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Antigen/MHC-Specific T Cells Are Preferentially Exported from the Thymus in the Presence of Their MHC Ligand

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Summary

Transgenic mice expressing a T cell receptor heterodimer specific for a fragment of pigeon cytochrome c plus an MHC class II molecule (I-E^k) have been made. We find that H-2^k αβ transgenic mice have an overall increase in the number of T cells and express a 10-fold higher fraction of cytochrome c–reactive cells than H-2^b mice. Surface staining of thymocytes indicates that in H-2^b mice, T cell development is arrested at an intermediate stage of differentiation ($CD4^+8^+$, $CD3^{lo}$). Analyses of mice carrying these T cell receptor genes and MHC class II I-E_α constructs indicate that this developmental block can be reversed in H-2^b mice by I-E expression on cortical epithelial cells of the thymus. These data suggest that a direct T cell receptor–MHC interaction occurs in the thymus in the absence of nominal antigen and results in the enhanced export of T cells, consistent with the concept of "positive selection."

Introduction

T cell recognition of foreign antigens is mediated through the T cell receptor–CD3 complex. In all known helper (T_H) and cytotoxic (T_C) T cells, this complex consists of polymorphic α and β chain subunits noncovalently associated with at least five invariant CD3 polypeptides (Meuer et al., 1983; Samelson et al., 1985). Gene transfer of particular αβ pairs is generally sufficient to transfer the specificity of one T cell into another (Dembic et al., 1986; Saito and Germain, 1987). Unlike the antigen receptors of B lymphocytes (the immunoglobulin molecule), which bind to native antigen directly, αβ T cell receptors seem principally to recognize fragments of antigens embedded in MHC molecules (reviewed in Davis and Bjorkman, 1988). This phenomenon is referred to as MHC restriction (Zinkernagel and Doherty, 1974).

Many earlier experiments had indicated that, functionally, the T cell repertoire is biased towards recognition of self-MHC, and that this bias is "learned" during T cell development in the thymus (Zinkernagel et al., 1978; Bevan, 1977; Sprent, 1978; Kappler and Marrack, 1978; Fink and Bevan, 1978; von Boehmer et al., 1978; Singer et al., 1981). However, general agreement on these issues was never reached, as additional experiments, both with MHC class I–and class II–restricted T cell responses, suggested little, if any, bias in the T cell repertoire (Matzinger and Mirkwood, 1978; Stockinger et al., 1980; Ishii et al., 1981). The experimental designs used in all these studies were of two basic types: first, reconstitution of irradiated mice with MHC nonidentical bone marrow and/or thymuses, followed by testing of immune responses in these mice for their MHC restriction properties (Zinkernagel et al., 1978; Bevan, 1977; Sprent, 1978; Kappler and Marrack, 1978; von Boehmer et al., 1978); and second, depletion of normal mouse T cell populations for alloreactivity, followed by testing of the remaining cells for responses restricted to that foreign MHC type (Bennink and Doherty, 1978; Stockinger et al., 1980).

One explanation that has been suggested to account for the failure of some experiments to detect a bias towards self-MHC recognition in the T cell repertoire is the possible variability in construction of bone marrow chimeras (Singer, 1988). If donor bone marrow was not adequately depleted of mature T cells, the resulting chimeras would contain donor MHC-specific T cells. Antigen-specific T cell responses in such mice would then be expected to be restricted to both donor and recipient MHC molecules. An additional source of controversy surrounding some of the reconstitution experiments involved the extent of chimerism achieved in the mice, and consequently, the identity and origin of the antigen-presenting cells responsible for priming the T cell responses for which assays were performed. To overcome these objections, some researchers opted for more elaborate experimental protocols, including transfer of T cell populations from the reconstituted recipients into additional mice in order to have a defined priming environment (Sprent, 1978; Fink and Bevan, 1978). Others examined only those T cell responses that could be elicited in the absence of in vivo priming (Stockinger et al., 1980; Singer et al., 1981). The net result of these efforts seems to be an uneasy acceptance of the phenomenon of positive selection, with only limited information about the specificity of the effect due to the heterogeneity of the T cells that could respond to a given antigen.

Among those groups supporting the concept of positive selection, an additional controversy has surrounded the identity of thymic cells responsible for imprinting this bias toward self-MHC recognition (Sprent et al., 1988). For MHC class II–specific T cells, two types of murine thymic

cells are candidates for this function, in that they express class II molecules: cortical epithelial cells or bone marrow–derived macrophage/dendritic cells. The experiments addressing this issue have analyzed T cells from irradiated and bone marrow–reconstituted mice for their MHC specificity. Several groups have shown for both MHC class I- (von Boehmer et al., 1988) and class II–restricted (Zinkernagel, 1982; Lo and Sprent, 1986) T cell responses that both normal and deoxyguanosine-treated thymus grafts (in which macrophage and dendritic cells have been destroyed, but not epithelial cells) result in the positive selection of T cells with affinity for the MHC expressed on the thymus graft. In conflict with these results, Longo and Schwartz (1980) have provided equally convincing evidence that, from the regeneration of peripheral T cells in long term irradiation bone marrow chimeras, the bone marrow–derived cells (macrophages and dendritic cells) are responsible for positive selection in the thymus. To date, this controversy has remained unresolved.

Many of the objections described above can be overcome through the use of mice that are transgenic for specific T cell receptor heterodimer pairs. In this way, a single $\alpha\beta$ T cell receptor of known antigen–MHC specificity can be expressed on a large fraction of T lymphocytes, and the cells expressing this receptor can be followed throughout T cell development. As the expression of the introduced T cell receptor is extremely high in the transgenics, no priming at all (either in vitro or in vivo) is necessary to elicit a response. Furthermore, physical rather than functional assays for measuring receptor frequency can also be utilized, overcoming the objection that the observed bias toward self-MHC recognition is simply the result of suppression in peripheral tissues, not positive selection. Transgenic mice also provide an opportunity for accurate measurement of the extent of positive selection, in addition to identifying where and when during T cell development the selection process is occurring. But most importantly, these studies eliminate the heterogeneity of the response being analyzed and allow the influence of thymic MHC type to be examined with respect to a single T cell receptor of known specificity.

We have developed a transgenic model utilizing T cell receptor α and β chain genes encoding a receptor specific for a class II MHC molecule plus a known peptide. This system allows us to express, and detect the expression of, each transgene either separately or together. By two independent assays—frequency analysis and surface staining—we find increased expression of this T cell receptor in mice of the MHC type that expresses the restricting element for the receptor. In similar transgenic experiments, both Teh et al. (1988) and Sha et al. (1988b) observed a decrease in transgenic T cell receptor–positive cells in mice of a noncompatible MHC type; in addition, they observed predominant expression of the transgene-encoded receptor on $CD8^+$ T cells. We observe the reciprocal of this class I–specific T cell receptor–CD8 pairing, as almost all of the peripheral $\alpha\beta$—positive cells in our transgenics are $CD4^+$. As a result of this selection, the periphery of the $\alpha\beta$ transgenics exhibit an extreme bias toward CD4 expression; this bias is also seen, though to a lesser extent, in the β only transgenics. Furthermore, our experiments show that in transgenic mice expressing an alternative MHC haplotype, the total number of peripheral T cells is significantly reduced.

In addition, transgenic mice can also be used to address the issue of the identity of thymic cells influencing T cell development. Of particular relevance here is the fact that lines of mice have been developed that express particular MHC class II molecules in defined thymic cell types (van Ewijk et al., 1988; Widera et al., 1987). Specific deletions in the 5′ flanking regions of the class II E_α gene, and introduction of these constructs into mice, have generated transgenic lines expressing the E_α gene either in thymic macrophage/dendritic cells or predominantly in cortical epithelium. By crossing the T cell receptor transgenes into mice expressing these E_α transgenes, the role of each of these class II–positive thymic cell types has been examined. We find that expression of class II I-E molecules on thymic cortical epithelial cells, but not on medullary epithelial cells, macrophages, and dendritic cells, is sufficient for positive selection.

Results

Expression of a Functional T Cell Receptor in Transgenic Mice

To generate transgenic mice expressing functional 2B4 specificity (Hedrick et al., 1982; Samelson et al., 1983; Buus et al., 1986), the rearranged α and β chain genes of 2B4 were introduced separately into the germline of (C57Bl/6J × C3H/HeJ)F_2 mice (Brinster et al., 1981; Constantini and Lacey, 1981; Hogan et al., 1986; Wagner et al., 1981a, 1981b). Mice expressing the 2B4 α chain have been described previously (Ivars et al., 1988; Berg et al., 1988). Briefly, transgenic lines were generated with two different α chain constructs (Figure 1A). The first, 2B4α, leads to expression of this α chain on 3%–5% of peripheral T cells, the majority of which also express endogenous α chains (Berg et al., 1988). In contrast, mice carrying the second construct, 2B4αE_H, which contains a 687 nucleotide fragment encompassing the immunoglobulin heavy chain enhancer (Gillies et al., 1983; Banerji et al., 1983), express the 2B4 α chain exclusively on 70%–80% of peripheral T cells (Berg et al., 1988).

On the basis of these results, we inserted the same immunoglobulin heavy chain enhancer fragment into a rearranged genomic clone of the 2B4 β chain gene (Figure 1B). This clone contained 4.5 kb of 5′ and 3 kb of 3′ flanking sequence from the β chain locus. Four transgenic lines expressing this 2B4βE_H construct were generated. All experiments described below were carried out with one such line which has integrated ~8 copies of the transgene and expresses the 2B4 β chain on 90% of peripheral T cells.

To assess whether the α and β chain transgenes encoded a functional T cell receptor, 2B4α^+ mice were crossed to 2B4βE_H^+ mice, and the resulting unimmunized progeny (all H-2^{kxb}) were tested for primary in vitro responses to cytochrome c by a proliferation assay (Bradley et al., 1980). Only mice carrying both the α and β chain

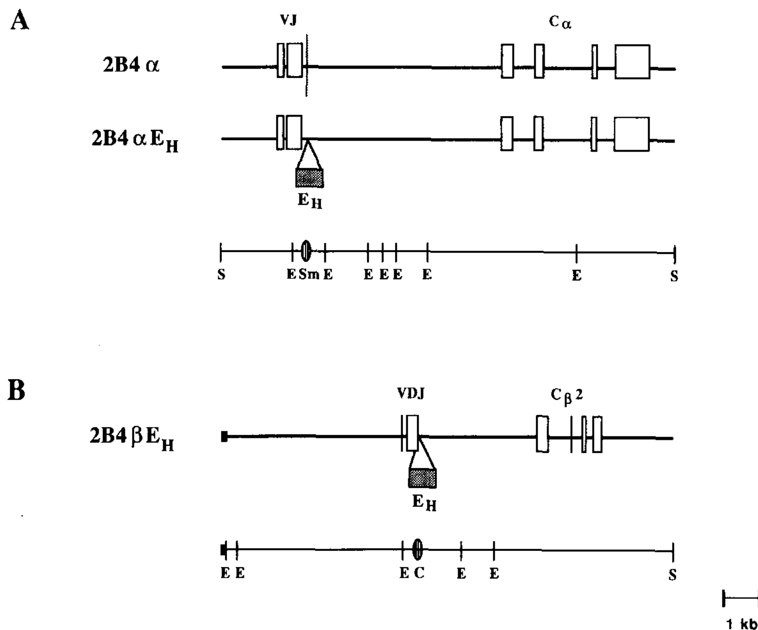


Figure 1. T Cell Receptor α and β Chain Constructs

Maps of the rearranged 2B4 α (A) and β (B) chain constructs used for injection into fertilized mouse eggs, indicating the position of the immunoglobulin heavy chain enhancer fragment introduced into both α and β chains. The line downstream of the 2B4 α VJ segment indicates the site where the rearranged VJ sequences were joined to a genomic clone of the C_α region, deleting approximately 55 kb of intervening sequence. The black square at the left end of the $2B4\beta E_H$ construct represents 120 bp of bacterial vector sequence that was present on the DNA used for injection. Below the constructs are partial restriction maps indicating the following sites: S, SalI; E, EcoRI; Sm, SmaI; C, ClaI. The striped oval on each map indicates the restriction site used for insertion of the heavy chain enhancer fragment.

transgenes responded in this assay; T cells from the other three types of progeny from this cross ($\alpha^+\beta E_H^-$, $\alpha^-\beta E_H^+$, and $\alpha^-\beta E_H^-$) all failed to proliferate in response to cytochrome c (data not shown).

Recently, it has been found that T cell receptors using the $V_\beta3$ gene have an intrinsic reactivity to non-MHC-linked antigens, Mls-2^a and -3^a, particularly in combination with H-2^k or H-2^d. Consequently, T cells carrying such receptors are deleted in strains of mice expressing this combination of genes (Pullen et al., 1988). As one of the parental strains used to generate our transgenic mice (C3H/HeJ) carries the $V_\beta3$-deleting Mls-2^a and -3^a alleles (Pullen et al., 1989), all of our intital progeny deleted 2B4β-positive cells, particularly those of the H-2^k haplotype (Berg et al., 1989). Repeated backcrossing of transgenic mice to Mls-2^b and -3^b B10 and B10.BR strain mice (Pullen et al., 1989) over three to four generations produced progeny that were free of this effect, as judged by CD4:CD8 ratios and $V_\beta3$ expression in peripheral T cells. Only those mice that were judged free of this "negative" selection effect (by the above criteria) were used for the analyses presented here.

Influence of MHC Type on the Frequency of Cytochrome c–Reactive Cells

To determine the role of the MHC in the development of T cells carrying this class II–restricted T cell receptor in the absence of Mls-2^a and -3^a alleles, 2B4α (H-2^b) or $2B4\alpha E_H^+$ (H-2^b) and $2B4\beta E_H^+$ (H-2^{kxb}) transgenics were crossed to generate $\alpha^+\beta E_H^+$ or $\alpha E_H^+\beta E_H^+$ mice of either the H-2^{kxb} or H-2^b haplotype. The H-2^b haplotype was chosen as the conterpart to H-2^k since it completely lacks I-E expression (due to a defect in the E_α gene [Mathis et al., 1983]). The frequency of cytochrome c–reactive T cells in these unimmunized mice was compared by limiting dilution analysis. In this assay, serial dilutions of a T cell population are plated out in replicate wells with antigen-presenting cells plus or minus cytochrome c. Individual wells are then assayed for the presence of IL-2 as a measure of T cell activation. The dilution of cells at which 0.37 of the wells give a negative response is a measure of the frequency of responding cells in the original population (Henry et al., 1980).

One problem in such analyses is the background responses of the H-2^b mice for the antigen-presenting cells alone. Since the goal of these experiments is to examine the frequency of T cells expressing the 2B4 receptor in a mouse completely lacking the restricting MHC molecule for that receptor, it is unavoidable that T cells from these mice will respond to the foreign MHC on the antigen-presenting cells. To minimize this alloreactive response, we used antigen-presenting cells from mice of the B10.A(5R) strain. These mice express K^bI-A^bI-$E^{k/b}D^d$, and consequently share half of their MHC genes with H-2^b mice. In addition, B10.A(5R) mice express a hybrid I-E molecule composed of I-E_α^k paired with I-E_β^b, which binds the carboxy-terminal peptide of moth cytochrome c; this combination, moth cytochrome c plus I-$E^{k/b}$, is stimulatory for the 2B4 T cell receptor (Fink et al., 1986). The alloreactive response of H-2^b T cells against these antigen-presenting cells is measurable in the control wells that lack cytochrome c. The value obtained was subtracted from the frequency of responding cells to antigen presenting cells plus cytochrome c, to yield the frequency of cytochrome c–specific cells.

The data presented in Figure 2 represent a composite derived from limiting dilution analyses performed on three $\alpha^+\beta E_H^+$-2^{kxb}, four $\alpha^+\beta E_H^+$ H-2^b (left panel), four $\alpha E_H^+\beta E_H^+$ H-2^{kxb}, and three $\alpha E_H^+\beta E_H^+$ H-2^b mice (right panel). For two of the $\alpha^+\beta E_H^+$ H-2^{kxb} and one of the $\alpha^+\beta E_H^+$ H-2^b mice, two independent experiments on the same mouse are shown, as an indication of the reproducibility of the

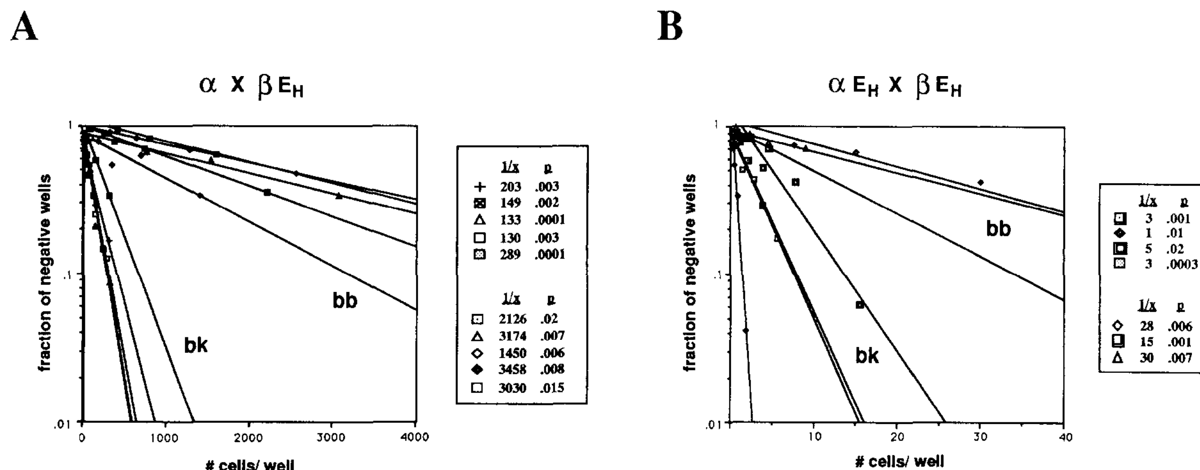


Figure 2. Limiting Dilution Analysis of Cytochrome c-Responsive Cells

Precursor frequency analysis of cytochrome c-responsive cells in H-2^{kxb} versus H-2^b transgenic mice was performed. The number of T cells per well is plotted versus the log of the fraction of negative wells for: (A) $\alpha^+\beta E_H^+$ transgenics; (B) $\alpha E_H^+\beta E_H^+$ transgenics. The compilation of data shown represents analyses of three $\alpha^+\beta E_H^+$ H-2^{kxb} mice (two individulas analyzed twice each), four $\alpha^+\beta E_H^+$ H-2^b mice (one individual analyzed twice), four $\alpha E_H^+\beta E_H^+$ H-2^{kxb} mice, and four $\alpha E_H^+\beta E_H^+$ H-2^b mice. The dilution of cells giving 0.37 negative wells was used as an estimate of the frequency of responding cells in the original population. These values as well as the p values for each regression are shown beside the graphs. For two of the five $\alpha^+\beta E_H^+$ H-2^b analyses, a significant response against the antigen-presenting cells was seen (see Experimental Procedures for description).

experiments. From this analysis, we found that in the $\alpha^+\beta E_H^+$ H-2^{kxb} transgenics the frequency of cytochrome c-reactive T cells was between 1/130 and 1/289; in contrast, only 1/1450 to 1/3458 T cells from the H-2^b transgenics responded in this assay (Figure 2). Similarly, the $\alpha E_H^+\beta E_H^+$ H-2^{kxb} transgenics showed a frequency of 1 to 1/5 cytochrome c-responsive cells, while in the H-2^b mice, only 1/15 to 1/30 T cells responded to cytochrome c (Figure 2). As all H-2^{kxb} transgenics showed on average a 5- to 10-fold higher frequency of cytochrome c-reactive T cells than transgenics expressing only the H-2^b haplotype, it seems likely that this difference results from segregation of MHC alleles, rather than any other non-MHC-linked genes. Furthermore, these results indicate that MHC expression alone, in the absence of antigen, results in a profound bias in the repertoire of functional T cell receptors found in the peripheral immune system.

Analysis of T Cell Receptor Expression by Cell Surface Staining

To examine whether the MHC-dependent functional bias in T cell receptor expression reflected a true physical difference in frequency of 2B4-positive cells, FACS analysis of transgenic mice was performed. For these experiments, 2B4$\alpha E_H^+\beta E_H^+$ mice of both the H-2^{kxb} and the H-2^b MHC types were analyzed. Our initial observations indicated that high levels of expression of the 2B4 T cell receptor in H-2^{kxb} mice had a profound effect on levels of $CD4^+$ versus $CD8^+$ peripheral T cells. Staining of peripheral blood T cells from a nontransgenic littermate with anti-CD4 and anti-CD8 antibodies illustrates the normal proportion of $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ T cells (Figures 3A and 3B). Transgenic mice expressing only the αE_H construct have an overall decrease in the total numbers of T cells, yet, excluding $CD4^-8^-$ cells, maintain a normal ratio of $CD4^+$:$CD8^+$ cells; in addition, these mice have an abnormally high level of $CD4^-CD8^-$ T cells (Ivars et al., 1988; see Figures 3A and 3B). Surprisingly, βE_H^+ transgenics showed a marked increase in $CD4^+$ T cells, resulting in a 4-to 5-fold excess of $CD4^+$ over $CD8^+$ T cells (Figures 3A and 3B), indicating that 2B4β, paired with presumably random endogenous α chains, has a significantly higher affinity for class II than for class I MHC in an H-2^k mouse. An even more striking effect is seen in $\alpha E_H^+\beta E_H^+$ transgenics, where there is a 12-fold excess of $CD4^+$ over $CD8^+$ T cells (Figures 3A and 3B). We observe this phenomenon consistently, although quantitatively there is some variability. The CD4:CD8 ratio in βE_H^+ H-2^{kxb} transgenics is on average 4.8 ± 0.3 (mean ± SEM; $n = 12$) and 12 ± 1.7 ($n = 8$) in $\alpha E_H^+\beta E_H^+$ H-2^{kxb} mice. This extreme bias toward $CD4^+$ T cells is not observed, however, in H-2^b mice, where neither βE_H^+ nor $\alpha E_H^+\beta E_H^+$ transgenics has a disproportionate increase in $CD4^+$ T cells. On average, the CD4:CD8 ratio in βE_H^+ H-2^b transgenics is 2.3 ± 0.1 ($n = 14$) and in $\alpha E_H^+\beta E_H^+$ H-2^b transgenics is 1.7 ± 0.3 ($n = 6$) (Figure 3B). Taken together, these results indicate that the MHC specificity of a T cell receptor determines whether CD4 or CD8 is expressed on the mature T cell, and suggests that this process is dependent on a direct T cell receptor–MHC interaction in the thymus. Figure 3B also demonstrates the striking decrease in the total numbers of T cells generated in the $\alpha E_H^+\beta E_H^+$ H-2^b transgenics.

To analyze directly the influence of MHC on T cells expressing the 2B4 T cell receptor, peripheral T cells were stained with monoclonal antibodies that recognize the α and β chains of this receptor. For the α chain, we used the A2B4-2 antibody (Samelson et al., 1983), which is highly

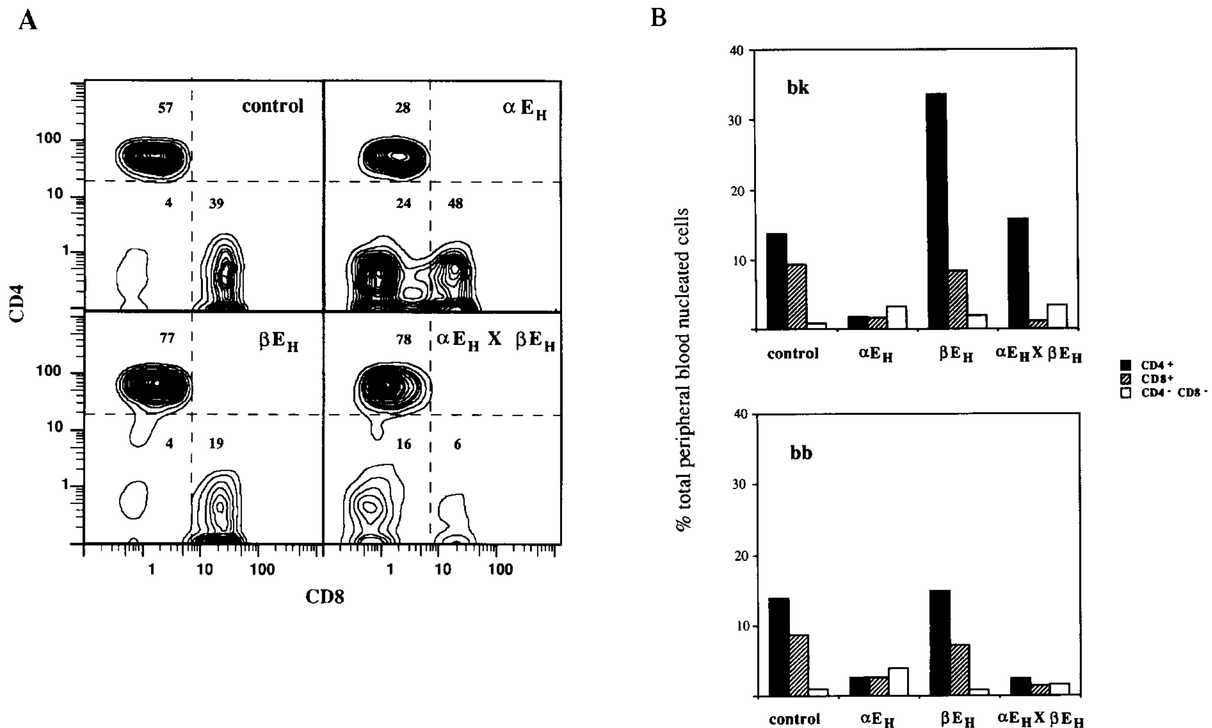


Figure 3. CD4 versus CD8 Expression on Peripheral Blood Cells of Transgenic Mice

(A) CD5-positive cells from peripheral blood of four individual mice, all H-2^{kxb}, were stained with anti-CD4 versus anti-CD8. As indicated on the figure, the four samples are a normal control, an αE_H^+ transgenic, a βE_H^+ transgenic, and an $\alpha E_H^+\beta E_H^+$ transgenic. The numbers in each quadrant indicate the percentage of total T cells of each phenotype. Cells were stained with directly conjugated anti-CD4-PE and anti-CD8-FITC.

(B) A diagrammatic representation of the data from the four individual mice shown in (A), plus comparable data from four mice expressing only H-2^b at the MHC. This graph shows the fraction of $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ T cells as the percentage of total peripheral blood (after removal of red blood cells). These data demonstrate the relative numbers of T cells being produced in each mouse.

specific for the rearranged 2B4 α chain (A. Korman, unpublished data; Gascoigne et al., 1987; Saito and Germain, 1987). For the β chain, we used the KJ25 antibody (Pullen et al., 1988), which is specific for all T cell receptors using the $V_\beta 3$ gene segment in their β chain. In nontransgenic littermates, $V_\beta 3$ is expressed on 3%–4% of peripheral T cells (data not shown). Since $2B4\beta E_H^+$ transgenics express $V_\beta 3$ on over 90% of their T cells, the endogenous $V_\beta 3$ expression in these mice is negligible.

Co-staining of total peripheral blood lymphocytes from an H-2^{kxb} $\alpha E_H^+\beta E_H^+$ transgenic with A2B4-2 and KJ25 demonstrated that a significant proportion of cells were expressing the 2B4 T cell receptor (22% of peripheral blood, 83% of $Ly1^+$ [$CD5^+$] cells [T cells]; Figure 4). Of these, 90% are $CD4^+$, 3% are $CD8^+$, and 7% are $CD4^-CD8^-$. In contrast, these same T cell receptor transgenes expressed in an H-2^b mouse result in only 6% $\alpha E_H^+\beta E_H^+$ cells in peripheral blood, of which 25% are $CD4^+$, 25% are $CD8^+$, and 50% are $CD4^-CD8^-$. Furthermore, these positive cells have in general 2-fold less surface T cell receptor than positive cells in the H-2^{kxb} mouse (Figure 4). However, the most striking contrast between the H-2^{kxb} and the H-2^b transgenic is in the number of $2B4^+\alpha E_H^+\beta E_H^+$ $CD4^+$ cells: 19.5% versus 1.5% of total peripheral blood. This difference, expressed as the percentage of total T cells, is 75% versus 15%, which correlates well with the quantitative results from the limiting dilution experiments.

MHC Antigens Influence the Fate of T Cells in the Thymus

Earlier experiments with bone marrow chimeras had indicated that positive selection of class I–restricted T cells occurred in the thymus (Zinkernagel et al., 1978; Fink and Bevan, 1978). Since the $\alpha E_H^+\beta E_H^+$ transgenics express such high levels of the 2B4 receptor, any effects on T cell development should be detectable by comparing an H-2^{kxb} thymus with one of an H-2^b transgenic.

To address this possibility, $\alpha E_H^+\beta E_H^+$ transgenic mouse thymuses were first stained with anti-CD4 and anti-CD8 antibodies. Remarkably, the H-2^b thymus showed a 5-fold reduction in single $CD4^+$ T cells relative to the H-2^{kxb} transgenic thymus (Figure 5A). Staining of these thymuses with KJ25 versus A2B4-2 indicated that the reduction in single-positive T cells in the H-2^b thymus correlated with the virtual absence of bright $2B4\alpha E_H^+\beta E_H^+$ T cells such as those seen in the H-2^{kxb} thymus (Figure 5B). Nonetheless, the majority of $CD4^+CD8^+$ T cells in the H-2^b thymus express both the 2B4 α and 2B4 β chains, but only at the low levels characteristic of immature $CD4^+8^+$ thymocytes (Figure 5B). The comparative staining of H-2^{kxb} versus H-2^b $\alpha E_H^+\beta E_H^+$ thymuses has

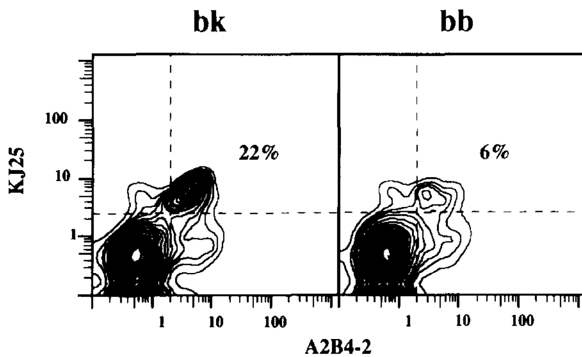


Figure 4. Expression of the 2B4 T Cell Receptor in H-2^{kxb} versus H-2^b $\alpha E_H^+\beta E_H^+$ Transgenic Mice

Total peripheral blood cells (after removal of red blood cells) from an H-2^{kxb} (left) versus an H-2^b (right) $\alpha E_H^+\beta E_H^+$ transgenic were stained with KJ25 (anti-$V_\beta 3$) followed by anti-hamster–Texas Red plus directly conjugated A2B4-2-FITC. The percentage of total peripheral blood lymphocytes expressing both α and β chains of the receptor is indicated in the upper righthand corner of each profile. Values for each of the T cell subsets were obtained by four-color staining of peripheral blood with two additional antibodies: directly conjugated anti-CD4-PE, and anti-CD8-biotin followed by avidin-APC. Similar results have been obtained in five independent experiments.

been repeated in four independent experiments, with similar results. In conclusion, these data indicate that the block in T cell maturation due to the absence of positive selection results in the failure of $CD4^+CD8^+$ thymocytes, expressing low levels of the 2B4 T cell receptor, to differentiate further.

I-E Expression on Cortical Epithelial Cells Is Sufficient for Positive Selection

To address the issue of whether the MHC molecule recognized by the 2B4 receptor, I-E, is responsible for positive selection of the receptor, and if so, on which thymic cell type I-E expression is necessary, 2B4 $\alpha E_H^+\beta E_H^+$ H-2^b transgenic mice were crossed to several lines of transgenic mice carrying either the wild-type or variants of the MHC class II I-E_α gene (Le Meur et al., 1985; Dorn et al., 1987; van Ewijk et al., 1988). Since these E_α transgenics are on an H-2^b background that lacks endogenous E_α expression, all surface I-E expression in these mice results from expression of the transgene-encoded E_α gene. Three lines of E_α transgenic mice were used for these experiments. First, E_α16, which carries a 9 kb clone of the wild-type E_α gene and expresses surface I-E in a pattern identical to endogenous class II. Second, WEΔX21.16 (ΔX), which carries a mutant of this E_α gene that has a deletion removing the upstream regulatory element referred to as the "X box"; in the thymus, ΔX transgenics express I-E exclusively on medullary epithelial cells, dendritic cells, and macrophages. Third, WEΔY301.54 (ΔY) mice carry the same E_α construct with a deletion removing the upstream regulatory "Y box" element; in the thymus, these transgenics express I-E predominantly on cortical epithelial cells, although a small fraction (1%) of dendritic cells in the medulla are I-E positive.

As a first step in these experiments, it was necessary to ascertain whether the $E_\alpha^k E_\beta^b$ molecule would have any effect on development of T cells expressing the 2B4 receptor. As mentioned above, the 2B4 receptor was originally derived from a B10.A mouse and is specific for pigeon cytochrome c plus I-E^k; however, it also cross-reacts on moth cytochrome c plus $E_\alpha^k E_\beta^b$, although with reduced efficiency. Thus, it seemed reasonable that $E_\alpha^k E_\beta^b$ expression would be sufficient to allow maturation of 2B4-positive T cells. Peripheral blood lymphocytes from 2B4 $\alpha E_H^+\beta E_H^+$ H-2^b transgenics also carrying the wild-type E_α transgene were stained with the A2B4-2 (anti-2B4α) and KJ25 (anti-$V_\beta 3$) antibodies as well as anti-CD4 and

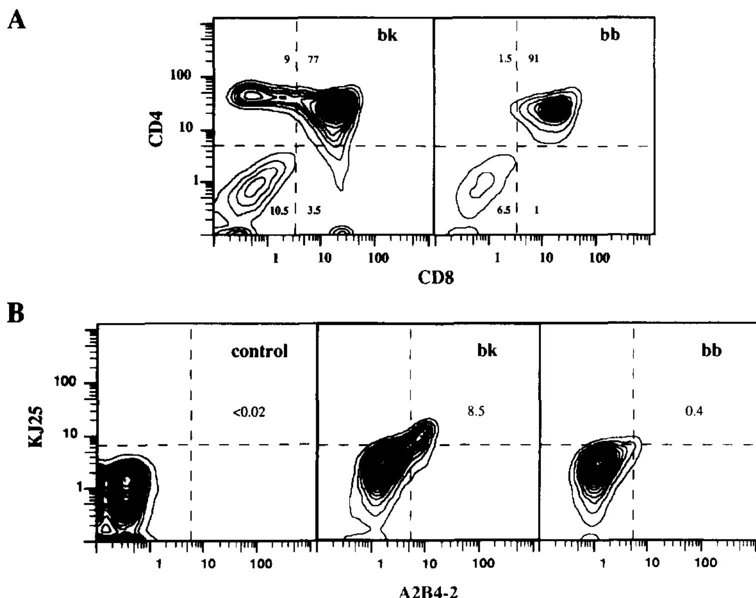
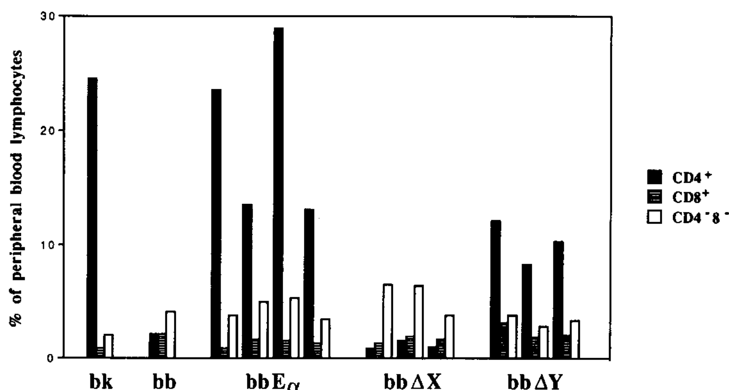


Figure 5. Staining of Thymocytes from H-2^{kxb} versus H-2^b $\alpha E_H^+\beta E_H^+$ Transgenic Mice

(A) Thymocytes from an H-2^{kxb} (left) and an H-2^b (right) $\alpha E_H^+\beta E_H^+$ transgenic mouse were stained with directly conjugated anti-CD4-PE and anti-CD8-FITC. The numbers on each profile indicate the percentage of total thymocytes with each phenotype.

(B) Thymocytes from an H-2^{kxb} nontransgenic control (left), and H-2^{kxb} $\alpha E_H^+\beta E_H^+$ transgenic (center), and an H-2^b $\alpha E_H^+\beta E_H^+$ transgenic (right) were stained with KJ25 (anti-$V_\beta 3$) followed by anti-hamster–Texas Red plus directly conjugated A2B4-2-FITC. The number on each profile indicates the percentage of cells staining brightly with both α and β chain antibodies.

A



B

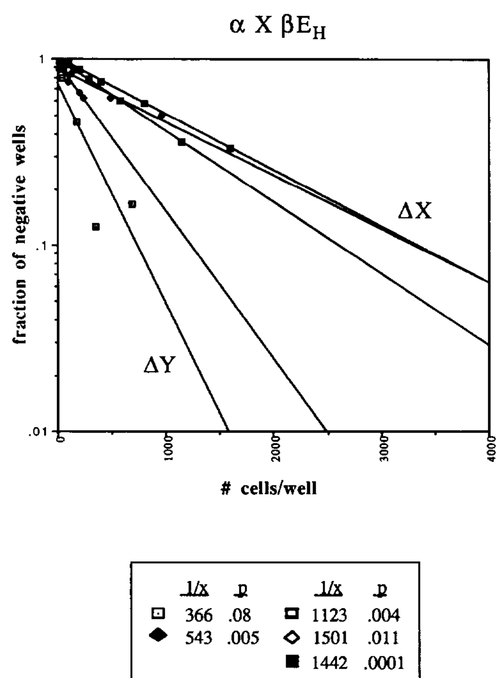


Figure 6. Expression of the 2B4 Receptor in Transgenic Mice of Varying MHC Types

(A) Peripheral blood (after lysis of red blood cells) from $\alpha E_H^+\beta E_H^+$ transgenic mice of varying MHC types was analyzed by four-color FACS analysis; the level of $\alpha E_H^+\beta E_H^+$ cells in each T cell subset is indicated as a percentage of total peripheral blood lymphocytes. For this analysis, peripheral blood lymphocytes were stained with KJ25 followed by anti-hamster-Texas Red, directly conjugated A2B4-2-FITC, directly conjugated anti-CD4-PE, and anti-CD8-biotin followed by avidin-APC.

(B) Precursor frequency analysis of $\alpha^+\beta E_H^+\Delta X$ versus $\alpha^+\beta E_H^+\Delta Y$ transgenic mice was performed. For each analysis, the number of T cells per well versus the log of the fraction of negative wells was plotted. Data from two ΔY transgenics and two ΔX transgenics (one individual analyzed twice) are shown. Below the graph the estimated frequency of responding cells and the p value for each regression are indicated. For one of the ΔX analyses, a significant response against the antigen-presenting cells was seen (see Experimental Procedures for description).

anti-CD8. As shown in Figure 6A, the high frequency of 2B4-positive cells in these mice, particularly those of the $CD4^+$ subset, exactly parallels that seen in H-2^{kxb} transgenics. In constrast, $\alpha E_H^+\beta E_H^+$ H-2^b transgenics express relatively low levels of the 2B4 receptor, with the majority of receptor-positive cells being $CD4^-8^-$ (Figure 6A). Having established that the $E_\alpha^k E_\beta^b$ molecule could select the 2B4 receptor, 2B4 $\alpha E_H^+\beta E_H^+$ H-2^b transgenics were crossed to ΔX and ΔY transgenics. Expression of the 2B4 receptor on peripheral blood lymphocytes of the resulting $\alpha E_H^+\beta E_H^+\Delta X$ or $\alpha E_H^+\beta E_H^+\Delta Y$ transgenics was then examined. The data shown in Figure 6A, from three mice of each type, demonstrate positive selection of the 2B4 receptor in ΔY but not in ΔX transgenics. Interestingly, the extent of positive selection observed, as measured by the percentage of peripheral 2B4 αβ—postive, $CD4^+$ cells, appears significantly less in the ΔY than in the $E_\alpha16$ transgenics, or normal H-2^{kxb} αβ transgenic mice.

As an additional test for positive selection, 2B4 $\alpha^+\beta E_H^+$ H-2^b transgenics were crossed to ΔX and ΔY transgenics, and peripheral blood lymphocytes from the resulting $\alpha^+\beta E_H^+\Delta X$ and $\alpha^+\beta E_H^+\Delta Y$ mice were analyzed for cytochrome c–reactive T cells by limiting dilution analysis. For two $\alpha^+\beta E_H^+\Delta Y$ transgenics, the frequency of cytochrome c–reactive cells ranged from 1/366 to 1/543 of T cells (Figure 6B), a frequency about 2- to 3-fold lower than that found in the comparable H-2^{kxb} transgenics (see Figure 2). In the $\alpha^+\beta E_H^+\Delta X$ transgenics, one of which was analyzed in two independent experiments, the frequency of cytochrome c–reactive cells ranged from 1/1123 to

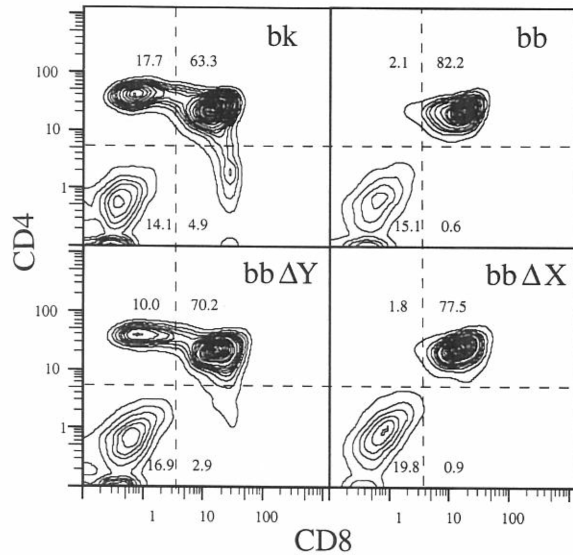


Figure 7. CD4 versus CD8 Staining of Thymocytes from ΔX and ΔY $\alpha E_H^+\beta E_H^+$ Transgenic Mice

Thymocytes for $\alpha E_H^+\beta E_H^+$ transgenic mice of four MHC types (H-2^{kxb}, H-2^b, H-2^b + ΔY, and H-2^b + ΔX) were stained with anti-CD4 and anti-CD8. The percentage of thymocytes in each subset is indicated on each profile. Cells were stained with directly conjugated anti-CD4-PE and anti-CD8-FITC.

1/1501 (Figure 6B). These values are slightly higher than, but still in the same range as, values obtained in the $\alpha^+\beta E_H^+$ H-2^b transgenics (see Figure 2). In summary, $\alpha^+\beta E_H^+$ H-2^b transgenics, also expressing the ΔY transgene, have a 3-fold higher frequency of cytochrome c–reactive T cells in the periphery than $\alpha^+\beta E_H^+$ H-2^b transgenics expressing the ΔX transgene.

As the ΔX and ΔY transgenics also differ in expression of I-E on peripheral B cells and macrophages (van Ewijk et al., 1988), it was possible that the differences in 2B4 receptor expression observed on peripheral T cells resulted not from a difference in selection in the thymus, but rather, from a difference in peripheral expansion of 2B4-positive T cells. To address this possibility, thymocytes from these mice were stained with anti-CD4 and anti-CD8, and analyzed by FACS. As shown in Figure 7, the absence of single $CD4^+8^-$ thymocytes in H-2^b transgenics, resulting from the absence of positive selection, is mirrored precisely in ΔX transgenics; H-2^{kxb} as well as ΔY transgenics, however, produce large numbers of single $CD4^+8^-$ thymocytes. These results indicate that the differences observed in peripheral levels of 2B4 receptor expression do result from a difference in selection in the thymus, and not from an inequity in peripheral expansion.

Discussion

The concept of positive selection has always been somewhat paradoxical. A mature T cell is stimulated to respond only by the proper juxtaposition of an antigen and an MHC molecule, yet this type of selection necessitates a stimulatory interaction between the T cell receptor and an MHC molecule in the thymus before exposure to antigen. Although the details of this interaction are not known, the fact that this type of selection occurs now seems beyond doubt. In particular, the T cell receptor transgenic experiments of Teh et al. (1988) and Sha et al. (1988b), in class I MHC-specific systems, indicated a strong effect of H-2 type in the successful export of the transgenic T cell receptor-positive cells to the periphery. Kisielow et al. (1988b) carried this analysis further by showing that only those recombinant MHC strains that expressed the original restricting element for the receptor were capable of positive selection. Our results presented here confirm and extend these findings in a number of important ways: we see marked evidence for selection in H-2^k versus H-2^b transgenic mice in an MHC class II–dependent system; this selection is entirely dependent on the expression of the original restricting element (I-E), and I-E expression on cortical epithelial cells in the thymus is required to produce this effect.

The studies of MHC class I– and class II–specific T cell receptors introduced into transgenic mice also demonstrate that the MHC specificity of a given T cell receptor is responsible for determining the CD4 versus CD8 phenotype of mature T cells. In our mice, over 90% of peripheral 2B4 positive cells express the CD4 molecule; Teh et al. (1988) and Sha et al. (1988a; 1988b) both demonstrate a similarly high percentage of transgene positive cells expresssing CD8. Since CD8 expression correlates with class I MHC recognition, and CD4 expression correlates with class II MHC recognition (Swain, 1983), these results indicate that the MHC specificity of a given T cell receptor determines, either by instruction or by selection, whether the CD4 or CD8 molecule is expressed by the cell.

The data presented here are also consistent with conclusions drawn from studies utilizing in vivo treatment of mice with anti-T cell receptor (McDuffie et al., 1986; Marrack et al., 1988) or anti-MHC class II antibodies (Kruisbeek et al., 1985). These studies indicated that the appearance of T cell receptor/CD3 dull $CD4^+CD8^+$ thymocytes was independent of T cell receptor specificity (McDuffie et al., 1986), and suggested that the maturation of such cells to T cell receptor–CD3 bright, $CD4^+CD8^-$ T cells required interactions between the T cell receptor and class II MHC (Marrack et al., 1988), as well as between CD4 and class II MHC (Kruisbeek et al., 1985). The fact that in the transgenic mice the 2B4 T cell receptor is preferentially expressed on $CD4^+$ T cells only when the proper MHC molecule is present on the appropriate cells in the thymus strongly supports this interpretation. Furthermore, staining of thymuses from $\alpha E_H^+\beta E_H^+$ H-2^{kxb} versus H-2^b transgenic mice highlights the combined role of T cell receptor specificity and MHC type in allowing maturation to T cell receptor–CD3 bright $CD4^+$ or $CD8^+$ thymocytes.

Our transgenic studies also indicate that positive selection occurs late in the $CD4^+8^+$ stage, since 2B4-positive thymocytes develop to a relatively mature stage ($CD4^+8^+$, T cell receptor/$CD3^{lo}$, $CD5^{hi}$) in the absence of positive selection (i.e., in H-2^b transgenic mice). Interestingly, thymocyte death due to negative selection seems to occur

at roughly the same stage (Kappler et al., 1987, 1988; MacDonald et al., 1988b, 1988c; Pullen et al., 1988; White et al., 1989; Fowlkes et al., 1988; Berg et al., 1989; Pircher et al., 1989). In $\alpha\beta$ transgenic mice, $CD4^+8^+$ thymocytes expressing a self-reactive receptor are actively eliminated, leading to a 10- to 40-fold reduction in the overall number of thymocytes (Kisielow et al., 1988a; Sha et al., 1988b; Berg et al., 1989). In contrast, the lack of positive selection, which also leads to the death of immature thymocytes, has a very different appearance, suggesting that these two types of cell death result from distinct mechanisms.

Limiting dilution analysis on $\alpha^+\beta E_H^+$ and $\alpha E_H^+\beta E_H^+$ transgenics shows only a 10-fold difference in 2B4 expression in an H-2^{kxb} versus an H-2^b MHC background. These results are consistent with earlier observations from chimeric mice studies, which often showed detectable levels of reactivity to antigens presented on foreign MHC molecules (Sprent, 1978; Bevan, 1977; Fink and Bevan, 1978). The reasons for this outcome might be different in the two systems, however. In the experiments with chimeric mice, a heterogeneous population of T cells is responding—a population that might contain detectable numbers of cells that cross-react on antigen plus the MHC of another strain. In the H-2^b as compared with the H-2^{kxb} transgenic mice, it appears that most of the 2B4 T cell receptor–positive cells are not allelically excluded for β chain expression (15% of the $KJ25^+$ cells also stain with the F23.1 monoclonal antibody, compared with <5% in the H-2^{kxb} mice). In contrast, we find no evidence for expression of a second α chain on the surface of 2B4 receptor-positive cells in either H-2^b or H-2^{kxb} transgenic mice (costaining of T cells with A2B4-2 and anti-CD3 antibodies fails to detect cells with excess CD3 over 2B4 α chain on their surface, data not shown). In light of these results, it is possible that 2B4 receptor-positive cells in the H-2^b transgenic mice might be selected on the basis of a second T cell receptor present on the cell surface, carrying the 2B4 receptor out of the thymus as an innocent bystander. A similar observation has been made in the class I–restricted T cell receptor transgenics of von Boehmer and colleagues. In these mice, however, cells expressing the transgene-encoded receptor in the inappropriate MHC background are often found to be expressing a second α chain on their surface (H. von Boehmer, personal communication).

Finally, we have presented evidence, using transgenics carrying MHC class II I-E_α mutants, that I-E expression on thymic cortical epithelial cells is necessary to select the 2B4 receptor for maturation. The ΔY mutant, which expresses I-E on cortical epithelium as well as on a small number (~1%) of macrophage/dendritic cells, does select the 2B4 receptor, while the ΔX mutant, which expresses I-E exclusively on medullary macrophage/dendritic cells and epithelial cells is incapable of positive selection. Both ΔX and ΔY mutant lines express similar levels of I-E on the surface of positive cells (van Ewijk et al., 1988). In the accompanying paper (Benoist and Mathis, 1989), we reach the identical conclusion from an analysis of endogenous $V_\beta6$ expression in these same Eα transgenics. Thus, in both cases, medullary class II expression is not sufficient for positive selection, and we suggest that the cortical epithelial cells of the thymus have a specialized role in this process. We do observe, however, a slightly lower level of expression of the 2B4 receptor in the periphery of mice expressing the ΔY mutant compared with the wild-type E_α^k transgene. This might indicate that thymic bone marrow–derived cells do play an accessory role in positive selection. Alternatively, this difference might reflect the deficiency in I-E expression in the periphery of ΔY mice, inhibiting peripheral expansion of 2B4-positive cells.

In conclusion, we have found that T cell development in the thymus is highly dependent on the presence in the cortical region of the thymus of the appropriate MHC molecule. Positive selection apparently results from interactions of the T cell receptor with specific self-MHC molecules in the absence of the nominal antigen. One mystery that remains is the precise nature of the MHC complex presented on the surface of cortical epithelial cells which is responsible for this type of selection. One way of approaching this question is to analyze the ability of naturally occurring MHC I-E variants to select the 2B4 receptor, as a way of mapping the critical residues of the MHC molecule involved in this selection process. In addition, site-directed mutants of both the I-E molecule and the T cell receptor α and β chains will be useful in ascertaining the molecular details of this interaction. Thus, T cell receptor transgenic mice can provide a valuable system for future investigations into the molecular biology and biochemistry of this developmental process.

Experimental Procedures

T Cell Receptor Constructs

The 2B4 α chain construct was derived by joining a 2.4 kb rearranged 2B4 VJ_α segment to a 9 kb fragment containing the C_α gene segment as described previously (Berg et al., 1988). A 0.7 kb fragment carrying the immunoglobulin heavy chain enhancer (Banerji et al., 1983; Gillies et al., 1983) was inserted into the JC intron, generating 2B4αE_H. The 2B4βE_H construct was generated as follows: a genomic clone of the rearranged 2B4 β chain containing 4.5 kb of 5′ flanking sequence was cleaved at the EcoRV site in the 3′ untranslated exon of C_β, and an XhoI linker was inserted at this site. The resulting 9.3 kb fragment extending from the 5′ end of the clone to this site was then ligated to a 3.0 kb fragment carrying the 3′ end of the $C_\beta2$ locus from AKR mouse DNA. The resulting reconstructed 2B4 β gene was cloned into pUC9, and the immunoglobulin heavy chain enhancer (Banerji et al., 1983; Gillies et al., 1983) was inserted into the unique ClaI site immediately downstream of the $J_\beta2.5$ segment. The resulting 2B4βE_H construct contains 4.5 kb of 5′ and 3.0 kb of 3′ flanking DNA. The $E_\alpha16$, ΔX, and ΔY transgenics have been described previously (Le Meur et al., 1985; Dorn et al., 1987; van Ewijk et al., 1988). The animals used here came from the ninth backcross generation on C57Bl/6 mice (original transgenic founders were [C57Bl/6 × SJL]F_2).

Transgenic Mice

The 2B4 α chain transgenic mice have been described previously (Ivars, 1988; Berg et al., 1988). The 2B4βE_H construct was digested with PvuI and SalI leaving 120 bp of bacterial vector sequences attached to the T cell receptor gene. This DNA was injected into [C57Bl/6J × C3H/HeJ]F_2 eggs and implanted into foster mothers (Wagner, 1981a, 1981b; Constantini and Lacy, 1981; Brinster, 1981; Hogan et al., 1986). Resulting progeny mice were screened for integration of the β chain DNA by Southern blot analysis of tail DNA. Four independent lines of 2B4βE_H transgenics were generated, with copy numbers

ranging from 1 to 10. All experiments described here were done with a single line carrying approximately eight copies of the transgene. Mice from this line were crossed to 2B4α or $2B4\alpha E_H$ transgenics, which were derived from the same strain background as the $2B4\beta E_H$ transgenics, but had already been backcrossed onto C57Bl/10J for three to four generations. $\alpha^+\beta^+$ transgenics were derived either from these intercrosses or from crossing $\alpha^+\beta^+$ transgenics to C57Bl/10J mice.

FACS Analysis

For fluorescence-activated cell sorter (FACS) analysis, peripheral blood nucleated cells, lymph node cells, or thymocytes were used where indicated. Red blood cells were removed from peripheral blood by ammonium chloride lysis. Cells were stained with the following monoclonal antibody reagents: KJ25 (anti-$V_\beta3$; Pullen et al., 1988), either as supernatant or biotinylated; FITC-conjugated A2B4-2 (Samelson, 1983); PE-conjugated GK1.5 (Dialynas et al., 1984) for detecting mouse CD4 (Becton-Dickinson); FITC-conjugated or biotinylated 53-6.7 (Ledbetter and Herzenberg,1979) for detecting mouse CD8 (Becton-Dickinson); 500A2 (Havran et al., 1987) for detecting mouse CD3 epsilon; allophycocyanin-conjugated 53-7.3 (Ledbetter and Herzenberg, 1979) for detecting mouse CD5. Secondary reagents were avidin–Texas Red (Cappel), anti-hamster-FITC (Caltag), anti-hamster–Texas Red (Caltag), and avidin allophycocyanin (Biomeda), as indicated in each figure. FACS analyses were performed essentially as described by Hayakawa et al. (1983). Initial attempts to co-stain peripheral T cells with both the α- and β-specific antibodies suggested that they physically blocked each other. To circumvent this problem, subsaturating concentrations of antibody were used to obtain binding of the two antibodies to different T cell receptor heterodimers on the same cell surface.

Limiting Dilution Analysis

Limiting dilution analyses were performed according to the protocol of Miller and Stutman (1982). Peripheral blood cells from the transgenic mice were treated with ammonium chloride to lyse red blood cells; in a few cases, lymph node cells from the transgenics were used as a source of T cells. Antigen-presenting cells were prepared as follows: peritoneal exudate cells from untreated B10.A(5R) mice were treated with anti-thy1.2 (30H12; Ledbetter, 1979) plus complement (Cedarlane) to remove T cells. Twenty-four to forty-eight replicate wells of 2-fold dilutions of transgenic cells were plated with 10^4 antigen presenting cells plus or minus 10 μM moth cytochrome c peptide per well. Cells were cultured for 5 days at 37°C. Supernatants from each well were then screened for the presence of lymphokines on HT-2 indicator cells (Watson, 1979). The number of positive and negative wells at each dilution of cells was determined, taking as the cutoff the mean plus three standard deviations of the incorporation in the absence of cytochrome c peptide.

For each experiment, the dilution of cells was plotted versus the log of the fraction of negative wells at that dilution, and a linear regression was calculated; this regression was used to estimate the frequency of responding cells in the original population assuming that the dilution that gives 0.37 negative wells is equivalent to one positive cell per well. The number of T cells in each population was determined by staining with anti-CD5, anti-CD4, and anti-CD8, and analysis by flow cytometry. In all cases but three, no significant response in the absence of cytochrome c peptide was observed. For two $\alpha^+\beta E_H^+$ H-2^b mice and one $\alpha^+\beta E_H^+$ H-2^b ΔX mouse, a small but significant allo-response against the B10.A(5R) antigen-presenting cells was seen. For these three cases, the slope of the regression line generated by this allo-response was subtracted from the slope of the regression line generated in the presence of cytochrome c, to produce a line whose slope reflects the frequency of cytochrome c–specific responding cells. For the first two cases, this calculated line is shown in Figure 2; for the third case, this calculated line is shown in Figure 7.

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References

Banerji, J., Olson, L., and Schaffner, W. (1983). A lymphocyte-specific cellular enhancer is located downstream of the joining region in immunoglobulin heavy chain genes. Cell *33*, 729–740.

Bennink, J. R., and Doherty, P. C. (1978). T cell populations specifically depleted of alloreactive potential cannot be induced to lyse H-2 different virus-infected target cells. J. Exp. Med. *148*, 128–135.

Benoist, C., and Mathis, D. (1989). Positive selection of the T cell repertoire: where and when does it occur? Cell, in press.

Berg, L. J., Fazekas de St. Groth, B., Ivars, F., Goodnow, C. C., Gilfillan, S., Garchon, H.-J., and Davis, M. M. (1988). Expression of T-cell receptor alpha-chain genes in transgenic mice. Mol. Cell. Biol. *8*, 5459–5469.

Berg, L. J., Fazekas de St. Groth, B., Pullen, A. M., and Davis, M. M. (1989). Phenotypic differences between αβ and β T cell receptor transgenic mice undergoing negative selection. Nature, in press.

Bevan, M. (1977). In a radiation chimera, host H-2 antigens determine immune responsiveness of donor cytotoxic T cells. Nature *269*, 417–418.

Bradley, L. M. (1980). Cell proliferation. In Selected Methods in Cellular Immunology, B. B. Mishell and S. M. Shiigi, eds. (San Francisco: W. H. Freeman and Co.), pp. 153–172.

Brinster, R. L., Chen, H. Y., Trumbauer, M., Senear, A. W., Warren, R., and Palmiter, R. D. (1981). Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. Cell *27*, 223–231.

Buus, S., Colon, S., Smith, C., Freed, J. H., Miles, C., and Grey, H. M. (1986). Interaction between a "processed" ovalbumin peptide and Ia molecules. Proc. Natl. Acad. Sci. USA *83*, 3968–3971.

Constantini, F., and Lacy, E. (1981). Introduction of a rabbit β-globin gene into the mouse germline. Nature *294*, 92–94.

Davis, M. M., and Bjorkman, P. J. (1988). T cell antigen receptor genes and T cell recognition. Nature *334*, 395–401.

Dembic, Z., Haas, W., Weiss, S., McCubrey, J., Kiefer, H., von Boehmer, H., and Steinmetz, M. (1986). Transfer of specificity by murine alpha and beta T cell receptor genes. Nature *320*, 232–238.

Dialynas, D. P., Quan, Z. S., Wall, K. A., Pierres, A., Quintans, J., Loken, M. R., Pierres, M., and Fitch, F. W. (1984). Characterization of the murine T cell surface molecule, designated L3T4, identified by a monoclonal antibody GK1.5: similarity of L3T4 to the human Leu3/T4 molecule. J. Immunol. *131*, 2445–2451.

Dorn, A., Durand, B., Marfing, C., Le Meur, M., Benoist, C., and Mathis, D. (1987). Conserved major histocompatibility complex class II boxes—X and Y—are transcriptional control elements and specifically bind nuclear proteins. Proc. Natl. Acad. Sci. USA *84*, 6249–6253.

Fink, P. J., and Bevan, M. J. (1978). H-2 antigens of the thymus determine lymphocyte specificity. J. Exp. Med. *148*, 766–775.

Fink, P. J., Matis, L. A., McElligott, D. L., Bookman, M., and Hedrick, S. M. (1986). Correlations between T cell specificity and the structure of the antigen receptor. Nature *321*, 219–226.

Fowlkes, B. J., Schwartz, R. H., and Pardoll, D. M. (1988). Deletion of self-reactive thymocytes occurs at a $CD4^+8^+$ precursor stage. Nature *334*, 620–623.

Gascoigne, N. R. J., Goodnow, C. C., Dudzik, K. I., Oi, V. T., and Davis, M. M. (1987). Secretion of a chimeric T-cell receptor–immunoglobulin protein. Proc. Natl. Acad. Sci. USA *84*, 2936–2940.

Gillies, S. D., Morrison, S. L., Oi, V. T., and Tonegawa, S. (1983). A

tissue-specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene. Cell *33*, 717–728.

Havran, W. L., Poenie, M., Kimura, J., Tsien, R., Weiss, A., and Allison, J. P. (1987). Expression and function of the CD3-antigen receptor on murine CD4$^+$8$^+$ thymocytes. Nature *330*, 170–173.

Hayakawa, K., Hardy, R. R., Parks, D. R., and Herzenberg, L. A. (1983). The "Ly-1 B" cell subpopulation in normal, immunodefective, and autoimmune mice. J. Exp. Med. *157*, 202–218.

Hedrick, S. M., Matis, L. A., Hecht, T. T., Samelson, L. E., Longo, D. L., Heber-Katz, E., and Schwartz, R. H. (1982). The fine specificity of antigen and Ia determinant recognition by T cell hybridoma clones specific for cytochrome c. Cell *30*, 141–152.

Henry, C., Marbrook, J., Vann, D. C., Kodlin, D., and Wofsky, C. (1980). Limiting dilution analysis. In Selected Methods in Cellular Immunology, B. B. Mishell and S. M. Shiigi, eds. (San Francisco: W. H. Freeman and Co.), pp. 138–152.

Hogan, B., Constantini, F., and Lacy, E. (1986). Manipulating the Mouse Embryo: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Ishii, N., Baxevanis, C. N., Nagy, Z. A., and Klein, J. (1981). Responder T cells depleted of alloreactive cells react to antigen presented on allogenic macrophages from nonresponder strains. J. Exp. Med. *154*, 978–982.

Ivars, F., Berg, L. J., Fazekas de St. Groth, B., Goodnow, C. C., Garchon, H.-J., Gilfillan, S., and Davis, M. M. (1988). The expression of T cell receptor α chain genes in transgenic mice. In The T Cell Receptor, M. M. Davis and J. Kappler, eds. (New York: Alan R. Liss, Inc.), pp. 187–197.

Kappler, J. W., and Marrack, P. (1978). The role of H-2 linked genes in helper T cell function. J. Exp. Med. *148*, 1510–1522.

Kappler, J. W., Roehm, N., and Marrack, P. (1987). T cell tolerance by clonal elimination in the thymus. Cell *49*, 272–280.

Kappler, J. W., Staerz, U., White, J., and Marrack, P. C. (1988). Self-tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex. Nature *332*, 35–40.

Kisielow, P., Bluthmann, H., Staerz, U. D., Steinmetz, M., and von Boehmer, H. (1988a). Tolerance in T cell receptor transgenic mice involves deletion of nonmature CD4$^+$8$^+$ thymocytes. Nature *333*, 742–746.

Kisielow, P., Teh, H. S., Bluthmann, H., and von Boehmer, H. (1988b). Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. Nature *335*, 730–733.

Kruisbeek, A. M., Mond, J. J., Fowlkes, B. J., Carmen, J. A., Bridges, S., and Longo, D. L. (1985). Absence of the Lyt-2$^-$, L3T4$^+$ lineage of T cells in mice treated neonatally with anti-IA correlates with absence of intrathymic I-A bearing antigen presenting cell functions. J. Exp. Med. *161*, 1029–1047.

Ledbetter, J. A., and Herzenberg, L. A. (1979). Xenogenic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. *47*, 63–90.

Le Meur, M., Gerlinger, P., Benoist, C., and Mathis, D. (1985). Correcting an immune response deficiency bycreating E_α gene transgenic mice. Nature *316*, 38–42.

Lo, D., and Sprent, J. (1986). Identity of cells that imprint H-2 restricted T cell specificity in the thymus. Nature *319*, 672–676.

Longo, D. L., and Schwartz, R. H. (1980). T cell specificity for H-2 and Ir gene phenotype correlates with the phenotype of antigen presenting cells. Nature *287*, 44–46.

MacDonald, H. R., Lees, R. K., Schneider, R., Zinkernagel, R. M., and Hengartner, H. (1988a). Positive selection of CD4$^+$ thymocytes controlled by MHC class II gene products. Nature *336*, 471–473.

MacDonald, H. R., Schneider, R., Lees, R. K., Howe, R. C., Acha-Orbea, H., Festenstein, H., Zinkernagel, R. M., and Hengartner, H. (1988b). T cell receptor V_β use predicts reactivity and tolerance to Mls^a-encoded antigens. Nature *332*, 40–45.

MacDonald, H. R., Hengartner, H., and Pedrazzini, T. (1988c). Intrathymic deletion of self-reactive cells prevented by neonatal anti-CD4 antibody treatment. Nature *335*, 174–176.

Marrack, P., Kushnir, E., Born, W., McDuffie, M., and Kappler, J. (1988). The development of helper T cell precursors in mouse thymus. J. Immunol. *140*, 2508–2514.

Mathis, D., Benoist, C., Williams, V., Kanter, M., and McDevitt, H. (1983). Several mechanisms can account for defective E_α expression in different mouse haplotypes. Proc. Natl. Acad. Sci. USA *80*, 273–277.

Matzinger, P., and Mirkwood, G. (1978). In a fully H-2 incompatible chimera, T cells of donor origin can respond to minor histocompatibility antigens in association with either donor or host H-2 type. J. Exp. Med. *148*, 84–92.

McDuffie, M., Born, W., Marrack, P., and Kappler, J. W. (1986). The role of the T cell receptor in thymocyte maturation: effects in vivo of anti-receptor antibody. Proc. Natl. Acad. Sci. USA *83*, 8728–8732.

Meuer, S. C., Fitzgerald, K. A., Hussey, R. E., Hodgdon, J. C., Schlossman, S. F., and Reinherz, E. L. (1983). Clonotypic structures involved in antigen-specific human T cell function. Relationship to the T3 molecular complex. J. Exp. Med. *157*, 705–719.

Miller, R. A., and Stutman, O. (1982). Enumeration of IL 2-secreting helper T cells by limiting dilution analysis, and demonstration of unexpectedly high levels of IL-2 production per responding cell. J. Immunol. *128*, 2258–2264.

Pircher, H., Mak, T. W., Lang, R., Balhausen, W., Ruedi, E., Hengartner, H., Zinkernagel, R., and Burki, K. (1989). T cell tolerance to Mls^a encoded antigens in T cell receptor V_β8.1 chain transgenic mice. EMBO J. *8*, 719–727.

Pullen, A. M., Marrack, P., and Kappler, J. W. (1988). The T cell repertoire is heavily influenced by tolerance to polymorphic self-antigens. Nature *335*, 796–801.

Pullen, A. M., Marrack, P., and Kappler, J. W. (1989). Evidence that Mls-2 antigens which delete $V_\beta3^+$ T cells are controlled by multiple genes. J. Immunol. *142*, 000–000.

Saito, T., and Germain, R. N. (1987). Predictable acquisition of a new MHC recognition specificity following expression of a transfected T-cell receptor beta-chain gene. Nature *329*, 256–259.

Samelson, L. E., Germain, R. N., and Schwartz, R. H. (1983). Monoclonal antibodies against the antigen receptor on a cloned T-cell hybrid. Proc. Natl. Acad. Sci. USA *80*, 6972–6976.

Samelson, L. E., Harford, J. B., and Klausner, R. D. (1985). Identification of the components of the murine T cell antigen receptor complex. Cell *43*, 223–231.

Sha, W. C., Nelson, C. A., Newberry, R. D., Kranz, D. M., Russell, J. H., and Loh, D. Y. (1988a). Selective expression of an antigen receptor on CD8-bearing T lymphocytes in transgenic mice. Nature *335*, 271–274.

Sha, W. C., Nelson, C. A., Newberry, R. D., Kranz, D. M., Russell, J. H., and Loh, D. Y. (1988b). Positive and negative selection of an antigen receptor on T cells in transgenic mice. Nature *336*, 73–76.

Singer, A. (1988). Experimentation and thymic selection. J. Immunol. *140*, 2481–2483.

Singer, A., Hathcock, K. S., and Hodes, R. J. (1981). Self recognition in allogenic radiation bone marrow chimeras. J. Exp. Med. *153*, 1286–1301.

Sprent, J. (1978). Restricted helper function of F_1 parent bone marrow chimeras controlled by K-end of H-2 complex. J. Exp. Med. *147*, 1838–1842.

Sprent, J., Lo, D., Gao, E.-K., and Ron, Y. (1988). T cell selection in the thymus. Immunol. Rev. *101*, 173–190.

Stockinger, H., Pfizenmaier, K., Hardt, C., Rodt, H., Rollinghoff, M., and Wagner, H. (1980). H-2 restriction as a consequence of intentional priming: T cells of fully allogenic chimeric mice as well as of normal mice respond to foreign alloantigens in the context of H-2 determinants not encountered on thymic epithelial cells. Proc. Natl. Acad. Sci. USA *77*, 7390–7394.

Swain, S. L. (1983). T cell subsets and the recognition of MHC class. Immunol. Rev. *74*, 129–142.

Teh, H. S., Kisielow, P., Scott, B., Kishi, H., Uematsu, Y., Bluthmann, H., and von Boehmer, H. (1988). Thymic major histocompatibility complex antigens and the αβ T cell receptor determine the CD4/CD8 phenotype of T cells. Nature *335*, 229–233.

van Ewijk, W., Ron, J., Monaco, J., Kappler, J., Marrack, P., Le Meur, M., Gerlinger, P., Durand, B., Benoist, C., and Mathis, D. (1988). Compartmentalization of MHC class II gene expression in transgenic mice. Cell *53*, 357–370.

von Boehmer, H., Haas, W., and Jerne, N. K. (1978). Major histocompatibility complex-linked immune response is acquired by lymphocytes of low responder mice differentiating in thymus of high responder mice. Proc. Natl. Acad. Sci. USA *75*, 2439–2442.

von Boehmer, H., Karjalainen, K., Pelkonen, J., Borgulya, P., and Rammensee, H.-G. (1988). The T cell receptor for antigen in T cell development and repertoire selection. Immunol. Rev. *101*, 21–37.

Wagner, E., Stewart, T., and Mintz, B. (1981a). The human β-globin gene and a functional viral thymidine kinase gene in developing mice. Proc. Natl. Acad. Sci. USA *78*, 5016–5020.

Wagner, T. E., Hoppe, P. C., Jollick, J. D., Scholl, D. R., Hodinka, R. L., and Gault, J. B. (1981b). Microinjection of a rabbit β-globin gene into zygotes and its subsequent expression in adult mice and their offspring. Proc. Natl. Acad. Sci. USA *78*, 6376–6380.

Watson, J. (1979). Continuous proliferation of murine antigen-specific helper T lymphocytes in culture. J. Exp. Med. *150*, 1510–1519.

White, J., Herman, A., Pullen, A. M., Kubo, R., Kappler, J. W., and Marrack, P. (1989). The Vβ-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. Cell *56*, 27–35.

Widera, G., Burkly, L. C., Pinkert, C. A., Böttger, E. C., Cowing, C., Palmiter, R. D., Brinster, R. L., and Flavell, R. A. (1987). Transgenic mice selectively lacking MHC class II (I-E) antigen expression on B cells: an in vivo approach to investigate Ia gene function. Cell *51*, 175–187.

Zinkernagel, R. M. (1982). Selection of restriction specificities of virus-specific cytotoxic T cells in the thymus: no evidence for a crucial role of antigen-presenting cells. J. Exp. Med. *156*, 1842.

Zinkernagel, R. M., and Doherty, P. C. (1974). Restriction of in vitro T cell mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semi-allogeneic system. Nature *248*, 701–702.

Zinkernagel, R. M., Callahan, G. N., Althage, A., Cooper, S., Klein, P. A., and Klein, J. (1978). On the thymus in the differentiation of "H-2 self recognition" by T cells: evidence for dual recognition? J. Exp. Med. *147*, 882–896.