

Thymus-independent Positive and Negative Selection of T Cells Expressing a Major Histocompatibility Complex Class I Restricted Transgenic T Cell Receptor α/β in the Intestinal Epithelium

By Philippe Poussier,* Hung Sia Teh,† and Michael Julius§

From the *Department of Medicine, McGill University, Montreal, Quebec, Canada H3A 1A1; the †Department of Microbiology, University of British Columbia, Vancouver, British Columbia, Canada V6T1S5; and the §Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada H3A 2B4

Summary

We demonstrate that in the mouse intestinal epithelium the selection of T lymphocytes expressing a transgenic T cell receptor α/β (TCR- α/β) specific for male antigen (H-Y) in the context of H-2D^b depends on the differential expression of H-Y and H-2D^b in situ. In H-2D^b transgenic males, there is no reduction in the number of intestinal intraepithelial lymphocytes (IEL), and the four main subsets of IEL expressing TCR- α/β , defined by the differential expression of CD4, CD8 α , and CD8 β , are present. Moreover, the level of expression of CD8 α and CD8 β on CD8⁺ IEL subsets is unaltered. The frequency of CD8 α ⁺ IEL expressing CD8 β , in H-2D^b male mice, however, is significantly decreased and these cells do not express the transgenic TCR. In contrast, virtually all CD8 α ⁺ β ⁻ IEL in the same animals express the transgenic TCR. Still, these potentially autoreactive cells are refractile to H-Y/H-2D^b stimulation in vitro. Both H-2D^b and H-2D^d transgenic females contain high frequencies of cells expressing the transgenic TCR among CD8 α ⁺ β ⁻ and CD8 α ⁺ β ⁺ IEL. However, two possibly related phenotypic features are peculiar to H-2D^b female mice. The frequency of CD8 α ⁺ IEL expressing CD8 β is increased in these mice and, while in H-2D^d females the level of the transgenic TCR α chain expressed on CD8 α ⁺ β ⁺ IEL is uniformly low, some of the CD8 α ⁺ β ⁺ IEL in H-2D^b females express a high level of both transgenic TCR chains. It is important to note, the ability of CD8 α ⁺ β ⁺ IEL to respond to H-Y/H-2D^b stimulation in vitro is restricted to those coexpressing a high level of both transgenic TCR chains. The analysis of athymic radiation chimeras using adult thymectomized recipients of distinct H-Y/H-2 haplotypes, reconstituted with bone marrow from H-2D^b transgenic females, demonstrates that all IEL subsets present in unmanipulated transgenic animals develop in the absence of a thymus. These IEL are phenotypically identical to those found in unmanipulated transgenic animals sharing the H-Y/H-2 haplotype of athymic recipients. Taken together, these results demonstrate that in the absence of male antigen, expression of H-2D^b in the intestinal epithelium results in the positive selection of functional IEL specific for male antigen, in situ. When both H-Y and H-2D^b are expressed in the intestinal epithelium, CD8 α ⁺ β ⁺ IEL expressing the transgenic TCR are negatively selected, while the frequency of nonfunctional CD8 α ⁺ β ⁻ IEL expressing the transgenic TCR is increased. Thus, while the functional T cell repertoire generated in the thymus and in the intestinal epithelium of these transgenic animals is similar, the supporting mechanisms are distinct.

It has recently been demonstrated that the mouse intestinal epithelium is a thymus-independent site of T-lymphopoiesis (1, 2). Phenotypic analysis of intestinal intraepithelial lymphocytes (IEL)¹ reveals that in adult animals, the majority express TCR- α/β (3). Further, these IEL can be divided into

four major subsets based on their differential expression of CD4, CD8 α , and CD8 β (4, 5). Two IEL subsets are phenotypically identical to mature thymus-derived T cells and express either CD4 or CD8 $\alpha\beta$ (4). Two additional subsets are unique to the intestinal epithelium, expressing either CD8 α alone or in combination with CD4. While the level of TCR- α/β expressed is comparable among these four subsets, only the CD4⁺ and CD8 α ⁺ β ⁺ subsets respond to anti-TCR-

¹ Abbreviation used in this paper: IEL, intestinal intraepithelial lymphocytes.

α/β stimulation (2). These two subsets are depleted of potentially self-reactive T cells in conventional animals, and using athymic radiation chimeras, it has been demonstrated that deletion occurs *in situ* and is dependent on the genotype expressed by the recipient (2). In contrast, analysis of both conventional animals and radiation chimeras indicate that the $CD4^+8\alpha^+$ and $CD8\alpha^+$ subsets contain high frequencies of potentially self-reactive T cells, but are refractile to anti-TCR- α/β stimulation (2, 4). It remains unclear whether phenotypic and functional differences among IEL subsets reflect products of distinct lineages, or alternatively, different stages of IEL development along a common pathway.

Thus, while available evidence supports the conclusion that potentially autoreactive TCR- α/β^+ IEL are negatively selected *in situ*, there is no evidence indicating that positive selection occurs at this site (6). To further characterize the development and selection of IEL expressing TCR- α/β , we have analyzed the phenotype and function of IEL expressing a transgenic TCR- α/β specific for male antigen (H-Y) in the context of H-2D^b (7). These analyses were performed in unmanipulated transgenic mice and in athymic radiation chimeras reconstituted with T-depleted bone marrow of H-2D^b transgenic females. Our results demonstrate that positive selection of functional H-Y specific IEL occurs, and does so exclusively in the intestinal epithelium of female mice expressing H-2D^b, within the $CD8\alpha^+\beta^+$ subset.

A number of features distinguish negative selection processes occurring in the intestinal epithelium from those occurring in the thymus of transgenic male H-2D^b animals. In contrast to T cell development in the thymus, there is no reduction in the number of IEL, rather, the proportions of IEL subsets are altered. Male H-2D^b animals contain fourfold fewer $CD8\alpha^+\beta^+$ IEL than do females of the same haplotype. Moreover, in contrast to $CD8\alpha^+\beta^+$ thymocytes, $CD8\alpha^+\beta^+$ IEL do not express the transgenic TCR in male mice. Strikingly, most $CD8\alpha^+\beta^-$ IEL in the male coexpress transgenic TCR α and β chains. Although the level of $CD8\alpha$ expression is not downregulated in this subset, these cells do not respond to antigen.

Taken together the results demonstrate that while the functional T cell repertoire generated in the intestinal epithelium is identical to that generated intrathymically in these transgenic animals, the supporting mechanisms are distinct.

Materials and Methods

Mice. H-2D^b and H-2D^d transgenic mice originated from the Basel Institute for Immunology (a gift from Dr. H von Boehmer, Basel, Switzerland) and from the University of British Columbia (Vancouver, Canada). These animals were housed and bred in our animal facilities, in Montreal. IEL were analyzed from transgenic mice at 4–20 wk of age. 6–8-wk-old C57BL/6 and BALB/c mice were purchased from Charles River Breeding Laboratories, Inc. (St-Constant, Que., Canada). Ly5.1 congenic C57BL/6 mice were bred in our animal facilities.

Isolation of IEL. IEL were prepared, as previously described (2). In some experiments, $CD4^+$ IEL were depleted by incubation of total IEL with anti- $CD4$ mAb (RL1724H; reference 8) and rabbit complement (Cedarlane Laboratories, Ltd., Mississauga, Ont.,

Canada) for 60 min at 37°C. Dead cells were removed by discontinuous Percoll gradient centrifugation, as previously described (2). Less than 1% of rescued viable cells expressed $CD4$.

mAbs, Three-color Immunofluorescence, and FACS® Analysis. The mAbs used in this study were purified from hybridoma culture supernatants on protein G-Sepharose and then conjugated with FITC or biotin. These mAbs include anti- $CD3\epsilon$ (145-2C11; reference 9), anti-TCR- α/β (H57.597; reference 10), anti-TCR- γ/δ (GL3; reference 11), anti- $CD8\alpha$ (53.6.7.2; reference 12), anti- $CD4$ (GK1.5; reference 13), anti- $CD8\beta$ (53.5.8; reference 14), anti-V β 8.2 (F23.2; reference 15), which recognizes the transgenic TCR β chain, anti-transgenic TCR α chain (T3.70; reference 16), anti-mIg (187; reference 17), anti-Ly5.1 (AZO.1.7), and anti-Ly5.2 (104.2). Both anti-Ly5 mAbs were a gift from Dr. S. Kimura (Memorial Sloan-Kettering Cancer Center, New York). PE-conjugated anti- $CD4$ (GK1.5) and anti- $CD8\alpha$ (53.6.7.2) were purchased from Becton Dickinson & Co. (Mountain View, CA) and Cedarlane Laboratories, Ltd., respectively. Anti- $CD8\beta$ (53.5.8) was custom-labeled with PE by Molecular Probes Inc. (Eugene, OR). Streptavidin-PE/Texas red Tandem™ was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Cells were stained and washed in PBS + 5% FCS at room temperature. Each staining step was carried out for 10 min and followed by three washes. IEL were incubated first with biotinylated mAb, followed by streptavidin-PE/Texas red Tandem™. PE-labeled mAb and FITC-conjugated mAb were then added simultaneously. Viable cells were gated using forward and side angle scatter.

Cell Sorting and Functional Analysis of IEL Subsets. $CD4$ -depleted IEL from transgenic mice were incubated with PE-labeled anti- $CD8\alpha$ and a mixture of FITC-conjugated mAbs specific for $CD8\beta$, TCR- γ/δ , $CD4$, and mIg, simultaneously. Cells reacting with PE-labeled anti- $CD8\alpha$ only, that is $CD4^-8\alpha^+\beta^-$ IEL expressing TCR- α/β , were then sorted using a FACStar® (Becton Dickinson & Co.). To purify $CD4^-8\alpha^+\beta^+$ IEL, all of which express TCR- α/β , $CD4$ -depleted IEL were incubated with PE-labeled anti- $CD8\beta$ and FITC-conjugated anti- $CD4$, simultaneously, and $CD8\beta^+$ cells were then sorted. To purify $CD4^-8\alpha^+\beta^+$ IEL expressing low and high levels of the transgenic TCR α chain (all of which coexpress the transgenic TCR β chain), $CD4$ depleted IEL from H-2D^b transgenic females were incubated with biotinylated T3.70, followed by streptavidin-PE/Texas red Tandem™ and PE-labeled anti- $CD8\beta$. $CD8\beta^+$ IEL expressing low and high levels of the transgenic TCR α chain were then sorted. Subsequent to washing, 3×10^4 sorted IEL (>99% pure) were co-cultured with 5×10^5 irradiated (2,000 rad) female or male C57BL/6 splenocytes in 0.2 ml of IMDM (2) containing 10% FCS, as previously described (2, 16). 18 h later, recombinant mouse IL-2 containing supernatant (2) was added to a final concentration of 0.5% (vol/vol). Cultures were pulsed at 66 h with 1 μ Ci of [³H]thymidine, harvested 6 h later, and the amount of thymidine incorporated was assessed using liquid scintillation spectroscopy. The kinetics of induction of DNA synthesis was analyzed at 24, 48, 72, and 96 h, for each IEL subset using various concentrations of Con A. 1 μ g/ml of Con A at 72 h gave optimal levels of thymidine incorporation for all IEL subsets.

Preparation and Analysis of Hematopoietic Chimeras. 8–10-wk-old Ly5.1 congenic C57BL/6 males and females, as well as BALB/c females, were thymectomized. 2 wk later these animals were exposed to 1,100 rad of radiation delivered from a ¹³⁷Cs source (Gamma cell 40; Atomic Energy of Canada Ltd., Ottawa, Ontario, Canada). Within 24 h, irradiated recipients were injected intravenously with 10^7 T-depleted bone marrow cells isolated from Ly5.2, H-2D^b transgenic female mice. Depletion of bone marrow

T lymphocytes was achieved using a combination of anti-Thy1.2 (HO.13.4; reference 18), anti-CD4 (RL1724H; reference 8), anti-CD8 (3.168; reference 19) mAbs, and rabbit complement (Cedarlane Laboratories, Ltd.). Less than 0.1% of rescued viable cells expressed CD3. 12–16 wk after reconstitution, suspensions of lymphocytes from the spleen and intestinal epithelium were analyzed on a FACScan[®] by three-color immunofluorescence using mAbs specific for mIg, TCR- α/β , and either H-2D^b and H-2D^d or Ly5.1 and Ly5.2, depending on the strain combination. T lymphocytes of donor origin accounted for <1% of the splenocytes. IEL contained 10–25% residual recipient cells, which were depleted before phenotypic analyses. Residual cells of BALB/c recipients were lysed using anti-H-2^d antiserum (a gift from Michel Seman, University of Paris, France) and guinea pig complement (Cedarlane Laboratories, Ltd.), while those of Ly5.1 recipients were negatively selected using biotinylated anti-Ly5.1 mAb, streptavidin conjugated magnetic particles and a magnetic activated cell sorter, MACS[®] (Becton Dickinson & Co.). In either case, less than 1% of the rescued cells were of recipient origin. In some experiments, CD4⁺ IEL of donor origin were depleted with anti-CD4 mAb and complement, as described above. Lymphocytes of donor origin were then analyzed flow cytometrically using three-color immunofluorescence, as described above.

Statistics. Statistical analysis of significance was performed by using the unpaired Student's *t* test. All results presented are expressed as mean \pm 1 SEM.

Results

Phenotypic Analysis of IEL in Male and Female Transgenic Animals. Expression of the H-Y/H-2D^b specific TCR- α/β

in male H-2D^b mice results in severe thymic atrophy, which is associated with the depletion of CD4⁺8⁺ thymocytes (7). In contrast, the number of IEL in these same animals is unaffected. Thus, $25.6 \pm 4.3 \times 10^6$ ($n = 18$), $21.5 \pm 3.5 \times 10^6$ ($n = 25$), and $19.8 \pm 1.8 \times 10^6$ ($n = 19$) IEL were isolated from H-2D^b males, H-2D^b females, and H-2D^d females, respectively. Moreover, the four IEL subsets characterized by the differential expression of CD4 and CD8 α are present (Fig. 1). However, their proportion is altered in H-2D^b transgenic animals, as a consequence of the high frequency of CD4⁺8⁺ IEL in both males and females, and an increased frequency of CD4⁺8⁻ IEL in males (Fig. 1). The proportion of CD4⁺8⁺ IEL in H-2D^b transgenic animals varies from $10.7 \pm 2.1\%$ in females ($n = 31$; range: 2.5–31.3%) to $12.1 \pm 2.1\%$ in males ($n = 35$; range: 2.4–34.3%). In H-2D^d transgenic females, the frequency of this IEL subset, $6.2 \pm 1.0\%$ ($n = 23$; range: 1.5–9.9%), is significantly lower ($p < 0.05$; Fig. 1). CD4⁺8⁻ IEL account for $26.0 \pm 1.6\%$ of TCR- α/β ⁺ IEL in H-2D^b transgenic males ($n = 35$; range: 14.2–33.5%). This subset is also present, but at reduced frequencies, in H-2D^b female transgenics [$4.1 \pm 0.3\%$ ($n = 31$); range: 0.5–7.9%] and H-2D^d transgenic females [$1.8 \pm 0.2\%$ ($n = 23$); range: 0.8–6.4%]. As for the double negative cells in the thymus and periphery of H-2D^b male animals (7), all of the double negative IEL coexpress transgenic TCR α and β chains, but they do not respond to antigen (data not shown). While it is striking that this subset is amplified exclusively in the thymus and

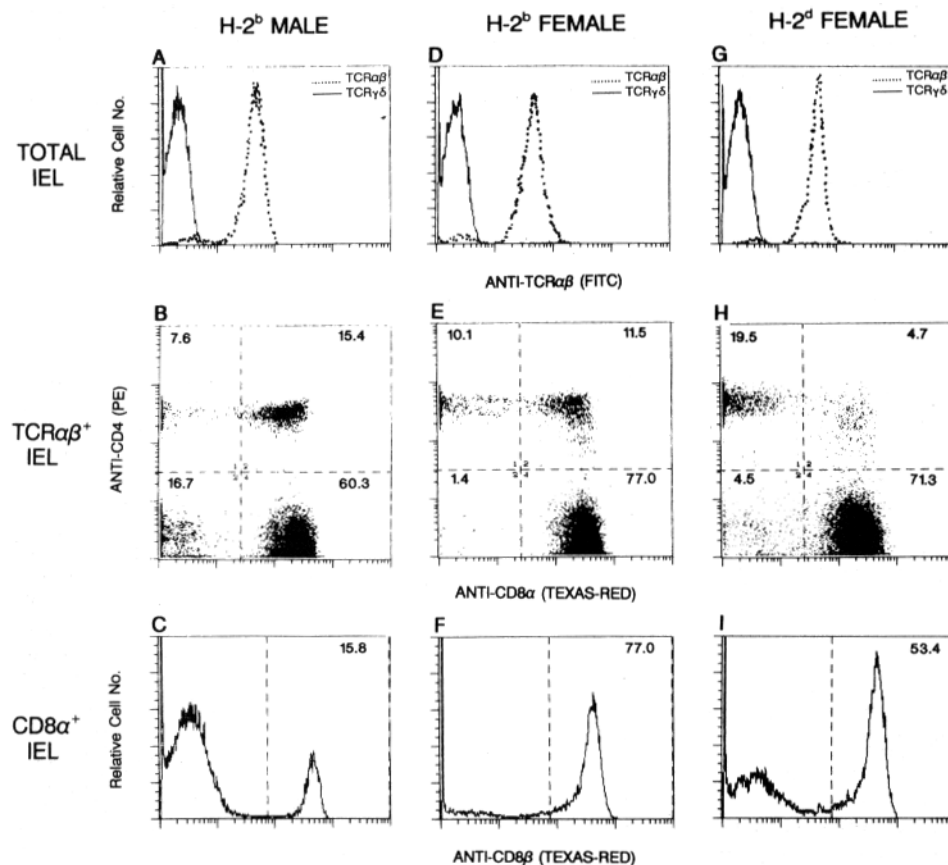


Figure 1. Phenotypic analysis of IEL from 10-wk-old H-2D^b and H-2D^d transgenic mice. Cells were isolated and stained, as described in Materials and Methods. (A, D, and G) Staining profiles of total IEL with anti-TCR- α/β and anti-TCR- γ/δ . (B, E, and H) Expression of CD4 and CD8 α by TCR- α/β ⁺ IEL. (C, F, and I) Staining profiles of CD4⁺8⁺, TCR- α/β ⁺ IEL with anti-CD8 β .

intestinal epithelium of animals that express both the antigen and restriction element recognized by the transgenic TCR, the development and fate of these cells remain obscure (7, 20).

Another feature common to the two sites of T lymphopoiesis in these transgenic animals is the reduced frequency of T cells expressing TCR- γ/δ . These cells account for the majority of IEL in young (6–10 wk) conventional animals (3, 21). As previously described for intrathymic T cell development in these transgenic animals (22), the frequency of IEL expressing TCR- γ/δ is reduced by an order of magnitude (Fig. 1), ranging from $5.0 \pm 0.9\%$ in H-2D^b males ($n = 26$) to $2.1 \pm 0.5\%$ in H-2D^b females ($n = 26$), and $1.2 \pm 0.1\%$ in H-2D^d females ($n = 16$). The paucity of TCR- γ/δ ⁺ IEL, observed in both young (4–6 wk) and old (>20 wk) transgenic animals, suggests that the presence of transgenes encoding rearranged TCR- α/β prevents the rearrangement and/or expression of endogenous genes encoding TCR- γ/δ during IEL development.

Two features distinguish intrathymic from intrainestinal T cell development in these transgenic animals. H-2D^b transgenic males contain high frequencies of CD8 α ⁺ β ⁺ thymocytes that express the transgenic TCR (7, 16). However, the level of CD8 expression is reduced 10-fold compared with that observed in H-2D^b females (7, 16), and correlates with the inability of these cells to respond to antigen (7). In contrast, the level of CD8 α and CD8 β expressed on IEL subsets is identical in male and female H-2D^b transgenics (Fig. 1). As previously described (4), TCR- α/β ⁺, CD8⁺ IEL can be subdivided based on the differential expression of CD8 β , and, moreover, the expression of CD8 β correlates with that of functional TCR-CD3 complexes (2). In H-2D^d transgenic females ($n = 21$), CD8 α ⁺ β ⁺ IEL account for $48.1 \pm 2.1\%$ (range: 38.8–57.7%) of CD8 α ⁺ IEL, a

proportion similar to that observed in conventional animals (Fig. 1; reference 3). The frequency of CD8 α ⁺ IEL expressing CD8 β is markedly different in H-2D^b transgenic animals. In H-2D^b transgenic females ($n = 26$), $74.6 \pm 2.9\%$ (range: 63.3–84.1%) of CD8 α ⁺ IEL express CD8 β , a proportion significantly higher ($p < 0.005$) than that observed in H-2D^d transgenic females (Fig. 1). In contrast, the frequency of CD8 α ⁺ β ⁺ IEL is reduced in H-2D^b males ($n = 29$), and account for only $18.7 \pm 1.6\%$ (range: 11.6–26.6%) of CD8 α ⁺ IEL, ($p < 0.005$ vs. H-2D^b and H-2D^d transgenic females, Fig. 1).

We then analyzed the surface expression of the transgenic TCR α and β chains in CD8 α ⁺ β ⁺ and CD8 α ⁺ β [−] IEL subsets of male and female transgenic animals. Previous studies in conventional animals and in athymic radiation chimeras, have demonstrated that the frequencies of CD8 α ⁺ β [−] IEL expressing TCR V β s specific for I-E and Mls1⁺ do not differ between animals that express these molecules or not (2, 23). This apparent lack of influence of self-antigen on the TCR repertoire of CD8 α ⁺ β [−] IEL does not apply to H-Y Ag in this transgenic TCR system. In H-2D^b males, on average $87.9 \pm 3.6\%$ ($n = 6$) of CD8 α ⁺ β [−] IEL coexpress the transgenic TCR α and β chains. The frequency of CD8 α ⁺ β [−] IEL expressing the transgenic TCR is lower ($p < 0.05$) in female mice, ranging from $29.0 \pm 4.7\%$ in H-2D^b females ($n = 10$) to $47.6 \pm 6.6\%$ in H-2D^d ($n = 6$) females (Fig. 2). It is important to note that virtually all CD8 α ⁺ β [−] IEL express the transgenic TCR β chain, in all three sorts of animals analyzed. Taken together these results indicate that the presence of rearranged TCR- α/β transgenes results in a more profound allelic exclusion of endogenous TCR- β genes than TCR- α genes in CD8 α ⁺ β [−] IEL.

While virtually all CD8 α ⁺ β [−] IEL are potentially au-

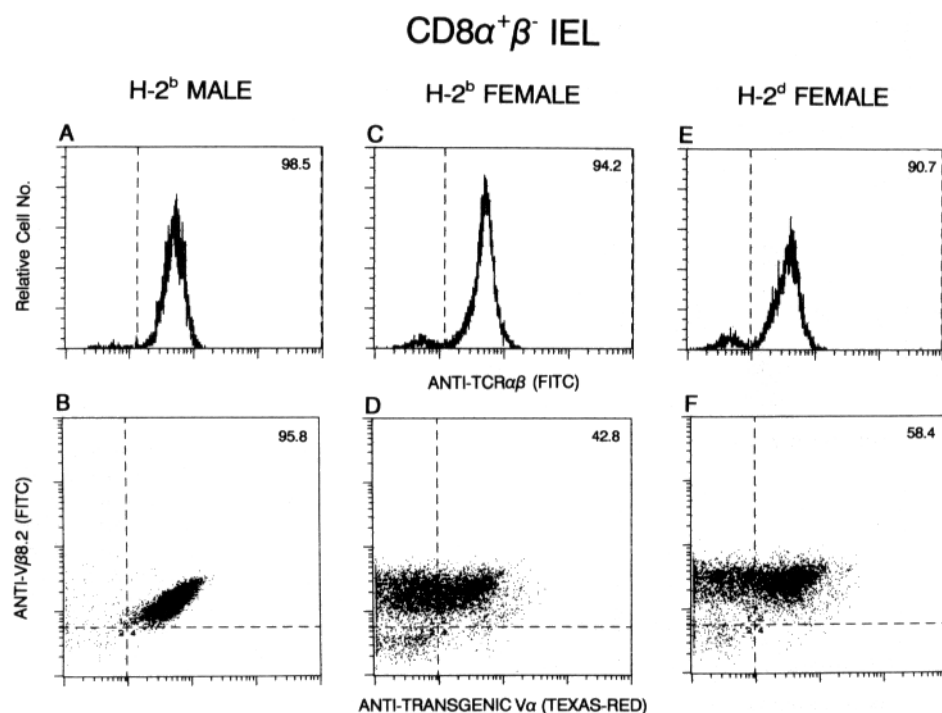


Figure 2. Coexpression of the TCR α and β chains by CD4[−]8 α ⁺ β [−] IEL of 15-wk-old H-2D^b and H-2D^d transgenic mice. CD4-depleted IEL were incubated with a mixture of FITC-labeled mAbs specific for CD8 β , CD4, TCR- γ/δ , and mlg. Unlabeled IEL, that is CD4[−]8 α [−] and CD4[−]8 α ⁺ cells, were then sorted using a FACStar[®]. Sorted cells were >99% pure. An aliquot of purified cells was simultaneously incubated with PE-labeled anti-CD8 α and FITC-conjugated anti-TCR- α/β . The remaining cells were incubated with biotinylated-T3.70, followed by streptavidin-PE/Texas red Tandem[™]. PE-labeled anti-CD8 α and FITC-conjugated F23.2 were then simultaneously added. (A, C, and E) Staining profiles of CD4[−]8 α ⁺ β [−] IEL with anti-TCR- α/β . (B, D, and F) Expression of transgenic TCR α and β chains by CD4[−]8 α ⁺ β [−] IEL.

toreactive in H-2D^b male mice, CD8 α ⁺ β ⁺ IEL in the same animals are devoid of cells expressing the transgenic TCR (Fig. 3). Rearrangement and expression of endogenous TCR- β genes in these cells is demonstrated by their reactivity with H57, a pan TCR β chain specific mAb (10). In contrast, high proportions of CD8 α ⁺ β ⁺ IEL coexpress the transgenic TCR α and β chains in both H-2D^b (42.2 \pm 3.1%) and H-2D^d (42.7 \pm 6.2%) females (Fig. 3). However, one phenotypic feature of CD8 α ⁺ β ⁺ IEL expressing the transgenic TCR distinguishes H-2D^b from H-2D^d females (Fig. 3). In the former, all of these cells express a low level of the transgenic TCR α chain (Fig. 3), while in the latter, two populations of CD8 α ⁺ β ⁺ IEL are distinguished, based on the level of expression of the transgenic TCR α chain (Fig. 3). One population is similar to that found in H-2D^d females, while the other, which accounts for 43.7 \pm 8.3% of the CD8 α ⁺ β ⁺ IEL expressing the transgenic TCR, expresses three- to fourfold higher levels of the transgenic TCR α chain (Fig. 3).

In H-2D^b males, the reduced frequency of CD8 α ⁺ β ⁺ IEL, and the lack of cells expressing the transgenic TCR within this IEL subset, are consistent with negative selection of functionally autoreactive cells during intraintestinal T cell development. The lack of symptoms of intestinal autoimmunity in the same animals suggests that CD8 α ⁺ β ⁺ IEL expressing the transgenic TCR cannot respond to male antigen. Fur-

ther, two possibly related phenotypic features peculiar to H-2D^b female mice are striking and consistent with positive selection of functional T cells. The frequency of CD8 α ⁺ β ⁺ IEL is increased, and some of them coexpress a high level of both transgenic TCR chains. These results led us to determine whether CD8 α ⁺ β ⁺ IEL expressing the transgenic TCR respond to H-Y/H-2D^b stimulation, and in addition, to determine whether different levels of transgenic TCR expression by CD8 α ⁺ β ⁺ IEL correlate with differential antigen responsiveness.

Functional Analysis of CD8 α ⁺ β ⁺ and CD8 α ⁺ β ⁺ IEL in Male and Female Transgenic Animals. CD8 α ⁺ β ⁺ and CD8 α ⁺ β ⁺ IEL subsets from transgenic animals were purified by FACS[®], and their proliferation in response to Con A and H-Y was assessed. As illustrated in Fig. 4, both CD8 α ⁺ β ⁺ and CD8 α ⁺ β ⁺ IEL subsets of transgenic mice synthesize DNA in response to Con A, with stimulation indices ranging from 51 to 415. In contrast, and despite the high frequencies of cells expressing the transgenic TCR, CD8 α ⁺ β ⁺ are refractile to H-Y/H-2D^b stimulation (Fig. 4). It is important to note, this lack of proliferation is observed despite the addition of exogenous IL-2 to cultures. As expected, CD8 α ⁺ β ⁺ IEL of H-2D^b males fail to respond to H-Y Ag (Fig. 4). In transgenic females, the ability of CD8 α ⁺ β ⁺ IEL to respond to H-Y Ag correlates with the presence of cells coexpressing a high level of both transgenic

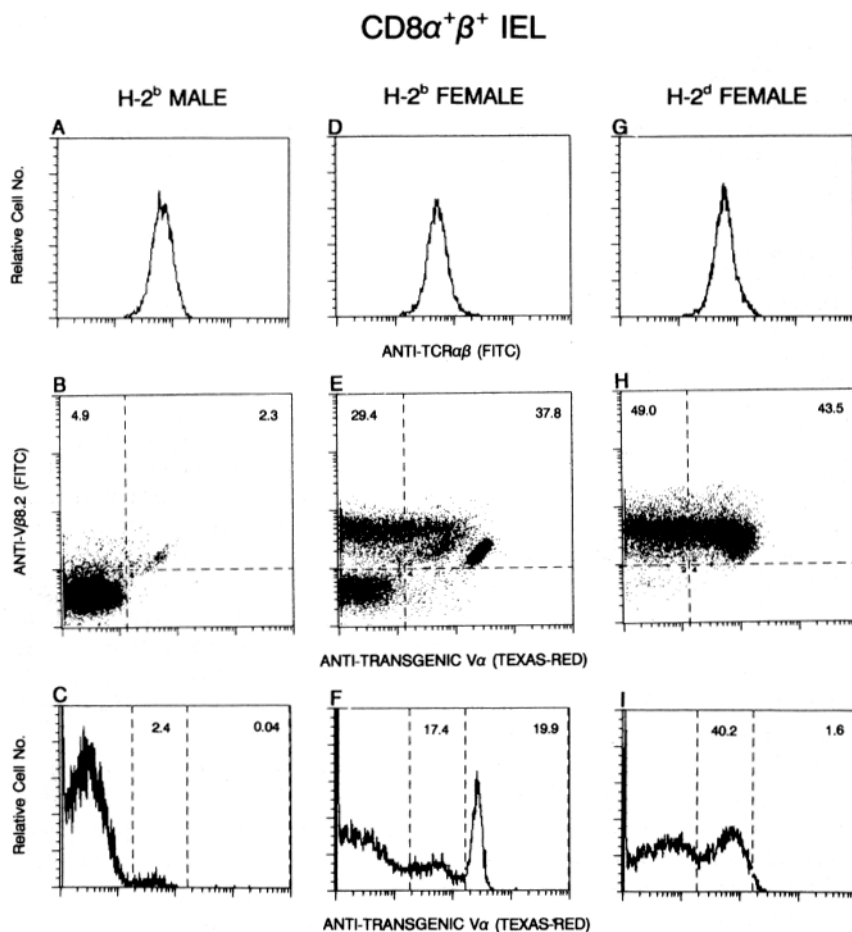


Figure 3. Coexpression of the transgenic TCR α and β chains by CD4⁺8 α ⁺ β ⁺ IEL of 15-wk-old H-2D^b and H-2D^d transgenic mice. CD4-depleted IEL were isolated and stained, as described in Materials and Methods. (A, D, and G) Staining profiles of CD4⁺8 α ⁺ β ⁺ IEL with anti-TCR- α/β . (B, E, and H) Expression of transgenic TCR α and β chains by CD4⁺8 α ⁺ β ⁺ IEL. (C, F, and I) Staining profiles of CD4⁺8 α ⁺ β ⁺ IEL with T3.70, a monoclonal antibody that recognizes the transgenic TCR α chain.

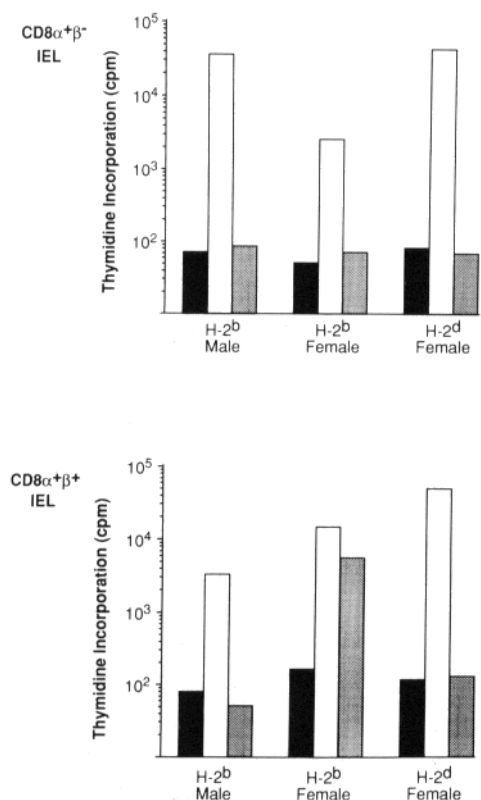


Figure 4. Differential responsiveness of $CD4^{-}8\alpha^{+}\beta^{-}$ and $CD4^{-}8\alpha^{+}\beta^{+}$ IEL expressing TCR- α/β to Con A and to male antigen. IEL subsets were purified, as described in Materials and Methods. 3×10^4 IEL were co-cultured in the presence of exogenous IL-2 and FCS with 5×10^5 irradiated splenocytes of C57BL/6 females for 66 h in IMDM containing $1 \mu\text{g/ml}$ of Con A. Ag stimulation was performed by co-culture of the same number of IEL with 5×10^5 irradiated splenocytes from C57BL/6 males. Cultures were then pulsed with $[^3\text{H}]$ thymidine and harvested 6 h later. Bars represent the mean level of thymidine incorporation from four to 10 individual experiments. (Top) Comparison of DNA synthesis induced by Con A and H-Y Ag in $CD4^{-}8\alpha^{+}\beta^{-}$ IEL expressing TCR- α/β . The mean background levels of thymidine incorporation \pm SEM were 71 ± 13 , 51 ± 9 , and 84 ± 12 cpm in transgenic H-2D^b males, H-2D^b females, and H-2D^d females, respectively. The mean levels of thymidine incorporation \pm SEM induced by Con A in the same subsets were $34,407 \pm 2,302$, $2,563 \pm 226$, and $42,880 \pm 4,288$ cpm, respectively. (Bottom) Comparison of DNA synthesis induced by Con A and H-Y Ag in $CD4^{-}8\alpha^{+}\beta^{+}$ IEL. The mean background levels of thymidine incorporation \pm SEM were 80 ± 9 , 163 ± 22 , and 118 ± 45 cpm in transgenic H-2D^b males, H-2D^b females, and H-2D^d females, respectively. The mean levels of thymidine incorporation \pm SEM induced by Con A in the same IEL subsets were $3,304 \pm 197$, $14,755 \pm 656$, and $47,771 \pm 2,505$, respectively, while those induced by Ag were 51 ± 5 , $5,522 \pm 223$, and 130 ± 19 , respectively. (■) Background; (□) Con A; (▨) H-2^b male.

TCR chains (Fig. 4). Thus, the levels of thymidine incorporation induced by H-Y/H-2D^b in $CD8\alpha^{+}\beta^{+}$ IEL of H-2D^b female mice are 35-fold higher than those observed in the same IEL subset of H-2D^d females (Fig. 4). This suggests that those $CD8\alpha^{+}\beta^{+}$ IEL expressing low levels of transgenic TCR- α , present in both H-2D^d and H-2D^b females, are not responding. This was directly assessed by sorting $CD8\alpha^{+}\beta^{+}$ IEL based on differential expression of trans-

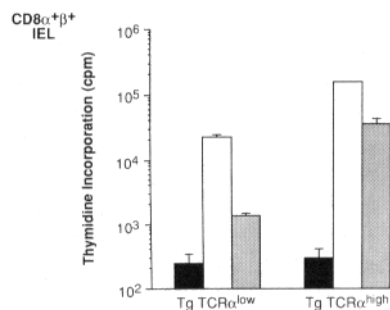


Figure 5. Differential responsiveness of $CD4^{-}8\alpha^{+}\beta^{+}$ IEL expressing low and high levels of the transgenic TCR α chain to Con A and H-Y antigen. IEL subsets were purified from transgenic H-2D^b female mice, as described in Materials and Methods. 2×10^4 IEL were cocultured in the presence of exogenous IL-2 and FCS with 5×10^5 irradiated splenocytes of C57BL/6 females for 66 h in IMDM containing $1 \mu\text{g/ml}$ of Con A. H-Y stimulation was performed by coculture of the same number of IEL with 5×10^5 irradiated splenocytes of C57BL/6 males. Cultures were then pulsed with $[^3\text{H}]$ thymidine and harvested 6 h later. Bars represent the mean level of thymidine incorporation from three to eight individual experiments. (Left) Comparison of DNA synthesis induced by Con A and H-Y Ag in $CD4^{-}8\alpha^{+}\beta^{+}$ IEL coexpressing the transgenic β chain and a low level of the transgenic α chain. The mean levels of thymidine incorporation \pm SEM were 250 ± 93 , $22,893 \pm 1,991$, and $1,370 \pm 100$ when IEL were cultured in the presence of female APCs alone, female APCs + Con A, and male APCs, respectively. (Right) Comparison of DNA synthesis induced by Con A and H-Y Ag in $CD4^{-}8\alpha^{+}\beta^{+}$ IEL coexpressing the transgenic β chain and a high level of the transgenic α chain. The mean levels of thymidine incorporation \pm SEM were 293 ± 113 , $151,512 \pm 2,655$, and $35,514 \pm 7,288$ when IEL were cultured in the presence of female APCs alone, female APCs + Con A, and male APCs, respectively. (■) Background; (□) Con A; (▨) Male H-2^b.

genic TCR α chain and determining their responsiveness to antigen. As illustrated in Fig. 5, DNA synthesis induced by Con A is not influenced by the level of expression of the transgenic α chain, and results in indices of stimulation of 92–512. In contrast, levels of thymidine incorporation induced by H-Y/H-2D^b stimulation in $CD8\alpha^{+}\beta^{+}$ IEL expressing a high level of the transgenic α chain are 25-fold higher than those observed in $CD8\alpha^{+}\beta^{+}$ IEL expressing a low level of the transgenic α chain (Fig. 5).

Thus, the differential responsiveness of $CD8\alpha^{+}\beta^{-}$ and $CD8\alpha^{+}\beta^{+}$ IEL subsets to anti-TCR stimulation that was previously reported (2), is paralleled by the differential ability of these IEL subsets to respond to their specific antigen, in vitro. Functional male specific IEL are not detected in H-2D^b male mice, consistent with the negative selection of these cells when they develop in the presence of both antigen and H-2D^b. Functional, male specific IEL are found in H-2D^b, but not H-2D^d female mice, and are restricted to the $CD8\alpha^{+}\beta^{+}$ IEL subset expressing high levels of transgenic TCR. The selection of this subset in the absence of antigen, thus correlates with the expression of H-2D^b.

Thymus-independent Positive and Negative Selection of T Lymphocytes Expressing the Transgenic TCR in the Intestinal Epithelium. Previous studies in conventional mice using athymic radiation chimeras demonstrate that T lymphocytes found in the intestinal epithelium develop in situ (1, 2). To determine whether the major phenotypic differences between T

cells found in the thymus and intestinal epithelium of the same transgenic animal reflect distinct and independent processes of T cell development and selection, we prepared athymic radiation chimeras reconstituted with transgenic bone marrow. Strain combinations were chosen such that cells of donor and recipient origin could be distinguished. Thus, Ly5.1 C57BL/6 (H-2^b) males and females, as well as BALB/c (H-2^d) females, were thymectomized, lethally irradiated, and reconstituted with T-depleted bone marrow isolated from H-2D^b transgenic female mice (H-2^b, Ly5.2). 12–16 wk after reconstitution >90% of the splenocytes were of donor origin, and <1% of these cells were T lymphocytes (data not shown). 10–25% of the IEL were of recipient origin, and were depleted before phenotypic analysis.

The phenotypic features of donor-derived IEL isolated from the different recipients are summarized in Table 1. In contrast to donor-derived splenocytes in these chimeras, the vast majority of IEL are T lymphocytes, which are predominantly TCR- α/β^+ . The total number of IEL is not affected by the genotype of the host, and is comparable to that obtained in unmanipulated transgenic animals (Table 1). The frequency of the four subsets of TCR- α/β^+ IEL, defined by the differential expression of CD4 and CD8 α , are similar to those found in unmanipulated transgenic animals sharing the same H-Y/H-2 haplotype, with the exception of the double positive subset. The frequency of this subset in athymic radiation chimeras was comparable and low, independent of the H-Y/H-2 haplotype of the recipient (data not shown). Importantly, the proportion of CD8 α^+ IEL expressing CD8 β is significantly increased in H-2D^b female recipients, and decreased in H-2D^b male recipients, relative to that found in H-2D^d female recipients (Table 1). The level of CD8 α and CD8 β expressed on CD8 $^+$ IEL subsets is not affected by the genotype of the host (data not shown). These results confirm that the intestinal epithelium is a site of thymus-independent T lymphopoiesis at which all IEL subsets found in an intact animal are generated (1, 2). Moreover, they demonstrate that the depletion of CD8 $\alpha^+\beta^+$ IEL in H-2D^b male mice, and the increased proportion of these cells in H-2D^b female mice, are induced by the environment in which these IEL develop.

We then analyzed the differential expression of the transgenic TCR in CD8 $\alpha^+\beta^-$ and CD8 $\alpha^+\beta^+$ IEL subsets derived from the same hematopoietic precursors, but developing in genetically different hosts. As illustrated in Fig. 6, cells coexpressing the transgenic TCR α and β chains account for 17–59% of CD8 $\alpha^+\beta^-$ IEL developing in H-2D^b and H-2D^d female recipients, a proportion similar to that found in unmanipulated transgenic female mice (Fig. 2). However, when the same precursors develop in H-2D^b male recipients, virtually all CD8 $\alpha^+\beta^-$ IEL express the transgenic TCR (Fig. 6). This phenotype is identical to that observed in unmanipulated transgenic H-2D^b males and suggests that the increased proportion of potentially autoreactive CD8 $\alpha^+\beta^-$ IEL results as a consequence of the coexpression of both H-Y and H-2D^b in the intestinal epithelium.

Similarly, expression of the transgenic TCR in CD8 $\alpha^+\beta^+$ IEL is strictly dependent on the genotype of the recipient in which these cells develop (Fig. 7). When IEL precursors derived from H-2D^b female mice develop in H-2D^b male recipients, CD8 $\alpha^+\beta^+$ IEL are devoid of cells expressing the transgenic TCR (Fig. 7). However, when the same precursors mature in female recipients, the proportion of CD8 $\alpha^+\beta^+$ IEL coexpressing transgenic TCR α and β chains is high, ranging from $61.6 \pm 11.8\%$ in H-2D^b females ($n = 6$) to $39.7 \pm 4.9\%$ in H-2D^d females ($n = 4$) (Fig. 7). While the MHC haplotype of the female recipient does not affect the frequency of CD8 $\alpha^+\beta^+$ IEL expressing the transgenic TCR, it does influence the selection of IEL expressing a high level of the transgenic α chain. As illustrated in Fig. 7, these functional, male specific, CD8 $\alpha^+\beta^+$ IEL (Fig. 5) develop exclusively in H-2D^b female recipients. In H-2D^d females, the level of the transgenic chain expressed on CD8 $\alpha^+\beta^+$ IEL expressing the transgenic TCR is uniformly low, a phenotypic feature associated with marginal responsiveness to H-Y/H-2D^b stimulation, (Fig. 5). These results demonstrate that selection of CD8 $\alpha^+\beta^-$ and CD8 $\alpha^+\beta^+$ IEL expressing the transgenic TCR occurs *in situ*, in a thymus independent manner, and is determined by the differential expression of H-Y and H-2D^b in the intestinal epithelium.

Table 1. Phenotype of Donor Derived IEL Isolated from Athymic Radiation Chimeras

	Recipient		
	H-2D ^b male $n = 5$	H-2D ^b female $n = 6$	H-2D ^d female $n = 4$
IEL Number ($\times 10^6$)	15.3 ± 1.6	17.4 ± 1.7	14.8 ± 3.0
TCR- α/β^+ IEL (%)	71.5 ± 2.6	74.0 ± 6.6	67.4 ± 2.8
CD4 $^+$ 8 α^+ , TCR- α/β^+ IEL	11.5 ± 3.7	85.1 ± 4.1	44.8 ± 3.7
Expressing CD8 β (%)			

IEL isolated from radiation chimeras were stained with antibodies to H-2^b and H-2^d or Ly5.1 and Ly5.2. The proportion of donor cells expressing H-2^b or Ly5.2 was determined flow cytometrically. The yield of donor IEL was calculated by multiplying the total number of cells isolated by the proportion of donor cells. After depletion of recipient IEL, phenotypic analysis of donor-derived IEL was performed, as described in Materials and Methods. Numbers represent means \pm SEM.

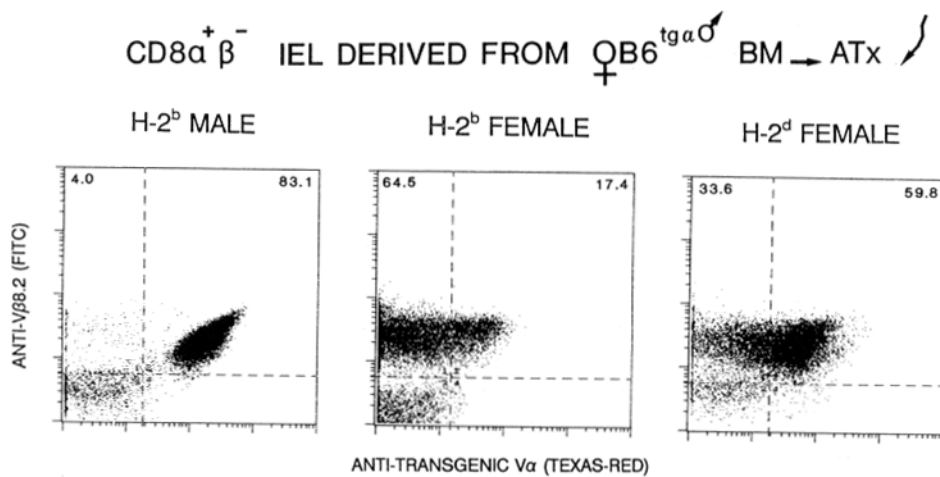


Figure 6. Coexpression of transgenic TCR α and β chains in $CD8\alpha^+\beta^-$ IEL derived from the bone marrow of H-2D^b transgenic females and isolated from athymic radiation chimeras expressing different H-Y/H-2 haplotypes. Cells obtained from three representative chimeras 15 wk after reconstitution were isolated, depleted of residual host cells and stained, as described in Materials and Methods. The H-Y/H-2 haplotype of the recipients is indicated on the top of each dot blot.

Discussion

The results presented here demonstrate that IEL expressing a transgenic TCR specific for H-Y/H-2D^b are either positively or negatively selected in situ, depending on the differential expression of H-Y and H-2D^b in the intestinal epithelium. Data obtained from unmanipulated transgenic animals and athymic radiation chimeras provide concordant results. They confirm previous reports demonstrating that all heretofore characterized IEL subsets are generated in the gut in a thymus independent fashion (1, 2). Further, they extend previous observations demonstrating in situ negative selection of IEL expressing potentially autoreactive MHC class II dependent/specific TCR (I-E and Mls-1^a) (2), to include negative selection of IEL expressing MHC class I restricted

TCR. Finally, they provide the first demonstration that positive selection of IEL also occurs in situ.

There is a potential caveat that merits comment, although it does not fundamentally alter the conclusions of this study. Results obtained using athymic radiation chimeras to demonstrate extrathymic T cell development, do not formally exclude the existence of an as yet unidentified site of T lymphopoiesis, through which IEL precursors transit en route to the intestinal epithelium. The best evidence in support of the T lymphopoietic capacity of the intestinal epithelium comes from recent experiments in which ectopic fetal grafts of either intestine or thymus were implanted under the kidney capsule of athymic radiation chimeras (24). The results demonstrate that both organs have a comparable capacity to repopu-

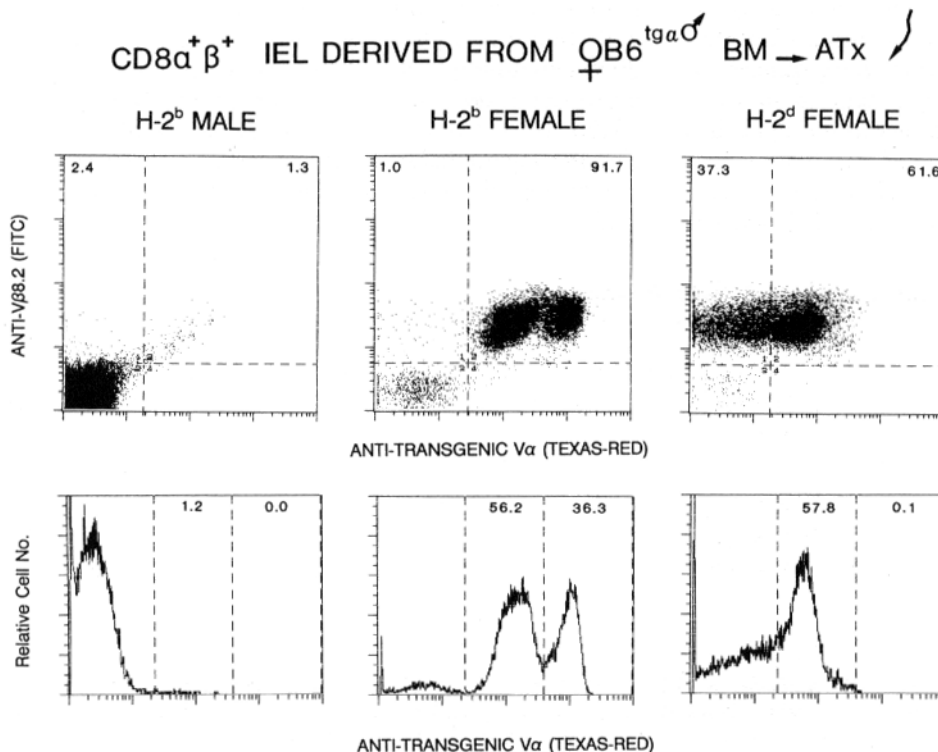


Figure 7. Coexpression of transgenic TCR α and β chains in $CD8\alpha^+\beta^+$ IEL derived from the bone marrow of H-2D^b transgenic females and isolated from athymic radiation chimeras expressing different H-Y/H-2 haplotypes. Cells obtained from three representative chimeras 15 wk after reconstitution were isolated, depleted of residual host cells and stained, as described in Materials and Methods. The H-Y/H-2 haplotype of the recipient is indicated on the top of each dot blot. (Top) Differential expression of the transgenic TCR α and β chains in donor-derived $CD8\alpha^+\beta^+$ IEL. (Bottom) Staining profiles of donor-derived $CD8\alpha^+\beta^+$ IEL with T3.70, a mAb specific for the transgenic TCR α chain.

late secondary lymphoid tissue with T cells. Importantly, both the kinetics of repopulation, as well as the phenotype of peripheral T cells derived from either graft, were identical (24).

There are both parallels and differences related to the selection of IEL and thymocytes expressing this transgenic TCR. Negative selection of functional T cells occurs exclusively in male H-2D^b animals. However, there are striking differences in the phenotypic outcome of this process at the two sites of T lymphopoiesis. While intrathymic deletion is associated with atrophy of the organ (7), the absolute number of IEL rescued in the deleting strain is not altered, rather, the proportions of the various IEL subsets change. The most significant alteration is that the number of CD8 $\alpha^+\beta^+$ IEL is reduced roughly fivefold in unmanipulated transgenic H-2D^b males, and even more dramatically in H-2D^b male recipients of bone marrow from H-2D^b transgenic females. Further, the mechanisms supporting negative selection in the thymus and gut are clearly distinct. At the former site, CD8⁺ T cells expressing the transgenic TCR are present at high frequencies, however, the level of CD8 expression is reduced by an order of magnitude (7). This is a central point, since function of T cells expressing the transgenic TCR requires the expression of this coreceptor (25). In contrast, none of the CD8 $\alpha^+\beta^+$ IEL in H-2D^b males express either the transgenic TCR α or β chains. Thus, while intrathymic selection processes, in circumstances where this TCR is self-reactive, result in the rescue of functionally compromised T cells expressing the transgenic TCR, deletion of these cells developing in the gut appears to be complete.

The most striking phenotypic difference among subsets of developing thymocytes and IEL, is the presence in the latter of a CD8⁺ population that expresses a homodimeric form of the CD8 α chain (4, 26). As previously described (2, 4), this IEL subset contains high frequencies of potentially self-reactive T cells. Importantly, these CD8 $\alpha^+\beta^-$ IEL may not express functional TCR, in that they are refractile to stimulation with mAb specific for TCR- α/β , *in vitro* (2). Two insights into this subset are provided by the results presented in this study. Virtually all of the CD8 $\alpha^+\beta^-$ IEL in H-2D^b males coexpress the transgenic TCR α and β chains. This phenotype is not observed in any of the transgenic female animals analyzed, nor in H-2D^d transgenic males (data not shown), and thus correlates with the expression of the putative H-Y peptide in combination with the appropriate restriction element in the gut. This, in turn, suggests that the increased proportion of this subset reflects some consequence of antigen recognition. Importantly, the level of CD8 expression, albeit CD8 α , is not down-regulated in this subset. Nonetheless, these potentially self-reactive T cells do not respond to antigen *in vitro*, consistent with the absence of any detectable deleterious effects of their presence *in vivo*. Thus, the inability of CD8 $\alpha^+\beta^-$ IEL to respond to anti-TCR (2), can now be extended to a more physiologically relevant stimulus. However, a fundamental question remains. Since lineage relationships among developing IEL are not established, it remains unclear whether the phenotype of the CD8 $\alpha^+\beta^-$ subset in H-2D^b male transgenics is a conse-

quence of antigen-induced developmental arrest, or antigen-induced anergy.

In contrast to the results presented here, it is important to consider that the high frequency of CD8 $\alpha^+\beta^-$ IEL expressing potentially self-reactive TCR- α/β observed in previous studies (2, 4), are not affected by the presence of ligand in the gut. A number of differences merit consideration. To date, with one exception (6), only IEL expressing MHC class II restricted/dependent TCR- α/β have been analyzed (2, 4), and importantly, they have been detected using mAb specific for the TCR β chain exclusively (2, 4). It is not implausible that selection processes for IEL expressing MHC class I restricted TCR are different. Recent analyses of the consequences of intrathymic selection of T cells expressing a transgenic TCR that can recognize MHC class II, or MHC class I, are consistent with this possibility (27). Alternatively, the ability to analyze coexpression of appropriate TCR α and β chains has revealed a developmentally intermediate phenotype within the CD8 $\alpha^+\beta^-$ subset of IEL.

The consequences of positive selection in the gut also appear to be mediated by distinct processes. As for intrathymic selection of this transgenic TCR- α/β , we demonstrate that selection of functional H-Y specific IEL occurs in H-2D^b females. However, in marked contrast to intrathymic T cell development, selection processes in the gut do not correlate with an increase in the proportion of IEL that express the transgenic TCR. Thus, the frequency of IEL expressing the transgenic TCR in either the CD8 $\alpha^+\beta^-$, or CD8 $\alpha^+\beta^+$ subsets is not affected by the MHC haplotype expressed in the gut. Rather, the level of membrane expression of the transgenic TCR appears to be critical.

Functional H-Y specific IEL are found exclusively within the CD8 $\alpha^+\beta^+$ subset in H-2D^b females. The CD8 $\alpha^+\beta^-$ subset in these animals, while containing a high frequency of cells that express the transgenic TCR, do not respond to antigen. Again, this result parallels those obtained in previous studies, in which this subset was shown to be refractile to anti-TCR stimulation (2). Of note here, is that despite the absence of H-Y in H-2D^b females, this subset is still unresponsive, consistent with the notion that they are developmentally immature, rather than anergic.

The observation that the CD8 $\alpha^+\beta^+$ IEL in H-2D^b females express two discrete levels of the transgenic TCR α chain is novel to T cell development in the gut. It is the absence of IEL, which express high levels of transgenic TCR- α , that distinguishes this subset in H-2D^b and H-2D^d females, and thus its presence correlates with some positive selection event that is dependent on the expression of the appropriate restriction element in the gut. Importantly, the level of TCR β chain expression, assessed using a pan specific mAb, is not bimodal, and uniformly high, among CD8 $\alpha^+\beta^+$ IEL in females of both strains. This result suggests two things, assuming that TCR- β is not expressed in the absence of TCR- α . These IEL express a mixture of endogenous and transgenic TCR α chains, both of which are able to pair with the transgenic TCR β chain, and further, that the expression of H-2D^b in the gut provides a selective advantage for those

IEL expressing more of the transgenic TCR α chain. Whether this selection event occurs within the CD8 $\alpha^+\beta^+$ subset itself, or within some as yet undefined precursor population, remains to be determined.

In closing, it is of note that there has been one previous report in which the expression of this transgenic TCR in the intestinal epithelium was analyzed (6). There are striking differences in the results obtained by these investigators, and those presented herein. They reported the absence of expression of the transgenic TCR chain in the CD8 $\alpha^+\beta^-$ IEL subset of H-2D^b or H-2D^d females, as well as the observation that high frequencies of TCR- γ/δ^+ IEL are present in these transgenic animals. Moreover, that a significant propor-

tion of IEL coexpressed transgenic TCR- α/β and endogenous TCR- γ/δ . We have no explanation for these discrepant findings.

The results presented here demonstrate striking differences in the selection processes occurring in the thymus and the intestinal epithelium, which, nonetheless, result in the generation of functionally identical available T cell repertoires. Both the phenotypic and functional differences that distinguish T cell development at these two independent sites of T lymphopoiesis, coupled with previous results demonstrating restricted circulation patterns of peripheral T cells and IEL (2), support the conclusion that thymus- and gut-derived T cells contribute to two distinct immune systems.

We wish to thank Rozie Arnaoutelis and Vicky Kottis for technical assistance, Jessie Blais for help in the preparation of the manuscript, and Claude Cantin of the Clinical Research Institute of Montreal, for cell sorting.

This work was supported by a grant from the National Cancer Institute of Canada.

Address correspondence to Dr. Philippe Poussier, The Wellesley Hospital Research Institute, 160 Wellesley Street East, Toronto, Ontario M4Y 1J3.

Received for publication 16 June 1993 and in revised form 1 September 1993.

References

- Mosley, R.L., D. Styre, and J.R. Klein. 1990. Differentiation and functional maturation of bone marrow-derived intestinal epithelial T cells expressing membrane T cell receptor in athymic radiation chimeras. *J. Immunol.* 145:1369.
- Poussier, P., P. Edouard, C. Lee, M. Binnie, and M. Julius. 1992. Thymus-independent development and negative selection of T cells expressing T cell receptor α/β in the intestinal epithelium: evidence for distinct circulation patterns of gut- and thymus-derived T lymphocytes. *J. Exp. Med.* 176:187.
- Lefrançois, L. 1991. Phenotypic complexity of intraepithelial lymphocytes of the small intestine. *J. Immunol.* 147:1746.
- Guy-Grand, D., N. Cerf-Bensussan, B. Malissen, M. Malassis-Seris, C. Briottet, and P. Vassalli. 1991. Two gut intraepithelial CD8⁺ lymphocyte populations with different T cell receptors: a role for the gut epithelium in T-cell differentiation. *J. Exp. Med.* 173:471.
- Maloy, K.J., A.M. Mowat, R. Zamoyska, and I.N. Crispe. 1991. Phenotypic heterogeneity of intraepithelial T lymphocytes from mouse small intestine. *Immunology.* 72:555.
- Rocha, B., H. von Boehmer, and D. Guy-Grand. 1992. Selection of intraepithelial lymphocytes with CD8 α/α co-receptor by self-antigen in the murine gut. *Proc. Natl. Acad. Sci. USA.* 89:5336.
- Kisielow, P., H. Bluthmann, U.D. Staerz, M. Steinmetz, and H. von Boehmer. 1988. Tolerance in T-cell receptor transgenic mice involves deletion of nonmature CD4⁺8⁺ thymocytes. *Nature (Lond.)* 333:742.
- Ceredig, R., J.W. Lowenthal, M. Nabholz, and H.R. MacDonald. 1985. Expression of interleukin-2 receptors as a differentiation marker on intrathymic stem cells. *Nature (Lond.)* 314:98.
- Bluestone, J.A., D. Pardoll, S.O. Sharrow, and B.J. Fowlkes. 1987. Characterization of murine thymocytes with CD3-associated T-cell receptor structures. *Nature (Lond.)* 326:82.
- Kubo, R., W. Born, J.W. Kappler, P. Marrack, and M. Pigeon. 1989. Characterization of a monoclonal antibody which detects all murine T-cell receptors. *J. Immunol.* 142:2736.
- Goodman, T., and L. Lefrançois. 1989. Intraepithelial lymphocytes. Anatomical site, not T cell receptor form, dictates phenotype and function. *J. Exp. Med.* 170:1569.
- Ledbetter, J.A., and L.A. Herzenberg. 1979. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.* 47:63.
- Sprent, J., and M. Schaefer. 1985. Properties of purified T cell subsets. I. In vitro responses to class I vs. class II H-2 Alloantigens. *J. Exp. Med.* 162:2068.
- Ledbetter, J.A., W.E. Seaman, T.T. Tsu, and L.A. Herzenberg. 1981. Lyt-2 and Lyt-3 antigens are two different polypeptide subunits linked by disulfide bonds. *J. Exp. Med.* 153:1503.
- Kappler, J.W., U. Staerz, J. White, and P. Marrack. 1988. Self-tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex. *Nature (Lond.)* 332:35.
- Kisielow, P., H.S. Teh, H. Bluthmann, and H. von Boehmer. 1988. Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. *Nature (Lond.)* 335:730.
- Yelton, D.E., C. Desayard, and M.D. Scharf. 1981. Use of monoclonal anti-mouse immunoglobulins to detect mouse antibody. *Hybridoma.* 7:5.
- Marshak-Rothstein, A., P. Fink, T. Gridley, D.H. Raulet, M.J. Bevan, and M.L. Teffer. 1979. Properties and applications of monoclonal antibodies directed against determinants of the Thy-1 locus. *J. Immunol.* 122:2491.

19. Sarmiento, M., A.L. Glasbrook, and F.W. Fitch. 1980. IgG or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing Lyt2 antigen block T-cell mediated cytotoxicity in the absence of complement. *J. Immunol.* 125:1665.
20. Takahama, Y., E.W. Shores, and A. Singer. 1992. Negative selection of precursor thymocytes before their differentiation into CD4⁺ CD8⁺ cells. *Science (Wash. DC)*. 258:653.
21. Goodman, T., and L. Lefrançois. 1988. Expression of the γ - δ T cell receptor on intestinal CD8⁺ intraepithelial lymphocytes. *Nature (Lond.)*. 333:855.
22. von Boehmer, H., M. Bonneville, I. Ishida, S. Ryser, G. Lincoln, R.T. Smith, H. Kishi, B. Scott, P. Kisielow, and S. Tonegawa. 1988. Early expression of a T cell receptor transgene suppresses rearrangement of the V γ 4 gene segments. *Proc. Natl. Acad. Sci. USA*. 85:9759.
23. Rocha, B., P. Vassalli, and D. Guy-Grand. 1991. The V β repertoire of mouse gut homodimeric α CD8⁺ intraepithelial T cell receptor α/β ⁺ lymphocytes reveals a major extrathymic pathway of T cell differentiation. *J. Exp. Med.* 173:483.
24. Mosley, R.L., and J.R. Klein. 1992. Peripheral engraftment of fetal intestine into athymic mice sponsors T cell development: direct evidence for thymopoietic function of murine small intestine. *J. Exp. Med.* 176:1365.
25. von Boehmer, H., H. Hemgartner, M. Nabholz, W. Lernhardt, M.H. Schreier, and W. Haas. 1979. Fine specificity of a continuously growing killer cell clone specific for HY antigen. *Eur. J. Immunol.* 9:592.
26. Parrott, D.M.V., C. Tait, S. MacKenzie, A.M. Mowat, M.D.J. Davies, and H.S. Micklem. 1983. Analysis of the effector functions of different populations of mucosal lymphocytes. *Ann. N.Y. Acad. Sci.* 409:307.
27. Pircher, H., K. Brki, R. Lang, H. Hengartner, and R.M. Zinkernagel. 1989. Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. *Nature (Lond.)*. 342:559.