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Role of Gut Cryptopatches in Early Extrathymic Maturation of Intestinal Intraepithelial T Cells

Takatoku Oida,*† Kenji Suzuki,* Masanobu Nanno,‡ Yutaka Kanamori,§ Hisashi Saito,¶ Eiro Kubota,¶ Shingo Kato,* Mamoru Itoh,† Shuichi Kaminogawa,† and Hiromichi Ishikawa2*†

Lympho-hemopoietic progenitors residing in murine gut cryptopatches (CP) have been shown to generate intestinal intraepithelial T cells (IEL). To investigate the role of CP in progenitor maturation, we analyzed IEL in male mice with a truncated mutation of common cytokine receptor γ-chain (CRγ-γ-/Y) in which CP were undetectable. IEL-expressing TCR-γδ (γδ-IEL) were absent, and a drastically reduced number of Thy-1
dbCD4+ and Thy-1
dbCD8ab+ αβ-IEL were present in CRγ-γ-/Y mice, whereas these αβ-IEL disappeared from athymic CRγ-γ-/Y littermate mice. Athymic CRγ-γ-/Y mice possessed a small TCR- and αββ-integrin-negative IEL population, characterized by the disappearance of the extrathymic CD8α+ subset, that expressed pre-Tα, RAG-2, and TCR-β but not CD3ε transcripts. These TCR-IEL from athymic CRγ-γ-/Y mice did not undergo Dβ-Iβ and Vδ-Jδ joinings, despite normal rearrangements at the TCR-β and -δ loci in thymocytes from euthymic CRγ-γ-/Y mice. In contrast, athymic severe combined immunodeficient mice in which CP developed normally possessed two major TCR-αβε, CD8αε+ and CD8ε-IEL populations that expressed pre-Tα, RAG-2, TCR-β, and CD3ε transcripts. These findings underscore the role of gut CP in the early extrathympic maturation of CD8αε+ IEL, including cell-surface expression of αβε integrin, CD3ε gene transcription, and TCR gene rearrangements. The Journal of Immunology, 2000, 164: 3616–3626.

Numerous intraepithelial T cells (IEL)1 bearing either TCR-αβ (αβ-IEL) or TCR-γδ (γδ-IEL) are localized between columnar epithelial cells of the mouse small intestine and are believed to maintain the anatomical front of the intestine under constant immune surveillance. However, IEL have a number of cellular and behavioral characteristics that distinguish them from thymocytes and other peripheral T cells (1–5). For example, IEL are enriched with TCR-γδ T cells (6, 7), and virtually all γδ-IEL and about one-third of αβ-IEL, unlike thymus-derived T cells that use ζ-chain as part of their CD3 complex, express the unique CD8εαβ homodimer (8–11) instead of the CD8βε heterodimer and can use the Fc receptor γ-chain (12–14) in place of the ζ-chain. Accumulating evidence indicates that these CD8αε+ IEL are potentially capable of developing somewhere in the intestinal mucosa without passing through the thymus (9, 15–20). Moreover, the presence of lymphoid cells with properties of precursor T cells among IEL (1, 9, 19, 21–24) and the expressions of recombination activating gene-1 (RAG-1) (9, 21) and RAG-2 (25) by a subset of IEL support the notion that T lineage-committed precursors may enter the epithelium and undergo all steps of TCR gene rearrangement and subsequent differentiation into mature IEL in situ.

Recently, however, we identified multiple tiny clusters (~1500) filled with ~1000 c-kit+ IL-7R+ Thy-1+ lympho-hemopoietic progenitors in crypt lamina propria (LP) of the mouse small intestine (cryptopatches; CP) (26) and corroborated that c-kit-Lin− (Lin−; CD3, B220, Mac-1, Gr-1, and TER119) cells separated by flow cytometry from CP cells were capable of reconstituting αβ- and γδ-IEL in irradiated SCID mice (27). In contrast, cells from Peyer’s patches (PP) and mesenteric lymph nodes (MLN), which belong in the same intestinal immune compartment but lack c-kit-Lin− cells, failed to do so. These findings indicate that CP are the key extrathympic anatomical sites in which precursor T cells develop to provide mature IEL and lead to the view that T lineage-committed precursors concentrating in gut CP and those residing in the IEL compartment represent at least two distinct intermediates along the extrathymic IEL lineage pathway, the more immature of which settles in CP.

Three athymic systems, namely congenitally athymic nude mice, neonatally thymectomized mice, and adult thymectomized, lethally irradiated, and hemopoietic stem cell-reconstituted mice, demonstrated that the generation of most peripheral T cells is wholly dependent on the thymus. Thus, mice that lack CP are extremely valuable not only for assessment of thymus-independent (TI) CD8αε+ IEL as the true descendants of progenitors residing in CP but also for dissection of precursor IEL maturation in CP. Because it is impossible to obtain experimentally manipulated mice lacking CP by surgical excision of every gut CP residing...
Materials and Methods

Mice

C57BL/6J Cjcl (B6), BALB/cA Cjcl (Bc), athymic (nu/nu) nude, and C.B-17/Scid Cjcl SCID (scid/scid) mice were purchased from the CLEA Japan (Tokyo, Japan). IL-7R α-chain-deficient (7Rα-/-) (34), RAG-2-/- (35), and TCR-Cβ-/- (36) mice have been described previously (26). IL-2R β-chain-deficient (2Rβ-/-) mice (37) that had been backcrossed seven times to B6 mice were a generous gift from Dr. H. Suzuki (Nagoya University School of Medicine, Nagoya, Japan), and heterozygous WT female mice carrying a truncated mutation of the CRγ (CRγ-T/- mice) (31) that had been backcrossed more than 20 times to B6 mice were kindly provided by Dr. K. Sugamura (Tohoku University School of Medicine, Sendai, Japan). athymic (nu/nu) nude, C.B-17/Scid Cjcl SCID, athymic (nu/nu) nude, and C.B-17/Scid Cjcl SCID (scid/scid) mice were purchased from the CLEA Japan (Tokyo, Japan). IL-7R α-chain-deficient (7Rα-/-) (34), RAG-2-/- (35), and TCR-Cβ-/- (36) mice have been described previously (26). IL-2R β-chain-deficient (2Rβ-/-) mice (37) that had been backcrossed seven times to B6 mice were a generous gift from Dr. H. Suzuki (Nagoya University School of Medicine, Nagoya, Japan), and heterozygous WT female mice carrying a truncated mutation of the CRγ (CRγ-T/- mice) (31) that had been backcrossed more than 20 times to B6 mice were kindly provided by Dr. K. Sugamura (Tohoku University School of Medicine, Sendai, Japan).

Flow cytometry and cell sorting

A single lymphoid cell suspension was prepared and nucleated cells were counted using a hemocytometer. IEL were isolated as described (39), and CP cells were isolated according to a newly devised method described elsewhere (27). In brief, with the aid of transillumination stereomicroscope, we isolated a tiny fragment of the small intestine containing one CP using an amputated and tapered 21-gauge needle. Lymphoid cells were incubated first with biotinylated mAb and then with streptavidin-PE (Becton Dickinson) and FITC-conjugated second mAb. Stained cells were suspended in staining medium (Hanks solution without phenol red, 0.02% NaN3, and 2% FCS) and analyzed using FACScan with LYSYS II software (Becton Dickinson). Dead cells were excluded by PI gating. Lymphoid cells were incubated with anti-Fcγ II/III receptor mAb (2.4G2; Pharmingen) before staining to block nonspecific binding of labeled mAbs to FcR. CD8α+ and CD8- subpopulations of TCR-IEL from athymic SCID mice were sorted by FACSVantage (Becton Dickinson).

Semi-quantitative RT-PCR analysis of mRNA levels

Total RNA was prepared from various lymphocytes with an RNeasy Mini Kit (Qiagen, Chatsworth, CA). RNA samples were treated with DNase (RT grade) (Nippon Gene, Toyama, Japan) to remove contaminating genomic DNA and repurified. Serial dilutions of each RNA sample were reverse transcribed with 5 μM random hexamers, 1 mM dNTP, 20 U of RNase inhibitor (Takara, Kyoto, Japan), and 100 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Rockville, MD) in a volume of 20 μl at 42°C for 30 min. PCR was conducted in a volume of 100 μl containing all reverse transcriptase products, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 1 μM of each primer, and 2.5 U of Taq DNA polymerase (Takara). Amplification for 35 cycles was performed for 15 s at 94°C, 30 s at 60°C, and 1 min at 72°C. The PCR products were run on 2% agarose gel and visualized by ethidium bromide staining. PCR primers and length of fragment PCR products were: pre-Tea, 5'-GGTCAAGCCTC TACCATAAGG-3' and 5'-CCGAAAGCGATTTGAAAGGA-3', 449 bp (40); CD3e, 5'-ATGGCGAAAGACGTCCT-3' and 5'-GAATACAG

Antibodies

The following mAbs described elsewhere (26) were used for immunohistochemical staining: anti-c-kit mAb (ACK-2), anti-CD3 mAb (145-2C11), anti-CD4 mAb (9GK.5), and anti-CD8α mAb (53-6.7). Anti-CD103 (αεβ2) mAb (2E7; Pharmingen, San Diego, CA) and biotinylated anti-Ly5.2 mAb (104; Pharmingen) were also employed in this study. The following FITC-conjugated and biotinylated mAbs were used for flow cytometric analysis: anti-CD3 mAb (145-2C11; Pharmingen), anti-αβ mAb (H57-597; Pharmingen), anti-γδ- MAb (GL3; Pharmingen), anti-Thy-1.2 mAb (30-H12; Becton Dickinson, San Jose, CA), anti-CD4 mAb (RM4-5; Pharmingen), anti-CD8α mAb (53-6.7; Becton Dickinson), and anti-CD8β mAb (53-5.8; Pharmingen). We also used FITC-conjugated anti-B220 mAb (RA3-6B2; Pharmingen), FITC-conjugated anti-aE mAb (2E7, Becton Dickinson), FITC-conjugated anti-CD19 mAb (Becton Dickinson), FITC- conjugated anti-IgM mAb (II/41; Pharmingen), and biotinylated anti-c-kit mAb (ACK-4; a gift from Dr. S. Nishikawa, Kyorai University, Kyoto, Japan).

Immunohistochemical procedure

Immunohistochemical staining was as described previously (26). In brief, longitudinally opened small intestine ~10 mm in length was embedded in OCT compound (Tissue-Tek; Miles, Elkhart, IN) at ~80°C. The tissue sections were cut at 5 μm, deparaffinized by xylene and ethanol at 60°C, and rehydrated by sequential washing with PBS. Sections were blocked with 5% bovine serum albumin in PBS for 30 min at room temperature. Endogenous peroxidase activity was blocked with 0.3% H2O2 in PBS for 10 min at room temperature. Sections incubated either with isotype-matched normal rat IgG or with nonimmune hamster serum showed only minimal background staining.

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along the length of the intestine, we determined genetically manipulated mutant mice that lack CP.

Mice carrying null mutation at the common cytokine receptor γ-chain (CRγ) exhibit generalized lymphoid abnormality associated with a variety of immunological disorders (28–30). In the null mutant mice, development of IEL is severely diminished and PP are not detected (28, 29). We examined extensively tissue sections of small intestine prepared from male mice with a truncated mutant of the CRγ chain (CRγ-T/- mice) that showed a phenotype similar to that of the null mutant mice in terms of development of the lymphocyte population (31, 32) and verified that CP were undetectable. Furthermore, not only γδ- but also αβ-IEL disappeared from the IEL compartment of athymic (nu/nu) CRγ-T/- mice leaving a small population of TCR IEL that expressed c-kit, Thy-1, B220, CD4, and CD8αβ molecules. Remarkably, these TCR IEL did not appear to contain the CD8αβ subset. Thus, the absence of TCR CD8αβ IEL in athymic CRγ-T/- mice contrasts sharply with the phenotype of putative TCR IEL precursors present in young wild-type (WT) (19), athymic (nu/nu) nude (9), SCID (9, 33), RAG-1-/- (23, 24), Ick-/- × fyn-/- (23), CD3γ-/- (24), and CD3ε-/- (24) mice, a predominant fraction of which expresses the CD8αβ homodimer.

We (26) have previously demonstrated that the development of CP is unaltered in athymic nude, SCID, TCR-β-/- × -ε-/-, and RAG-2-/- mice and is comparable with that of normal B6 mice. In this study, we confirmed that athymic (nu/nu) SCID mice, which lacked αβ- and γδ-IEL but, unlike athymic CRγ-T/- mice, in which CP developed normally, possessed the major TCR CD8αβ subset in their IEL compartment. Further comparative analysis of TCR IEL from athymic CRγ-T/- mice and those from athymic SCID mice with respect to the cellular and genetic levels of events associated with T cell development revealed other noteworthy distinctions between these two putative IEL precursors. Overall, the data are consistent with the view that maturation of precursor IEL in the small intestine proceeds sequentially in CP followed by intestinal epithelium and suggest an early and indispensable role of gut CP in the generation of an extrathymic subset of IEL-expressing CD8αβ homodimer.
GTCCCGCT-3', 383 bp (41); RAG-2, 5'-CACATCCACAAGCAG GAAGTACAC-3', 349 bp (42); TCR-Cα, 5'-GAGCAATTTAGCTACTGCG-3', 349 bp (42); TCR-β, 5'-AGGCCACAGGTCAGCTGC-3', 472 bp (42); TCR-CD8a, 5'-GGTTGCACTATTGACC-3', 472 bp (42); TCR-CD8b, 5'-TTGAAAT ACTGTTGACATCATGAAA-3', 349 bp (43).

Semiquantitative PCR analysis of TCR gene rearrangements

Genomic DNA was prepared from various lymphocytes with a QIAamp Blood Kit (Qiagen). For the analysis of TCR Dβ-Jβ gene rearrangement, we conducted a nested PCR to amplify exactly the rearranged DNA sequences. PCR primers were designed to be positioned 5' to the Dβ2 gene segment and 3' to the Jβ2.2 gene segment for both external (E) and internal (I) primers that are capable of amplifying the rearranged Dβ2-Jβ2.1 and Dβ2-Jβ2.2 sequences. Moreover, to minimize the amplification of germ-line sequence (1072-bp fragment), which competitively inhibits amplifications of rearranged Dβ2-Jβ2.1 (458-bp fragment) and Dβ2-Jβ2.2 (289-bp fragment) sequences, genomic DNA was first digested with EcoRI and EcoRV restriction enzymes (Takara), both of which have one recognition site in the germine DNA sequence extending from the Dβ2 to Jβ2 segment but have none in the rearranged Dβ2-Jβ2.1 and Dβ2-Jβ2.2 sequences.

Serial dilutions of the digested DNA samples were subjected to the first PCR consisting of 20 cycles of 30 s at 94°C and 2 min at 68°C in a volume of 50 µl containing 1 µM of Dβ2 (E) and Jβ2.2 (E) primers, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, and 1.25 U of Taq polymerase. The PCR products were further digested with EcoRI and EcoRV and purified. Subsequently, one-tenth of the purified products were subjected to the second PCR with Dβ2 (I) and Jβ2.2 (I) primers for 20 cycles under the same conditions employed for the first round PCR. This method allowed us to determine nearly a two-order smaller amount of rearranged sequences in WT thymocytes (data not shown). For the analysis of TCR Vβ, Dβ-Jb gene rearrangement, serial dilutions of DNA samples were amplified by PCR with Vβ8 and Jb1 primers for 35 cycles. Each cycle consisted of 30 s at 94°C, 30 s at 61°C, and 1 min at 72°C. In this case, the unrearranged germine Vβ8-Jb1 sequence was too long for PCR amplification. PCR primers used were: Dβ2-E-Jβ2.2 (E), 5'-CACTGCAAGCAAACCTCTC GCCAC-3' and 5'-GGCTCTCAGGACAACAAAACCTCGTCTG-3', this study; Dβ2-I-Jβ2.2 (I), 5'-GTAGGCGACGTGGGAAAGAATCT-3' and 5'-GCTCTCTGTGCTACCACTGAC-3', K. Hozumi, Tokai University School of Medicine, unpublished observation; and Vβ8-Jb1, 5'-CCGGCT TCTGGTTGAACTTCC-3' and 5'-CAGTCACCTGGTCCTTGGC-3', −168 bp (44).

Results

CRγ−/− mice lack CP, γδ-IEL, CD8αα+ αβ-IEL, and Thy-1+ αβ-IEL

To explore the developmental events that proceed in CP toward IEL generation, we hoped to find mice that lack CP and to characterize IEL that emerge in the absence of CP. We (26) have previously reported that CP are undetectable in 7Rab−/− mice. However, although γδ-IEL are absent owing to the selective blockade of TCR-γ gene rearrangements (34), we have noticed only slightly decreased development of TI as well as thymus-dependent (TD) αβ-IEL subsets in 7Rα−/− mice (Fig. 1B; data not shown). With these observations in mind, we reinvestigated hundreds of cryosections prepared from the small intestines of 7Rα−/− mice by immunohistochemistry and verified that conspicuously emaciated CP filled with c-kit+ cells and decreased by >16-fold in number were present in the mutant intestine (Fig. 1A). As it has been reported (45), 2Rβ−/− mice exhibited a dramatic reduction of TI CD8αα+ IEL (Fig. 1B). This finding raised the possibility that the development of CP might be hampered in the 2Rβ−/− condition. However, we found that 2Rβ−/− mice have barely decreased CP filled with c-kit+ cells (Fig. 1A). Severely diminished development of IEL in CRγ mutant mice has also been reported (28, 29). Surprisingly, we found that lymphoid cell aggregates filled with a meaningful number of c-kit+ cells, namely CP, were hardly detectable in the intestinal LP of CRγ−/− mice and that most c-kit+ cells were localized individually throughout the length of the small intestine, although two to three c-kit+ cells settled together in several locations (Fig. 1A). These results indicate that the CRγ-mediated signaling is needed for the generation of lymphoid and/or stromal CP cells. Concomitantly, a conspicuous decrease in the total number of IEL was also observed in CRγ−/− mice (Fig. 1B).

As shown in Fig. 1C, flow cytometric analysis on IEL isolated from CRγ−/− revealed that mutant intestine retained Thy-1+ αβ-IEL expressing either CD4 or CD8αβ molecules but lacked γδ-IEL and Thy-1+ as well as CD8αα+ αβ-IEL. Because the recombination of TCR-γ genes is blocked in 7Rα−/− mice (34) and the CRγ mutation also inhibits 7Rα-mediated signaling pathway (28–31), CRγ−/− mice completely lacked γδ-IEL, and all CD3+ IEL in this mutant animals were αβ-IEL (Fig. 1, B and C). To

FIGURE 1. Immunochemical characterization of CP and flow cytometric analysis of IEL in B6, 7Rα−/−, 2Rβ−/−, and CRγ−/− mice. A, Representative immunohistochemical visualization of c-kit+ lymphocytes in the small intestinal CP (×400). Arbitrarily chosen duodenal, jejunal, and ileal tissue fragments from different mice (n = 4–6) were examined. We checked 50–60 fragments from each strain of mice, and 54 fragments from B6 mice equivalent to one and three-quarters of a small intestine gave 2745 CP. This number basically agreed with our previous CP enumeration of ∼1500 CP per small intestine in adult B6 mice (26). Based on the same calculation, the numbers of CP per intestine in the other mice are also shown. B, Absolute numbers of γδ- and αβ-IEL and the composition of Thy-1+ and CD8αα+ αβ-IEL subsets from B6, 7Rα−/−, 2Rβ−/−, and CRγ−/− mice (n = 5 or 7) were determined by two-color flow cytometry. The composition (%) of CD8αα+ αβ-IEL was calculated from (%CD8αα+ αβ-IEL – %CD8β− αβ-IEL). C, Two-color flow cytometric analysis was performed on IEL isolated from B6 and CRγ−/− mice. Percentage of positive cells in the corresponding quadrants is shown.
B220

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IEL are evident in villous epithelia of the small intestine from WT (nu/)

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g

1

IEL are not B

lineage cells (data not shown). Immunohistochemical analysis of tissue sections of intestinal villi confirmed that a small number of IEL detected by flow cytometry such as CD3\(^+\), CD4\(^+\), and CD8\(^+\) IEL in euthymic CR\(\gamma\)\(^{-}\)/Y mice and CD4\(^+\) and CD8\(^+\) IEL in athymic CR\(\gamma\)\(^{-}\)/Y mice were indeed localized in the intestinal epithelium rather than in the villous LP (Fig. 2B).

Consistent with the results obtained from CR\(\gamma\)\(^{-}\)/Y (31, 32) and CR\(\gamma\) null mutant (28, 29) mice, PP and peripheral lymph nodes, except markedly atrophied MLN, were not detectable in athymic CR\(\gamma\)\(^{-}\)/Y mice at necropsy. We also examined MLN cells and spleen cells isolated from athymic CR\(\gamma\)\(^{-}\)/Y mice and found that a TCR\(^-\) lymphocyte population that expressed c-kit, Thy-1, CD4, and/or CD8\(\alpha\) Percentage of positive cells in the corresponding quadrants is shown. B, Representative immunohistochemical visualization of IEL expressing CD3, CD4, or CD8\(\alpha\) (\(\times 400\)). Arrowheads indicate IEL. Although not shown by arrowheads, a considerable number of CD3\(^+\) and CD8\(\alpha\) IEL are evident in villous epithelia of the small intestine from WT (nu/+) CR\(\gamma\)\(^{-}\)/Y mice.

compare WT and CR\(\gamma\)\(^{-}\)/Y B6 mice with respect to the phenotype of CD8-IEL, the expression of Thy-1, CD4, CD8\(\alpha\), and CD8\(\beta\) molecules restricted to the CD8-IEL from WT mice was also shown (Fig. 1C, bottom panel).

Distinctive TCR\(^-\) IEL are localized in the intestinal epithelium of athymic CR\(\gamma\)\(^{-}\)/Y mice

Histogenesis of CP and generation of CD8\(\alpha\)-IEL expressing CD8\(\alpha\) homodimer are almost completely blocked in CR\(\gamma\)\(^{-}\)/Y mice, implying that CP are indispensable for the TI pathway of CD8-IEL development. In an attempt to further explore this issue, we produced athymic CR\(\gamma\)\(^{-}\)/Y mice and analyzed IEL that migrated to epithelial destinations in the absence of the thymus and CP. As shown in Fig. 2A, euthymic WT CR\(\gamma\)\(^{-}\)/Y mice have CD8-IEL and CD8\(\alpha\)-IEL and euthymic CR\(\gamma\)\(^{-}\)/Y mice have CD8-IEL but lack CD8\(\alpha\)-IEL. In contrast to these littermates, athymic CR\(\gamma\)\(^{-}\)/Y mice lacked both CD8-IEL and CD8\(\alpha\)-IEL, leaving a small TCR\(^-\) IEL population, a subpopulation of IEL in athymic CR\(\gamma\)\(^{-}\)/Y B6 mice with respect to the phenotype of CD8-IEL, whereas a significant fraction of IEL but not MLN cells expresses c-kit, Thy-1, CD4, and/or CD8\(\alpha\). Percentage of positive cells in the corresponding quadrants is shown. C, Athymic (nu/nu) SCID mice in which CP develop normally possess a major TCR\(^-\) IEL population expressing CD8\(\alpha\), and athymic (nu/nu) nude mice in which CP develop normally (26) possess major TCR\(^-\) and TCR\(^+\) populations expressing CD8\(\alpha\). Percentage of positive cells in the corresponding quadrants is shown. D, Two-color (CD8\(\alpha\) vs CD8\(\beta\) flow cytometric profiles of IEL from various mice carrying and not carrying CP. The profile of IEL from athymic (nu/nu) CR\(\gamma\)\(^{-}\)/Y mice was essentially the same as that of IEL from B/c athymic nude mice depicted in the figure. Percentage of positive cells in the corresponding rectangular regions is shown.

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Major TCR\(^{-}\) IEL from athymic CR\(^{\gamma/\nu}\) mice fail to express but major TCR\(^{-}\) IEL from athymic SCID mice express \(\alpha\beta\)-integrin

IEL are known to express \(\alpha\beta\)-integrin (54–60), and it has been shown that \(\alpha\beta\)-integrin recognizes E-cadherin on gut epithelial cells (55, 59, 60). In fact, ~90% IEL from euthymic WT CR\(^{\gamma/\nu}\) mice expressed \(\alpha\beta\)-integrin (Fig. 4, A and B), whereas 98% PP cells were \(\alpha\beta\)-integrin negative (Fig. 4A). Interestingly, a significant population (16%) of CP cells from these mice expressed \(\alpha\beta\)-integrin (Fig. 4A). However, this \(\alpha\beta\)-integrin lymphocyte subset is most likely the unavoidable LP lymphocyte and/or IEL contaminant present in the CP cell preparation because c-kit\(^{+}\) CP cells were \(\alpha\beta\)-integrin by immunohistochemistry (data not shown). Provided that CP are important lymphoid tissues in which early maturation of precursor IEL takes place, it can be assumed that the expression of \(\alpha\beta\)-integrin on IEL is also influenced by the absence of CP. Consistent with this proposition, most TCR\(^{-}\) common leukocyte alloantigen-positive (Ly5.2\(^{+}\)) IEL (~80%) were \(\alpha\beta\)-integrin in athymic CR\(^{\gamma/\nu}\) mice that lacked CP (Fig. 4B). In contrast, most TCR\(^{-}\) Ly5.2\(^{+}\) IEL (~80%) expressed high levels of \(\alpha\beta\)-integrin in athymic SCID mice that possessed CP (Fig. 4B). To confirm \(\alpha\beta\)-integrin expression in situ, we used anti-\(\alpha\beta\)-integrin and anti-Ly5.2 mAbs to stain frozen sections of the small intestine. As shown in the lower panel of Fig. 4B, IEL are identifiable by anti-Ly5.2 but not by anti-\(\alpha\beta\)-integrin mAbs in the small intestinal villi of athymic CR\(^{\gamma/\nu}\) mice, whereas they are identifiable by both mAbs in those of athymic SCID mice. Note that although the pictures are not enough for accurate quantitation of IEL, Ly5.2\(^{+}\) IEL of athymic CR\(^{\gamma/\nu}\) mice are about 10% of WT euthymic CR\(^{\gamma/\nu}\) mice, endorsing the results presented in Table I. Taken together, these results underline the role of CP in converting precursor IEL into the \(\alpha\beta\)-integrin state.

Pre-\(\alpha\)- and CD3e gene transcripts in TCR\(^{-}\) IEL

Putative TCR\(^{-}\) IEL precursors in athymic CR\(^{\gamma/\nu}\) mice are distinct from those in athymic SCID mice in that the majority of former TCR\(^{-}\) IEL do not express CD8\(\alpha\)- and \(\alpha\beta\) molecules. However, the phenotype of these TCR\(^{-}\) IEL does not necessarily prove their T cell commitment but instead may represent cells with characteristics of NK, dendritic cell, and/or mast cell progenitors (1, 48, 53, 61). To determine whether these two TCR\(^{-}\) IEL populations include T cell precursors, we investigated whether these cells express pre-\(\alpha\) and CD3e-specific mRNA. As internal standard for the mRNA and cDNA preparations, the intensities of the actin RT-PCR products, corresponded in all experiments to mRNA concentrations that were within the linear range of the template titration curve (Fig. 5).

The exclusively T lineage-specific surrogate TCR-\(\alpha\)-chain (62), namely pre-\(\alpha\), is expressed in immature thymocytes before

Table I. Absolute numbers of IEL per small intestine (\(\times10^{6}\))\(*

<table>
<thead>
<tr>
<th>Mice (n)</th>
<th>(\alpha\beta)-IEL</th>
<th>(\gamma\delta)-IEL</th>
<th>TCR(^{+})</th>
<th>CD4(^{-})CD8(^{-})</th>
<th>CD4(^{+})CD8(^{-})</th>
<th>CD4(^{-})CD8(^{+})</th>
<th>CD8aa(^{+})</th>
<th>CD8ab(^{+})</th>
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<td>nu+/ CR(^{\gamma/\nu}) (5)</td>
<td>31.0 ± 3.0</td>
<td>30.0 ± 7.8</td>
<td>6.3 ± 1.5</td>
<td>9.4 ± 1.5</td>
<td>4.1 ± 0.7</td>
<td>2.2 ± 1.5</td>
<td>35.0 ± 3.0</td>
<td>18.3 ± 1.5</td>
</tr>
<tr>
<td>nu+/ CR(^{\gamma/\nu}) (5)</td>
<td>4.5 ± 1.0</td>
<td>&lt;0.1</td>
<td>2.0 ± 0.3</td>
<td>1.9 ± 0.4</td>
<td>3.6 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>&lt;0.1</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>nu+/ SCID (7)</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>6.0 ± 4.0</td>
<td>3.5 ± 0.5</td>
<td>1.6 ± 0.5</td>
<td>0.7 ± 0.3</td>
<td>&lt;0.1</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>nu+/ (6)</td>
<td>0.8 ± 0.4</td>
<td>0.8 ± 2.5</td>
<td>12.0 ± 3.5</td>
<td>8.1 ± 2.0</td>
<td>0.7 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>11.8 ± 3.6</td>
<td>0.3 ± 0.2</td>
</tr>
</tbody>
</table>

\(*\) Two-color flow cytometric analyses were performed on IEL isolated from indicated mice. For analysis of TCR expression, IEL were incubated first with biotinylated anti-\(\alpha\) mAb and then with streptavidin-PE and FITC-conjugated anti-\(\delta\) mAb. For analysis of CD4 and/or CD8 expression, IEL were incubated first with biotinylated anti-CD8a mAb and then with streptavidin-PE and FITC-conjugated anti-CD4 mAb or with streptavidin-PE and FITC-conjugated anti-CD8b mAb. Dead cells were excluded by PI gating.
earlier reports, pre-Tα gene rearrangement but is absent from mature αβ and γδ T cells (62, 63). Expression of pre-Tα-specific mRNA is also found in extrathymic sites of T cell development such as the IEL compartment of athymic nude mice (62). Consistent with these earlier reports, pre-Tα transcripts were abundant in thymocytes from euthymic WT CRγ−/− mice and euthymic CRγ−/− mice but were almost undetectable in MLN cells from WT CRγ−/−, athymic CRγ−/−, and athymic SCID mice (Fig. 5). However, it should be pointed out that the signals for pre-Tα transcripts were detectable, although weak, in MLN cells from euthymic CRγ−/− (Fig. 5). This observation might reflect the increased number of hemopoietic progenitor cells in the peripheral lymphoid tissues such as in the spleen of these mutant mice (31). In any event, semiquantitative RT-PCR analysis with serially diluted templates showed that a significant amount of pre-Tα was similarly expressed in IEL from euthymic and athymic CRγ−/− mice and in both CP cells and IEL from euthymic WT CRγ−/− and athymic SCID mice (Fig. 5). This indicated that TCR− IEL from athymic CRγ−/− and athymic SCID mice and CP cells from athymic SCID mice include a comparable number of T lineage-committed precursors.

CD3ε-specific mRNA-encoding TCR-associated molecules are expressed in the earliest T-committed mouse fetal thymocytes (41, 53, 64) and represent a marker to define whether immature lymphoid cells are committed to T cell lineages. RT-PCR analysis of mRNA in lymphocytes from athymic SCID mice revealed that CD3ε transcripts were found in TCR− IEL at high levels and in CP cells, albeit at 25-fold reduced levels, but were undetectable in MLN cells (Fig. 5). In contrast, the same CD3ε transcripts were not detected even in mRNA extracted from a large number (6250 cells) of athymic CRγ−/− IEL (Fig. 5). Taken together, these data demonstrate that most TCR− IEL from athymic CRγ−/− mice do not express CD8α (Fig. 3D) and include cells that express pre-Tα but not CD3ε transcripts, whereas that TCR− IEL from athymic SCID mice are comprised of two major CD8α+ and CD8− subpopulations (Fig. 3D) that express pre-Tα and CD3ε transcripts.

**TCR− IEL and CP lymphocytes express RAG-2 transcripts**

Proteins encoded by RAG-1 and -2 are essential in TCR and Ig gene rearrangements and are present in T and B lineage cells of the early stages but not in other lympho-hemopoietic cells. RT-PCR (9, 19) as well as in situ hybridization (21) analyses of IEL revealed the expression of RAG-1 mRNA by a small and confined subset of IEL. RAG-2 transcripts are also detectable by RT-PCR in IEL from the small intestine but not the large intestine (25). Thus, it is important to explore not only TCR− IEL but also cells that reside in CP for the expression of RAG-1 and/or -2 genes because CP were shown to be responsible for generating IEL (27). For this purpose, we determined RAG-2 transcripts by semiquantitative

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**FIGURE 4.** Major TCR− IEL from athymic CRγ−/− mice that lack CP fail to express αβ integrin. A. The expression of αβ on lymphoid cells isolated from PP, CP, and intestinal intraepithelial compartments of WT CRγ−/− mice. B. The upper panel shows a representative histogram of αβ expression on the leukocyte common alloantigen (Ly5.2)-positive IEL from WT CRγ−/− (with CP), athymic CRγ−/− (without CP), and athymic SCID (with CP) mice, and the lower panel verifies immunohistochemically the flow cytometric profiles shown in the upper panel. Two-color flow cytometric analysis by using biotinylated anti-Ly5.2 mAb and FITC-conjugated anti-αβ mAb revealed that most of the IEL from WT CRγ−/− mice (99%), athymic CRγ−/− mice (95%), and athymic SCID mice (97%) expressed Ly5.2 molecules. Note that anti-αβ mAb but not anti-Ly5.2 mAb can hardly visualize IEL in the villous epithelia of the small intestine from athymic CRγ−/− mice, whereas both mAbs visualize almost the same numbers of IEL in the villous epithelia of the small intestine from athymic SCID mice. Arrowheads indicate IEL. Although not shown by arrowheads, numerous αβ+ and Ly5.2− IEL are evident in the villous epithelia of the small intestine from WT CRγ−/− mice.

**FIGURE 5.** Semiquantitative RT-PCR analysis of pre-Tα and CD3ε mRNA levels in CP cells, IEL, MLN cells and thymocytes from WT (nu/nu) CRγ−/−, euthymic (nu/+ ) CRγ−/−, athymic (nu/nu) CRγ−/−, and athymic (nu/nu) SCID mice. Serial 5-fold dilutions of RNAs equivalent to the indicated numbers of cells were reverse transcribed, and the cDNA products were PCR amplified, electrophoresed in agarose gels, and visualized with ethidium bromide. CP cells and IEL from all four strains of mice express comparable levels of pre-Tα gene. In contrast, while the signal for CD3ε transcripts is high in IEL from WT CRγ−/− and euthymic CRγ−/− mice that include TCR+ lymphocytes, and also in TCR− IEL from athymic SCID mice carrying CP, the same signal is almost undetectable in TCR− IEL from athymic CRγ−/− mice not carrying CP. Note that β-actin-specific mRNA levels are comparable in all RNA preparations.
RT-PCR analysis and compared mRNA of gut lymphocytes with those of thymocytes from WT and RAG-2<sup>−/−</sup> mice, with the latter two mRNA serving as positive and negative templates, respectively.

mRNA from 50 WT thymocytes displayed a strong signal for RAG-2 transcripts, whereas mRNA from 6250 RAG-2<sup>−/−</sup> thymocytes failed to display any detectable signals (Fig. 6). Under this condition, low levels of RAG-2 transcripts were constantly detected in an amount of mRNA equivalent to 6250 lymphocytes contained in 50 thymocytes from WT mice and those contained in 6250 thymocytes from RAG-2<sup>−/−</sup> mice were used for positive and negative control RNAs, respectively. Note that β-actin-specific mRNA levels are comparable in all RNAs equivalent to RNAs contained in two cells.

FIGURE 6. CP cells and IEL express low levels of RAG-2 gene. Amounts of RNAs equivalent to RNAs contained in 6250 CP cells and IEL but not MLN cells from WT CR<sup>Y<sup>+/+</sup></sup>, athymic CR<sup>Y<sup>−/−</sup></sup>, and athymic SCID mice exhibit low levels of RT-PCR signals for RAG-2 transcripts. RNAs equivalent to RNAs contained in 50 thymocytes from WT mice and those contained in 6250 thymocytes from RAG-2<sup>−/−</sup> mice were used for positive and negative control RNAs, respectively.

Rearrangement and expression of TCR genes in TCR<sup>−/−</sup> IEL and CP cells

To test further the proposition that TCR<sup>−/−</sup> IEL and CP cells include lymphoid precursors committed to the T cell lineage, we used nested as well as standard DNA-PCR strategies to examine the status of TCR-β and -δ gene rearrangements among these cells. The nested PCR analysis was capable of diminishing markedly the unarranged germline DJβ2-Jβ2.2 band and thus allowed us to titrate much more accurately the template DNA containing a small number of rearranged DJβ2-Jβ2.1 and DJβ2-Jβ2.2 gene segments (see Materials and Methods). The rearranged DJβ2-Jβ2.1 and DJβ2-Jβ2.2 bands were not detected in TCR<sup>−/−</sup> IEL from athymic CR<sup>Y<sup>−/−</sup></sup> mice, unlike thymocytes and IEL from euthymic CR<sup>Y<sup>−/−</sup></sup> mice and athymic nude mice that exhibited DJβ2-Jβ2.1 and DJβ2-Jβ2.2 rearrangements (Fig. 7A). Likewise, a standard DNA-PCR analysis revealed that cells undergoing V64-J61 rearrangement were also not detected in TCR<sup>−/−</sup> IEL from athymic nude mice, whereas cells undergoing the rearrangement

FIGURE 7. Semiquantitative DNA-PCR analysis of TCR gene rearrangements and semiquantitative RT-PCR analysis of TCR-Cβ mRNA levels in CP cells, IEL, and thymocytes from euthymic (nu/+<sup>+</sup>) CR<sup>Y<sup>+</sup></sup>, athymic (nu/nu) CR<sup>Y<sup>−/−</sup></sup>, athymic (nu/nu) SCID, and athymic (nu/nu) nude mice. A, Serial 5-fold dilutions of genomic DNAs equivalent to DNAs extracted from the indicated numbers of cells were subjected to TCR gene rearrangement analysis using indicated PCR primers as described in Materials and Methods. Subsequently, PCR products were electrophoresed in agarose gels and visualized with ethidium bromide. For the amplification of rearranged DJβ2-Jβ2.1 (458-bp fragment) and DJβ2-Jβ2.2 (289-bp fragment) sequences, we employed a newly devised PCR strategy as described in Materials and Methods to minimize the amplification of 1072-bp signals corresponding to germline DJβ2-Jβ2.2 DNA, which competitively inhibited the amplification of a minute number of rearranged DNA sequences. DJβ2-Jβ2.1, DJβ2-Jβ2.2, and V64-J61 rearrangements were absent among IEL from athymic CR<sup>Y<sup>−/−</sup></sup> mice, whereas rearrangements were present in CP cells from athymic nude mice and also among IEL from euthymic CR<sup>Y<sup>−/−</sup></sup> and athymic nude mice. CP cell and IEL DNAs from athymic SCID mice were used as negative control DNAs. B, Serial 5-fold dilutions of RNAs equivalent to RNAs extracted from the indicated numbers of cells were reverse transcribed, and the cDNA products were PCR amplified, electrophoresed in agarose gels, and visualized with ethidium bromide. Not only CP cells and IEL from athymic SCID mice but also IEL from athymic CR<sup>Y<sup>−/−</sup></sup> mice express the transcript of the TCR-Cβ gene, indicating that the signals are most likely the germline transcript because these cells do not undergo rearrangement of the TCR-β gene. CP cell, IEL, and thymocyte RNAs from TCR-Cβ<sup>−/−</sup> mice were used as negative control RNAs. Note that β-actin-specific mRNA levels are comparable in all RNA preparations.
were detected not only in thymocytes (68, 69) but also in IEL that include thymus-derived αβ T cells (Fig. 1, B and C, Fig. 2A, and Table I) from euthymic CRγ−/− mice as well as in CP cells and IEL from athymic nude mice (Fig. 7A). As depicted in Fig. 7A, neither IEL nor CP cells from athymic SCID mice exhibit DJβ2-Jβ2.1, DJβ2-Jβ2.2, and Vβ4-Jβ1 rearrangements (negative control).

RT-PCR analysis of mRNA levels revealed that TCR-β transcripts were found in TCR− IEL from athymic CRγ−/− mice and even in TCR− IEL and CP cells from athymic SCID mice on condition that the same transcripts were not detected in any lymphoid cells from TCR-β gene-deficient mice (negative control) (Fig. 7B). Thus, TCR-β transcripts detected in IEL from athymic CRγ−/− mice and in IEL and CP cells from athymic SCID mice are germline TCR-β transcripts because these cells do not undergo rearrangement of TCR-β gene (Fig. 7A). Collectively, these findings indicate that TCR− IEL that migrate into the intestinal compartment of athymic CRγ−/− mice in the absence of CP include cells expressing the germline TCR-β transcripts that do not rearrange the gene encoding TCR-β-chain.

Both CD8αa+ and CD8− IEL from athymic SCID mice express pre-Tα, CD3ε, and germline TCR-β/Cα transcripts

TCR− IEL from athymic SCID mice contain pre-Tα, CD3ε, and germline TCR-β/Cα transcripts (Figs. 5 and 7B), and the generation of TI CD8αa+ IEL in these mice appears to be dependent on CP (Fig. 3D). However, because TCR− IEL from athymic SCID mice include two major CD8αa+ and CD8− subsets (Fig. 3C), it is possible that the CD8αa+ IEL are not T lineage-committed precursors. To determine whether T lineage-committed cells are present in TCR−CD8αa+ IEL from athymic SCID mice, we purified CD8αa+ and CD8− IEL by flow cytometry. As shown in Fig. 8, RT-PCR analysis of mRNA extracted from CD8αa+ IEL (purity, 99.4%) and CD8− IEL (purity, 99.5%) revealed that pre-Tα, CD3ε, and germline TCR-β/Cα transcripts were expressed by these two sorted IEL to the same extent, indicating that both subpopulations include T lineage-committed precursors.

Discussion

In the small intestine of 7Rαa−/− mice, CP were reduced drastically in numbers and average size. γδ-IEL are absent from epithelial compartment of 7Rαa−/− mice (34), whereas αβ-IEL from 7Rαa−/− were only 2-fold less than those from WT B6 mice and were comprised of every five discriminable (11) TD and TI subsets (data not shown). By contrast, 2Rβ−/− mice, in which signaling through IL-2R and IL-15R is compromised (37, 45, 70), exhibited slightly reduced development of CP in terms of numbers. Although a conspicuous decrease in the total number of γδ-IEL was noted in 2Rβ−/− mice, absolute numbers of αβ-IEL were basically comparable to those of WT B6 mice. Strikingly, however, the population size of major TI CD8αa+ IEL subset (8–11) was reduced drastically in the 2Rβ−/− condition (45) (Fig. 1B). Because analyses of lymphoid cells in mice lacking the 2Rβ (45, 71) or IL-15Rα (72) chain have indicated the essential role of signaling through IL-15R rather than IL-2R in the creation of a permissive extrathymic microenvironment for the differentiation of lymphocyte subsets such as NK T cells, NK cells, and CD8αa+ IEL, it is likely that deficiency of IL-15R-mediated signaling is attributable to the malfunction of 2Rβ−/− CP in support of TI IEL maturation.

In the present study, we verified that the small intestine of CRγ−/− mice, in which signal transductions from IL-2, IL-4, IL-7, IL-9, and IL-15 receptors are compromised (28, 29, 31), was devoid of CP and did not contain γδ-IEL and TI CD8αα− αβ-IEL but did contain a small number of TD CD4+ and CD8αβ+ αβ-IEL-expressing Thy-1 molecules. In fact, these remaining TD αβ-IEL subsets disappeared completely from the IEL compartment of athymic CRγ−/− mice. Regarding the gut microenvironment of CRγ−/− mice, we assume that deficiencies of IL-7R- and IL-15R-mediated signaling pathways reduce synergistically and/or additively the histogenesis of CP and, consequently, result in the disappearance of CP. Truncated CRγ expressed in these mutant mice could also abolish signal transductions from IL-2R, IL-4R, and IL-9R. However, these signalings might not be involved in the histogenesis of CP or in maturation of TI IEL (30, 72, 73), although these possibilities remain to be ruled out.

From the point of view of the correlation between CP development and generation of TI IEL in these three mutant mice, the present findings appear to be rather conflicting. Nonetheless, given that CP are indispensable for early TI IEL maturation, it is conceivable that CP in the 7Rαa−/− intestine are functionally intact even if histogenesis is markedly diminished, whereas CP in the 2Rβ−/− intestine might be functionally crippled even if histogenesis remains nearly the same in its numerical and immunohistochemical appearances (Fig. 1A). The role of thymus in the generation of peripheral CD8αβ+ T cells has been well established to date. In this context, the following observations are noteworthy. First, MHC class I mutant mice do not have peripheral CD8αβ+ T cells but their thymus is almost normal in terms of size and appearance. Thus, even though MHC class I mutant mice have a normal appearing thymus, it must not function normally because it does not produce CD8αβ+ T cells. Second, IL-7R mutant mice have much reduced but still a significant number of peripheral CD8αβ+ T cells, although their thymus displays a drastic reduction in its cellularity. Third, athymic nude mutant mice do not have thymus and peripheral CD8αβ+ T cells. Based simply on these
observations, we cannot conclude that the correlation is seen between thymus development and generation of peripheral CD8αβ+ T cells. However, because T cell development in the thymus has been extensively studied by many investigators, we now know that the development of most peripheral CD8αβ+ T cells is wholly dependent on the thymus. But in any case, much yet remains to be learned about the cellular mechanism of precursor IEL maturation in CP before we conclusively establish the correlation between CP development and generation of TI IEL. This issue, for instance, could be explored by analysis of various types of bone marrow chimeric animals produced between WT, 7R−/−, 2Rβ−/−, and CRγ−/− mice.

TCR− IEL from athymic CRγ−/− mice without CP and those from athymic SCID mice with CP exhibited abundant signals for pre-Tα and germline TCR-Cβ transcripts, markers of T cell commitment (41, 53, 62) and a weaker but comparable level of signal for RAG-2 transcripts, and included cells that expressed c-kit, Thy-1, CD4, and CD8αβ molecules. Outstanding differences between these two TCR− IEL revealed in the present study concerned the development of the CD8αα+ subset, cell-surface expression of αβ2 integrin, and transcription of CD3α-specific mRNA (Figs. 3D, 4B, and 5). On the basis of these parameters, athymic CRγ−/− and athymic SCID mice could be classified into incapable and capable mutant strains, respectively, indicating that at least these three events take place or are determined to take place in CP during the early stages of IEL maturation.

Although the in vivo function of αβ2 integrin remains to be determined, it has been reported that IEL receive signals for activation through αβ2 molecules triggered by E-cadherin on the epithelial cells rather than use this integrin as a homing receptor for the intestinal epithelium (55, 59, 60) and that a decrease of about 2-fold in the number of IEL is observed in α--deficient BALB/c mice (74). We revealed that major TCR− Ly5.2+ IEL from athymic CRγ−/− mice without CP failed to express αβ2, whereas major TCR− Ly5.2− IEL from athymic SCID mice with CP expressed αβ2, indicating an important role of CP in the expression of αβ2 integrin on these putative TCR− IEL precursors.

The fact that mRNA for CD3α molecules was hardly detectable in IEL and MLN cells of athymic CRγ−/− mice, whereas the same mRNA was detectable in cells from CP and IEL but not MLN compartments of athymic SCID mice (Fig. 5), favors a scheme approving the sequential in situ maturation of precursor IEL in CP followed by intraepithelium. Compartmentalization of T lineage committed precursors in gut CP was also verified in the present study by showing: 1) TCR− CP cells and TCR− IEL from athymic SCID mice contained a comparable amount of pre-Tα-specific (Fig. 5) and germline TCR-Cβ-specific (Fig. 7B) mRNA; and 2) although our previous immunohistochemical study failed to demonstrate the presence of RAG-1-bearing cells in CP (26), a low level of RAG-2 transcripts relative to that seen for thymocytes from WT mice was detected by RT-PCR analysis in lymphoid CP from WT and athymic SCID mice (Fig. 6) as well as athymic nude mice (data not shown). Taken together, the results indicate that T cells mature in CP only in small numbers and/or at a slow rate. Moreover, both CD8αα+ and CD8+ IEL subsets from athymic SCID mice displayed a comparable level of signal for CD3α transcripts (Fig. 8), and most CP cells (>99%) from the same animals did not express CD8α molecules (data not shown). These findings support our contention that the commitment of putative IEL precursors to express CD8α molecules is also achieved during early IEL maturation in CP, whereas actual cell-surface expression of CD8α starts after migration of such cells into the epithelium.

Endorsing this scenario, our preliminary immunohistochemical analysis of the small intestines from irradiated and WT bone-marrow reconstituted athymic CRγ−/− mice revealed the emergence of donor-derived TCR− IEL during an early and confined time period after reconstitution in the restricted epithelial areas beneath which histogenesis of CP filled with donor-derived lymphoid cell was detected (our unpublished observation).

It is also important that both Δβ-Δβ and Vβ-Δβ-Jα gene rearrangements were hardly detectable in TCR− IEL of athymic CRγ−/− mice (Fig. 7A). Because thymocytes and TD IEL from euthymic CRγ−/− mice not only displayed abundant CD3ε signals but also included cells that undergo Δβ-Jα and Vβ-Dβ-Jα joining, the machinery necessary to carry out these genetic events is not crippled by the mutation and is retained by TCR− IEL of athymic CRγ−/− mice, suggesting that the permissive gut microenvironment in which immature IEL drive such machinery is canceled in the CRγ−/− condition most likely due to the lack of CP. Consistent with the status of TCR-β gene rearrangement in the athymic/euthymic CRγ−/− condition, it has recently been demonstrated that major CD3− CD8αα CD16− IEL from CD3ε gene-deficient mice fail to undergo Δβ-Jα joining despite normal rearrangements at the TCR-β locus in thymocytes from these animals (24). However, unlike TCR− IEL of athymic CRγ−/− mice, it should be pointed out that Vβ-Δβ-Jα rearrangements were detected in sorted CD3− CD8αα CD16− IEL from CD3ε-deficient mice (24). All in all, their data (24) in conjunction with our present findings illuminate an early role of CD3ε in IEL maturation and provide another distinction between TD and TI IEL by establishing that TCR-β gene rearrangement is controlled differentially in the thymus and intestine.

In conclusion, the majority of TCR− IEL isolated from athymic CRγ−/− mice retain a similar distinguishing characteristic from the recently described B220− fetal liver T lymphoid progenitors (40) and HSAlowc-kit+Thy-1− CD3+ T/NK progenitor cells present in the fetal blood and spleen (53) and perhaps represent the most primitive gut T lineage-committed precursors passed directly in small numbers into the epithelium without prior differentiation in CP, i.e., their development is at a standstill before the onset of TCR gene rearrangements, CD3ε gene transcription, and αβ2 expression but after expression of pre-Tα and germline TCR-Cβ-specific mRNA.

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References


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