mice with anti-CD8 mAb, D^b-Smcy3 tetramers, and anti-BV8.2 (or anti-CD3) mAb. As shown in Figure 4A, similar levels of CD8, BV8, and CD3 were observed among tetramer-positive and -negative populations in male TCR-β Tg mice. Similar results were obtained in female animals (Figure 4A). To test the possibility that male-specific cells achieve a low-avidity phenotype by expressing two distinct functional TCRs, we crossed TCR-β Tg mice with $TCR\alpha^{-/-}$ animals and stained the PBL of male $TCR\alpha^{+/-}$ TCR-β Tg mice with D^b-Smcy3 tetramers (Figure 4B). Despite the fact that expression of dual TCRα chains is prevented in $TCR\alpha^{+/-}$ mice, the frequency of tetramer stained cells in male $TCR\alpha^{+/-}$ TCR-β Tg mice was very similar to that observed in male $TCR\alpha^{+/+}$ TCR-β Tg animals (Figures 1B and 4B). Taken together, these findings indicate that the low-avidity phenotype of male Smcy3-specific T cells does not originate from a reduced level of CD8 or TCR surface expression or from the expression of a second TCR. Rather, these results strongly suggest that the low-avidity phenotype of male Smcy3-specific T cells is due to low-affinity TCRs.

To test this hypothesis, we stained splenocytes from TCR-β Tg mice with D^b-Smcy3 tetramers and the clonotypic T3.70 antibody. This reagent has been shown to react against the B6.2.16 anti-H-Y αβ TCR although the exact spectrum of TCRs recognized by this antibody is unknown (Teh et al., 1989). In female TCR-β Tg mice, the T3.70 antibody stained 1%–2% of CD8 T cells and 4%–5% of Smcy3-specific T cells (Figure 4C). In males, these numbers were reduced by 5- to 7-fold and 2- to 3-fold, respectively. Thus, we conclude that male and female Smcy3-specific T cells do not display identical TCR repertoires and that the T3.70 antibody preferentially binds to a subpopulation of high-affinity TCRs in female mice.

In order to confirm the correlation between T cell avidity and tetramer staining intensity in our system, we measured the fluorescence intensity of tetramer staining among female Smcy3-specific T cells that have been induced to proliferate with various doses of peptide. We found that anti-Smcy3 T cells that were able to proliferate upon stimulation with low peptide concentrations displayed high fluorescence intensity of tetramer staining (Figure 4D). Importantly, these results could not been accounted for by differences in the level of expression of TCR or CD8 (data not shown). Finally, staining of the same cultures with the T3.70 antibody revealed that $T3.70^+$ T cells preferentially proliferate upon stimulation with low peptide concentrations (Figure 4E).

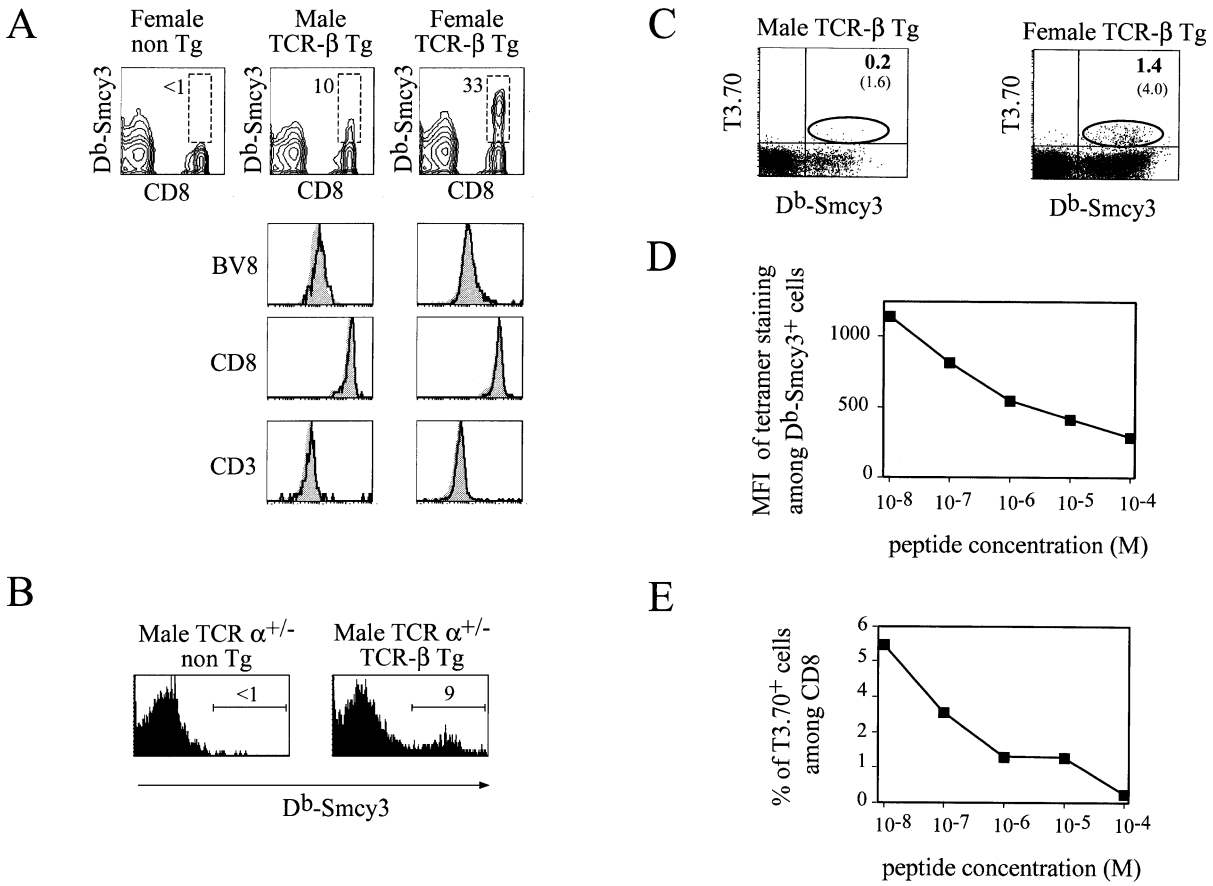

Figure 4. Origin of the Low-Avidity Phenotype of Male Smcy3-Specific T Cells

(A) Comparison of TCR and CD8 expression level among D^b-Smcy3 tetramer-positive and -negative T cell populations. PBL from female non-Tg B6 mice and male and female TCR-β Tg mice were triple stained with D^b-Smcy3 tetramers, anti-CD8 and anti-BV8 (or anti-CD3) mAbs. Gates used to delimit the tetramer-positive populations are shown as well as the percentage of tetramer-positive cells among the CD8 subset. Histograms show the level of BV8, CD8, and CD3 on gated $CD8^+D^b$-$Smcy3^+$ cells (fine line) and gated $CD8^+D^b$-$Smcy3^-$ cells (filled) from either male TCR-β Tg mice (left column) or female TCR-β Tg mice (right column). Note that the levels of the surface markers analyzed were similar for all these cell populations.

(B) Smcy3-specific T cells are present in male $TCR\alpha^{+/-}$ TCR-β Tg mice. PBL from male $TCR\alpha^{+/-}$ non-Tg (left histogram) or $TCR\alpha^{+/-}$ TCR-β Tg mice (right histogram) were stained with an anti-CD8 Ab and D^b-Smcy3 tetramers. Histograms are gated on $CD8^+$ cells.

(C) The T3.70 clonotypic antibody preferentially binds to a fraction of female Smcy3-specific T cells. PBL from male or female TCR-β Tg mice were triple stained with D^b-Smcy3 tetramers, anti-CD8 and T3.70 mAbs. Profiles are gated on $CD8^+$ cells. Numbers under brackets are the percentage of $T3.70^+$ cells among D^b-Smcy3 tetramer-positive cells.

(D) Relationship between T cell avidity and MHC tetramer binding. Splenocytes from female TCR-β Tg mice were induced to proliferate by incubation with various doses of Smcy3 peptide. After 2 days, cells were washed extensively and cultured for 3 additional days for TCR reexpression. Figure shows the mean fluorescence intensity of tetramer staining among tetramer-positive cells as a function of the peptide concentration used for stimulation.

(E) $T3.70^+$ T cells preferentially proliferate upon stimulation with low peptide concentrations. The same cultures as in (D) were stained with the T3.70 antibody. Shown are the frequencies of T3.70-positive CD8 T cells as a function of the peptide concentration used for stimulation.

Peculiar TCRs Are Absent in Male but Not in Female TCR-β Tg Mice

To test if the clonal deletion could be detected at the molecular level in the preimmune repertoire, we compared Smcy3-specific TCR repertoire in male versus female TCR-β Tg mice. To this end, tetramer-positive and -negative cells derived from male or female TCR-β Tg mice were sorted (Figure 5A). Semiquantitative analysis of TCRAV transcripts was performed on these samples using a panel of TCRAV primers that detects most AV families. We found that tetramer-negative populations from either female or male TCR-β Tg mice displayed a very diverse usage of TCRAV segments (Figure 5B). By contrast, the Smcy3-specific T cell repertoire of female or male TCR-β Tg mice was heavily skewed toward AV9 usage. Noteworthily, the α chain of the original B6.2.16 CTL clone from which originates the β chain used in the Tg mice also uses AV9 (previously named Vα B6.2.16, Vα13) segment (Uematsu, 1992).

We further analyzed sorted male and female preimmune Smcy3-specific T cells with respect to their CDR3α size distributions using the Immunoscope technology. Figure 6A shows the AV9-CDR3α size distributions in male and female Smcy3-specific T cells. Female Smcy3-specific TCRs mostly displayed CDR3α regions of 9, 10, or 11 aa. In males, Smcy3-reactive T cells with a 10 aa long CDR3α were virtually absent (see arrows in Figure 6A, left panels). To quantitate the differences

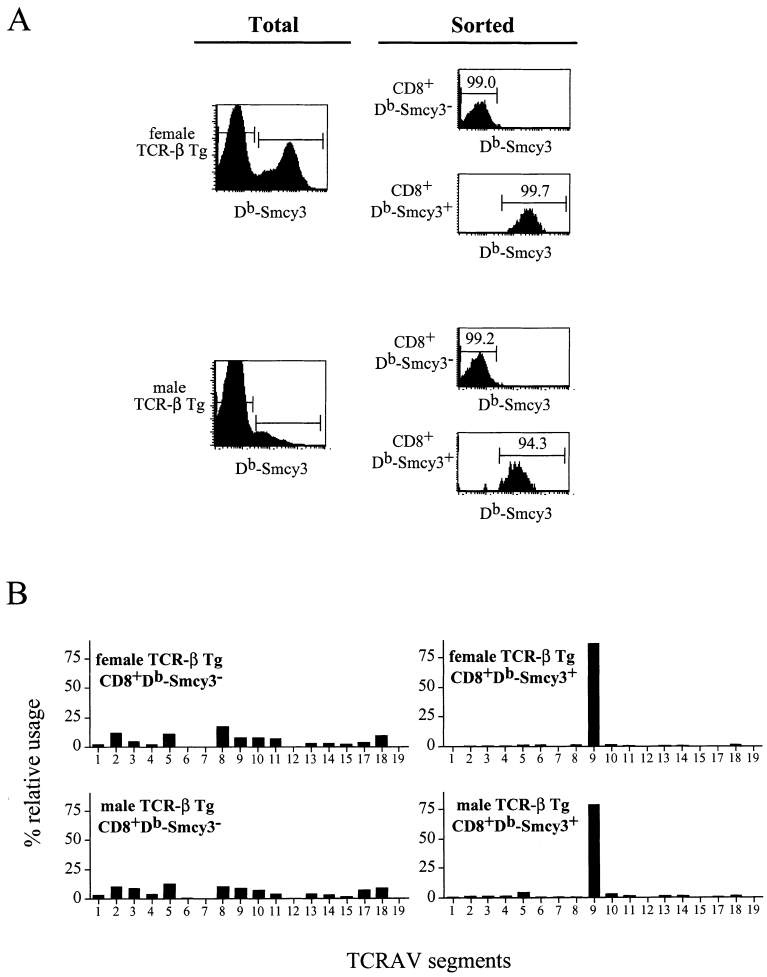

Figure 5. TCRAV Repertoire of Smcy3-Reactive T Cells

(A) Pooled lymph nodes cells and splenocytes from either female or male TCR-β Tg mice were double stained using D^b-Smcy3 tetramers and an anti-CD8 mAb. $CD8^+D^b$-$Smcy3^-$ and $CD8^+D^b$-$Smcy3^+$ cell populations were sorted. Cell purity was checked immediately after sorting.

(B) Semiquantitative PCR analysis of TCRAV usage was performed on the indicated sorted population. Similar results were obtained in two independent experiments. In contrast to the $CD8^+D^b$-$Smcy3^-$ populations, $CD8^+D^b$-$Smcy3^+$ cells mostly use TCRAV9 segment.

between male and female repertoires, profiles were further normalized so that their total surface area was proportional to the frequency of T cells stained with D^b-Smcy3 tetramers. The normalized profiles shown in Figure 6A indicate that the number of T cells with a 10 aa long CDR3α was reduced by 18-fold in male as compared to female mice. The number of TCRs with an 11 aa long CDR3α was also largely reduced (3- to 4-fold decrease) whereas the subset of T cells bearing a 9 aa long CDR3α appeared less affected (2-fold decrease). Thus, susceptibility to clonal deletion is largely dependent on the size of the CDR3α loop. Because the original anti-H-Y αβ-TCR uses AV9 and AJ57 segments (previously named Jα27), we then focused the Immunoscope analyses on AV9-AJ57 TCRs. In female TCR-β Tg mice, Smcy3-specific TCRs bearing AV9-AJ57 segments used CDR3α of 9, 10, or 11 aa, while only CDR3α of 9 aa were observed in male animals (Figure 6B). This is consistent with the observation that the anti-H-Y αβ-TCR is deleted in male animals since this particular TCR uses an 11 aa long CDR3α (Kisielow et al., 1988). We also analyzed the TCR repertoire that binds to the T3.70 clonotypic antibody. AV9-AJ57 Immunoscope profiles performed on sorted T3.70-positive and -negative CD8 T cell populations from female TCR-β Tg mice are shown in Figure 6C. Interestingly, a single CDR3α size (11 aa) was compatible with the binding to the T3.70 antibody, indicative of a high degree of specificity for this reagent. Finally, we sorted female Smcy3-specific T cells that displayed either high or low to intermediate levels of tetramer staining. The contribution of T cells displaying AV9-AJ57 segments with a 9 aa long CDR3 was increased in the D^b-$Smcy3^{low/int}$ population and reduced in the D^b-$Smcy3^{high}$ fraction, whereas opposite results were observed for T cells bearing an 11 aa long CDR3α (Figure 6D). These results provide evidence that male Smcy3-specific T cells represent the low-affinity fraction of the corresponding female population.

To refine our repertoire analysis, we sequenced the TCRα chains of the sorted preimmune Smcy3-specific T cell populations derived from male and female TCR-β Tg mice. A total of 211 TCRα sequences was performed: 140 distinct amino acid sequences were obtained, indicative of a large degree of polyclonality in the specific TCR repertoire. As predicted by Immunoscope analyses, the vast majority (88%) of the CDR3α sequences displayed a length of 9 to 11 aa. Figure 6E summarizes TCRAJ usage in Smcy3-specific T cells as a function of their CDR3α size. Female and male D^b-$Smcy3^+$ cells preferentially use five TCRAJ segments (AJ32, AJ45, AJ52, AJ56, and AJ57). Usage of particular AJ segments was significantly reduced in male as com-

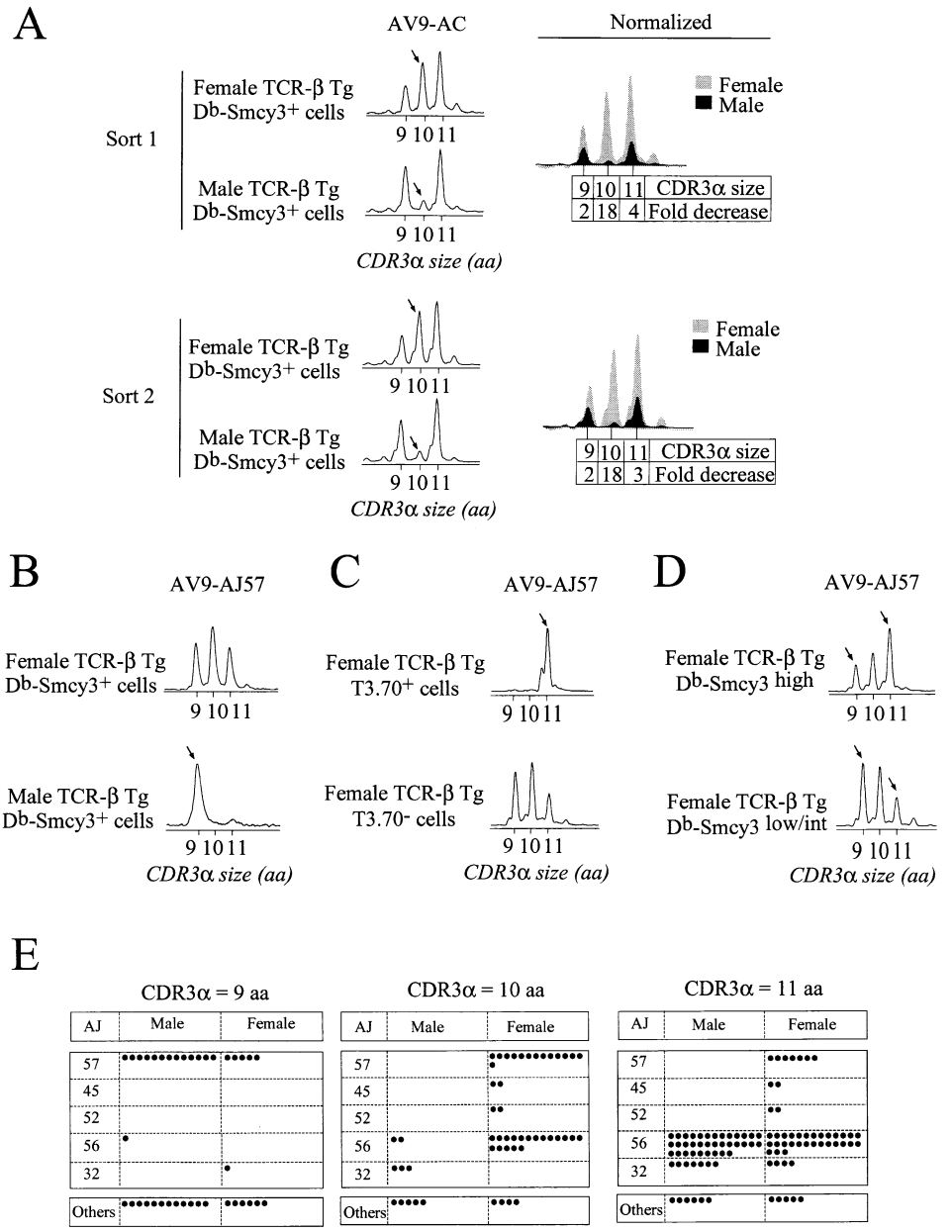

Figure 6. Susceptibility to Clonal Deletion Is Highly Dependent on the Size of the CDR3α Loop and on TCRAJ Usage

(A) CDR3α size distribution analysis (Immunoscope) of Smcy3-specific T cells derived from female and male TCR-β Tg mice. cDNA from the indicated sorted cell population was subjected to PCR using AV9- and AC-specific primers followed by a run-off reaction with a nested fluorescent AC-specific primer. The AV9-AC size distributions analyzed with the Immunoscope software corresponding to two independent sorts are shown. To quantitate the differences between male and female repertoires, profiles were further normalized so that their total surface area was proportional to the frequency of T cells stained with D^b-Smcy3 tetramers. Based on the normalized profiles, we could calculate the reduction in the number of specific T cells displaying a given CDR3 size in male as compared to female animals. The indicated fold decrease corresponds to the ratio of a peak surface area in the normalized female profile to the corresponding peak surface area in the male profile. Figure shows that the impact of clonal deletion varies depending on the CDR3α size and is maximal for T cells bearing a 10 aa long CDR3α.

(B–D) Immunoscope profiles were performed using AV9- and AJ57-specific primers on (B) sorted male and female Smcy3-specific T cells, (C) clonotypic T3.7-positive and -negative T cell populations sorted from female TCR-β Tg mice, and (D) sorted female Smcy3-specific T cells displaying either low to intermediate or high levels of tetramer staining. Note that the contribution of T cells displaying a 9 aa (respectively, 11 aa) long CDR3α is increased in the D^b-Smcy3$^{low/int}$ (respectively, D^b-Smcy3^{high}) population.

(E) TCRAJ usage in preimmune Smcy3-specific T cells from male or female TCR-β Tg mice. cDNA from sorted D^b-Smcy3$^+$ cell populations were amplified by PCR using AV9- and AC-specific primers. PCR products were cloned in *E. coli* and sequenced. Each dot represents the sequence information from a single colony. TCRAJ usage is shown as a function of the length of the CDR3α loop.

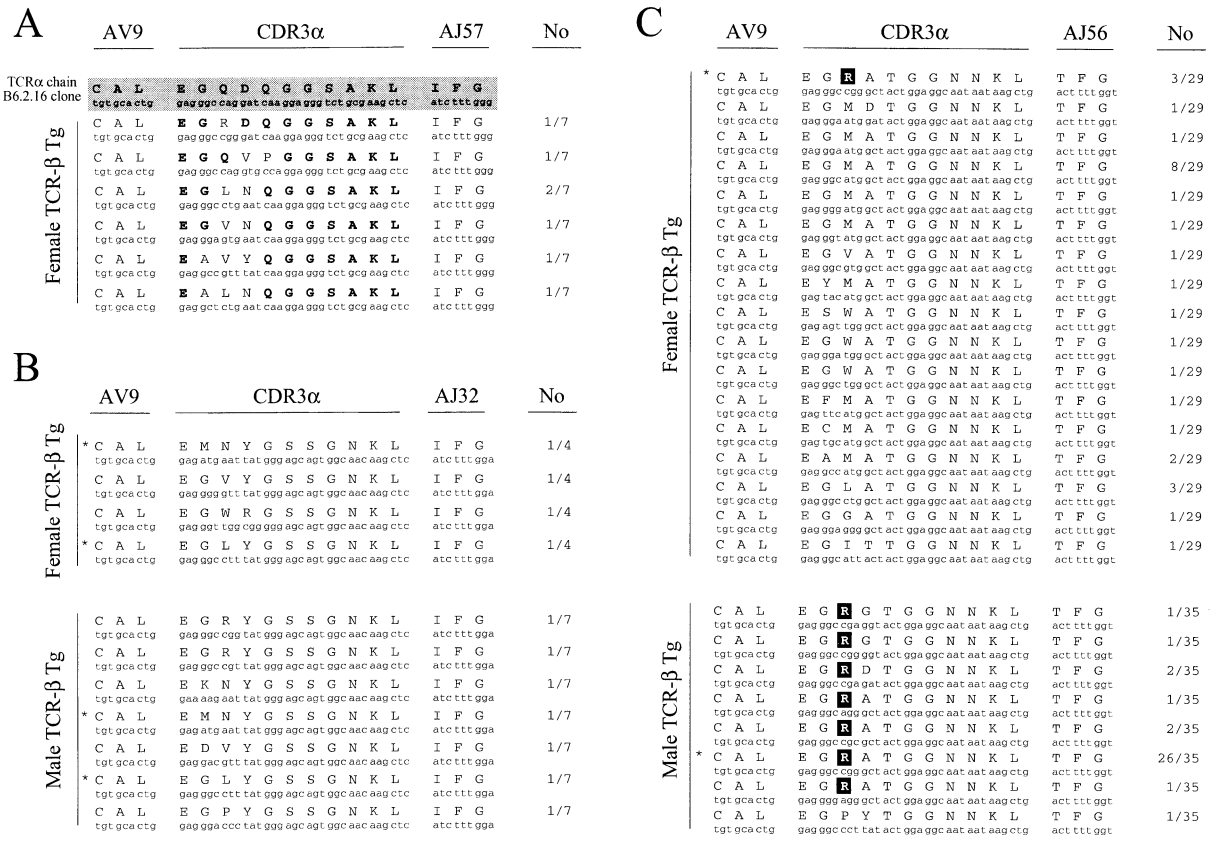

Figure 7. TCRα Sequences Derived from Male and Female Smcy3-Specific T Cells

Figure shows nucleotide and deduced amino acid sequences of the TCRα chains expressed by female and male preimmune sorted D^b-Smcy3$^+$ T cell populations.

(A) TCRα rearrangements (AV9-AJ57, CDR3α = 11aa) similar to that of the original anti-H-Y B6.2.16 CTL clone are found in female but not in male D^b-Smcy3$^+$ T cells.

(B) Comparison of the TCRα rearrangements displaying AV9-AJ32 combination and an 11 aa long CDR3 in male and female mice. Asterisks indicate that identical nucleotides sequences were found in both male and female D^b-Smcy3$^+$ cells.

(C) Comparison of the TCRα rearrangements displaying AV9-AJ56 combination and an 11 aa long CDR3 in male and female mice. Boxes indicate the presence of an arginine in position 3 of the CDR3α.

pared to female Smcy3-reactive T cells. This reduction concerned AJ56 and AJ57 segments in the residual male cells that display a 10 aa long CDR3α and the AJ57 segment in male cells with an 11 aa long CDR3α. These results further confirmed the absence of TCRα rearrangements similar to that of the anti-H-Y TCR in male mice. In contrast, several TCRα chains that were nearly identical to that of the original anti-H-Y CTL clone were observed in female mice (listed in Figure 7A).

As shown in Figure 6E, three privileged T cells populations displaying a particular AV-AJ combination and a given CDR3 size were shared between male and female D^b-Smcy3$^+$ cells: AV9-AJ57, CDR3 = 9 aa ; AV9-AJ32, CDR3 = 11 aa ; and AV9-AJ56, CDR3 = 11 aa. We therefore compared TCRα sequences of male and female Smcy3-reactive cells belonging to these three subsets. As shown in Figure 7B, rearrangements using AV9-AJ32 combination and an 11 aa long CDR3α were identical or highly homologous in male and female Smcy3-specific T cells, although the relatively small number of sequences analyzed in this particular subset precluded a more detailed analysis of amino acid usage within the CDR3. The same analysis with AV9-AJ57 expressing T cells displaying a 9 aa long CDR3α yielded similar results (data not shown). In contrast, a clear bias was observed in the junctional regions derived from male D^b-Smcy3$^+$ cells using the AV9-AJ56 combination together with an 11 aa long CDR3α. The vast majority (90%, 26/29) of the sequences derived from female D^b-Smcy3$^+$ cells used a nonpolar residue in position 3 of the CDR3α (methionine 59%, tryptophan 10%, leucine 10%, valine 3%, isoleucine 3%, glycine 3%). The remaining sequences (10%, 3/26) used a positively charged residue (arginine) at this position. In sharp contrast, 97% (34/35) of the sequences derived from male D^b-Smcy3$^+$ cells displayed a positively charged residue (arginine) in position 3 of the CDR3α. This observation suggests that the third amino acid of the CDR3α could play a key role in modulating TCR affinity. Taken together, these analyses identify a correlation between particular TCRα primary structures and susceptibility to clonal deletion.

Discussion

Although it is well documented that some self-reactive T cells escape clonal deletion in the thymus and in the

periphery, little is known on the overall efficiency of clonal deletion mediated by a particular self-peptide on a polyclonal TCR repertoire. In the present report, we thoroughly quantified the impact of self-tolerance mediated by a self-peptide on the preimmune repertoire. We found that as many as 25%–40% of T cells reactive to a self-peptide escaped clonal deletion in the thymus and in the periphery. In addition, our results identify the imprint of negative selection on a peptide-specific T cell population in terms of reactivity and TCR structure.

In this study, we made use of mice transgenic for the β chain of an anti-H-Y CTL clone recognizing the male-specific D^b-Smcy3 complex. This experimental system offers three major advantages. First, Smcy3-reactive T cells are present in elevated frequency in TCR-β Tg mice. While other studies have analyzed the residual self-reactive T cells following immunization, our system enables the characterization of self-specific T cells in nonimmunized animals, a unique approach to distinguish clonal elimination from anergy. Second, the diversity provided by the TCRα chain should allow these animals to generate a set of TCRs displaying heterogeneous affinities for the D^b-Smcy3 complex as it would be expected to occur in normal mice. Third, the comparison between female and male Smcy3-specific T cell repertoires allowed us to assess how a physiological self-ligand shapes the self-reactive T cell repertoire. Preimmune Smcy3-specific T cells were directly probed using the technology of MHC-peptide tetramers, which enables not only ready enumeration of peptide-specific cells but also estimation of their relative avidity/affinity based on the strength of tetramer binding (Altman et al., 1996; Crawford et al., 1998; Yee et al., 1999).

Using two independent approaches, we found that ~30% of $CD8^+$ cells derived from female TCR-β Tg mice were reactive to the D^b-Smcy3 complexes (Figures 1 and 2). The high frequency of Smcy3-specific T cells in female TCR-β Tg mice could be due to an important contribution of this particular β chain to the recognition of the D^b-Smcy3 complex, a preferential positive selection of this specificity or both. Under the former hypothesis, it would be expected that the pairing of the β-Tg chain with a diverse set of TCRα chains could provide Smcy3 specificity. The finding that TCRAV usage among Smcy3-specific T cells is highly restricted toward a single AV segment further supports the idea that Smcy3-reactive T cells are favored during thymic positive selection. In this respect, a previous study has shown that the introduction of a TCR-β transgenic chain enlightens the imprint of positive selection on the mature repertoire (Sant'Angelo et al., 1998).

Taking advantage of the increased frequency of Smcy3-specific T cells in female TCR-β Tg mice, we thought to identify Smcy3-specific T cells that have escaped clonal deletion in male TCR-β Tg mice. One of the most striking observations of this study was that a large proportion of Smcy3-reactive T cells persists in male animals as detected by tetramer staining experiments and functional assays. By comparing male and female Smcy3-specific T cell populations, we estimated that, in males, as many as 25%–40% of self-reactive T cells have not been subjected to clonal elimination in the thymus and the periphery. It is clear that the extent of clonal deletion varies depending on the level of expression of the considered self-peptide and may be maximal for highly expressed transgenic neo-self-antigen. Our results illustrate that, at least for some physiological self-peptides, a large subset of the preselection self-reactive repertoire ultimately escapes clonal deletion.

We found that differences between male and female anti-Smcy3 T cells are already apparent in CD8 SP thymocytes, indicating that most of the deletion occurs in the thymus. This is reminiscent of the central deletion observed in male anti-H-Y αβ-TCR Tg mice (Kisielow et al., 1988). We believe that the Smcy3 peptide itself is likely to mediate most of (if not all) the clonal deletion observed, although it remains formally possible that other male-specific peptides contribute to the elimination of some male Smcy3-reactive T cells.

Despite the high number of self-reactive T cells in male TCR-β Tg mice, these animals appeared perfectly healthy and did not show any obvious signs of autoimmunity. Collectively, our results suggest that male Smcy3-specific T cells that escape clonal deletion do not react with the endogenous level of D^b-Smcy3 expression as predicted by the observation that the threshold of antigenic stimulation is lower for negative selection than for activation of mature T cells (Pircher et al., 1991; Vasquez et al., 1994). In this respect, we could deduce from our results (Figures 2B and 2C) that the peripheral female Smcy3-specific T cell population comprises three distinct subsets: (1) a population of high-avidity T cells (10%) that has the ability to react with the endogenous level of D^b-Smcy3 complexes on male cells and that is subjected to clonal deletion in male animals (Figure 2C); (2) a population (50%–65%) of intermediate avidity T cells that is also subjected to clonal deletion in male mice but that does not react with male cells in vitro; and (3) a population (25%–40%) of low-avidity T cells that persists in male animals. The finding that more than half of the Smcy3-specific T cells are not reactive against male cells in vitro but are yet deleted in males is likely to reflect the margin of safety built during the deletion process. Nonetheless, male Smcy3-specific T cells were perfectly capable of in vitro proliferation and differentiation into effectors when stimulated with high doses of peptide (Figure 3). While it is clear that, due to the process of positive selection, thymocytes have an intrinsic ability to interact with self-peptide:self-MHC complexes, the fraction of the mature T cell population capable of acquiring effector functions when facing high doses of self-peptides is unknown. In this respect, our results suggest that the number of circulating T cells capable of functional reactivity toward self-peptides may be substantially higher than previously thought.

The low-avidity phenotype of male Smcy3-specific T cells was reflected by: (1) a reduced intensity of tetramer staining and (2) a requirement of high peptide densities for stimulation in functional assays (Figures 1D, 2, and 3). This phenotype does not originate from reduced surface level of TCR or CD8 or from the expression of two functional TCRs (Figures 4A and 4B). Although several reports have shown that these mechanisms could prevent self-specific T cells from being deleted (Teh et al., 1989; Schonrich et al., 1991; Zal et al., 1996; Girgis et al., 1999), it should be noted that these results were observed in TCR-αβ Tg models in which increased competition for survival is likely to occur. Our results indicate that, in

the context of an oligoclonal population of self-specific T cells harboring various TCRs, the outcome of negative selection is mostly dependent on TCR affinity.

We have also identified the molecular imprint of clonal deletion on the TCRα repertoire. Three major TCR features were found to modulate susceptibility to clonal deletion. First, a striking correlation between the extent of negative selection and the length of the CDR3α loop was observed (Figure 6A): the impact was maximal for T cells bearing a 10 aa long CDR3α, high for those with an 11 aa long CDR3α, and moderate for those with a CDR3α length of 9 aa. This, together with the observation that particular CDR3 sizes have been shown to be highly selected during some antigen-specific T cell responses (McHeyzer-Williams and Davis, 1995), indicates that the length of CDR3 loops can be as important (if not more) as their amino acid composition in modulating TCR affinity. Second, we found that negative selection was preferentially directed toward T cells bearing particular TCRAJ segments. In particular, usage of TCRAJ57 segment was altered in male Smcy3-reactive T cells displaying a 10 or an 11 aa long CDR3α (Figures 6B and 6E). It is noteworthy that our results are in perfect agreement with the observation that the anti-H-Y αβ-TCR (AV9-AJ57, CDR3α = 11 aa) is normally deleted in male animals (Kisielow et al., 1988). Third, subtle changes in the amino acid usage within the CDR3α region can apparently tip the balance of clonal deletion. In this respect, we found that virtually all TCRα rearrangements derived from male Smcy3-specific T cells using the AV9-AJ56 combination and an 11 aa long CDR3α use a positively charged residue in position 3, whereas nonpolar residues were predominantly found at this position in the TCRα chains of female Smcy3-reactive cells (Figure 7C). This raises the intriguing possibility that the presence of this positively charged residue could interfere with the recognition of the D^b-Smcy3 complex and thereby result in a decreased TCR affinity. In any case, these results illustrate how a negatively selecting ligand can narrow the structural diversity of self-specific TCRs.

On the other hand, T cells with particular TCRα features were shared between male and female TCR repertoires. This subset presumably corresponds to the low-affinity T cell population and includes TCRα chains using AV9-AJ56 combination and 11 aa long CDR3α displaying an arginine in position 3 as well as T cells using AV9-AJ57 and AV9-AJ32 combination and a 9 (respectively, 11) aa long CDR3α. Consequently, our results indicate that male-specific peptides (and in particular the Smcy3 peptide) are not necessary for the positive selection of this common subset of low-affinity TCRs. In addition, we found no clear evidence for the positive selection of unique TCRα structures in male. Overall, TCR sequence analyses strongly suggest that differences between male and female anti-Smcy3 TCR repertoires simply reflect the elimination of particular TCR structures (Figures 6 and 7).

In summary, this report provides a quantitative and qualitative estimate of the impact of a peptide-specific clonal deletion on a polyclonal preimmune repertoire. While previous studies have used TCR-β Tg mice to show that the preimmune repertoires bear the imprint of the positively selecting ligands (Sant'Angelo et al., 1997, 1998; Fukui et al., 1998), we report here the characterization of the imprint of a deleting ligand on the mature TCR repertoire. In particular, we found a correlation between particular TCRα primary structures and susceptibility to clonal deletion. Surprisingly, roughly one T cell reactive to a self-peptide out of three was found to escape negative selection in this system. Considering the whole set of self-peptides, this raises the possibility that the number of peripheral T cells functionally reactive to high densities of self-peptides could be much higher than previously thought. What fraction of this low-avidity T cell population has the ability to trigger autoimmunity under certain circumstances remains to be elucidated.

Experimental Procedures

Mice

B6.2.16 TCR-β Tg mice (C57BL/6 background) have been previously described (Uematsu et al., 1988) and were a gift from Dr. P. Pereira (Pasteur Institute, France). $TCR\alpha^{-/-}$ mice (C57BL/6 background) were obtained from CDTA (Orleans, France). C57BL/6 mice were obtained from IFFA-Credo (L'arbresle, France). B6.2.16 TCR-αβ Tg mice on a $Rag^{-/-}$ background were a gift from Dr D. Guy-Grand (Pasteur Institute, France). B6 Ly5.1 mice were a gift from I. Douagi (Pasteur Institute, France). All mice used in this study were 5–10 weeks old.

Antibodies and Flowcytometry

PE-labeled D^b-Smcy3 tetramers were prepared as previously described (Bousso et al., 1998). Smcy3 peptide (KCSRNRQYL) has been described previously (Markiewicz et al., 1998) and was purchased from Neosystem (Strasbourg, France). FITC-conjugated anti-CD8 antibody was purchased from Caltag (South San Francisco, CA). APC-conjugated anti-CD8, FITC-conjugated anti-CD69, anti-BV8.1-8.2, and anti-CD3ε were purchased from PharMingen (San Diego, CA). Biotinylated T3.70 Ab was a gift from Dr. D. Guy-Grand. For tetramer staining, cells were incubated with D^b-Smcy3 tetramers for 2 hr at 4°C, washed, and incubated with the indicated antibodies. Cell sorting was performed on a FACStar Plus (Beckton Dickinson, San Jose, CA). Cell purity was checked immediately after sorting.

In Vitro Stimulation Assays

For CD69 expression assays, splenocytes were cultured in the presence of various concentrations of the indicated peptide in 96-well plates at a concentration of 1.10^6 cells/well. After 24 hr, cells were washed and stained with anti-CD8 and anti-CD69 mAbs. Proliferation assays were performed by labeling splenocytes with the intracellular fluorescent dye CFSE (Molecular Probes, Eugene, OR) for 15 min at 37°C. Cells were then washed extensively and cultured in the presence of the indicated peptide concentration for 3 days. For cytotoxic assays, splenocytes were cultured for 3 days in the presence of 10 μM of Smcy3 peptide. Cells were assayed for cytotoxicity on peptide-pulsed EL4 cells in a standard 4 hr ^{51}Cr-release assay. Results are means of triplicates.

Semiquantitative Analysis of TCRAV Usage and CDR3 Size Distribution Analyses

Total RNA was prepared using the Trizol reagent (GIBCO-BRL, Gaithersburg, MD) with the addition of 20 μg/ml of glycogen (Boehringer Mannheim, Mannheim, Germany). cDNA was synthesized using M-MLV reverse transcriptase (GIBCO-BRL). TCRAJ57-specific primer was 5′-TGTCCCCTCCCCAAAGATGA-3′. Other primers used in this study as well as the nomenclature for TCRAV have been already described (Casanova et al., 1991; Arden et al., 1995; Apostolou et al., 1999). Semiquantitative PCR analysis of TCRAV usage was performed as previously described (Apostolou et al., 2000). For CDR3 size distribution analyses, cDNA was amplified using primers specific for TCRAC and TCRAV9 segments. PCR products were then subjected to a run-off reaction using a fluorescent TCRAC-specific or TCRAJ57-specific primer. Labeled products were re-

solved on an automated 373A sequencer (Perkin Elmer, Foster City, CA). The fluorescent intensity of each band was recorded and analyzed using Immunoscope software (Pannetier et al., 1993).

TCR Sequencing

The cDNA of the indicated samples were amplified by PCR using AV9- and AC-specific primers. Blunt PCR products were then cloned using the Zero Blunt Topo PCR Cloning kit (Invitrogen, Groningen, The Netherlands). PCR was carried out on individual colonies using RP and M13(-40) universal primers. Sequences were performed using the M13(-20) primer and the ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Perkin Elmer). Samples were run on a 3700 DNA Analyzer (Perkin Elmer). Nomenclature for TCRAJ segments was published previously (Koop et al., 1994).

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