

# Thymus-independent Development and Negative Selection of T Cells Expressing T Cell Receptor $\alpha/\beta$ in the Intestinal Epithelium: Evidence for Distinct Circulation Patterns of Gut- and Thymus-derived T Lymphocytes

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## Summary

We demonstrate that mouse intestinal intraepithelial lymphocytes (IEL) can be divided into subsets based on the differential expression of functional T cell receptor  $\alpha/\beta$  (TCR- $\alpha/\beta$ ) signaling complexes. Two subsets, CD4<sup>+</sup>8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup> and CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup>, are refractory to stimulation with anti-TCR- $\alpha/\beta$  and contain high frequencies of potentially self-reactive cells. In contrast, the CD4<sup>+</sup> and CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>+</sup> IEL subsets are responsive to anti-TCR- $\alpha/\beta$  and depleted of potentially self-reactive cells. The analysis of fetal liver radiation chimeras using adult thymectomized recipients demonstrates that the four TCR- $\alpha/\beta$ <sup>+</sup> IEL subsets are generated in normal numbers in the absence of the thymus. Moreover, expression of the major histocompatibility complex class II-encoded I-E molecule and Mls1<sup>a</sup> in the gut of the athymic host results in the negative selection of potentially self-reactive T cells expressing V $\beta$ 11 and V $\beta$ 6, respectively, from those IEL subsets that express functional TCR- $\alpha/\beta$  signaling complexes. Neither the spleen nor the Peyer's patches of athymic recipients contain T cells of donor origin. In contrast, normal numbers of phenotypically and functionally mature CD4<sup>+</sup> and CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>+</sup> T cells of donor origin are found in the lamina propria of chimeric animals. The phenotypic analysis of lymphocytes obtained from Ly5 congenic parabionts reveals that peripheral T cells migrate rapidly to the Peyer's patches and lamina propria, but not to the intestinal epithelium. Taken together, these results demonstrate that the intestinal epithelium is a thymus-independent site of T lymphopoiesis, where selection of the T cell repertoire involves the deletion of potentially self-reactive cells in situ. Moreover, the appearance of donor-derived, phenotypically mature T cells, exclusively in the lamina propria of athymic radiation chimeras, suggests that mature IEL expressing functional TCR- $\alpha/\beta$  migrate to this site.

Intestinal intraepithelial lymphocytes (IEL)<sup>1</sup> belong to the T cell lineage, and in adult mice, preferentially express TCR- $\alpha/\beta$  (1, 2). The function and fate of IEL is obscure, and moreover, the origin of the different IEL subsets expressing TCR- $\alpha/\beta$  remains controversial (3–6). The phenotypic heterogeneity of TCR- $\alpha/\beta$ <sup>+</sup> IEL is comparable with that of thymocytes (1, 2). Two subsets expressing CD8 $\alpha$  alone or in combination with CD4 are unique to the intestinal epithelium (4, 7). Two additional subsets are phenotypically identical to mature peripheral T cells expressing either CD4 or

CD8 $\alpha/\beta$  (4, 6). Recent results demonstrate that the CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup> IEL subset contains high frequencies of potentially self-reactive cells. Specifically, in mouse strains expressing I-E and Mls1<sup>a</sup>, the frequency of CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup> IEL expressing V $\beta$ 11 and V $\beta$ 6, respectively, is 10-fold higher than that found in the periphery of these animals (8). It remains unclear, however, whether these potentially autoreactive cells express functional TCR- $\alpha/\beta$  signaling complexes. In contrast, in these same mouse strains, the CD4<sup>+</sup>8<sup>-</sup> and CD4<sup>-</sup>8 $\alpha$ <sup>+</sup> $\beta$ <sup>+</sup> IEL subsets contain low frequencies of V $\beta$ 11<sup>+</sup> and V $\beta$ 6<sup>+</sup> cells, comparable with those found in the peripheral T cell pool (8). These phenotypic correlations led to the hypothesis that IEL phenotypically identical to peripheral T cells are thymus derived, while CD4<sup>-</sup>8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup> IEL develop in situ and are subject to distinct selection pressures (8).

<sup>1</sup> Abbreviation used in this paper: IEL, intraepithelial lymphocytes.

In this study we directly addressed questions concerning the origin, fate, and function of TCR- $\alpha/\beta^+$  IEL. We demonstrate that CD4 $^+$ 8 $^-$  and CD4 $^-$ 8 $\alpha^+$  $\beta^+$  IEL proliferate in response to anti-TCR- $\alpha/\beta$  in vitro. In contrast, CD4 $^+$ 8 $^+$  IEL and CD8 $\alpha^+$  $\beta^-$  IEL, shown here to contain high frequencies of potentially autoreactive cells, do not. Using radiation chimeras in which adult thymectomized recipients are reconstituted with fetal liver, we demonstrate that all IEL subsets are generated in the absence of a thymus. Moreover, deletion of autoreactive T cells in those IEL subsets responsive to anti-TCR- $\alpha/\beta$  is determined by the genotype of the host. Importantly, the number of IEL generated in athymic recipients is equivalent to that found in conventional animals. Neither the spleen nor Peyer's patches of these recipients contain T cells, however, the lamina propria was found to contain phenotypically mature CD4 $^+$  and CD8 $\alpha^+$  $\beta^+$  T cells of donor origin. In addition, using Ly5 congenic parabionts, we demonstrate that circulating T cells rapidly access the spleen, Peyer's patches, and the lamina propria, but not the intestinal epithelium. Thus, the results demonstrate that the intestinal epithelium is a thymus-independent site of T cell development in which deletion of potentially autoreactive cells occurs, and indicate that circulation of mature IEL is likely restricted to the lamina propria.

## Materials and Methods

**Mice.** C57BL/6, BALB/c, DBA/1, and (C57BL/6  $\times$  BALB/c)F $_1$  mice were purchased from Charles River (St. Constant, Quebec). (C57BL/6  $\times$  DBA/1)F $_1$  and Ly5.1 congenic C57BL/6 mice were bred in our animal facilities.

**Isolation of IEL.** The method was derived from that of Goodman and Lefrançois (9). Briefly, the small intestine was flushed with 0.15 M NaCl, opened longitudinally, cut into 5-mm pieces, and incubated for 20 min, with stirring, at 37°C in 100 ml of Ca $^{2+}$ - and Mg $^{2+}$ -free HBSS supplemented with 4 mM NaHCO $_3$  and 2 mM dithiothreitol. The supernatant containing IEL and epithelial cells was collected and the above procedure repeated. After washing in HBSS + 5% FCS, rescued cells were fractionated by discontinuous Percoll gradient centrifugation. Cells were resuspended in Percoll ( $\rho = 1.062$ ) and underlaid with Percoll ( $\rho = 1.109$ ). After centrifugation at 900 g for 10 min, the  $\rho = 1.109/1.062$  interface was harvested, washed, and then incubated for 60 min at 37°C in HBSS + 5% FCS. This was followed by a second discontinuous Percoll gradient, as described above. Cells at the  $\rho = 1.109/1.062$  interface contained >95% CD3 $\epsilon^+$  and <3% membrane immunoglobulin-positive (mlg $^+$ ) lymphocytes. In some experiments, CD4 $^+$  IEL were depleted by incubation of total IEL with anti-CD4 mAb (RL1724H; reference 10) and rabbit complement (Cedarlane, Mississauga, Ontario) for 60 min at 37°C. Dead cells were removed by discontinuous Percoll gradient centrifugation, as described above. Less than 1% of rescued viable cells expressed CD4.

**Isolation of Peyer's Patch Lymphocytes.** Peyer's patches were excised and incubated for 30 min, with stirring, at room temperature in 10 ml of Ca $^{2+}$ - and Mg $^{2+}$ -free HBSS supplemented with 4 mM NaHCO $_3$ , 2 mM dithiothreitol, and 5 mM EDTA. This procedure was repeated twice to assure the removal of overlying intestinal epithelium. Peyer's patches were then rinsed twice in RPMI 1640 + 5% FCS. This was followed by a 30-min incubation with stirring at 37°C in 5 ml of RPMI 1640 + 5% FCS containing 90 U/ml of collagenase (Gibco Laboratories, Burlington, Ontario).

25 ml of cold RPMI 1640 + 5% FCS was added to the supernatant to stop the digestion. Peyer's patches were rescued and gently disrupted by teasing through a stainless steel mesh. The resulting cell suspension was washed three times in PBS + 5% FCS.

**Isolation of Lamina Propria Lymphocytes.** After the isolation of IEL, the resulting pieces of gut were incubated for 30 min at room temperature, with stirring, in 25 ml of Ca $^{2+}$ - and Mg $^{2+}$ -free HBSS supplemented with 4 mM NaHCO $_3$  and 5 mM EDTA, and the supernatant was discarded. This procedure was repeated twice. Pieces of gut mucosa were washed twice in HBSS and then incubated for 20 min at room temperature, with stirring, in RPMI 1640 + 5% FCS. The resulting supernatant contained neither epithelial cells nor lymphocytes. The pieces of gut mucosa were then incubated for 30 min at 37°C with stirring in 15 ml of RPMI 1640 + 5% FCS containing 90 U/ml of collagenase, and the supernatant was collected. This procedure was repeated twice. The resulting cell suspensions were pooled and washed three times in PBS + 5% FCS. Cells were then resuspended in Percoll ( $\rho = 1.062$ ), and underlaid with Percoll ( $\rho = 1.109$ ). After centrifugation at 900 g for 10 min, the  $\rho = 1.109/1.062$  interface was harvested and washed twice in PBS + 5% FCS.

**mAbs, Three-Color Immunofluorescence, and FACS $^{\circ}$  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; 11), anti-TCR- $\alpha/\beta$  (H57.597; 12), anti-TCR- $\gamma/\delta$  (GL3; 13), anti-CD8 $\alpha$  (53.6.7.2; 14), anti-CD4 (GK1.5; 15), anti-CD8 $\beta$  (53.5.8; 16), anti-V $\beta$ 6 (44.22.1; 17), anti-V $\beta$ 11 (RR.3.15; 18), anti-mIg (187; 19), 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, NY). PE-conjugated GK1.5 and 53.6.7.2 were purchased from Becton Dickinson & Co. (San José, CA), and Cedarlane, respectively. Streptavidin PE/Texas red Tandem $^{\text{TM}}$  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 $^{\text{TM}}$ . 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.** IEL from 20-wk-old BALB/c mice were incubated with biotinylated anti-CD8 $\beta$ , then with Streptavidin-PE/Texas red Tandem $^{\text{TM}}$ . This was followed by the simultaneous addition of PE-conjugated anti-CD4 and FITC-labeled anti-CD8 $\alpha$ . CD4 $^+$ 8 $\alpha^-$  $\beta^-$  and CD4 $^+$ 8 $\alpha^+$  $\beta^-$  IEL were then sorted using a FACStar $^{\circ}$  (Becton Dickinson & Co.). To purify CD4 $^-$ 8 $\alpha^+$  $\beta^-$  and CD4 $^-$ 8 $\alpha^+$  $\beta^+$  IEL subsets, CD4-expressing IEL were depleted, as described above. The resulting cell suspension was stained first with biotinylated anti-CD8 $\beta$ , followed by streptavidin-PE/Texas red Tandem $^{\text{TM}}$ . PE-conjugated anti-CD8 $\alpha$ , FITC-labeled anti-TCR- $\gamma/\delta$ , and FITC-labeled rat anti-mouse Ig were then added simultaneously. The two FITC-labeled mAbs were used to gate out B lymphocytes and TCR- $\gamma/\delta^+$  IEL. TCR- $\alpha/\beta^+$  IEL expressing CD8 $\alpha$  alone or in combination with CD8 $\beta$  were then sorted using a FACStar $^{\circ}$ . Sorted cells were >99% pure. Subsequent to washing,  $5 \times 10^4$  sorted IEL were cocultured with  $5 \times 10^5$  irradiated (2,000 rad) syngeneic splenocytes in 0.2 ml of serum-free IMDM, as previously described (20). Anti-TCR- $\alpha/\beta$  mAb was added to final concentrations ranging from 0.01 to 10  $\mu$ g/ml, and Con A to a final concentration of 1  $\mu$ g/ml. Cultures were pulsed at 40 h with 1  $\mu$ Ci of [ $^3$ H]thymidine, harvested 6 h later, and the amount of thymidine incorpo-

rated was assessed using liquid scintillation spectroscopy. The kinetics of induction of DNA synthesis was analyzed at 20, 40, 60, and 80 h, for each IEL subset, at each concentration of stimulating mAb. 40-h cultures gave optimal levels of thymidine incorporation for all subsets.

**Preparation and Analysis of Hematopoietic Chimeras.** 8–10-wk-old (C57BL/6 × BALB/c)<sub>F1</sub> and (C57BL/6 × DBA/1)<sub>F1</sub> mice were thymectomized. 2 wk later, these animals were exposed to 1,300 rad of  $\gamma$  radiation delivered from a <sup>137</sup>Cs source (Gamma cell 40; Atomic Energy of Canada Ltd., Ottawa, Ontario). Within 24 h, irradiated recipients were injected with 2–4 × 10<sup>6</sup> day 12 fetal liver cells of C57BL/6 origin. 8–15 wk after reconstitution, suspensions of lymphocytes from the spleen, Peyer's patches, lamina propria, and intestinal epithelium were analyzed on a FACScan<sup>®</sup> using three-color immunofluorescence involving mAbs specific for mIg, TCR- $\alpha/\beta$ , and H-2D<sup>b</sup>, in combination with either H-2D<sup>d</sup> or H-2D<sup>a</sup> (Cedarlane) depending on the strain combination. T lymphocytes of donor origin represented <1% of the splenocyte population. Lymphocytes of the intestinal epithelium, Peyer's patches, and lamina propria contained variable proportions of residual recipient cells ranging from 5 to 20%, which were depleted before phenotyping using anti-H-2<sup>d</sup> (a gift from Michel Seman, University of Paris, France) or anti-H-2<sup>a</sup> antiserum and guinea pig complement (Cedarlane). Anti-H-2<sup>a</sup> antiserum was produced in our laboratory by immunizing C57BL/6 mice with DBA/1 splenocytes. In some experiments, CD4<sup>+</sup> 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.

**Parabiotic Mice.** Weight-matched 6–8-wk-old Ly5 congenic C57BL/6 female mice were joined laterally under general anesthesia, as previously described (21). Briefly, the mice were joined by suturing the peritoneal and abdominal wall muscles, as well as the connective tissue along the thorax. In addition, the scapulae were joined to insure that animals would not stress the soft tissue joining sites. The skin was closed from behind the ears to the femora. Parabionts were killed at various intervals after surgery. Lymphocytes from the spleen, Peyer's patches, lamina propria, and intestinal epithe-

lium of each parabiont were isolated and incubated with biotinylated mAbs specific for either of the two allelic forms of Ly5. This was followed by the addition of Streptavidin-PE/Texas red Tandem<sup>™</sup>. Various combinations of PE-labeled and FITC-labeled mAbs were then added simultaneously. The phenotype of lymphocytes expressing Ly5.1 or Ly5.2 in each parabiont was then determined flow cytometrically.

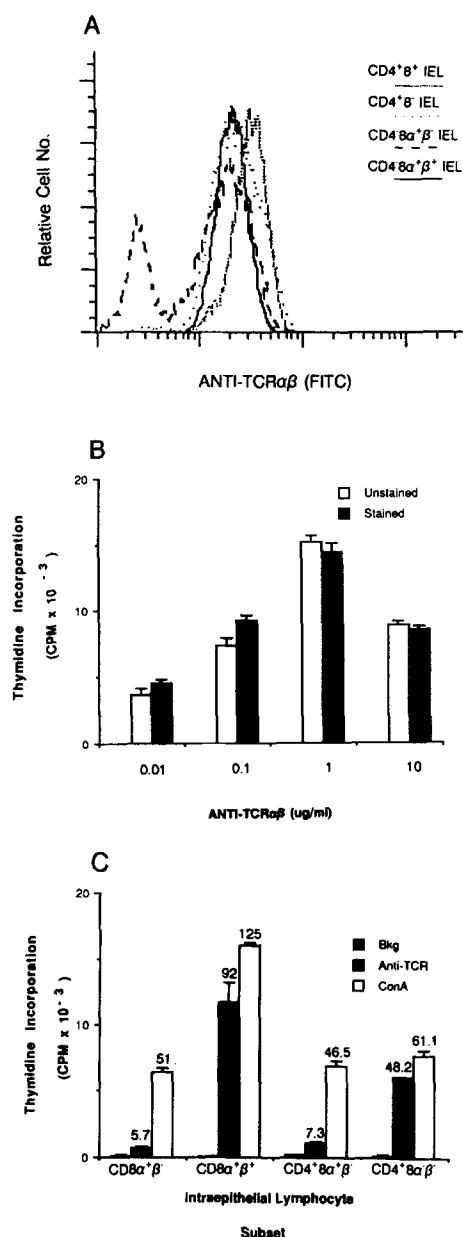
## Results

**High Frequencies of Potentially Autoreactive Cells in IEL Subsets Expressing Nonfunctional TCR- $\alpha/\beta$ .** We have analyzed the frequencies of V $\beta$ 11- and V $\beta$ 6-expressing cells within four IEL subsets obtained from various I-E- and Mls1<sup>a</sup>-expressing mouse strains. It has recently been reported that the CD8 $\alpha^+\beta^-$  IEL subset contains high frequencies of potentially self-reactive T cells (8). As shown in Table 1, we confirm and extend this observation for V $\beta$ 11-expressing cells to the double-positive, CD4<sup>+</sup>8 $\alpha^+\beta^-$  IEL subset. The frequency of V $\beta$ 11 expression is comparable and high among the four IEL subsets obtained from I-E<sup>-</sup>, B6 mice. In contrast, the CD4<sup>+</sup>8 $\alpha^+\beta^-$  and CD8 $\alpha^+\beta^-$  IEL subsets obtained from I-E-expressing BALB/c and CB6F1 mice contain 3–10-fold higher frequencies of V $\beta$ 11<sup>+</sup> cells than those observed in the CD4<sup>+</sup> and CD8 $\alpha^+\beta^+$  subsets from these animals (Table 1). The comparison of V $\beta$ 6 expression within various IEL subsets of Mls1<sup>a</sup> and Mls1<sup>b</sup> strains gave similar results. Thus, we observed high frequencies of V $\beta$ 6<sup>+</sup> IEL within the four IEL subsets obtained from B6 (Mls1<sup>b</sup>) mice. In contrast, in both Mls1<sup>a</sup>-expressing strains analyzed, DBA/1 and D1B6F1, the frequency of V $\beta$ 6-expressing cells among the CD8 $\alpha^+\beta^-$  IEL was 10-fold higher than that observed within the other three IEL subsets (Table 1). In contrast to results obtained for V $\beta$ 11, the frequency of V $\beta$ 6<sup>+</sup>, CD4<sup>+</sup>8 $\alpha^+$  IEL from Mls1<sup>a</sup> strains was not always higher than that observed in

**Table 1.** Distribution of V $\beta$ 11- and V $\beta$ 6-expressing Cells among IEL Subjects

Strain	Genotype	Frequency of V $\beta$ 11 <sup>+</sup> IEL			
		CD4 <sup>+</sup>	CD4 <sup>+</sup> 8 $\alpha^+$	CD8 $\alpha^+\beta^-$	CD8 $\alpha^+\beta^+$
C57BL/6	I-E <sup>-</sup>	5.4 ± 1.1	5.8 ± 1.2	7.6 ± 2.4	5.5 ± 0.5
BALB/c	I-E <sup>+</sup>	1.1 ± 0.3	2.9 ± 0.7	9.3 ± 1.2	0.9 ± 0.2
CB6F1	I-E <sup>+</sup>	0.9 ± 0.1	4.1 ± 0.6	10.1 ± 2.2	1.2 ± 0.1
		Frequency of V $\beta$ 6 <sup>+</sup> IEL			
		CD4 <sup>+</sup>	CD4 <sup>+</sup> 8 $\alpha^+$	CD8 $\alpha^+\beta^-$	CD8 $\alpha^+\beta^+$
C57BL/6	Mls1 <sup>b</sup>	4.1 ± 1.1	4.2 ± 0.8	6.5 ± 1.6	5.5 ± 1.8
DBA/1	Mls1 <sup>a</sup>	0.5 ± 0.03	0.6 ± 0.2	4.1 ± 0.4	0.5 ± 0.2
D1B6F1	Mls1 <sup>a</sup>	0.3 ± 0.1	1.2 ± 0.7	12.9 ± 1.9	1.4 ± 0.7

The numbers represent the mean frequency of V $\beta$ 6- and V $\beta$ 11-expressing cells ± SEM of three to seven experiments, in each of which IEL from two to five adult mice were pooled.



**Figure 1.** Differential responsiveness of IEL subsets to anti-TCR- $\alpha/\beta$ . (A) Staining profiles of CD4<sup>+</sup>8 $\alpha$ <sup>-</sup>, CD4<sup>+</sup>8 $\alpha$ <sup>+</sup>, CD4<sup>-</sup>8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup>, and CD4<sup>-</sup>8 $\alpha$ <sup>+</sup> $\beta$ <sup>+</sup> IEL subsets with anti-TCR- $\alpha/\beta$ . The negative peak in the TCR- $\alpha/\beta$  profile of CD4<sup>-</sup>8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup> IEL represents the frequency of cells expressing TCR- $\gamma/\delta$  in this subset. IEL were isolated and stained, as described in Materials and Methods. (B) Comparison of DNA synthesis induced by anti-TCR- $\alpha/\beta$  in unfractionated IEL, either unstained or preincubated with a mixture of fluorochrome-conjugated mAbs, as described in Materials and Methods.  $5 \times 10^4$  IEL were cocultured with  $5 \times 10^5$  irradiated syngeneic splenocytes for 40 h in IMDM containing the indicated concentration of anti-TCR- $\alpha/\beta$ . Cultures were then pulsed with [<sup>3</sup>H]thymidine and harvested 6 h later. Bars represent the mean level of thymidine incorporation from four to six individual experiments; one SEM is indicated. Thymidine incorporation in the absence of stimulation was similar in stained and unstained IEL:  $221 \pm 23$  and  $178 \pm 19$  cpm, respectively. (C) Anti-TCR- $\alpha/\beta$ - and Con A-induced DNA synthesis by purified TCR- $\alpha/\beta$ <sup>+</sup> IEL subsets. The four TCR- $\alpha/\beta$ <sup>+</sup> IEL subsets, as defined by the differential expression of CD4, CD8 $\alpha$ , and CD8 $\beta$ , were purified from total IEL by FACS<sup>®</sup>, as described in Materials and Methods. Sorted cells

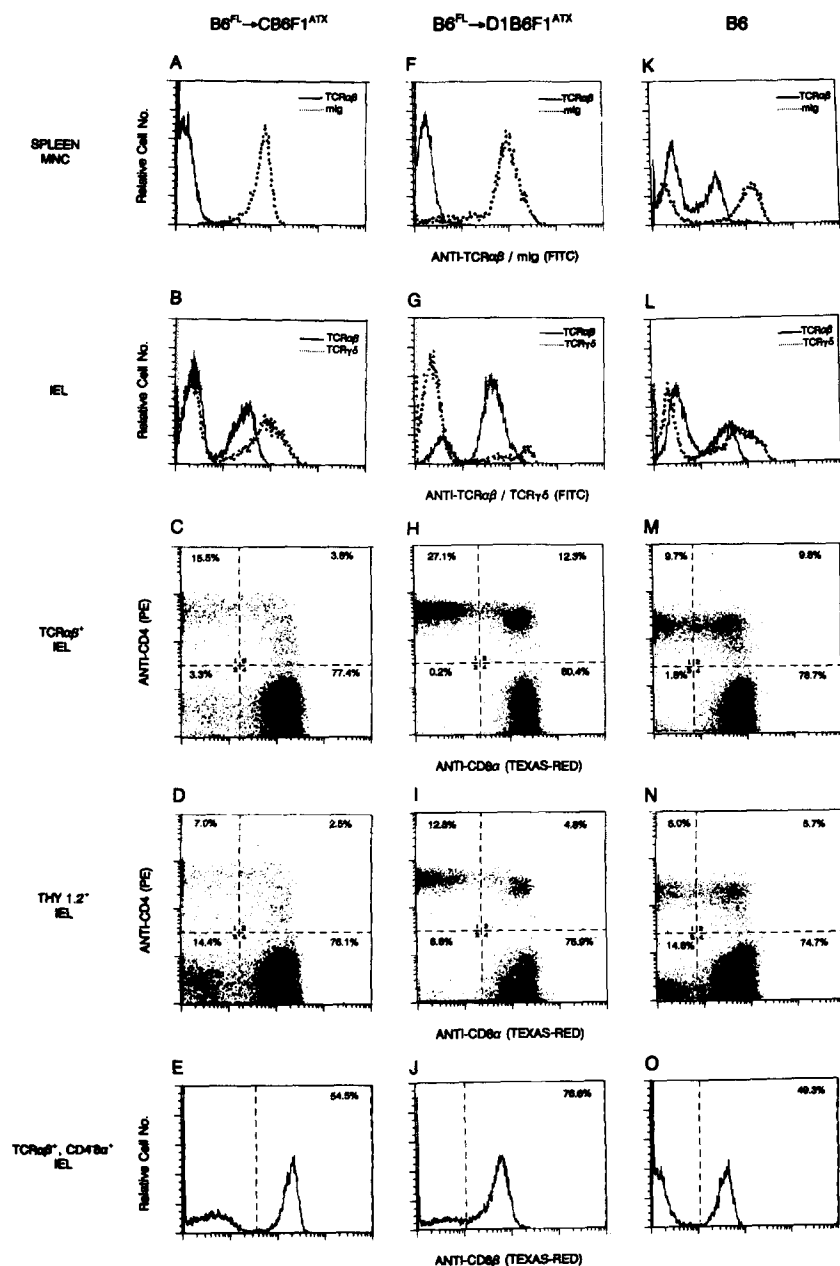
CD4<sup>+</sup> and CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>+</sup> subsets (Table 1). It is noteworthy that we have analyzed V $\beta$ 6 expression in I-E<sup>-</sup> strains. It has been demonstrated that deletion of V $\beta$ 6<sup>+</sup> T cells in Mls1<sup>a</sup> strains requires the expression of I-E (22). In agreement, we observe that the frequencies of V $\beta$ 6-expressing splenocytes in DBA/1 animals is consistently higher than those observed within IEL subsets of the same phenotype. Specifically, CD4<sup>+</sup> and CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>+</sup> DBA/1 splenocytes contain  $2.4 \pm 0.4\%$  and  $4.2 \pm 1.1\%$  V $\beta$ 6<sup>+</sup> cells, respectively (compare with Table 1).

In summary, the frequency of V $\beta$ 11<sup>+</sup> and V $\beta$ 6<sup>+</sup> IEL within the phenotypically mature subsets of I-E<sup>+</sup> and Mls1<sup>a</sup> animals, respectively, is low, consistent with the deletion of self-reactive cells during T cell development. However, the high frequencies of V $\beta$ 11<sup>+</sup> and V $\beta$ 6<sup>+</sup> IEL within the CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup> subset of healthy animals expressing I-E and Mls1<sup>a</sup>, respectively, suggest that these cells are either non-functional, or subject to distinct selection mechanisms that render them non-self-reactive.

To assess the functional status of IEL, we determined whether each of the four subsets would respond, in vitro, to stimulation with mAb specific for TCR- $\alpha/\beta$  (H57.597). As illustrated in Fig. 1 A, the level of TCR- $\alpha/\beta$  expression is comparable among the four IEL subsets, and identical to that of mature splenic T cells (Fig. 2 K). Anti-TCR- $\alpha/\beta$  induces DNA synthesis by unfractionated IEL over a broad range of concentrations (Fig. 1 B). Importantly, preincubation of unfractionated IEL with a mixture of fluorochrome-conjugated mAbs specific for CD4, CD8 $\alpha$ , and CD8 $\beta$  does not interfere with anti-TCR- $\alpha/\beta$ -induced DNA synthesis (Fig. 1 B). Using these mAbs, pure populations of each of the four IEL subsets were isolated by FACS<sup>®</sup>. Purified IEL subsets were then stimulated in vitro with either the mitogen Con A or the optimal concentration of anti-TCR- $\alpha/\beta$ . As shown in Fig. 1 C, the levels of thymidine incorporation induced by Con A are comparable among the four IEL subsets, with indices of stimulation ranging from 46- to 125-fold over background. In striking contrast, levels of thymidine incorporation induced by anti-TCR- $\alpha/\beta$  in the CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup> and CD4<sup>+</sup>8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup> subsets are 10–20-fold lower than those observed in the CD4<sup>+</sup> and CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>+</sup> subsets (Fig. 1 C).

These results indicate that IEL subsets can be divided based on the expression of functionally distinct TCR- $\alpha/\beta$  signaling complexes. Taken together with results indicating the differential expression of V $\beta$ 11 and V $\beta$ 6 within IEL subsets of I-E<sup>+</sup> and Mls1<sup>a</sup> strains, it becomes apparent that high frequencies of potentially self-reactive T cells are found predominantly in the two IEL subsets (CD4<sup>+</sup>8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup> and CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup>), which are refractory to anti-TCR- $\alpha/\beta$ -mediated

were >99% pure.  $5 \times 10^4$  sorted IEL were cocultured with  $5 \times 10^5$  irradiated syngeneic splenocytes in the presence of either 1  $\mu$ g/ml anti-TCR- $\alpha/\beta$  or 1  $\mu$ g/ml Con A. Bars represent the mean level of thymidine incorporation  $\pm$  SEM from a pool of 5–13 individual experiments. The mean stimulation index is indicated above each bar.



**Figure 2.** Phenotypic analysis of donor-derived splenocytes and IEL in athymic radiation chimeras. Cells obtained from chimeras 12 wk after reconstitution were isolated, depleted of residual host cells, and stained, as described in Materials and Methods. Cells from 12-wk-old B6 mice are also shown. (A, F, and K) Staining profiles of total splenocytes with anti-TCR- $\alpha/\beta$  and anti-mIg. (B, G, and L) Staining profiles of total IEL with anti-TCR- $\alpha/\beta$  and anti-TCR- $\gamma/\delta$ . (C, H, and M) Expression of CD4 and CD8 $\alpha$  by TCR- $\alpha/\beta$ <sup>+</sup> IEL. (D, I, and N) Expression of CD4 and CD8 $\alpha$  by Thy-1.2<sup>+</sup> IEL. Some CD4<sup>-</sup>8 $\alpha$ <sup>-</sup> and CD4<sup>-</sup>8 $\alpha$ <sup>+</sup> TCR- $\gamma/\delta$ <sup>+</sup> IEL express Thy-1.2 and account for the changes in the proportions of IEL subsets defined by the differential expression of CD4 and CD8 $\alpha$ . (E, J, and O) Staining profiles of CD4<sup>-</sup>8 $\alpha$ <sup>+</sup>, TCR- $\alpha/\beta$ <sup>+</sup> IEL with anti-CD8 $\beta$ .

stimulation. Conversely, potentially self-reactive IEL are depleted from the two phenotypically mature IEL subsets (CD4<sup>+</sup> and CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>+</sup>), which are responsive to anti-TCR- $\alpha/\beta$ .

**Thymus-independent Development and Negative Selection of TCR- $\alpha/\beta$ <sup>+</sup> IEL.** While the above results are consistent with the deletion of potentially autoreactive T cells from the functional IEL pool in situ, this and other studies (4, 5, 8) characterizing the phenotype and V $\beta$  repertoire of TCR- $\alpha/\beta$ <sup>+</sup> IEL are complicated by the presence of a full complement of peripheral T cells. In these circumstances, one cannot exclude the possibility that the phenotypically distinct IEL subsets observed reflect the differential homing of peripheral

T cells to the gut. As previously suggested, perhaps the phenotypically mature CD4<sup>+</sup> and CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>+</sup> IEL subsets are thymus-derived migrants (8). To directly assess the T lymphopoietic potential of the intestinal epithelium, we prepared radiation chimeras using adult thymectomized recipients. Strain combinations were chosen that would permit both discrimination between T cells of donor and host origin, and establish whether potentially self-reactive TCR- $\alpha/\beta$ <sup>+</sup> T cells developing in the gut are subject to negative selection in situ.

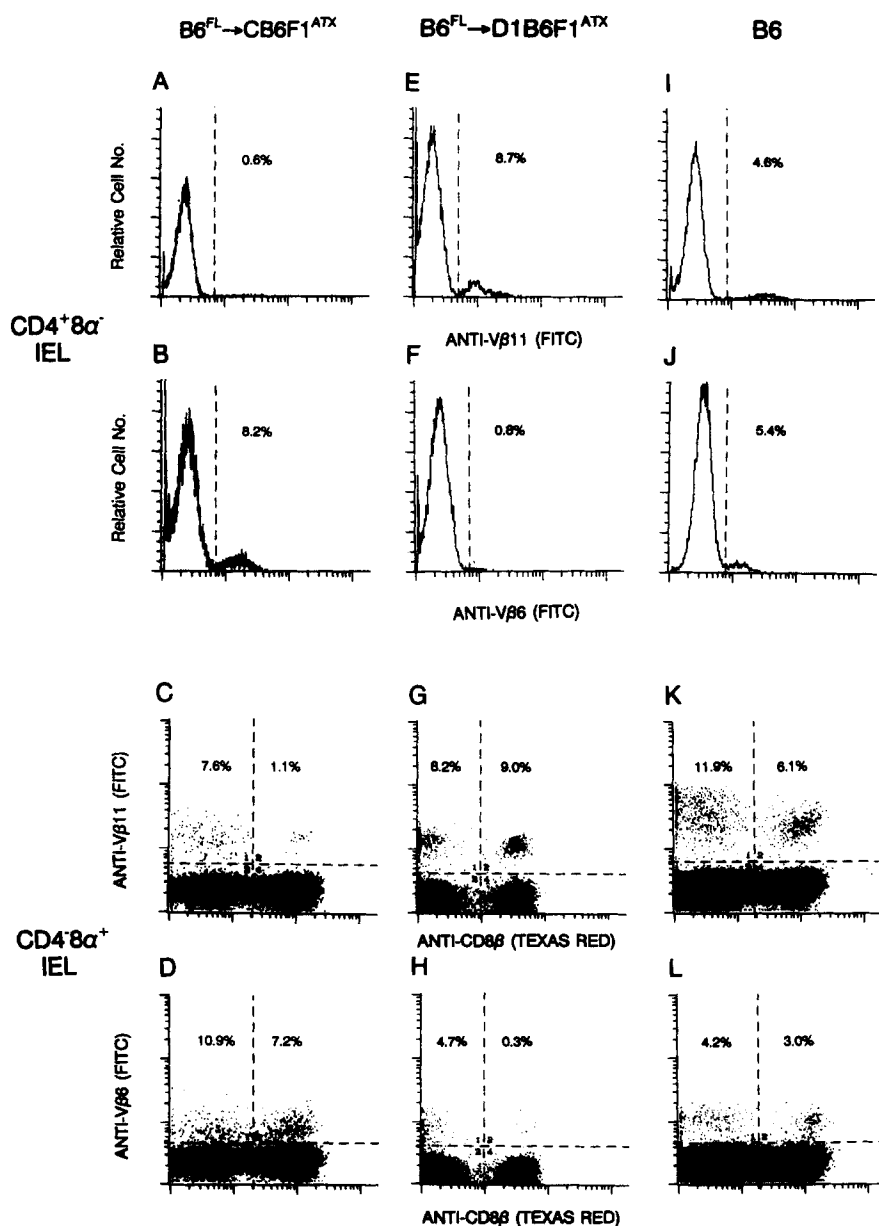
Two sets of chimeras were generated to allow for the independent analysis of V $\beta$ 6 and V $\beta$ 11 selection occurring within the intestinal epithelium. Lethally irradiated adult thymectomized CB6F<sub>1</sub> mice (H-2<sup>d/b</sup>, Mls1<sup>b</sup>, I-E<sup>+</sup>) were recon-

**Table 2.** Number of IEL and Lamina Propria Mononuclear Cells Isolated from Normal Mice and Radiation Chimeras

Donor	Lamina propria	Intestinal epithelium
		$\times 10^6$
B6	$8.2 \pm 1.1$	$16.2 \pm 3.6$
B6 <sup>FL</sup> →CB6F1 <sup>ATX</sup>	$6.1 \pm 1.4$	$22.2 \pm 4.0$
B6 <sup>FL</sup> →D1B6F1 <sup>ATX</sup>	$8.4 \pm 1.6$	$22.9 \pm 4.2$

Lymphocytes isolated from the radiation chimeras were stained with antibodies to H-2<sup>b</sup> and either H-2<sup>a</sup> or H-2<sup>d</sup>. The proportion of donor cells expressing H-2<sup>b</sup> exclusively was determined flow cytometrically. The yield of donor lymphocytes was calculated by multiplying the total number of cells isolated by the proportion of donor cells.

stituted with  $2-4 \times 10^6$  day 12 fetal liver cells from B6 mice (H-2<sup>b</sup>, Mls1<sup>b</sup>, I-E<sup>-</sup>). Alternatively, thymectomized D1B6F<sub>1</sub> mice (H-2<sup>a/b</sup>, Mls1<sup>a</sup>, I-E<sup>-</sup>) were reconstituted with  $2-4 \times 10^6$  day 12 fetal liver cells from B6 mice. 8-15 wk after reconstitution, >90% of the splenocytes from these chimeras expressed H-2<sup>b</sup> exclusively. Of these cells, 80-90% expressed membrane Ig (Fig. 2, A and F), <1% expressed TCR- $\alpha/\beta$  (Fig. 2, A and F), and <1% expressed Thy-1.2, CD4, or CD8 (not shown). As for the spleen, the majority (80-95%) of the IEL obtained from these chimeras expressed H-2<sup>b</sup>, exclusively. After depleting residual host IEL using anti-H-2<sup>d</sup> or anti-H-2<sup>a</sup> antiserum and complement, phenotypic analysis of donor-derived IEL was carried out using mAbs specific for TCR- $\alpha/\beta$ , TCR- $\gamma/\delta$ , CD4, CD8 $\alpha$ , CD8 $\beta$ , and Thy-1.2. In striking contrast to donor-derived splenocytes in these



**Figure 3.** Negative selection of V $\beta$ 6<sup>+</sup> and V $\beta$ 11<sup>+</sup> IEL in athymic radiation chimeras. IEL were isolated and stained, as described in Materials and Methods. Before staining donor lymphocytes obtained from radiation chimeras, residual recipient cells were depleted with anti-H-2<sup>d</sup> or anti-H-2<sup>a</sup> and complement. All CD4<sup>+</sup>8 $\alpha$ <sup>+</sup> $\beta$ <sup>+</sup> IEL express TCR- $\alpha/\beta$ . CD4<sup>+</sup>8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup> IEL contain cells expressing TCR- $\alpha/\beta$  or TCR- $\gamma/\delta$ . The proportion of CD4<sup>+</sup>8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup> IEL expressing TCR- $\alpha/\beta$  was determined flow cytometrically. The frequency of cells expressing V $\beta$ 6 or V $\beta$ 11 in the CD4<sup>+</sup>8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup> subset was corrected for the proportion of TCR- $\alpha/\beta$ <sup>+</sup> cells. In the eight B6→CB6F1<sup>ATXFL</sup> chimeras that were analyzed, the frequencies of V $\beta$ 11<sup>+</sup> cells within the CD4<sup>+</sup>8 $\alpha$ <sup>-</sup>, CD4<sup>+</sup>8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup>, and CD4<sup>+</sup>8 $\alpha$ <sup>+</sup> $\beta$ <sup>+</sup> subsets were  $0.58 \pm 0.1\%$ ,  $7.7 \pm 1.9\%$ , and  $1.1 \pm 0.3\%$ , respectively. In the six B6→D1B6F1<sup>ATXFL</sup> chimeras that were analyzed, the frequencies of V $\beta$ 6<sup>+</sup> cells within the same IEL subsets were  $0.8 \pm 0.1\%$ ,  $10.1 \pm 2.5\%$ , and  $0.8 \pm 0.3\%$ , respectively.

chimeras, the frequency of donor-derived IEL expressing TCR- $\alpha/\beta$  and TCR- $\gamma/\delta$  was comparable with that observed in conventional 12-wk-old B6 mice, and ranged from 30 to 70% and 20 to 60%, respectively (compare Fig. 2, B, G, and L).

As illustrated in Fig. 2, all four TCR- $\alpha/\beta^+$  IEL subsets were observed within the intestinal epithelium of these chimeras. The proportion of CD4 $^+$ , CD4 $^+8\alpha^+$ , and CD4 $^-8\alpha^+$  TCR- $\alpha/\beta^+$  IEL was comparable with that found in conventional B6 mice (compare Fig. 2, C, H, and M). Moreover, a large proportion of TCR- $\alpha/\beta^+$  IEL coexpressed CD8 $\alpha$  and CD8 $\beta$  (Fig. 2, E, J, and O). Finally, the expression of Thy-1.2 within IEL subsets was indistinguishable from that observed in conventional animals (compare Fig. 2, D, I, and N). It is important to note that the absolute number of donor-derived (H-2 $^b$ ) IEL isolated from athymic recipients 8–15 wk after reconstitution was comparable with that obtained from age-matched conventional H-2 $^b$  animals (Table 2). These results demonstrate that normal numbers of all of the heretofore described IEL subsets (1) are generated in the absence of the thymus.

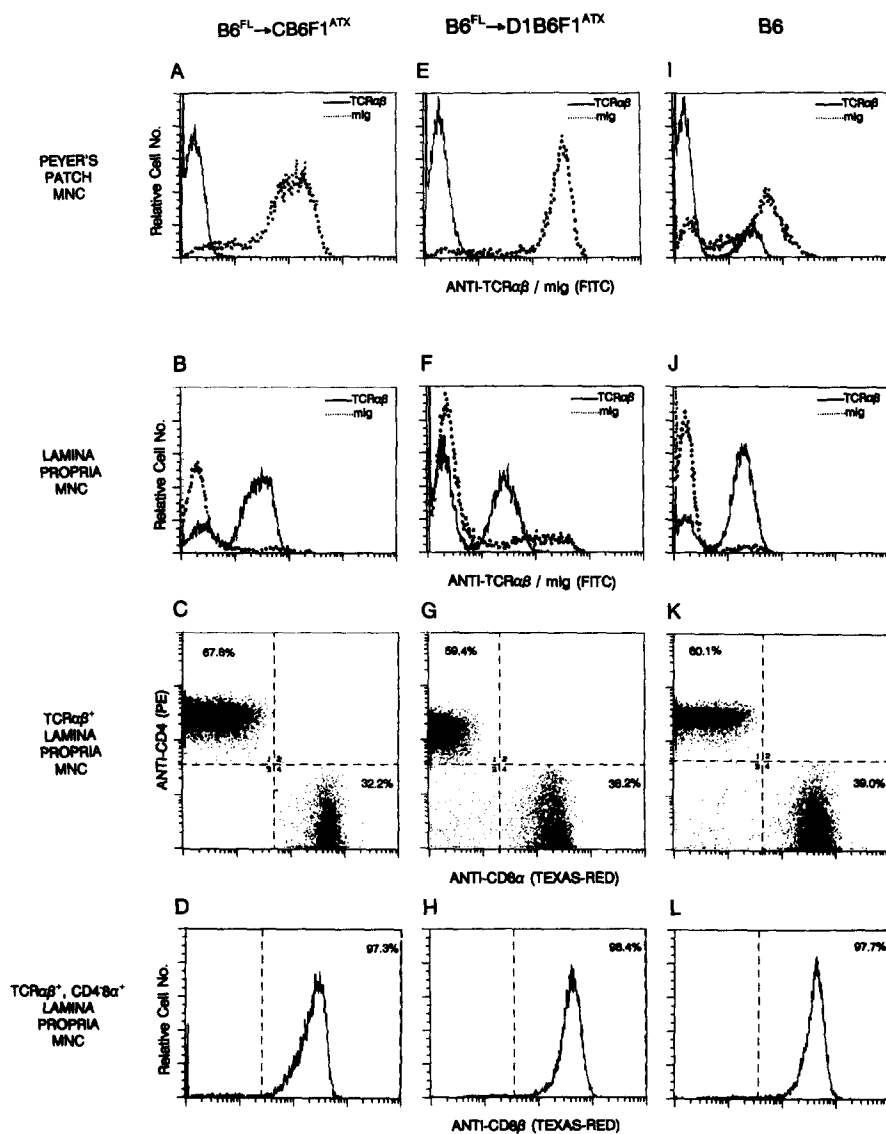
We then compared the frequencies of V $\beta$ 11- and V $\beta$ 6-expressing cells within the CD4 $^+$ , CD8 $\alpha^+\beta^-$ , and CD8 $\alpha^+\beta^+$  IEL subsets obtained from conventional B6 mice to those obtained from CB6F $_1^{ATX}$  and D1B6F $_1^{ATX}$  reconstituted with B6 hematopoietic precursors. As illustrated in Fig. 3, the frequency of V $\beta$ 6- and V $\beta$ 11-expressing cells within the phenotypically mature IEL subsets expressing functional TCR- $\alpha/\beta$  (CD4 $^+$  and CD8 $\alpha^+\beta^+$ ) is strictly dependent on the genotype of the recipient in which the precursor T cells develop. Among CD4 $^+$  IEL from conventional B6 mice (I-E $^-$ , Mls1 $^b$ ), 4.6% express V $\beta$ 11, and 5.4% express V $\beta$ 6 (Fig. 3, I and J). When B6-derived precursors mature in CB6F $_1^{ATX}$  recipients (I-E $^+$ , Mls1 $^b$ ), the frequency of V $\beta$ 11-expressing CD4 $^+$  IEL is reduced eightfold to 0.6% (Fig. 3 A), while the frequency of V $\beta$ 6 $^+$ , CD4 $^+$  IEL remains high (Fig. 3 B). Conversely, when B6-derived precursors mature in D1B6F $_1^{ATX}$  (I-E $^-$ , Mls1 $^a$ ), the frequency of V $\beta$ 6 $^+$ , CD4 $^+$  cells is reduced to 0.8% (Fig. 3 F), while the frequency of V $\beta$ 11 $^+$ , CD4 $^+$  cells remains high (Fig. 3 E). The same genotype-specific deletion pattern was observed within the CD8 $\alpha^+\beta^+$  IEL subset. Thus, the frequencies of V $\beta$ 11 $^+$  and V $\beta$ 6 $^+$ , CD8 $\alpha^+\beta^+$  IEL in thymectomized recipients expressing I-E and Mls1 $^a$ , respectively, were 6–10-fold lower than those observed in this subset obtained from conventional B6 mice (compare Fig. 3, C, D, G, and H with K and L).

In striking contrast, the frequencies of V $\beta$ 11 $^+$  and V $\beta$ 6 $^+$  IEL within the CD8 $\alpha^+\beta^-$  subset were not perturbed by the presence of their respective ligands in the host. The frequency of B6-derived V $\beta$ 11 $^+$  IEL in the CD8 $\alpha^+\beta^-$  subsets developing in I-E $^+$  and I-E $^-$  athymic recipients was comparable (Fig. 3, C and G) and similar to that observed in conventional B6 mice (Fig. 3 K). Similarly, the expression of Mls1 $^a$  in the host environment did not influence the frequency of V $\beta$ 6 $^+$ , CD8 $\alpha^+\beta^-$  IEL of B6 (Mls1 $^b$ ) origin (Fig. 3, D, H, and L). These results demonstrate that the expression of I-E and Mls1 $^a$  results in the deletion of cells expressing V $\beta$ 11 and V $\beta$ 6, respectively, from those IEL subsets that express functional antigen receptors, thus attesting to

their autoreactive potential. The persistence of potentially self-reactive cells within the CD8 $\alpha^+\beta^-$  IEL subset of healthy animals is consistent with the observation that these cells are refractory to anti-TCR- $\alpha/\beta$ -mediated stimulation (Fig. 1). Thus, while lineage relationships among IEL subsets remain to be established, the restricted expression of potentially autoreactive cells to IEL subsets expressing nonfunctional antigen receptors suggests that either they are at an earlier developmental stage or that they have distinct activation requirements that render them nondeleterious to the host.

*Normal Numbers of Phenotypically Mature Donor-derived T Cells Are Found in the Lamina Propria of Athymic Radiation Chimeras.* As predicted by the many studies involving adult thymectomized bone marrow or fetal liver radiation chimeras, peripheral lymphoid organs are virtually devoid of T cells derived from the inoculum (23). The striking observation in this and other studies (4) is that T cells developing in the gut of these animals do not migrate to the periphery. This may result from a disruption of T cell circulation patterns induced by experimental procedures. Alternatively, the intestinal epithelium may function as a primary T lymphopoietic organ that specifically services gut-related secondary lymphoid tissues. To directly address this question, we performed a phenotypic analysis of donor-derived lymphocytes obtained from the Peyer's patches and the lamina propria of chimeric animals.

As illustrated in Fig. 4 I, Peyer's patches of normal B6 mice contained 25% TCR- $\alpha/\beta^+$  cells and 65% mIg $^+$  lymphocytes. This was not the case for Peyer's patches isolated from ATX radiation chimeras which contained >90% mIg $^+$  cells and <2% TCR- $\alpha/\beta^+$  cells (compare Fig. 4 A, E, and I), indicating that this site is not readily accessed by donor-derived T cells. Strikingly, the lamina propria of these chimeric animals not only contained normal numbers of mononuclear cells (Table 2), but as illustrated in Fig. 4, their phenotype was identical to that observed in conventional mice. Thus, >60% of lamina propria lymphocytes derived from fetal liver inocula expressed TCR- $\alpha/\beta$  (Fig. 4, B, F, and J). In contrast to donor-derived IEL (Fig. 2), the frequency of TCR- $\gamma/\delta^+$  cells in the lamina propria was low, ranging from 1 to 5% (not shown). Moreover, the distribution of CD4 and CD8 among TCR- $\alpha/\beta^+$  T cells was identical to that found in conventional animals (Fig. 4, C, G, and K), and virtually all of the CD8 $\alpha^+$ , TCR- $\alpha/\beta^+$  T cells coexpressed CD8 $\beta$  (Fig. 4, D, H, and L). Thus, the phenotype of donor-derived T cells found in the lamina propria was distinct from that observed in the intestinal epithelium. In addition, the analysis of V $\beta$ 6 expression in populations of lamina propria T cells obtained from Mls1 $^a$ , I-E $^-$  recipients indicated that potentially autoreactive T cells had been deleted. Specifically, the frequencies of V $\beta$ 6 $^+$  cells in the CD4 $^+$  and CD8 $^+$  subsets obtained from the lamina propria of D1B6F $_1$  chimeras were 1.2  $\pm$  0.3% and 1.1  $\pm$  0.1%, respectively, while the frequencies of V $\beta$ 11 $^+$  cells in these subsets were 4.3  $\pm$  0.8% and 10.0  $\pm$  2.1%, respectively. Thus, in contrast to IEL rescued from these chimeras, lamina propria T cells are comprised exclusively of phenotypically mature T cell subsets containing low frequencies of potentially self-reactive T cells.



**Figure 4.** Phenotypic analysis of donor-derived lymphocytes isolated from the Peyer's patches and lamina propria of athymic radiation chimeras. At 12 wk after reconstitution, donor-derived cells were isolated and stained, as described in Materials and Methods. The phenotype of lamina propria and Peyer's patch lymphocytes obtained from conventional animals is shown for comparison. (*A*, *E*, and *I*) Staining profiles of total Peyer's patch lymphocytes with anti-TCR- $\alpha/\beta$  and mIg. (*B*, *F*, and *J*) Staining profiles of total lamina propria cells with anti-TCR- $\alpha/\beta$  and mIg. (*C*, *G*, and *K*) Staining of TCR- $\alpha/\beta^+$  lamina propria cells with anti-CD4 and anti-CD8 $\alpha$ . (*D*, *H*, and *L*) Staining of TCR- $\alpha/\beta^+$ , CD4-8 $\alpha^+$  lamina propria cells with anti-CD8 $\beta$ .

*Mature Thymus-derived T Lymphocytes Migrate Rapidly to the Spleen, Peyer's Patches, and Lamina Propria, but Not to the Intestinal Epithelium.* The experiments described up to this point have not addressed whether peripheral T cells have access to the gut. To determine the circulation pattern of peripheral T cells, we parabiosed 6–8-wk-old Ly5 (CD45) congenic B6 mice. The phenotype of lymphocytes from the spleen, Peyer's patches, lamina propria, and intestinal epithelium of each parabiont was then analyzed at various intervals after surgery using mAbs specific for Ly5.1, Ly5.2, TCR- $\alpha/\beta$ , TCR- $\gamma/\delta$ , CD4, CD8 $\alpha$ , CD8 $\beta$ , and mIg. As illustrated in Fig. 5, virtually no mixing of Ly5.1 $^+$  and Ly5.2 $^+$  lymphocytes was observed before 6 d after surgery. After 9 d, splenic and Peyer's patch lymphocytes had equilibrated, that is,  $\sim$ 50% of the cells within these organs in each parabiont expressed the inappropriate Ly5 allele (Fig. 5, *A*, *B*, *C*, and *D*). This level of mixing remained stable for the duration of the experiment. As in the spleen and Peyer's patches, mixing of

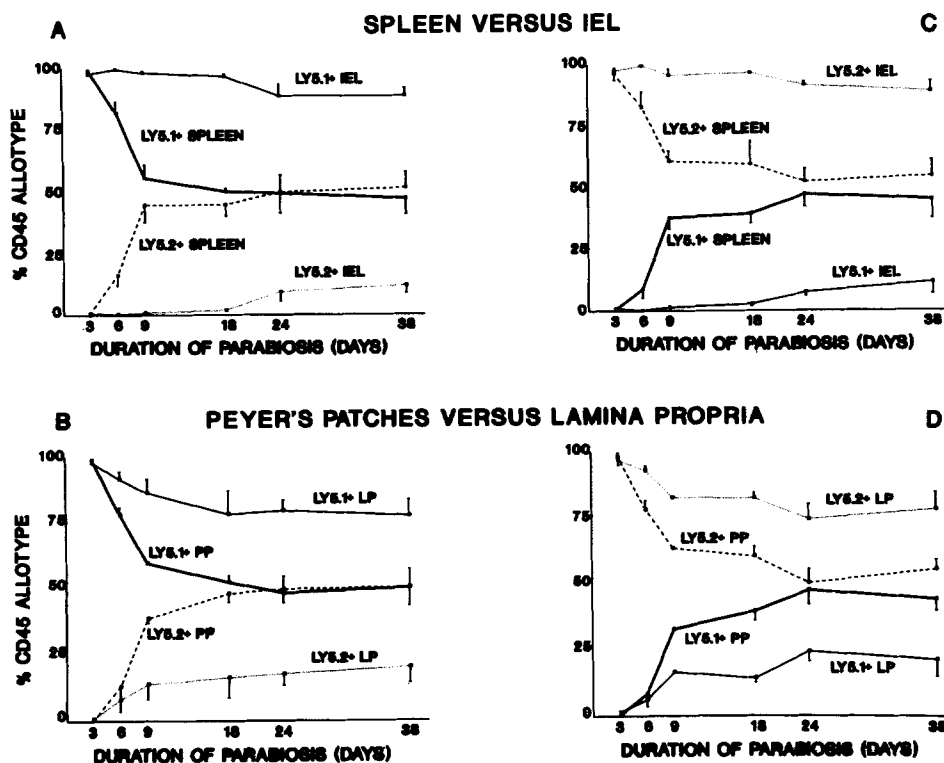
Ly5.1 $^+$  and Ly5.2 $^+$  lymphocytes in the lamina propria of each parabiont occurred with rapid kinetics, that is, by 9–18 d postsurgery, the maximum level of mixing was observed. However, in contrast to the equilibration observed in spleen and Peyer's patches, the proportion of cells expressing the inappropriate Ly5 phenotype in lamina propria reached a maximum of 25% and remained stable at this level for the duration of the experiment (Fig. 5, *B* and *D*).

The kinetics of mixing within the intestinal epithelium were strikingly different. At the time when lymphocytes in the spleen, Peyer's patches, and lamina propria had reached maximum levels of mixing, virtually no exchange was observed within the IEL compartment. Thus, at 9–18 d postsurgery,  $<$ 1% of the IEL in each parabiont expressed the inappropriate Ly5 allele (Fig. 5, *A* and *C*). The lack of mixing within this compartment extended to 3 wk postsurgery (Fig. 5, *A* and *C*). At this time the proportion of IEL expressing the inappropriate Ly5 allele began to increase, reaching 5–13%



## LY5.1 PARABIONT

## LY5.2 PARABIONT



**Figure 5.** Kinetics of mononuclear cell circulation in parabionts. The proportion of lymphocytes expressing Ly5.1 or Ly5.2 in the spleen, intestinal epithelium, Peyer's patches, and lamina propria of Ly5 congenic B6 parabionts was assessed at various times after joining. Cells were isolated and stained, as described in Materials and Methods. Shown are the mean values  $\pm$  SEM from three to five individual parabionts. (A and C) Changes observed in the spleen and the intestinal epithelium. (B and D) Changes observed in the lamina propria and Peyer's patches.

by day 38 (Fig. 5, A and C). These results demonstrate the differential circulation pattern of lymphocytes to these four anatomical sites, and minimally characterize the intestinal epithelium as a site of restricted access. Further insight was gained by phenotypic analysis of lymphocytes expressing the inappropriate Ly5 allele at each of these four anatomical sites.

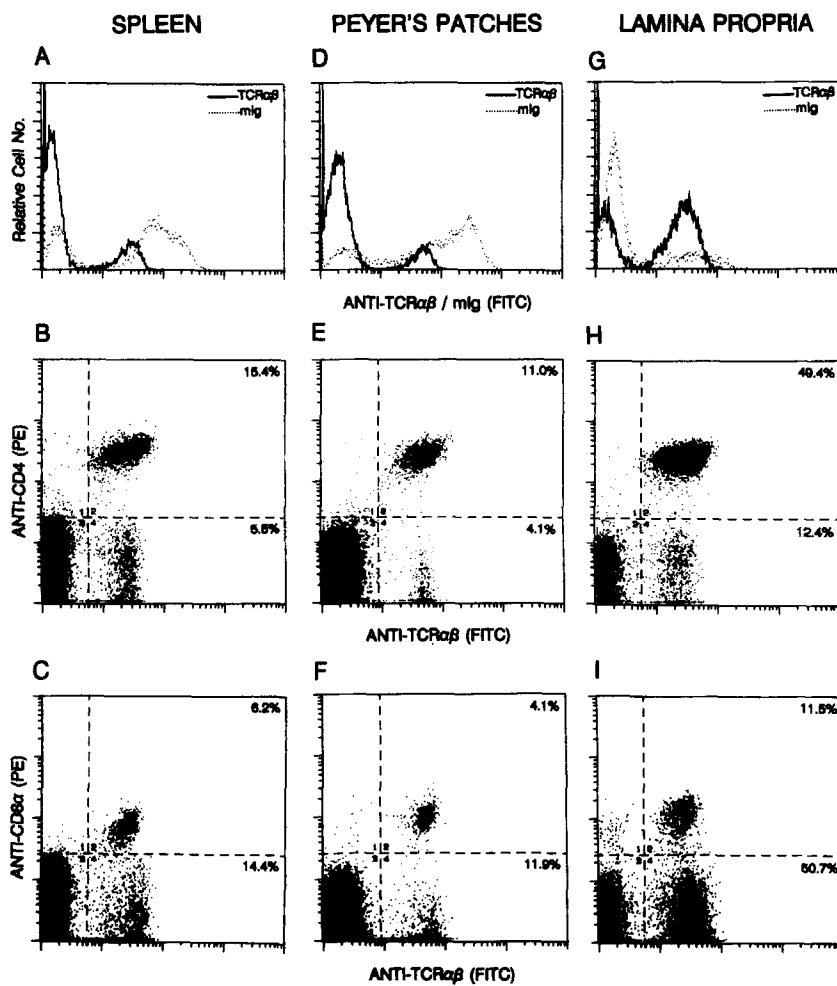
As illustrated in Fig. 6, Ly5.2<sup>+</sup> lymphocytes within the spleen, Peyer's patches, and lamina propria of an Ly5.1 parabiont, at 24 d postsurgery, are comprised of B lymphocytes and phenotypically mature TCR- $\alpha/\beta$ <sup>+</sup> T cells expressing either CD4 or CD8. The proportion of Ly5.2<sup>+</sup> B cells ranged from 25% in the lamina propria (Fig. 6 G) to 50% in the spleen (Fig. 6 A) and 70% in the Peyer's patches (Fig. 6 D). The proportion of Ly5.2<sup>+</sup> TCR- $\alpha/\beta$ <sup>+</sup> cells varied from 15–20% in the spleen and Peyer's patches (Fig. 6, A and D) to 62% in the lamina propria (Fig. 6 G). While the spleen and Peyer's patches contained <1% TCR- $\gamma/\delta$ <sup>+</sup> cells, the lamina propria contained 5% Ly5.2<sup>+</sup> TCR- $\gamma/\delta$ <sup>+</sup> cells (not shown). Importantly, the ratio of CD4/CD8 expression within the Ly5.2<sup>+</sup> TCR- $\alpha/\beta$ <sup>+</sup> subset in the spleen, Peyer's patches, and lamina propria was roughly equivalent ranging from 2.7 to 4.0 (Fig. 6). Also noteworthy is that all of the CD8 $\alpha$ <sup>+</sup>, TCR- $\alpha/\beta$ <sup>+</sup> cells within these three organs coexpressed CD8 $\beta$  (not shown).

The phenotype of the Ly5.2<sup>+</sup> IEL at 24 d postsurgery was markedly different. Less than 1% were mIg<sup>+</sup>, while CD3<sup>+</sup> lymphocytes were comprised of 67% TCR- $\gamma/\delta$ <sup>+</sup> cells and 32% TCR- $\alpha/\beta$ <sup>+</sup> cells (Fig. 7 A). The TCR- $\alpha/\beta$ <sup>+</sup>

cells were >80% CD8<sup>+</sup>, so that the CD4/CD8 ratio was roughly 0.1 (compare Fig. 7, C and F), similar to that observed in IEL from conventional animals (Fig. 2 M). Importantly, and in contrast to TCR- $\alpha/\beta$ <sup>+</sup>, CD8<sup>+</sup> cells observed in the spleen, Peyer's patches, and lamina propria, 30% of TCR- $\alpha/\beta$ <sup>+</sup>, CD8<sup>+</sup> IEL did not express CD8 $\beta$  (compare Fig. 7, C and D). Finally, and unique to Ly5.2<sup>+</sup> cells isolated from the intestinal epithelium, a significant proportion of CD4<sup>+</sup> cells coexpressed CD8 $\alpha$  (Fig. 7 E), a phenotype observed in IEL isolated from conventional animals (Fig. 2 M). Thus, in addition to being a site of restricted access, as determined by the kinetics of appearance of lymphocytes expressing the inappropriate Ly5 allele, the intestinal epithelium contains lymphocytes phenotypically distinct from those found in the other anatomical sites.

### Discussion

The results presented demonstrate that all of the TCR- $\alpha/\beta$ -expressing IEL subsets observed in conventional animals can develop from hematopoietic precursors present in fetal liver, in the absence of a thymus. Four main subsets are generated. The two single-positive subsets, CD4<sup>+</sup> and CD8 $\alpha\beta$ <sup>+</sup>, are phenotypically identical to mature peripheral T cells and express functional TCR complexes, in that DNA synthesis can be induced by mAbs specific for TCR- $\alpha/\beta$ . The deletion of V $\beta$ 6- and V $\beta$ 11-expressing cells from these two subsets correlates with the expression of Mls1<sup>a</sup> and



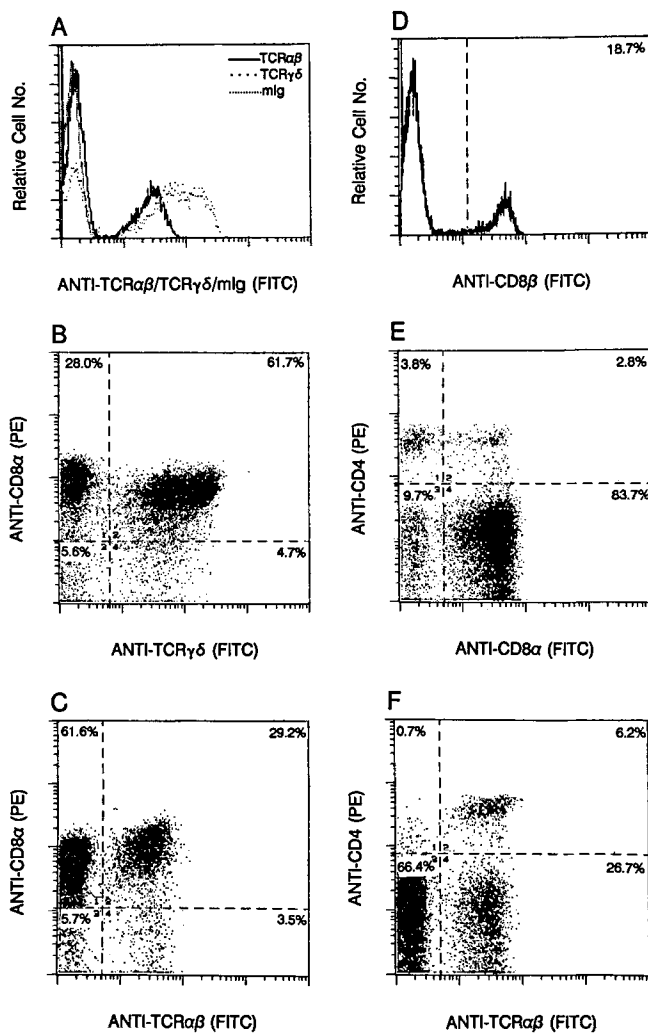
**Figure 6.** Phenotypic analysis of Ly5.2<sup>+</sup> lymphocytes present in the spleen, Peyer's patches, and lamina propria of an Ly5.1 parabiont. At 24 d after joining, cells were isolated and stained, as described in Materials and Methods. (A, D, and G) Anti-TCR- $\alpha/\beta$  and anti-mIg staining profiles of total Ly5.2<sup>+</sup> lymphocytes obtained from the spleen, Peyer's patches, and lamina propria. (B, E, and H) Staining of these populations with anti-TCR- $\alpha/\beta$  and anti-CD4. (C, F, and I) Staining of these populations with anti-TCR- $\alpha/\beta$  and anti-CD8 $\alpha$ .

I-E (24), respectively, in the host environment. The two other lymphocyte subsets, CD4<sup>+</sup>8 $\alpha$ <sup>+</sup> and CD4<sup>-</sup>8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup>, are found exclusively in the intestinal epithelium, and share two properties that distinguish them from mature T cells. The frequency of potentially self-reactive V $\beta$  in the CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup> IEL subset is roughly 10-fold higher than that observed in CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>+</sup> or CD4<sup>+</sup> IEL subsets. This was also observed for V $\beta$ 11 expression in CD4<sup>+</sup>8 $\alpha$ <sup>+</sup> IEL. In addition, neither CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup> nor CD4<sup>+</sup>8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup> IEL can be stimulated to synthesize DNA in response to anti-TCR- $\alpha/\beta$ . Hence, high frequencies of cells expressing potentially self-reactive TCR- $\alpha/\beta$  are restricted to IEL subsets that do not have functional antigen receptors.

Two observations support the conclusion that IEL develop in a thymus-independent fashion. The lack of T lymphocytes in the periphery of athymic radiation chimeras makes it unlikely that functional thymic remnants persist. Moreover, the use of hematopoietic stem cells devoid of T lymphocytes to reconstitute irradiated recipients obviates problems of contaminating mature T cells.

Importantly, the number of IEL and lamina propria T cells rescued from athymic radiation chimeras was identical to that

observed in conventional animals. Moreover, these were the only two anatomical sites that were reconstituted with donor-derived T cells. We cannot formally exclude the possibility that these cells originate in an as yet uncharacterized site of T lymphopoiesis. However, several considerations support the hypothesis that IEL differentiate within the intestinal epithelium and that functionally mature cells migrate to the lamina propria. As for T cell development in the thymus, the rearrangement and expression of self-reactive TCRs at any site of T lymphopoiesis must be unavoidable. The phenotypic and functional analogies between IEL and thymocyte subsets are striking. In the thymus, T cells expressing potentially self-reactive V $\beta$  are restricted to the CD4<sup>+</sup>8<sup>+</sup> subset and deleted from the CD4<sup>+</sup> and CD8<sup>+</sup> subsets (17, 18, 25). Moreover, double-positive and single-positive thymocytes express functionally distinct antigen receptors (26). The results presented here demonstrate the existence of functionally analogous IEL subsets. It is noteworthy that all CD8 $\alpha$ <sup>+</sup>, TCR- $\alpha/\beta$ <sup>+</sup> thymocytes and peripheral T cells coexpress CD8 $\beta$  (27). In contrast, of the two putatively immature CD8 $\alpha$ <sup>+</sup> IEL subsets, the CD4<sup>-</sup>8 $\alpha$ <sup>+</sup> subset does not express CD8 $\beta$ , while only 5% of the CD4<sup>+</sup>8 $\alpha$ <sup>+</sup> subset is CD8 $\beta$ <sup>+</sup> (1, 6,



**Figure 7.** Phenotypic analysis of Ly5.2<sup>+</sup> IEL present in an Ly5.1 parabiont. At 24 d after joining, cells were isolated and stained, as described in Materials and Methods. (A) Staining profiles with anti-TCR- $\alpha/\beta$ , anti-TCR- $\gamma/\delta$ , and anti-mIg. Panels (B, C, D, E, and F) Staining of the Ly5.2<sup>+</sup> IEL with the indicated mAb.

7). Despite these phenotypic differences, the inverse correlation between expression of self-reactive  $V\beta$ s and functional antigen receptors among IEL subsets provides a compelling argument that the intestinal epithelium contains a mixture of mature and developing T cells.

In contrast, donor-derived T lymphocytes rescued from the lamina propria of athymic radiation chimeras are phenotypically mature. They are comprised exclusively of CD4<sup>+</sup> and CD8 $\alpha^+\beta^+$  T cells present in ratios identical to those found within the periphery of conventional animals. Moreover, the frequency of cells expressing self-reactive  $V\beta$ s at this site is low and comparable with that observed in the periphery of haplotype-matched conventional animals. This suggests that lamina propria T cells in the chimeras are derived from a population that had already passed through the negative selection process. Thus, while the details of T cell maturation in the gut are as yet uncharacterized, taken together,

results using athymic radiation chimeras suggest that T cells expressing potentially self-reactive antigen receptors are generated in the intestinal epithelium and deleted in situ from phenotypically and functionally mature IEL subsets, which then migrate to the lamina propria.

Lineage relationships among IEL subsets developing in the absence of a thymus have not been formally demonstrated, however, the analogies with thymocyte development are striking. Of note are two possibly related features that distinguish the two processes. Immature, double-positive thymocytes express 10-fold lower levels of TCR- $\alpha/\beta$  than do mature single-positive thymocytes (28). It has been suggested that this is the result of an active process mediated by the thymic environment, since the level of TCR- $\alpha/\beta$  expressed on double-positive thymocytes cultured in vitro without stimulus increases 5–10-fold within hours (28). In contrast, double-positive and CD8 $\alpha^+\beta^-$  IEL, the two putatively immature IEL subsets, express high levels of TCR- $\alpha/\beta$ . While the biochemical basis for the inability of these IEL subsets to respond to anti-TCR- $\alpha/\beta$  is not established, it is clear that alternative mechanisms for downregulation of T cell responsiveness operate in the gut. The second distinct feature is the relative paucity of double-positive IEL. Assuming that the phenotypic progression of T cells developing in the gut is not totally dissimilar to that of thymocytes, this observation could be explained by the high level of TCR expression in all IEL subsets. Recent results using animals transgenic for MHC class I-restricted self-reactive TCR- $\alpha/\beta$  demonstrate that the high level of TCR expressed on phenotypically immature thymocyte subsets correlates with reduced frequencies of double-positive cells, indicating that negative selection has occurred at an earlier developmental stage (29, 30). In contrast, when levels of transgenic TCR expression are low, the frequency of double-positive thymocytes is unperurbed, and the presence of potentially self-reactive T cells within this subset is easily demonstrated (30, 31).

The differential expression of  $V\beta 6$  by thymus-derived T cells and IEL in Mls1<sup>a</sup>, I-E<sup>-</sup> animals demonstrates that deletion of  $V\beta 6$ -expressing cells in the intestinal epithelium of Mls1<sup>a</sup> strains does not require the I-E molecule (22). The effective deletion of  $V\beta 6^+$  IEL observed in conventional animals, as well as in athymic radiation chimeras, may result from the high level of TCR expression on putatively immature CD4<sup>-</sup>8 $\alpha^+\beta^-$  IEL. In addition to reinforcing the notion that distinct negative selection processes occur in the intestinal epithelium, this observation also suggests that there is a barrier that prohibits the equilibration of T cells in the periphery of an animal with those in the intestinal epithelium. This is confirmed by the analysis of parabiotic animals.

While circulation of peripheral T cells through the spleen, Peyer's patches and lamina propria of each parabiont was evident as early as 6 d after joining the animals, it took in excess of 3 wk before lymphocytes expressing the inappropriate Ly5 allele were observed in the intestinal epithelium. Moreover, the proportion of immigrant IEL at this time was low, increasing to 5–15% by 6 wk. At this point, we cannot exclude the possibility that the intestinal epithelium is a site

of restricted access for mature peripheral T cells, which, one could argue, lack the appropriate homing receptors. However, if this were the case, one would have to account for the striking phenotypic differences between peripheral T cells and IEL expressing the inappropriate Ly5 allele present in the same parabiont. Thus, the presence of CD4<sup>+</sup>8 $\alpha$ <sup>+</sup> and CD4<sup>-</sup>8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup> IEL subsets would require the differential reexpression or loss of CD8 $\alpha$ , CD4, and CD8 $\beta$  by peripheral T cells depending on their initial phenotype. Moreover, the reexpression of self-reactive V $\beta$ s by preselected thymus-derived T cells would have to be postulated. Alternatively, the slow rise in the proportion of IEL expressing the inappropriate Ly5 allele is consistent with the kinetics with which hematopoietic precursors derived from each parabiont would seed the intestinal epithelium of the other, and begin to differentiate. Given the capacity of fetal liver cells to repopulate the intestinal epithelium of radiation chimeras, it is clear that T cell precursors have access to this compartment.

The function of IEL, and how or whether they interact with thymus-derived T cells, remains to be determined. However, some insights are provided if one considers the differential circulation patterns of peripheral T cells observed in parabionts and the anatomical sites that are reconstituted with

T cells in athymic radiation chimeras. In parabionts, the kinetics of appearance of T cells expressing the inappropriate Ly5 allele was identical and rapid in the spleen and Peyer's patches. These two lymphoid compartments, however, were devoid of donor-derived T cells in radiation chimeras. This result suggests not only that T cells resident in the spleen and Peyer's patches of conventional animals are thymus derived, but also demonstrates that IEL do not originate from these compartments. The lamina propria of athymic radiation chimeras, however, was reconstituted with T cells and, as discussed above, these cells are phenotypically mature and express a V $\beta$  repertoire characteristic of a postselection population. In parabionts, the rapid kinetics of reciprocal circulation to the lamina propria suggests that thymus-derived peripheral T cells also have access to this site. It is noteworthy in this context that the proportion of lamina propria T cells expressing the inappropriate Ly5 allele rose rapidly, but stabilized at a level between 25 and 30% for the duration of the 6 wk analyzed. This suggests the existence of a homeostatic process, perhaps regulated in part by the presence of gut-derived T cells at the same site. It is not implausible that thymus-derived and gut-derived T cells contribute to distinct immune systems that interact within the lamina propria.

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