

The MHC Reactivity of the T Cell Repertoire Prior to Positive and Negative Selection

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Summary

T cell antigen receptors (TCRs) on mature T cells react with peptide antigens presented by self-MHC proteins and also frequently cross-react with foreign MHC proteins. The fundamental question whether MHC reactivity is inherent in the germline TCR sequences or is imposed by thymic selection was addressed here by inducing nonselective maturation of immature thymocytes in the absence of MHC molecules. MHC reactivity in the preselection repertoire is very high, but no higher than in the normal repertoire. Cross-reactivity of clones with multiple MHC molecules occurred to a similar extent in the preselection and MHC-selected repertoires. The results establish the MHC reactivity of the germline TCR repertoire, indicate the minimum fraction of immature thymocytes that must undergo negative selection, and suggest that some TCR-MHC contacts may be conserved.

Introduction

T cell antigen receptors (TCRs) recognize peptides of foreign protein antigens complexed with self-MHC (major histocompatibility complex) molecules. Indeed, the mature T cell repertoire is biased toward recognition of foreign peptides associated with self-MHC molecules, as opposed to those associated with nonself-MHC molecules. This characteristic is imposed by positive selection of developing T cells as they mature in the thymus (Jameson et al., 1995). At the same time, a negative selection process purges the T cell repertoire of cells that are reactive with complexes of thymic self-peptides with self-MHC molecules (Robey and Fowlkes, 1994).

It is thought that positive selection favors immature CD4⁺CD8⁺ thymocytes that bind weakly or strongly to self-MHC/peptide complexes, whereas negative selection induces the death of thymocytes that bind strongly to self-MHC/peptide complexes (Ashton-Rickardt et al., 1994; Hogquist et al., 1994). Operating together, positive and negative selection yield a repertoire of T cells that bind too weakly to self-MHC/self-peptide complexes for induction of immune responses. Binding contacts of the TCR with a foreign peptide in the groove of a self-MHC molecule may provide the added affinity necessary for

triggering of a low but sufficient fraction of T cells, often estimated as 10^{-5} – 10^{-4} T cells per foreign peptide.

A mysterious and striking feature of the T cell repertoire is its reactivity with cells bearing foreign MHC molecules (alloreactivity), manifested by frequencies of 10^{-2} – 10^{-1} T cells reacting to cells bearing any one foreign MHC molecule (Fischer-Lindahl and Wilson, 1977). Alloreactive T cells are included in the set that reacts to foreign peptide/self-MHC complexes (Bevan, 1977; Ashwell et al., 1986). Alloreactivity may result from the large number of MHC/cellular peptide complexes found on normal cells, estimated as >2000 (Hunt et al., 1992), to which a responding allogeneic T cell population is not tolerant. The frequency of T cells that reacts with any one such complex is thought to be very low, since the T cells were not positively selected to recognize this MHC molecule. Yet the additive frequency of T cells reactive with all such complexes on the allogeneic cell may be very high (Matzinger and Bevan, 1977).

While recent studies have shed much light on how positive and negative selection shape the repertoire (Robey and Fowlkes, 1994; Jameson et al., 1995), little is known concerning the starting point for selection, i.e., the preselection repertoire. A fundamental issue is whether and to what extent interactions with MHC molecules are ingrained in the germline sequences of TCRs. Is the preselection repertoire biased to recognize MHC molecules (Jerne, 1971), and if so, to what extent? Recently, it has been established that the TCR contacts the $\alpha 1$ and $\alpha 2$ domains of the MHC molecule (Hong et al., 1992; Ehrlich et al., 1993; Garboczi et al., 1996; Garcia et al., 1996; Sim et al., 1996). Although the sequences of the CDR1 and CDR2 regions of TCR α and β chains are germline encoded, many of the amino acids in the third CDRs are nongermline encoded, as a consequence of random processes operating during V(D)J recombination. Furthermore, the particular combination of α and β chains is randomly chosen in a given T cell, and it is unclear to what extent interactions between the chains could alter the conformation and, consequently, the MHC specificity of a TCR. In principle, these random components of the receptors could play a large role in determining their ultimate specificity for antigen as well as for MHC molecules.

On one extreme, the preselection repertoire might be completely random in its specificities; the propensity of TCRs on mature T cells to react with MHC molecules may result wholly as a consequence of positive selection, which demands MHC-reactive cells. In this scheme, only rare clones in the preselection repertoire should be subject to thymic selection. At the other extreme, the preselection repertoire might be even more strikingly MHC-reactive than the mature repertoire, including many clones that cross-react on several different or even all MHC molecules (Ignatowicz et al., 1996). In this scheme, many clones are subject to positive selection, and a large fraction of those are also subject to negative selection, including the MHC cross-reactive clones. These schemes and others could give rise to the observed mature repertoire. Resolving among these possibilities is central to understanding the relative roles of

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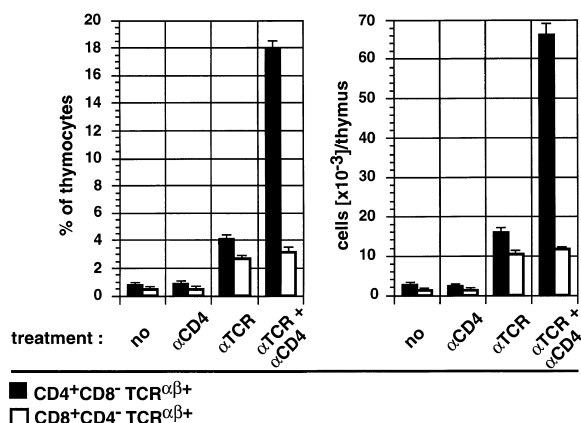


Figure 1. Development of CD4⁺CD8⁻ T Cells in MHC I^{II} Fetal Thymic Organ Culture Induced by Anti-TCR^{αβ} MAb and Enhanced by Anti-CD4 MAb

Day 16 fetal thymii from MHC I^{II} mice were placed in organ culture and treated with either 10 μg/ml anti-TCR^{αβ} MAb H57-597, 30% supernatant containing anti-CD4 MAb GK1.5, or both. After 6 days of culture, the percentages (left panel) and numbers (right panel) of CD4⁺CD8⁺ T cells in the respective organ cultures were determined. The data represent the mean of two independent experiments and the ranges.

positive and negative selection in shaping the ultimate T cell repertoire. These issues also bear on the TCR-MHC interaction per se, because inherent MHC reactivity of the preselection repertoire would suggest that there may be some conserved contacts between nonvariable portions of the TCRs and MHC molecules.

The available information concerning the specificity of the preselection repertoire is intriguing, but it is largely based on indirect analyses of selected T cells or their TCRs. It was shown that random combinations of TCR α and β chains, generated by fusions of hybridomas, were often alloreactive with another MHC molecule (Blackman et al., 1986). Because each of these chains had come from cells that may have been previously subjected to thymic selection, it was possible that their MHC specificity had been previously selected for. More recent studies examined the T cell repertoires in mice in which thymic selection was directed by one or a few peptide/MHC complexes (Ignatowicz et al., 1996; Miyazaki et al., 1996). As numerous mature T cells arose as a consequence of positive selection with one or a few complexes, it was proposed that the frequency of MHC-reactive precursors must be reasonably high (Ignatowicz et al., 1996). Furthermore, it was observed that many of the T cells arising from thymic selection by a single MHC/peptide complex exhibited a surprisingly high degree of reactivity to MHC-matched cells expressing diverse cellular peptides, as well as cross-reactivity to different allogeneic MHC molecules. The high frequency of these clones raised the possibility that many clones in the preselection repertoire are broadly MHC cross-reactive (Ignatowicz et al., 1996). Another study employed transgenic mice to achieve a situation where thymocytes underwent positive selection directed by the normal array of MHC/peptide complexes, but not negative selection. A high frequency of the resulting T cells reacted to self-MHC (Laufer et al., 1996). The MHC

reactivity of cells observed in all of these experiments could have been the result of positive selection.

We have directly examined the repertoire of T cell specificities that arises in the absence of MHC expression. In mice deficient for both MHC I and MHC II molecules (MHC I^{II} mice), T cell development is blocked at the CD4⁺CD8⁺TCR^{αβ}⁺ stage (Chan et al., 1993; Grusby et al., 1993). These cells represent the developing T cell receptor repertoire prior to positive or negative selection by MHC molecules. Like CD4⁺CD8⁺ thymocytes in normal mice, these cells are immature; they fail to proliferate in response to stimulation through the TCR in vitro (Robey and Fowlkes, 1994) and do not yield stable hybridomas at significant frequencies (data not shown). In fetal thymic organ cultures, these cells can be efficiently induced to mature by providing anti-TCR^{αβ} and anti-CD4 MAbs. Analysis of the specificity of these cells provides a quantitative analysis of the MHC reactivity of the preselection repertoire.

Results

An Organ Culture System to Stimulate Maturation of CD4⁺CD8⁺ Thymocytes in the Absence of MHC Expression

As a means to stimulate maturation of CD4⁺CD8⁺ thymocytes in MHC I^{II} mice, we tested the effects of adding soluble anti-TCR^{αβ} MAb (H57-597) to MHC I^{II} fetal thymic organ cultures (Takahama et al., 1994). After 6 days of organ culture, the antibody treatment resulted in an increase of 5- to 10-fold in the number of TCR^{αβ}⁺CD4⁺CD8⁻ and CD4⁺CD8⁺ thymocytes, compared to the number that appeared without addition of antibody (Figure 1). Since the number of mature T cells was still limiting, we investigated whether simultaneous stimulation of the CD4 coreceptor with anti-CD4 MAbs, along with stimulation of the TCR, would augment the differentiation of mature T cells in the organ cultures (Punt et al., 1993). The anti-CD4 MAb had no effect in the absence of anti-TCR^{αβ} MAb (Figure 1). In cultures containing both MAbs, there was no significant enhancement in the number of CD4⁺CD8⁺TCR^{αβ}⁺ thymocytes, but the appearance of CD4⁺CD8⁻ cells was enhanced by 4- to 5-fold, corresponding to an overall 27-fold increase in the number of these cells compared to cultures without antibodies (Figures 1 and 2, Table 1). The cultures treated with both antibodies contained as many, or more, CD4⁺CD8⁻ cells as MHC I^{II} thymus organ cultures run in parallel without antibody addition.

Most of the CD4⁺ T cells selected by the antibody mixture ("antibody-selected CD4⁺ T cells") expressed the TCR^{αβ} and were phenotypically mature (i.e., HSA⁺) (Figure 2). Furthermore, most of these cells exhibited the CD44(Pgp-1)⁺CD25⁻CD69⁻ cell surface phenotype of resting, naive T cells, strongly suggesting that they had not arisen by antibody-driven expansion of a few precursors (Figure 2). The failure of the CD4 antibody by itself to stimulate the differentiation of CD4⁺CD8⁻ thymocytes suggests that the effects of the anti-TCR antibody are specific. Sufficiently large numbers of CD4⁺CD8⁻ cells differentiated in these antibody-treated MHC I^{II} fetal thymic organ cultures for subsequent analysis of their function and specificity.

Table 1. Generation of CD4⁺CD8⁻ T cells in MHC I^qII^o FTOC by Antibody Treatment

	I ^q II ⁺ FTOC	I ^o II ^o FTOC	I ^q II ^o FTOC + α TCR/ α CD4
Number of Experiments	2	4	4
Cells/Thymus [$\times 10^{-3}$]	255.0–800.0	163.0–380.0	155.0–390.0
CD4⁺CD8⁻			
% of Thymocytes	13.8–18.9	0.7–2.9	17.7–59.0
Number per Thymus [$\times 10^{-3}$]	48.2–110.4	1.1–6.1	63.7–112.5
CD8⁺CD4⁻			
$\alpha\beta$ TCR ⁺ % of Thymocytes	1.1–3.2	0.3–1.1	2.9–7.8
Number per Thymus [$\times 10^{-3}$]	8.2–9.0	1.1–2.4	9.5–29.0
$\gamma\delta$ TCR ⁺ % of Thymocytes	1.5–4.1	3.9–5.6	2.3–4.6
Number per Thymus [$\times 10^{-3}$]	10.4–12.1	8.9–19.5	6.7–12.3
CD4⁺CD8⁺			
% of Thymocytes	42.2–77.1	67.2–84.4	7.6–66.0
Number per Thymus [$\times 10^{-3}$]	107.0–616.0	119.0–295.0	32.6–257.0

Cell suspensions from the indicated fetal thymic organ cultures (FTOC) were analyzed for expression of CD4, CD8, and T cell receptor $\alpha\beta$ or $\gamma\delta$ expression. The data represent the range of the indicated number of experiments. Essentially all of the CD4⁺CD8⁻ thymocytes in all groups expressed TCR $\alpha\beta$.

The Antibody-Selected CD4⁺ Thymocytes Represent the Preselection Repertoire

Because the anti-TCR $\alpha\beta$ MAb is not known to discriminate between different TCRs, the repertoire selected by the antibody mixture is likely to be representative of the preselection repertoire. This contention was supported

by analyses of the V β and V α usage (Figure 3). V β and V α frequencies among antibody-selected CD4⁺CD8⁻ cells were similar to those of CD4⁺CD8⁺ cells from MHC I^qII^o organ cultures, those that were treated with the antibody mixture, or control cultures that were not antibody treated (Figure 3). In some cases, these frequencies

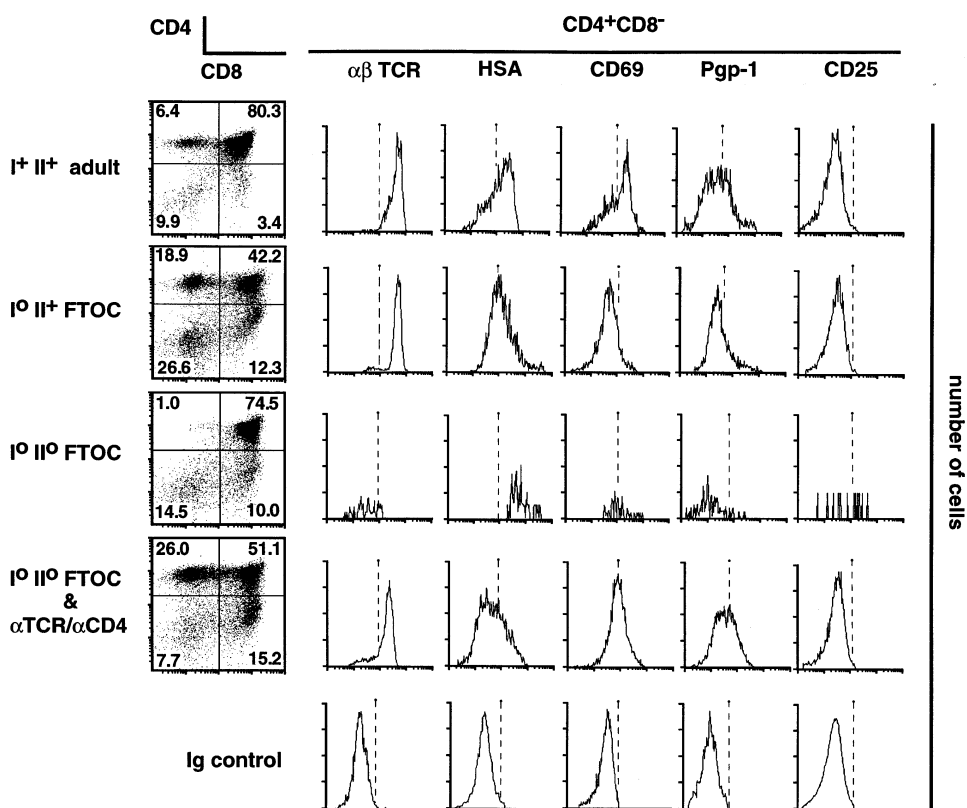


Figure 2. Phenotype of Antibody-Selected CD4⁺CD8⁻ T Cells Derived from MHC I^qII^o Thymic Organ Cultures

Flow cytometric analyses of gated CD4⁺CD8⁻ thymocytes that developed in fetal thymic organ cultures from MHC I^qII⁺ mice (MHC II–selected), from MHC I^qII^o mice (unselected), or from MHC I^qII^o mice with the addition of anti-TCR $\alpha\beta$ and anti-CD4 MAbs (antibody-selected). Shown for comparison are thymocytes from adult MHC I^qII⁺ mice. Thymocytes were stained for CD4, CD8, and either TCR $\alpha\beta$, HSA, CD69, CD44 (Pgp-1), CD25, or TCR $\gamma\delta$ (data not shown, see Table 1). Isotype-matched antibodies were used as negative controls. Note that a substantial fraction of the CD4⁺CD8⁻ thymocytes observed in these organ cultures were either TCR $\gamma\delta$ ⁺ (data not shown) or expressed TCR $\gamma\delta$ rather than TCR $\alpha\beta$ (Table 1).

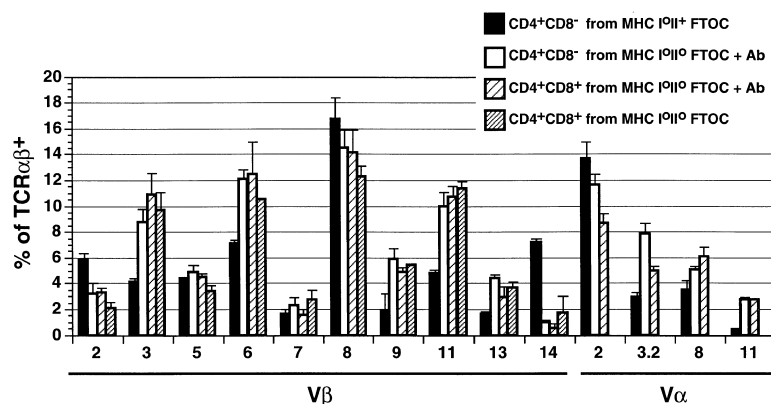


Figure 3. The TCR Variable Region Usage by Antibody-Selected $CD4^+CD8^-$ T Cells Differs from That of MHC II-Selected $CD4^+CD8^-$ T Cells

Organ cultures were prepared with day 16 fetal thymii from MHC I^lI^o or MHC I^lI^o mice. The MHC I^lI^o organ cultures were incubated with or without anti-TCR/anti- $CD4$ MAbs; the MHC I^lI^o organ cultures were incubated without antibodies. After 6 days, the proportion of gated $CD4^+CD8^-$ or $CD4^+CD8^+$ T cells expressing individual TCR $V\beta$ or $V\alpha$ elements were determined by three-color flow cytometry. Percentages given for $CD4^+CD8^-$ T cells were adjusted based on the proportion of cells that stained positively with anti-TCR $\alpha\beta$ MAb H57-597. The results represent the mean $\pm$ SD of 3 ($CD4^+CD8^-$ cells) or 2 ($CD4^+CD8^+$ cells) independent determinations.

differed considerably from those of $CD4^+CD8^-$ thymocytes from MHC II^+ fetal thymic organ cultures ("MHC II-selected $CD4^+$ T cells"). The MHC II-selected $CD4^+$ cells contained a higher number of $V\beta 2^+$ and, especially, of $V\beta 14^+$ $CD4^+CD8^-$ cells, suggesting that these cells are preferentially positively selected by IA^b . Previous findings suggested that $V\beta 14^+$ T cells are favored for positive selection by IA^b , compared to positive selection by the K^b and D^b class I molecules (Liao et al., 1989). T cells expressing $V\beta 3$, $V\beta 6$, $V\beta 9$, $V\beta 11$, and $V\beta 13$ were more frequent in the antibody-selected repertoire than in the MHC II-selected repertoire. The B6/B10 strains express the endogenous mammary tumor virus-encoded Mtv-8, Mtv-9, and Mtv-17 superantigens. Previous studies document that $V\beta 11^+$ and $V\beta 13^+$ T cells undergo clonal deletion in mice expressing these superantigens (Braun et al., 1995; Scherer et al., 1995), suggesting that the higher frequency of these cells in the antibody-selected repertoire is due to their failure to undergo superantigen-induced clonal deletion in the MHC $^-$ thymii. The higher frequency of $V\beta 3^+$, $V\beta 6^+$, and $V\beta 9^+$ cells in the antibody-selected repertoire could reflect a previously undetected superantigen reactivity of these $V\beta$ s, or could be due to inefficient positive selection of cells expressing these $V\beta$ s by IA^b MHC molecules.

As a means to address directly whether the antibody selection procedure operates independently of the MHC specificity of TCRs on selected cells, we determined whether the antibodies induce thymocytes expressing an MHC I-specific TCR to mature inappropriately into $CD4^+CD8^-$ thymocytes. Mice expressing a transgene-encoded TCR specific for HY antigen presented by the D^b class I molecule were crossed onto an MHC I^o background in order to prevent expression of the selecting D^b class I molecule. Cultures of HY-TCR transgenic/MHC I^o fetal thymii in the presence of anti-TCR and anti- $CD4$ MAbs resulted in a substantial increase in the number of $CD4^+CD8^-$ thymocytes, which stained with T3.70 MAb specific for the transgene-encoded TCR and were mature based on their HSA $^-$ phenotype (Figure 4). Control organ cultures lacking the MAbs contained some $CD4^+CD8^-$ cells, but these were largely T3.70 $^-$, indicating that they expressed endogenously encoded TCRs. These data indicated that the antibody mixture

can induce maturation of MHC I-specific thymocytes into $CD4^+CD8^-$ cells, even in the absence of the normal selecting ligand(s) for the corresponding TCRs.

MHC Reactivity of Antibody-Selected Thymocytes

A limiting dilution analysis (LDA) of proliferating $CD8^-$ (predominantly $CD4^+$) thymocytes was employed as one means to assess MHC reactivity of the selected thymocytes (Figure 5). An average of 1/498 of the antibody-selected $CD4^+$ T cells responded to H-2 a stimulator cells. H-2 a molecules fail to present endogenous superantigens (Herman et al., 1991), and therefore, it is likely that the observed responses were directed at MHC molecules rather than at superantigens (and see below). A similar frequency of antibody-selected T cells (1/546) also reacted against "self" H-2 b (MHC I^o) stimulator cells. These responses were specific for the A^b MHC II molecule, since there was no reaction against the H-2 b MHC I^lI^o stimulator cells that were used as the baseline for the LDAs. The absence of tolerance to H-2 b molecules, defined as self by the parents of the MHC I^lI^o mice, would be expected of the preselection repertoire. The

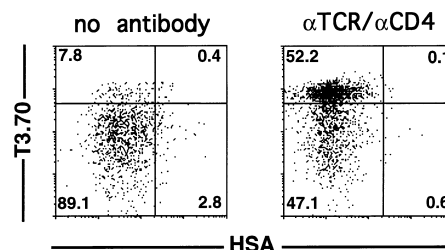
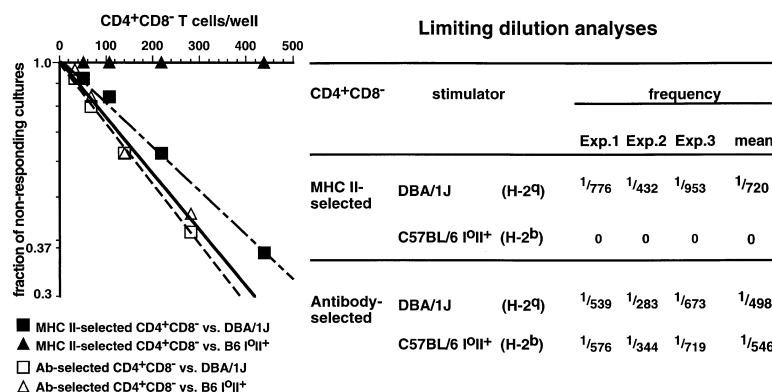


Figure 4. Antibody-Mediated Selection of $CD4^+CD8^-$ Thymocytes Expressing an MHC Class I-Specific Transgenic TCR in a Nonselective (MHC I^lI^o) Background

Day 16 fetal thymii from HY-TCR-transgenic, MHC I^lI^o mice were cultured alone (left panel) or with the addition of the anti-TCR/anti- $CD4$ MAb mixture (right panel). After 6 days, gated $CD4^+CD8^-$ T cells were analyzed for expression of the maturation marker HSA and the transgenic TCR, revealed by clonotypic MAb T3.70, by four-color flow cytometry. Averaging the results of three determinations, the antibody treatment resulted in a 4.6-fold increase in the absolute number of $CD4^+CD8^-$ T3.70 $^+$ thymocytes per thymic lobe, compared to control cultures.



for the frequency determinations for experiments 1–3 were: 1/578–1/940, 1/328–1/515, and 1/655–1/1242 for MHC II-selected cells versus H-2^b; 1/427–1/470, 1/263–1/408, and 1/534–1873 for antibody-selected cells versus H-2^q; and 1/402–1/601, 1/216–1/335, and 1/504–1/812 for antibody-selected cells versus H-2^b.

frequencies of MHC-reactive cells observed in the antibody-selected repertoire were similar to the frequency of control MHC II^b-selected CD8⁻ thymocytes that proliferated in response to allogeneic DBA/1J (H-2^q) stimulator cells (1/720). These frequencies are within the reported range (Ceredig et al., 1983; Itoh et al., 1990), but underestimate the frequency of MHC-reactive cells in the repertoire due to imperfect plating efficiencies in LDAs. As expected, the MHC II^b-selected thymocytes did not respond to self (H-2^b MHC I^q) stimulator cells.

To extend the analysis of the antibody-selected repertoire, a panel of T hybridomas was prepared from antibody-selected CD4⁺ T cells, or control MHC II-selected CD4⁺ T cells, after activating the cells with immobilized anti-TCR and costimulating anti-CD28 antibodies. As fusion partner, we employed the TCR α ⁻ and β ⁻ variant of BW5147 (White et al., 1989). Hybridomas that stained positively with anti-TCR $\alpha\beta$ and anti-CD4 MAbs and that produced IL-2 in response to anti-TCR $\alpha\beta$ stimulation were further tested for MHC reactivity. Each hybrid was stimulated with splenic cells from 10 different strains, representing 8 independent MHC haplotypes (Figure 6). Only 1–2 hybrids from either collection responded to negative control MHC I^qII^b stimulator cells, and these were discarded. A total of 38 reactive hybridomas was stained with a panel of 10 anti-V β antibodies, revealing positive staining in 28 instances (see Figure 6 for V β assignments). Most of the reactive hybrids were clonal, based on the observation that 27 of 28 reactive hybrids in both sets that stained with a given V β antibody stained uniformly with that antibody. No evidence was found for expression of multiple V β s by a given hybridoma clone, based either on costaining with 2 anti-V β antibodies or comparisons of the ratio of staining intensity of different hybridomas with a given anti-V β antibody versus anti-TCR β antibody. Hybridomas from set 1 of each panel were tested against both MHC I^qII⁺ and MHC I⁺II⁺ stimulator cells of the relevant H-2^b, H-2^d, or H-2^k haplotypes, and all proved to be MHC II reactive; MHC II reactivity is to be expected, based on the expression of the class II-specific CD4 coreceptor by the hybridomas.

An average of 5.7% of the hybrids representing the antibody-selected repertoire responded to any given

Figure 5. MHC-Reactive T Cells Are as Frequent in the Antibody-Selected Population as in the MHC II-Selected Population

Limiting dilution analysis (LDA) was employed to determine precursor frequencies of MHC-reactive T cells from antibody-treated MHC I^qII^b or untreated MHC I^qII⁺ organ cultures after depletion of CD8⁺ cells. Stimulator cells were irradiated spleen cells from MHC I^qII⁺ (H-2^b), DBA/1J (H-2^q), or I^qII^b (H-2^b) mice. The latter cells were used as negative controls to determine the background of the LDA, and essentially all of the wells yielded very low cpm. Results of three independent experiments and the mean are shown (right panel), as well as the graphic depiction of experiment 2 (left panel). The 95% confidence intervals

H-2 haplotype, excluding those that exhibited reactivity with endogenous superantigens (see below). In comparison, 5.4% of the hybrids representing the MHC II-selected repertoire reacted with a given H-2 haplotype, excluding the H-2^b (self) stimulator cells that did not stimulate any of these hybrids. In terms of MHC reactivity, there was no statistically significant difference between these hybridoma sets ($p > 0.1$ by χ^2 test). Hybrids representing each repertoire were reactive with each stimulating MHC haplotype tested, with the exception of the MHC II-selected hybrids responding to H-2^b (self) stimulator cells, where no responses were detected (Figure 6A). The antibody-selected repertoire was not tolerant to H-2^b stimulator cells, as a similar frequency of these hybrids reacted against H-2^b stimulator cells and non-H-2^b stimulator cells (Figure 6B). These results demonstrated that the predilection for MHC reactivity is high in the antibody-selected repertoire. However, in contrast to the predictions of Ignatowicz et al. (Ignatowicz et al., 1996), MHC reactivity in the antibody-selected repertoire was not substantially higher than observed in the MHC-selected repertoire.

If the observed responses represented reactivities to endogenous mammary tumor virus (Mtv) genome-encoded superantigens presented by MHC molecules, our conclusions would be misleading. We examined whether the hybrids that were stimulated with B10.BR (H-2^k) stimulator cells could also be stimulated with cells from an H-2^k mouse strain that had been bred free of endogenous superantigens (Scherer et al., 1995). Hybrids that respond to B10.BR but not to the Mtv⁻ H-2^k stimulator cells are likely to be superantigen-reactive. It was found that 3 of the 7 B10.BR-reactive hybrids representing the antibody-selected repertoire were superantigen reactive, as were 2 of the 6 B10.BR-reactive hybrids from the MHC II-selected repertoire (listed in Figure 6, bottom entries). One of the superantigen-reactive hybrids from the antibody-selected repertoire expressed V β 5.1 and/or V β 5.2, previously shown to react with E^k and either the Mtv-8 or Mtv-9 superantigens that are expressed in B6/B10 mice (Braun et al., 1995; Scherer et al., 1995). The other Mtv superantigen-reactive hybrids expressed V β 3, V β 8, or undetermined

MHC II-selected CD4 ⁺ hybridomas												
		C57BL/6										
		A ^b	A ⁱ	A ^q	A ^s	A ^d E ^d	A ^u E ^u	A ^v E ^v	A ^k E ^k			mtv
#	Vβ											A ^k E ^k
1		-	-	-	-	-	-	-	-			
2		-	-	-	-	-	-	-	-			
3		-	-	-	-	-	-	-	-			
4		-	-	-	-	-	-	-	-			
5		-	-	-	-	-	-	-	-			
6		-	-	-	-	-	-	-	-			
7		-	-	-	-	-	-	-	-			
8		-	-	-	-	-	-	-	-			
9		-	-	-	-	-	-	-	-			
10		-	-	-	-	-	-	-	-			
11		-	-	-	-	-	-	-	-			
12	3	-	-	-	-	-	-	-	-			
13		-	-	-	-	-	-	-	-			
14	x	-	-	-	-	-	-	-	-			
15		-	-	-	-	-	-	-	-			
16	2	-	-	-	-	-	-	-	-			
17		-	-	-	-	-	-	-	-			
18		-	-	-	-	-	-	-	-			
19		-	-	-	-	-	-	-	-			
20	6	-	-	-	-	-	-	-	-			
21	5	-	-	-	-	-	-	-	-			
22		-	-	-	-	-	-	-	-			
23		-	-	-	-	-	-	-	-			
24		-	-	-	-	-	-	-	-			
25	11	-	-	-	-	-	-	-	-			
26		-	-	-	-	-	-	-	-			
27	3	-	-	-	-	-	-	-	-			
28	11	-	-	-	-	-	-	-	-			
29		-	-	-	-	-	-	-	-			
30		-	-	-	-	-	-	-	-			
31		-	-	-	-	-	-	-	-			
32		-	-	-	-	-	-	-	-			
33		-	-	-	-	-	-	-	-			
34		-	-	-	-	-	-	-	-			
35		-	-	-	-	-	-	-	-			
36	7	-	-	-	-	-	-	-	-			
37		-	-	-	-	-	-	-	-			
38		-	-	-	-	-	-	-	-			
39	2	-	-	-	-	-	-	-	-			
40	8	-	-	-	-	-	-	-	-			
41	x	-	-	-	-	-	-	-	-			
42		-	-	-	-	-	-	-	-			
43		-	-	-	-	-	-	-	-			
44		-	-	-	-	-	-	-	-			
45		-	-	-	-	-	-	-	-			
46		-	-	-	-	-	-	-	-			
47	x	-	-	-	-	-	-	-	-			
48		-	-	-	-	-	-	-	-			
49	ND	-	-	-	-	-	-	-	-			
50		-	-	-	-	-	-	-	-			
51		-	-	-	-	-	-	-	-			
52		-	-	-	-	-	-	-	-			
53		-	-	-	-	-	-	-	-			
54		-	-	-	-	-	-	-	-			
55	x	-	-	-	-	-	-	-	-			
56		-	-	-	-	-	-	-	-			
57		-	-	-	-	-	-	-	-			
58		-	-	-	-	-	-	-	-			
mtv-superantigen reactive hybridomas												
59	8	-	-	-	-	-	-	-	-			
60	3&x	-	-	-	-	-	-	-	-			

Antibody-selected CD4 ⁺ hybridomas												
		C57BL/6										
		A ^b	A ⁱ	A ^q	A ^s	A ^d E ^d	A ^u E ^u	A ^v E ^v	A ^k E ^k			mtv
#	Vβ											A ^k E ^k
1		-	-	-	-	-	-	-	-			
2	7	-	-	-	-	-	-	-	-			
3		-	-	-	-	-	-	-	-			
4	7	-	-	-	-	-	-	-	-			
5	2	-	-	-	-	-	-	-	-			
6	5	-	-	-	-	-	-	-	-			
7		-	-	-	-	-	-	-	-			
8		-	-	-	-	-	-	-	-			
9		-	-	-	-	-	-	-	-			
10	x	-	-	-	-	-	-	-	-			
11		-	-	-	-	-	-	-	-			
12		-	-	-	-	-	-	-	-			
13		-	-	-	-	-	-	-	-			
14	2	-	-	-	-	-	-	-	-			
15	2	-	-	-	-	-	-	-	-			
16	3	-	-	-	-	-	-	-	-			
17	x	-	-	-	-	-	-	-	-			
18		-	-	-	-	-	-	-	-			
19		-	-	-	-	-	-	-	-			
20		-	-	-	-	-	-	-	-			
21		-	-	-	-	-	-	-	-			
22		-	-	-	-	-	-	-	-			
23		-	-	-	-	-	-	-	-			
24		-	-	-	-	-	-	-	-			
25		-	-	-	-	-	-	-	-			
26		-	-	-	-	-	-	-	-			
27	8	-	-	-	-	-	-	-	-			
28	8	-	-	-	-	-	-	-	-			
29	11	-	-	-	-	-	-	-	-			
30	x	-	-	-	-	-	-	-	-			
31		-	-	-	-	-	-	-	-			
32		-	-	-	-	-	-	-	-			
33		-	-	-	-	-	-	-	-			
34		-	-	-	-	-	-	-	-			
35		-	-	-	-	-	-	-	-			
36		-	-	-	-	-	-	-	-			
37		-	-	-	-	-	-	-	-			
38		-	-	-	-	-	-	-	-			
39		-	-	-	-	-	-	-	-			
40		-	-	-	-	-	-	-	-			
41	5	-	-	-	-	-	-	-	-			
42		-	-	-	-	-	-	-	-			
43		-	-	-	-	-	-	-	-			
44		-	-	-	-	-	-	-	-			
45		-	-	-	-	-	-	-	-			
46		-	-	-	-	-	-	-	-			
47		-	-	-	-	-	-	-	-			
48	x	-	-	-	-	-	-	-	-			
49		-	-	-	-	-	-	-	-			
50		-	-	-	-	-	-	-	-			
51		-	-	-	-	-	-	-	-			
52		-	-	-	-	-	-	-	-			
53	6	-	-	-	-	-	-	-	-			
54		-	-	-	-	-	-	-	-			
55		-	-	-	-	-	-	-	-			
56		-	-	-	-	-	-	-	-			
57	8	-	-	-	-	-	-	-	-			
58	6	-	-	-	-	-	-	-	-			
59		-	-	-	-	-	-	-	-			
60		-	-	-	-	-	-	-	-			
61	x	-	-	-	-	-	-	-	-			
mtv-superantigen reactive hybridomas												
62	3	-	-	-	-	-	-	-	-			
63	5	-	-	-	-	-	-	-	-			
64	x	-	-	-	-	-	-	-	-			

Figure 6. Pattern of MHC Reactivity of Hybridomas Prepared from Antibody-Selected and MHC II-Selected CD4⁺CD8⁻ Thymocytes. Hybridomas were stimulated with the indicated spleen cells, and IL-2 in the supernatant was tested with the CTLL-2 bioassay. Closed boxes indicate a positive reaction (cpm at least 4-fold over background with H-2^b MHC I^l spleen cells), (–) indicates a negative reaction (most of these were close to background), and open boxes were not tested. One or two hybrids from each panel secreted IL-2 upon challenge with H-2^b MHC I^l spleen cells, which served as negative control; these are not depicted. All of the hybridomas produced abundant IL-2 in response to plate-bound anti-TCRαβ (MAb H57-597) stimulation (data not shown). The data represent a compilation of several experiments. Expression by MHC reactive hybridomas of TCR Vβ 2, 3, 5, 6, 7, 8, 9, 11, 13, or 14 was determined by flow cytometry. Hybridomas expressing none of these Vβ elements are indicated with (x), and (ND) indicates not determined. The MHC II-selected hybridoma panel was derived from two sets of fusions (set 1: #1–20, and set 2: #21–60), whereas the antibody-selected hybridoma panel was derived from three sets of fusions (set 1: #1–17, set 2: #18–27, and set 3: #28–64).

Vβs. The Mtv/H-2^k-reactive hybrids were excluded from calculations of MHC reactivity. Since the IE^k class II molecule presents all tested endogenous superantigens very effectively (Herman et al., 1991), it is unlikely that

any of the remaining MHC reactive clones are reactive to B6/B10-derived superantigens.

A question of some interest is whether the antibody-selected repertoire contains a subset of clones that

Table 2. Distribution of MHC Reactivities in the Antibody-Selected Repertoire

Number of MHC Reactivities/Hybrid	Observed	Predicted from Poisson Distribution
0	42	38.6
1	14	17.7
2	2	4.1
3	2	0.6
4	1	0.1
Sum	61	61

The observed distribution is not significantly different from the predicted one, $\chi^2 = 1.09$, $p > 0.1$ (for statistical analysis, the 2, 3, and 4 reactivity groups were combined to validate the calculations). Mtv superantigen-reactive hybridomas were omitted for the calculations.

cross-react promiscuously with several or many MHC molecules. Hybrids reactive with up to 3 or 4 of the 8 tested H-2 haplotypes were present in both hybridoma sets. In most or all of these cases, multireactivity was due to cross-reactivity rather than lack of clonality, because 3 of 5 multireactive hybrids in each hybridoma set stained uniformly with one of the V β antibodies tested; the remaining 2 multireactive hybrids in each set did not stain with any of the V β antibodies tested. While MHC multireactivity was clearly observed, a statistical analysis suggests that it occurs largely at random. If the reactivities were distributed randomly, the pattern should follow the Poisson distribution, which predicts that the observed 28 MHC reactivities in the antibody-selected repertoire should be distributed among the 61 hybrids, as depicted in Table 2. As assessed by the χ^2 test, the observed distribution of reactivities was indistinguishable from the predicted distribution ($p > 0.1$, Table 2). Thus, there is no indication from this analysis that reactivity with multiple MHC molecules occurs at a higher than expected frequency in the preselection repertoire.

Discussion

Positive Thymic Selection Mediated by Anti-TCR and Anti-CD4 MABs

In line with an earlier report (Takahama et al., 1994), we observed that soluble anti-TCR $\alpha\beta$ MABs added to a fetal thymic organ culture from MHC-deficient mice can stimulate maturation of CD4 $^+$ thymocytes. In the earlier study, it was emphasized that the effectiveness of the treatment depended on the anti-TCR $\alpha\beta$ antibody being in a soluble and unaggregated form, or it would otherwise cause clonal deletion. Thus, a relatively low level of TCR signaling by the bivalent anti-TCR $\alpha\beta$ MAB appears to substitute for the low affinity multivalent interaction thought to stimulate positive selection in vivo. Our results extend the earlier findings, as we observed that not only CD4 $^+$, but also CD8 $^+$ thymocyte maturation was induced by anti-TCR $\alpha\beta$ MABs. We found that soluble anti-CD4 MABs strongly enhanced the anti-TCR-induced maturation of CD4 $^+$ thymocytes in MHC I b thymic organ cultures, as had been previously demonstrated in MHC $^+$ thymic organ cultures (Punt et al., 1993).

These CD4 $^+$ cells were of resting, naive cell surface phenotype, suggesting that they arose by maturation as opposed to postmaturation expansion of a few precursor cells. Significantly, the anti-CD4 MAB enhanced the differentiation of only CD4 $^+$ thymocytes, not CD8 $^+$ thymocytes. These data suggest that coreceptor signaling induced by the anti-CD4 antibody promotes positive selection, just as MHC-induced coreceptor signaling enhances positive selection in vivo. This system may therefore be useful for subsequent studies of TCR and coreceptor signaling in positive selection.

The Antibody-Selected Repertoire

Because the antibody-selected repertoire is created in MHC-deficient mice, it is likely to resemble the preselection repertoire. A caveat is that the MHC-deficient mice express very low levels of class I molecules (Bix and Raulet, 1992). However, these levels are too low to stimulate appreciable levels of T cell development (Zijlstra et al., 1990). Although it is formally possible that the mice could also express very low levels of an A α E β dimeric class II molecule, attempts to detect this molecule have met with failure (Cosgrove et al., 1991). In any case, several features of our data strongly support the contention that the antibody-selected repertoire is representative of the preselection repertoire. First, the V β and V α distribution was similar to that of CD4 $^+$ CD8 $^+$ thymocytes present in MHC I b thymic organ cultures, yet differed considerably in several respects from that of MHC II-selected CD4 $^+$ T cells. Second, as would be expected of the preselection repertoire, the antibody-selected repertoire contained clones reactive with H-2 b MHC molecules, which defines self for the parents of the MHC I b mice (Figures 5 and 6). Third, the antibody selection protocol caused the maturation of T cells expressing the MHC I-restricted HY-specific TCR into the CD4 $^+$ compartment, in the absence of the D b -selecting ligand for the HY-TCR. Taken together, these findings indicate that the antibody-selected repertoire is independent of MHC expression and representative of the preselection repertoire that exists in the fetal thymus.

MHC Reactivity of the Preselection Repertoire

Collectively, the results of the LDAs and hybridoma analysis suggest that the preselection repertoire exhibits a high reactivity with MHC-expressing cells. The LDAs and hybridoma analysis demonstrated a similar frequency of MHC-reactive cells in the antibody-selected and MHC II-selected repertoires. However, the LDAs yielded a 25-fold lower estimate of the frequency of MHC-reactive cells than the hybridoma analysis. In similar LDAs with TCR-transgenic CD8 $^+$ T cells, we observed that the plating efficiency was only about 10%, suggesting that the frequencies based on the hybridoma analysis are more accurate than those from the LDA (data not shown). Based on the hybridoma analysis, the average percentage of hybrids from the antibody-selected repertoire that were reactive with a given MHC class II $^+$ stimulator cell was 5.7%, similar to the 5.4% observed in the MHC II-selected repertoire. Correcting for the fact that some of the stimulator cells present two class II molecules (IA and IE), while others present only one (IA), an average

of 4.4% of the hybrids in the antibody-selected repertoire was reactive against a given class II molecule, compared to 3.4% in the MHC II-selected repertoire. From another point of view, more than 30% of the antibody-selected hybridomas reacted with at least one of the 8 MHC haplotypes in the panel, suggesting that most clones in the preselection repertoire are reactive with one or another of the 100 or so murine MHC II molecules available in nature (Gotze et al., 1980). Many of these hybridomas may also be MHC I-reactive, but were not observed, presumably because the hybridomas do not express CD8. These data argue strongly that MHC reactivity is inherent in the germline-encoded portions of the TCR, independent of positive and negative thymic selection.

The TCRs in the fetal repertoire contain smaller and less variable CDR3 regions due to the low levels of terminal deoxynucleotidyl transferase in the fetal thymus. Recent reports raise the possibility that the shorter CDR3 regions may confer greater MHC reactivity (Gilfillan et al., 1994; Gavin and Bevan, 1995). Therefore, the MHC reactivity of the fetal preselection repertoire, as assessed in our experiments, may be somewhat higher than that in the adult preselection repertoire.

In recent studies, it was proposed that the alloreactivity observed in the normal repertoire might represent only the "tip of the iceberg" of all alloreactivity (Ignatowicz et al., 1996). This suggestion was based on the observation that among clones positively and negatively selected by a single peptide/MHC complex, a large fraction exhibited alloreactivity, and an even larger fraction exhibited reactivity with cells expressing the selecting MHC molecule complexed with the full array of cellular peptides. This reactivity pattern could arise if these TCRs bind mostly to conserved MHC residues, with only a small contribution from interactions with peptide residues. It was suggested that this recognition pattern would also result in a higher than expected degree of reactivity with cells expressing other MHC molecules. However, our results suggest that cells with multiple MHC reactivities are not significantly more common in the preselection repertoire than would be predicted by chance, given the observed overall number of MHC reactivities. Furthermore, our data suggest that the frequency of MHC reactivities in general is not significantly elevated compared to the MHC-selected repertoire. Even if a larger sample were to show significantly increased MHC reactivity in the preselection repertoire, the effect is bound to be small based on the present analysis. Our data are in line with the conclusions of an analysis of apoptotic cells in the thymus, which failed to detect a high level of MHC-induced negative selection in the normal thymus (Surh and Sprent, 1994). To reconcile our results with those of Ignatowicz et al., we propose that thymic selection by a single peptide/MHC complex results in the enrichment of relatively rare clones in the preselection repertoire that exhibit strong interactions with conserved MHC residues, and therefore excessive cross-reactivity with different MHC molecules. The enrichment of these clones occurs because many of them are not induced to undergo negative selection by this particular peptide/MHC complex, although they would be deleted in a normal thymus. Thus,

we propose that a single peptide/MHC complex must select a relatively small fraction of the preselection repertoire, which accumulates under steady state conditions to represent a substantial number of CD4⁺ T cells. Based on our data, the highly MHC cross-reactive clones must be relatively rare, perhaps no more frequent than would be predicted by chance given the observed distribution of MHC reactivities in the antibody-selected repertoire.

It might be argued that the preselection repertoire contains many broadly MHC-reactive clones that were lost during the preparation of hybridomas. This could occur because MHC I (H-2^b) and β 2m molecules donated by the BW5147 fusion partner were expressed by the hybridomas; hybridomas reactive with these MHC I molecules might have been induced to undergo apoptosis and therefore would have been lost from the panel. It should first be noted that the results of Ignatowicz et al. were also obtained with a T hybridoma panel, so this mechanism cannot account for any differences in the results between that study and ours. Second, it is unlikely that the loss of hybridomas by this mechanism is seriously biasing the antibody-selected repertoire, based on the independent comparison of MHC reactivity from the LDA experiments. The similar frequencies of MHC-reactive clones in the LDAs performed with antibody-selected and MHC-selected repertoires supports the conclusion that the preselection repertoire exhibits a level of MHC reactivity similar to that of the MHC-selected repertoire and suggests that there cannot exist a large number of pan-MHC-reactive clones that were lost during hybridoma preparation.

The results suggest that a minimum of 5.7% of the preselection repertoire is potentially subject to negative selection in mice of a given haplotype, since that is the average percentage of clones that reacted with each MHC haplotype. The actual percentage is likely to be significantly higher than this, since our assays were not suitable for detecting MHC I-reactive clones. Furthermore, negative selection can delete clones that exhibit avidity for MHC below the threshold for triggering mature T cells (Pircher et al., 1991). MHC heterozygosity, which is the rule in outbred populations, should lead to a further doubling of the frequency of cells susceptible to negative selection. Taking all of these factors into account, we estimate that 20%–30% of thymocytes in a typical outbred animal might be susceptible to negative selection. In a hypothetical mouse expressing all MHC molecules of the species, the entire repertoire might be subject to clonal deletion, suggesting an evolutionary rationale for the distribution of a few highly polymorphic MHC molecules to each member of the species, rather than having each member express a large number of different MHC molecules. The fraction of the preselection repertoire that is subject to positive selection cannot be ascertained from our results.

The inherent MHC reactivity of TCRs leads to the inference that TCRs interact with MHC molecules in a specific way(s). Recent structural analyses of TCR–MHC/peptide complexes suggest that different TCRs may interact with their different MHC/peptide target complexes with a similar orientation (Brock et al., 1996; Garcia et al., 1996; Garboczi et al., 1996; Sant'Angelo et

al., 1996). Numerous contacts between the TCR and conserved MHC I residues were observed primarily in the CDR α 1, CDR α 2, and CDR β 3 regions of the TCR (Garboczi et al., 1996). Evidence that the TCR α CDR1/CDR2 regions interact with MHC molecules has also been recently provided (Sim et al., 1996). It will be interesting to determine whether specific contacts between TCR residues and conserved MHC residues underlie the TCR-MHC interaction and account for the inherent MHC reactivity of the T cell repertoire.

Experimental Procedures

Mice

The C57BL/6 (*H-2^b*), B10.BR (*H-2^k*), and BALB/c (*H-2^d*) β 2m-deficient (β 2m^{-/-}), C57BL/6 A β -deficient (A β ^{-/-}), and C57BL/6 A β ^{-/-} β 2m^{-/-} mice have been described before (Zijlstra et al., 1990; Grusby et al., 1993). The B6- β 2m^{-/-} and B6-A β ^{-/-} mice used to prepare MHC I^{fl} mice and as stimulator cells had each been backcrossed to B6 five times. HY-TCR-transgenic (*H-2^b*) (Teh et al., 1988) and *Mtv⁻* (*H-2^k*) (Scherer et al., 1995) mice were generously provided by Drs. H. von Boehmer (Basel Institute for Immunology, Basel, Switzerland) and P. Marrack and J. Kappler (University of Colorado, CO, USA). HY-TCR-transgenic mice were crossed with C57BL/6 β 2m^{-/-} (MHC I^{fl}) mice, and the pups were intercrossed to obtain HY-TCR⁺, MHC I^{fl} mice. The remaining strains were purchased from The Jackson Laboratories (Bar Harbor, ME, USA).

Antibodies

From CALTAG (San Francisco, CA, USA), we purchased anti-CD4-Phycoerythrin (PE) (CT-CD4), anti-CD8/Lyt-2-Fluorescein isothiocyanate (FITC) (CT-CD8 α), anti-CD44/Pgp-1-FITC (IM7.8.1), anti-CD25/IL-2R α -FITC (PC61.5.3), anti- $\gamma\delta$ TCR-FITC (GL.3), and Streptavidin-Tricolor (SA1006). From Pharmingen (San Diego, CA, USA), we purchased biotinylated anti-V β , anti-V α , and anti-CD69 (H1.2F3). From GIBCO (Grand Island, NE, USA), we purchased anti-CD8/Lyt-2-RED613 $\oplus$ (#53-6.7). Anti-CD24/HSA (M1/69.16.11. HL) and anti-TCR β (H57-597) hybridomas were purchased from ATCC (Rockville, MD, USA). Anti-HY-TCR (T3.70) (Teh et al., 1988) was provided by Dr. H. von Boehmer. The antibodies were purified from culture supernatants over Protein G Agarose (Boehringer, Indianapolis, USA) and either biotinylated or FITC-conjugated. For addition to fetal organ cultures, the purified MAb H57-597 in PBS was centrifuged for 4 hr at 55,000 rpm (Beckman TLS55), 4°C, and sterile-filtered.

Fetal Thymic Organ Culture (FTOC)

Thymii from embryonic day 16 or 17 fetuses were placed in fetal thymic organ cultures on 0.4 μ m membranes of 24 mm Transwell plates (Corning Costar Corp.) submersed in 2 ml DMEM (GIBCO, #11995) supplemented with 10% fetal calf serum (FCS; Sigma, St. Louis, MO, USA), 2-ME, nonessential aminoacids, and antibiotics. On days 1, 3, and 4 (or 5), the medium was replaced with medium containing 10 μ g/ml anti-TCR β MAb H57-597 and 30% culture supernatant from anti-CD4 MAb producing GK1.5 hybridoma, unless stated otherwise. On day 5 (or 6) fetal thymii were washed with medium and incubated for another day in the absence of the antibodies. For untreated organ cultures, the medium was changed accordingly. After a total of 6 or 7 days in culture, single cell suspensions of thymocytes were prepared and analyzed.

Flow Cytometric Analysis

For three-color analysis, thymocytes were first stained with anti-CD4-PE and either biotinylated anti-V β , anti-V α , or anti-CD69 and subsequently with anti-CD8-FITC and Streptavidin-Tricolor. Alternatively, anti-CD4-PE and anti-CD8-RED613 $\oplus$ were used in the first step, and either FITC-conjugated anti-CD44, -CD25, - $\gamma\delta$ TCR, -CD24, or -TCR β in the second step. For four-color analysis, cells were first stained with anti-CD4-PE, anti-CD8-Red613 $\oplus$, and T3.70-Biotin, and subsequently with Streptavidin-Tricolor and anti-HSA-FITC. The

cells were analyzed on a EPICS Excel flow cytometer (Coulter, Hialeah, FL, USA). 2–4 $\times$ 10⁴ events were accumulated using forward and side scatter gating to exclude nonviable cells. Graphics were generated using the WindMdi program (John Trotter, Salk Institute, USA).

Limiting Dilution Analysis

Thymocytes from organ cultures were depleted of CD8⁺ cells by use of anti-CD8 MAb AD4(15) ascites and rat anti-mouse IgM-coated Dynabeads M-450 (Dynal Inc., Great Neck, NY, USA). The fraction of CD4⁺CD8⁻ T cells in the CD8-depleted fraction was assessed by flow cytometry. Cultures were in 200 μ l DMEM/FCS medium supplemented as described (Ceredig et al., 1983) and containing 15 ng/ml rIL-2 (Chiron Corporation). Graded numbers of thymocytes were plated into round bottom 96-well plates (Costar Corporation) (24 wells per dilution), followed by the addition of 6–8 $\times$ 10⁵ irradiated (3000 rad) splenic stimulator cells from DBA/1J, C57BL/6 β 2m^{-/-}, or C57BL/6 A β ^{-/-} β 2m^{-/-} mice. After 6–7 days at 37°C, 5% CO₂, cultures were restimulated with 5 $\times$ 10⁵ stimulator cells and pulsed 2 days later with 1 μ Ci [³H]thymidine for 12–16 hr. Cultures in which the incorporated label exceeded by more than 3 SD the mean in negative control wells stimulated with MHC I^{fl} stimulator cells were defined as “positive.” The frequency of MHC-reactive thymocytes was calculated according to the minimum chi-square method by use of a computer program kindly provided by Dr. D. Cassel.

Production and Analysis of T Hybridomas

Thymocytes from organ cultures were depleted of CD8⁺ cells, as described above, and stimulated for 2 days in culture wells of 24-well plates that were coated with 10 μ g/ml anti-TCR $\alpha\beta$ MAb H57-597 and 1/400 dilution of anti-CD28 (37.51) ascites fluid (a gift of Dr. M. Krummel). Blasts were purified by Ficoll-Paque centrifugation, washed, fused to BW5147 $\alpha\beta$ ⁻ cells (White et al., 1989), and selection was performed according to standard protocols. Plating efficiency of HAT-resistant hybridomas was $\leq$ 10%, except for the first set (set 1) of hybrids derived from MHC I^{fl} fetal thymii, for which it was almost 100%. Clonality of this set of hybridomas was achieved by subsequent single cell cloning; clonality of most hybridomas in all of the sets was indicated by staining with anti-V β MAbs (see text). Hybridomas that stained strongly positive for CD4 and $\alpha\beta$ TCR and specifically released substantial IL-2 upon $\alpha\beta$ TCR stimulation by plate-bound MAb H57-597 were chosen for further analysis.

MHC reactivity was assessed by culturing 1 $\times$ 10⁵ hybridoma cells with 2 $\times$ 10⁵ irradiated splenic stimulator cells in 200 μ l of complete RPMI medium for 24 hr. Stimulator cells from MHC I^{fl} mice served as negative controls. For evaluation of IL-2 release, 100 μ l of the supernatant was added to 100 μ l containing 5 $\times$ 10³ CTLL-2 cells. The cultures were pulsed after 24 hr with 1 μ Ci [³H]thymidine for 6 hr. Hybridomas were designated MHC-reactive when the stimulation index in reference to the negative controls exceeded 4.0. The large majority of “negative” hybridomas yielded stimulation indices close to 1.0.

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