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Gut Cryptopatches: Direct Evidence of Extrathymic Anatomical Sites for Intestinal T Lymphopoiesis

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Summary

Athymic cytokine receptor γ chain mutant mice that lack the thymus, Peyer's patches, cryptopatches (CP), and intestinal T cells were reconstituted with wild-type bone marrow cells. Bone marrow–derived TCR$^-$ intraepithelial lymphocytes (IEL) first appeared within villous epithelia of small intestine overlying the regenerated CP, and these TCR$^-$ IEL subsequently emerged throughout the epithelia. Thereafter, TCR$^+$ IEL increased to a comparable number to that in athymic mice and consisted of TCRγδ and TCRαβ IEL. In gut-associated lymphoid tissues of wild-type mice, only CP harbored a large population of c-kit^{high}IL-7R$^+$CD44$^+$Thy-1$^{+/-}$CD4$^{+/-}$CD25$^{low/-}$$\alpha_E\beta_7^-Lin^-$ (Lin, lineage markers) lymphocytes that included cells expressing germline but not rearranged TCRγ and TCRβ gene transcripts. These findings provide direct evidence that gut CP develop progenitor T cells for extrathymic IEL descendants.

Introduction

Twenty years have passed since it was discovered that virtually all intraepithelial lymphocytes (IEL) distributed in the intestines of mice are T cells. During that period, it has been shown that mouse IEL expressing T cell receptor (TCR)αβ and TCRγδ form a population markedly different from T cells that develop in the thymus and are distributed in peripheral lymphoid tissues (Rocha et al., 1995; Mowat and Viney, 1997; Cruz et al., 1998; Klein, 1998; Lefrancois and Puddington, 1999; Poussier and Julius, 1999). In contrast to thymus-derived CD8αβ T cells, most γδ IEL and many αβ IEL express the unique CD8αα homodimer and develop without passing through the thymus (thymus-independent [TI] IEL). The detection, although weak, of mRNA for recombination activating proteins (RAG-1 and RAG-2) (Guy-Grand et al., 1991, 1992; Lin et al., 1994; Boll et al., 1995; Oida et al., 2000) and identification of a small number of T lineage–committed TCR$^-$ lymphocytes in IEL from wild-type (WT) mice (Guy-Grand et al., 1991, 1992; Lin et al., 1994; Hamad et al., 1997; Mowat and Viney, 1997; Page et al.,1998) give support to the concept of localized development of IEL in the epithelial layer in situ. Concurrently, it has recently been demonstrated that enterocyte-produced IL-7 is important (Watanabe et al., 1995; Laky et al., 1998) and sufficient (Laky et al., 2000) for the intestinal development of TI IEL. There may also be extrathymic T cells that develop in the human intestine, because CD8αα$^+$ T cells are present (Latthe et al., 1994), and T early α and T early β messages (Koningsberger et al., 1997) as well as pre-Tα transcripts (Howie et al., 1998) are detected in lymphocytes isolated from fetal intestine in the second trimester. Moreover, RAG-1 (Lundqvist et al., 1995; Lynch et al., 1995) and RAG-2 (Lynch et al., 1995) transcripts are detected in adult human IEL but not lamina propria (LP) lymphocytes.

We (Kanamori et al., 1996) have reported that multiple tiny clusters, filled with closely packed c-kit$^+$IL-7R$^+$Thy-1$^+$ lymphocytes, can be found throughout the murine small and large intestinal crypt LP (cryptopatches, CP). A large fraction of CP cells isolated from the small intestine displayed c-kit$^+$Lin$^-$ (Lin; CD3, B220, Mac-1, Gr-1, and TER-119) lympho-hemopoietic stem cell phenotype, and in vivo progeny studies revealed that they were capable of generating αβ and γδ IEL (Saito et al., 1998). Mayrhofer and coworkers (Mayrhofer and Brooks, 1995; Mayrhofer et al., 1999) also identified a similar structure in the rat and mouse small intestines, which they named lymphocyte-filled villi (LFV), and look upon LFV as a diffuse "gut thymus" that is the source of the population of TI T cells in these animals.

Given that CP are indispensable for maturation of TI IEL in gut mucosa, development of CD8αα$^+$ IEL must be selectively hampered in mice that lack CP. We (Oida et al., 2000) verified that the small intestine of male mice with a truncated mutation of the common cytokine receptor γ chain (CRγ$^{-/Y}$) (Ohbo et al., 1996) was devoid of CP and did not contain CD8αα$^+$ αβ and γδ IEL but did contain a small number of thymus-dependent (TD) CD4$^+$ and CD8αβ$^+$ αβ IEL. These TD αβ IEL subsets disappeared from the IEL compartment of athymic CRγ$^{-/Y}$ mice, leaving a small population of TCR$^-$ IEL that included cells expressing c-kit, Thy-1, B220, CD4,

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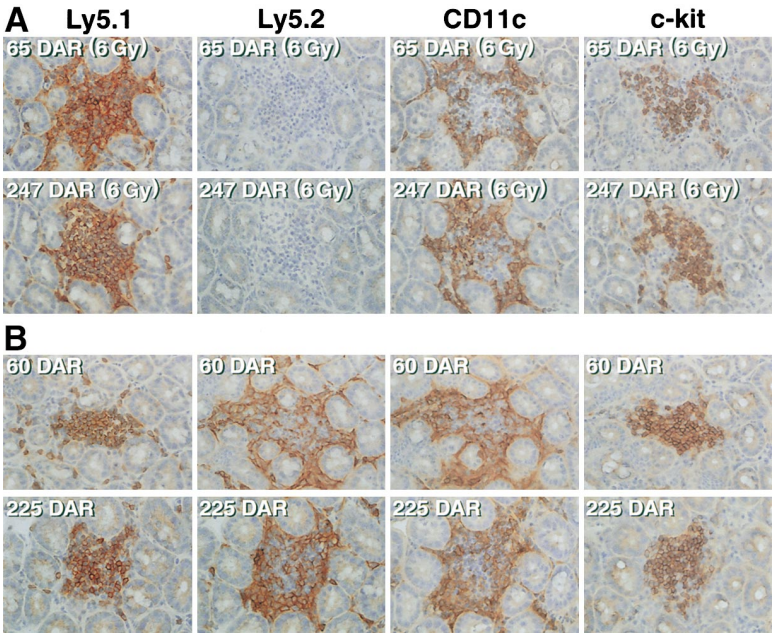

Figure 1. Origins of Dendritic CD11c$^+$ and Lymphoid c-kit$^+$ CP Cells Regenerated in Irradiated and Unirradiated BM Chimeric Mice

T cell–depleted BM cells from male B6 Ly5.1 mice were injected intraveneously into CR$\gamma^{-/Y}$ B6 Ly5.2 recipient mice that lacked CP, and regenerating duodenal, jejunal, and ileal CP on the consecutive tissue sections were examined by immunohistochemistry. Representative jejunal CP on the indicated days after reconstitution (DAR) are shown (×400). (A) CR$\gamma^{-/Y}$ B6 Ly5.2 recipient mice were irradiated 8 hr before reconstitution by BM cells. Note that both CD11c$^+$ and c-kit$^+$ cells in the regenerated CP are cells of donor BM origin. (B) CR$\gamma^{-/Y}$ B6 Ly5.2 recipient mice were unirradiated. Note that, in the regenerated CP, CD11c$^+$ cells are cells of host origin, whereas c-kit$^+$ cells are cells of donor BM origin.

and/or CD8$\alpha\beta$ but not CD8$\alpha\alpha$ molecules (Oida et al., 2000). A comparative study of TCR$^-$ IEL from athymic CR$\gamma^{-/Y}$ mice not possessing CP and those from athymic SCID mice possessing CP illuminated the key role of gut CP in the early extrathymic maturation of CD8$\alpha\alpha^+$ IEL, including cell surface expression of $\alpha_E\beta_7$ integrin, CD3ϵ gene transcription, and TCR gene rearrangements (Oida et al., 2000).

In view of these findings, we realized that it is important to confirm the restoration of CP and the subsequent appearance of IEL in villous epithelia overlying the regenerated CP by transplantation of WT bone marrow (BM) cells into athymic CR$\gamma^{-/Y}$ mice. Since it is also important to obtain definitive evidence that T lineage–committed precursors are present in CP, we took a closer look at c-kit$^+$Lin$^-$ CP cells of WT mice at the cellular and subcellular levels in the present study. Our results confirmed that the developmental pathway of TI IEL in the mouse gut mucosa is BM-derived IEL progenitor cells → c-kit^{high}TCR$^-$ CP cells → TCR$^-$ IEL → TCR$^+$ IEL. They also revealed that the conversion from TCR$^-$ to TCR$^+$ IEL, the final step, is unexpectedly slow.

Results

Reconstitution of the Intestinal CP and IEL in CR$\gamma^{-/Y}$ Mutant Mice with BM Cells

Our previous study (Oida et al., 2000) demonstrated that CR$\gamma^{-/Y}$ mice lacked Peyer's patches (PP), CP, $\gamma\delta$ IEL, and TI $\alpha\beta$ IEL. With these findings in mind, we aimed at reconstituting the missing gut-associated lymphoid tissues (GALT) by transplantation of T cell–depleted BM cells from WT Ly5.1 mice into 6 Gy-irradiated and unirradiated CR$\gamma^{-/Y}$ Ly5.2 mice. As a result, CP and all TI/TD $\alpha\beta$ and TI $\gamma\delta$ IEL subsets but not PP developed efficiently and reached absolute peaks almost equivalent to those of B6 mice by 60 days after reconstitution (DAR) (data not shown).

With respect to the origin of dendritic CD11c$^+$ CP cells (Kanamori et al., 1996) in the regenerated CP, however, the difference between irradiated and unirradiated CR$\gamma^{-/Y}$ recipients was striking (Figure 1). Judging by the expression of Ly5.1 and Ly5.2 alloantigens over 200 DAR, c-kit$^+$ CP cells in two types of BM chimera and CD11c$^+$ CP cells in 6 Gy-irradiated BM chimera were cells of donor BM origin. In sharp contrast, CD11c$^+$ CP cells in unirradiated BM chimera were entirely cells of mutant host origin.

Four Distinctive Cellular Events in the Reconstitution of TI IEL in BM Chimeric Mice

In an attempt to perform a more comprehensive time course study on the reconstitution of CP and TI IEL in the absence of thymus and thymus-derived cells, we produced athymic CRγ mutant mice and transplanted T cell–depleted WT Ly5.1$^+$ BM cells into 6 Gy-irradiated athymic CR$\gamma^{-/Y}$ mice. As shown in Figures 2A and 2B, a small number of tiny clusters filled with BM-derived Ly5.1$^+$ cells are first detected at 7 DAR in the small intestinal crypt LP of athymic CR$\gamma^{-/Y}$ hosts. At this time, the majority of cells residing in these clusters expressed CD11c molecules and displayed a dendritic profile (Figure 2B). Thereafter, a rapid and marked increase of these clusters both in number and size was observed (Figures 2A and 2B), and with this increase, the accumulation of BM-derived c-kit$^+$ cells started (Figure 2B). These c-kit$^+$ cells also expressed IL-7 receptor α chain (data not shown). During 10 to 15 DAR, reconstruction of clusters structurally and phenotypically comparable to those of athymic nude mice was completed, and, as verified in the preceding experiment (Figure 1A), double immunofluorescence analysis confirmed that CD11c$^+$ CP cells in the regenerated CP were cells of donor BM origin (Figure 2C).

Next, we investigated when and where BM-derived IEL appear in the villous epithelia of athymic CR$\gamma^{-/Y}$

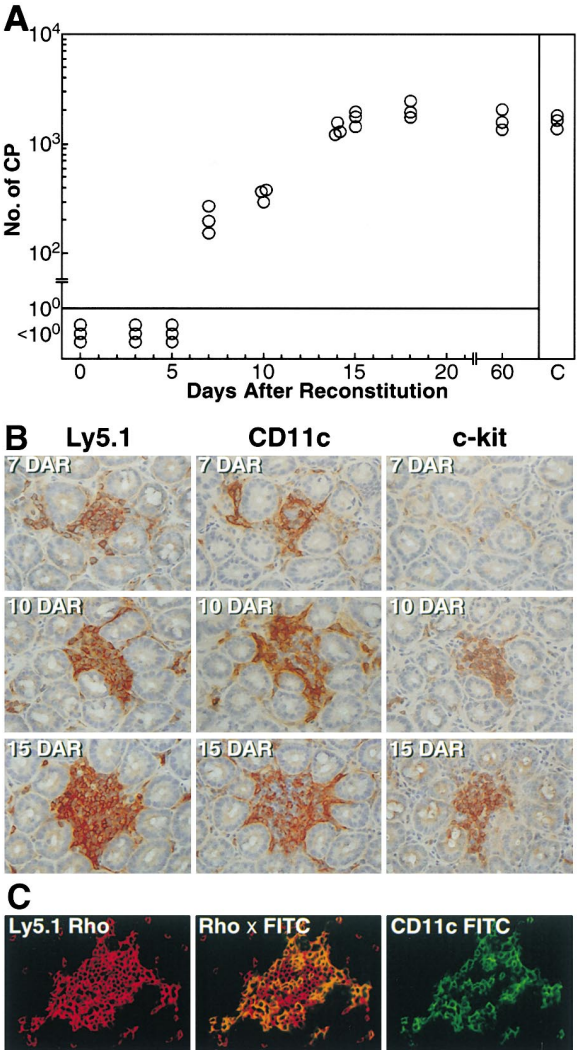

Figure 2. Neogenesis of CP in Irradiated Athymic CR$\gamma^{-/Y}$ Ly5.2 Mice that Received Ly5.1$^+$ BM Cells

T cell–depleted BM cells from male nu/+ (B6 × B/c)F1 (Ly5.1/2, H-2$^{b/d}$) mice were intraveneously injected into six Gy-irradiated athymic CR$\gamma^{-/Y}$ Ly5.2 recipient mice, and regenerating CP in the small intestines were enumerated and characterized by immunohistochemistry.

(A) The number of CP filled with BM-derived cells in the posttransplantation time frame was enumerated. We examined the entire small intestine from three recipient mice per analysis. C represents the number of CP in the small intestine of three control athymic (nu/nu) mice carrying heterozygous Ly5 (Ly5.1/2) alloantigens.

(B) Immunohistochemical characterization of regenerating CP on the consecutive tissue sections at 7, 10, and 15 DAR (×400). On 7 DAR, a small number of tiny clusters filled with BM-derived Ly5.1$^+$ cells that include CD11$^+$ but not c-kit$^+$ cells was detected. However, on 10 and 15 DAR, BM-derived nonoverlapping CD11$^+$ and c-kit$^+$ cell populations were detected in these clusters, i.e., CP. The tissue sections from control athymic CR$\gamma^{-/Y}$ Ly5.2 mice were devoid of cells stained with anti-Ly5.1 mAb (not shown).

(C) Anti-Ly5.1/anti-CD11c double immunofluorescence analysis of the regenerated CP on 60 DAR (×400). Dendritic CD11c$^+$ cells (green; right panel) are cells of donor BM origin (yellow; middle panel).

hosts. During 7 to 11 DAR, six to eight arbitrarily chosen sections had to be examined before we identified one CP filled with BM-derived c-kit$^+$ cells, indicating that the regenerated CP were few in number. At 14 DAR, however, CP filled with c-kit$^+$ cells became detectable at an average of two CP per section. Many thousand such sections were examined, and it was confirmed that IEL expressing Ly5.1 alloantigen tended to be located within the epithelial layer of villi around the regenerated CP (Figure 3, top left panel). Intriguingly, Ly5.1$^+$ cells were also detected in LP toward the tips of villi, whereas Ly5.1$^+$c-kit$^+$ cells constituted the distinctive population that occupied the regenerated CP (Figure 3, top panels). During 14 to 17 DAR, these Ly5.1$^+$ IEL rapidly repopulated all of the epithelial layers, but few if any mature TCR$^+$ (CD3$^+$) IEL were detected at this posttransplantation period (Figure 3, middle panels). Thereafter, not only Ly5.1$^+$ but also TCR$^+$ IEL increased gradually, and at 60 DAR, numerous BM-derived T cells were regenerated (Figure 3, bottom right panel).

Taking all of these results together, thymus-independent development of IEL in these radiation BM chimeric animals proceeds through at least four successive steps. The first is the appearance of CD11c$^+$ dendritic cell (DC) clusters at 7 DAR. The second step is characterized by the accumulation of c-kit$^+$ cells in these regenerating CP during 7 to 14 DAR as the number of CP increases. In the third step, the first significant appearance of BM-derived TCR$^-$ IEL at 14 DAR in the epithelial layers overlying the villi that contain regenerated CP is followed by the emergence of such TCR$^-$ IEL along the length of the small intestine on 17 DAR. Thereafter, conversion of TCR$^-$ to TCR$^+$ IEL, the final step, takes place very slowly until 60 DAR.

Flow Cytometric Analysis of Regenerating IEL in Radiation BM Chimeric Mice

To characterize the maturing IEL more precisely, we performed flow cytometric analysis on IEL isolated from these athymic radiation chimeras, and results are presented in Figure 4A. About 10^4 BM-derived Ly5.1$^+$ IEL were detected as early as 3 DAR. These Ly5.1$^+$ IEL increased exponentially up to 20 DAR and gradually thereafter, reaching a level equivalent to the number of IEL in control athymic mice at 40 DAR. It was extremely difficult to detect Ly5.1$^+$ IEL by immunohistochemistry before 10 DAR, but flow cytometric analysis showed about 10^5 Ly5.1$^+$ IEL on 10 DAR. The cause of this discrepancy between these two different techniques is most likely the much lower detection sensitivity of immunohistochemistry. The appearance of BM-derived CD8$\alpha\alpha^+$ and TCR$^+$ IEL was slower than that of BM-derived Ly5.1$^+$ IEL. For instance, at 14 DAR, CD8$\alpha\alpha^+$ IEL accounted for 17% and TCR$^+$ IEL for only 1.7% of Ly5.1$^+$ IEL (Figure 4A). The same results were obtained with immunohistochemistry, and almost no, if any, TCR$^+$ (CD3$^+$) IEL were identified at 14 to 17 DAR when detection of Ly5.1$^+$ was possible (Figure 3). However, by 40 to 60 DAR it was evident that absolute numbers of CD8$\alpha\alpha^+$ and TCR$^+$ IEL had reached or even exceeded the numbers in control athymic mice (Figure 4A).

At 60 DAR, donor BM cells efficiently generated T cells in the intestinal epithelium and gave rise to substantial

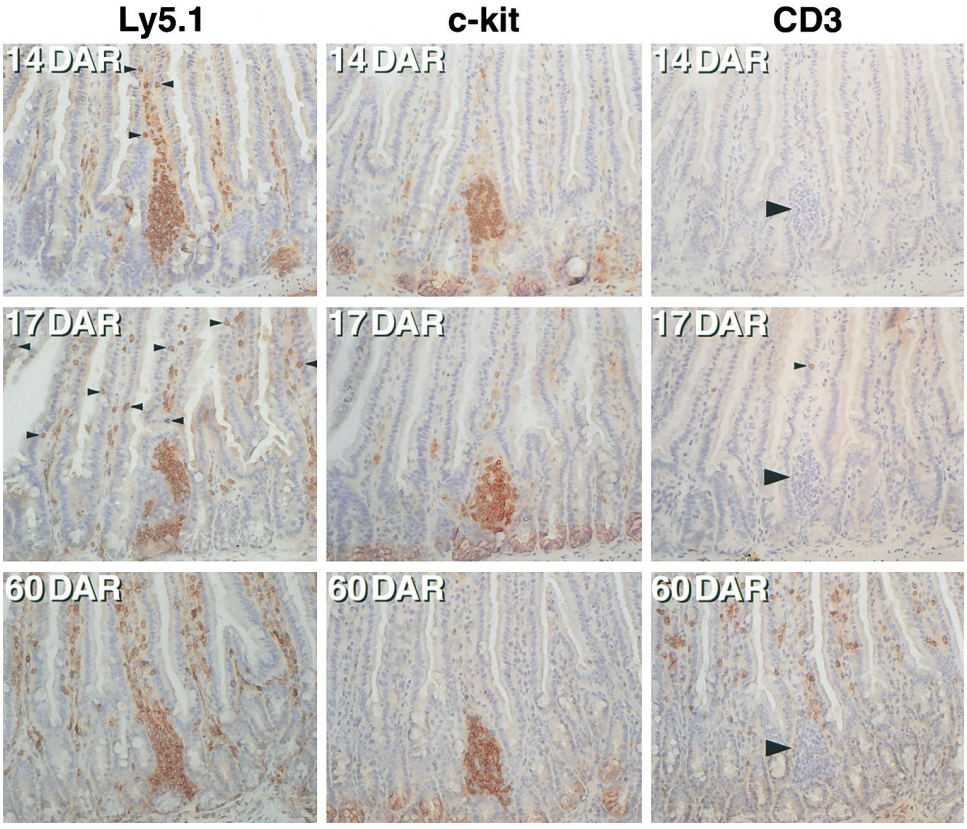

Figure 3. Reconstitution of IEL with Donor Ly5.1$^+$ BM Cells in Relation to the Regenerating CP

T cell–depleted BM cells from male nu/+ (B6 × B/c)F1 (Ly5.1/2, H-2$^{b/d}$) mice were injected intraveneously into 6 Gy-irradiated athymic CR$\gamma^{-/Y}$ Ly5.2 recipient mice, and the development of BM-derived IEL in relation to CP neogenesis was determined on consecutive tissue sections of recipient small intestines by immunohistochemistry. We examined the entire small intestine from 14 (14 DAR), 10 (17 DAR), and 3 (60 DAR) recipient mice, respectively, and the representative jejunal pictures are shown (×200). Small arrowheads indicate IEL and large arrowheads indicate CP. Note that on 14 DAR, BM-derived Ly5.1$^+$c-kit$^-$ IEL and also Ly5.1$^+$c-kit$^-$ LPL predominate in a villus overlying a regenerated CP filled with Ly5.1$^+$c-kit$^+$ cells (top panels), and these BM-derived Ly5.1$^+$c-kit$^-$ IEL colonize all of the epithelial layers on 17 DAR, but most of them still do not express CD3 (TCR) (middle panels). Eventually, numerous CD3$^+$ (TCR$^+$) IEL are generated in the epithelial and LP compartments of the recipient mice on 60 DAR (bottom panels).

B220$^+$ B cells in the mesenteric lymph node (MLN) and splenic compartments of these athymic radiation chimera, whereas they were incapable of reconstituting a meaningful number of T cells in any other lymphoid tissues of the recipient such as MLN and spleen (Figure 4B). These results support the contention that neither T lineage–committed CP cells nor TI IEL circulate to parts of the body other than the intestinal mucosa.

Finally, we addressed whether the missing GALT are regenerated from WT fetal liver cells in which thymus-derived T cells are absent (Lefrancois and Olson, 1997). Immunohistochemical and flow cytometric analyses performed at 7, 14, and 20 DAR (three to four mice per analysis) revealed that CP and IEL but not PP developed efficiently in 6 Gy-irradiated and fetal liver reconstituted athymic CR$\gamma^{-/Y}$ mice. These findings, in conjunction with the failure of BM cells to regenerate T cells in the MLN and splenic compartments of athymic CR$\gamma^{-/Y}$ recipients (Figure 4B), make the possibility that a minimum number of residual T cells present in anti-Thy-1.2 mAb and C-treated BM inoculum are responsible for the restoration of CP and IEL highly unlikely.

Phenotypic Analysis of BM-Derived IEL in Irradiated/Unirradiated Athymic CR$\gamma^{-/Y}$ Mice and in Irradiated Euthymic CR$\gamma^{-/Y}$ Mice

The data presented in the preceding sections indicate that proportional repopulation of gut epithelium by BM-derived IEL is nearly complete. In an attempt to explore this issue in more detail, generation of BM-derived TCR$\alpha\beta^+$, TCR$\gamma\delta^+$, CD4$^+$, CD8$\alpha\alpha^+$, and CD8$\alpha\beta^+$ IEL in irradiated/unirradiated athymic CR$\gamma^{-/Y}$ mice as well as in irradiated euthymic CR$\gamma^{-/Y}$ mice in the posttransplantation time frame was quantitated (Table 1). In these three types of chimeric animals, the extent of BM-derived IEL reconstitution after 60 DAR was extremely efficient, i.e., >98% in irradiated athymic hosts, >89% in unirradiated athymic hosts, and >98% in irradiated euthymic CR$\gamma^{-/Y}$ hosts, respectively, and $\gamma\delta$ IEL predominated over $\alpha\beta$ IEL. Absolute numbers of BM-derived IEL and the population size of TCR$\alpha\beta$, TCR$\gamma\delta$, CD4, CD8$\alpha\alpha$, and CD8$\alpha\beta$ expressing IEL subsets regenerated after 60 DAR in the small intestinal epithelia of irradiated and unirradiated athymic CR$\gamma^{-/Y}$ hosts ranged from comparable to slightly different although the differences were statistically insignificant. By contrast, when the

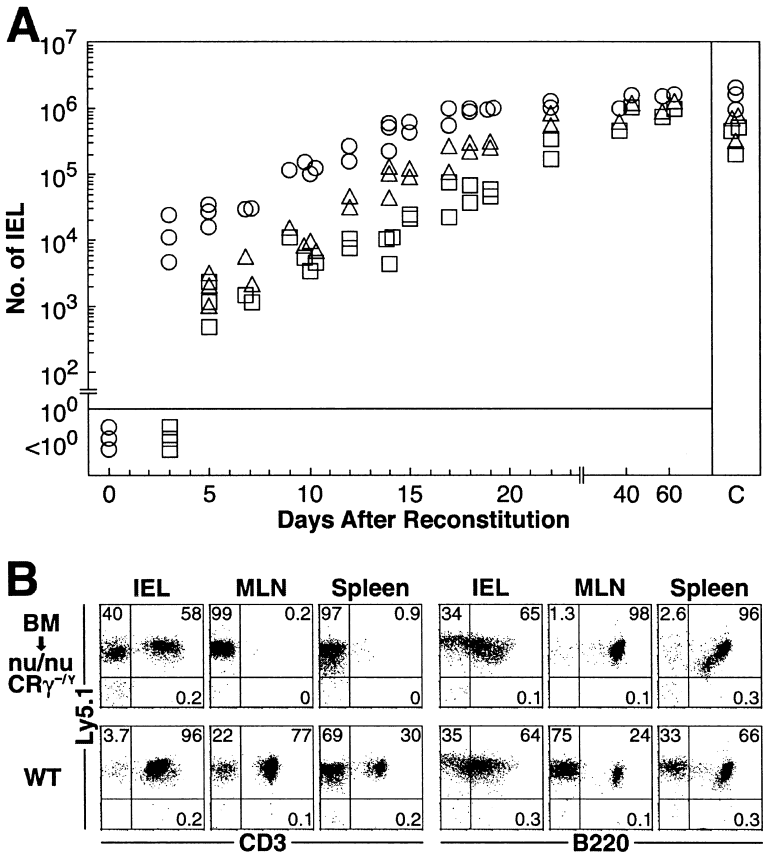

Figure 4. Flow Cytometric Analysis of Regenerating IEL after Reconstitution with BM Cells

T cell–depleted BM cells from male nu/+ (B6 × B/c)F1 (Ly5.1/2, H-$2^{b/d}$) mice were injected intraveneously into six Gy-irradiated athymic CR$\gamma^{-/Y}$ Ly5.2 recipient mice, and two-color flow cytometric analysis was performed on IEL isolated from the recipient small intestines.

(A) Generation of BM-derived Ly5.1$^+$ (open circles), Ly5.1$^+$CD8$\alpha\alpha^+$ (open triangles), and Ly5.1$^+$CD3$^+$ (open squares) IEL in the small intestinal epithelia of the recipient mice (two to three animals per analysis). IEL isolated from recipient mice on the indicated DAR and from three age-matched control athymic (nu/nu) mice carrying heterozygous Ly5 (Ly5.1/2) alloantigens (C) were examined. Absolute numbers of Ly5.1$^+$ and Ly5.1$^+$CD3$^+$ IEL subsets were calculated on the basis of total numbers of lymphoid cells recovered, and those of Ly5.1$^+$CD8$\alpha\alpha^+$ subset were calculated from (numbers of Ly5.1$^+$CD8α^+ IEL − numbers of Ly5.1$^+$CD8β^+ IEL).

(B) On 60 DAR, IEL, MLN cells, and spleen cells isolated from recipient mice and from age-matched control nu/+ (B6 × B/c)F1 (Ly5.1/2, H-$2^{b/d}$) mice were analyzed. Note that most of BM-derived lymphoid cells repopulated in the MLN and splenic compartments are B cells, whereas, in contrast to a marked generation of IEL, few if any BM-derived T cells are generated in these anatomical sites. A substantial fraction of IEL are B220 dull positive, as reported elsewhere (Oida et al., 2000), but they are cell surface IgM$^-$ (not shown).

reconstitution of BM-derived IEL in irradiated euthymic CR$\gamma^{-/Y}$ mice was compared after 60 DAR with that in irradiated/unirradiated athymic CR$\gamma^{-/Y}$ mice, the population size of donor IEL was about 2-fold larger in euthymic hosts. Second, on average, there were 3-fold more $\alpha\beta$ IEL and 2-fold more $\gamma\delta$ IEL in euthymic hosts. Third, absolute numbers of TI CD8$\alpha\alpha$ IEL and TD CD4 and TD CD8$\alpha\beta$ IEL in euthymic hosts were 1.5-fold, 2-fold, and 10-fold those in athymic hosts, respectively. Judging by the expression of TCR, most CD4 and CD8$\alpha\beta$ IEL recovered after 60 DAR from irradiated/unirradiated athymic CR$\gamma^{-/Y}$ hosts expressed TCR$\alpha\beta$, whereas, among CD8$\alpha\alpha$ IEL recovered from these chimeras, 65%–80% expressed TCR$\gamma\delta$, 4%–12% expressed TCR$\alpha\beta$, and 13%–25% were TCR$^-$ (data not shown). These findings are in line with the results of previous transplantation studies (Rocha et al., 1994; Lefrancois and Olson, 1997) in which the requirement of the thymus and/or thymus-derived T cells for the reconstitution of a large number of CD4$^+$ and CD8$\alpha\beta^+$ $\alpha\beta$ IEL was verified and, in addition, confirm the role of thymus in the expansion TI $\gamma\delta$ IEL (Guy-Grand et al., 1991; Lin et al., 1993; Lefrancois and Olson, 1994; Wang and Klein, 1994). However, the fact that a significant number of BM-derived CD4$^+$ and CD8$\alpha\beta^+$ $\alpha\beta$ IEL was invariably recovered from athymic CR$\gamma^{-/Y}$ recipient mice with or without irradiation (Table 1) is inconsistent with what was observed in the reconstitution models employing thymectomized and 4 Gy-irradiated *RAG*$^{-/-}$ mice (Rocha et al., 1994) and thymectomized and unirradiated c-kit mutant *W/W*^{v} mice (Lefrancois and Olson, 1997). In fact, these model experiments have demonstrated that the thymus is obligatory for the development of CD4$^+$, CD8$\alpha\beta^+$, and CD4$^+$CD8$\alpha\alpha^+$ $\alpha\beta$ IEL.

Only CP Contain a Large Population of c-kit^{high} T Lineage–Committed Precursor Cells among GALT of Wild-Type Mice

We isolated fragments of the small intestine containing one CP (CP$^+$) and those containing no CP (CP$^-$) from WT BALB/c (B/c) mice using an amputated and tapered 21 G needle (Figure 5A). Among cells that had been squeezed out of CP$^+$, CP$^-$, PP, and MLN tissues using fine stainless steel mesh examined, a large population (30%) of c-kit$^{high}\alpha_E\beta_7^-$Lin$^-$ (c-kit^{high}) was detected only in cells from CP$^+$ (Figure 5B). It was also evident that cells from CP$^+$, CP$^-$, and IEL but not PP and MLN included c-kit^{low} and $\alpha_E\beta_7^+$ and/or Lin$^+$ lymphocytes (Figure 5B). As shown in Figure 5C, these c-kit^{high} CP cells were found to express IL-7R and CD44 and contained Thy-$1^{+/-}$, CD4$^{+/-}$, and/or CD25$^{low/-}$ (Figure 5D, top panel) lymphocytes. A fragment having one CP looked like a fried egg, sunny-side up (Figure 5A), i.e., CP (the yolk) was surrounded by other intestinal mucosal tissues (the white). Although considerable time was required, isolation of cells from the yolk one by one with the aid of microsurgical pincettes in order to minimize cells from

Table 1. Characterization of IEL from BM Chimeric Mice[a]

Host	Host Number	Irradiation (6 Gy)	Days after Reconstitution	BM-Derived Ly5.1$^+$ IEL		Number of Cells in Various BM-Derived IEL Subsets ($\times 10^4$)							
				%	Number of Cells ($\times 10^4$)	TCR-$\alpha\beta$		TCR-$\gamma\delta$		CD4		CD8$\alpha\alpha$		CD8$\alpha\beta$	
	1	+	22	95	99.0	0.59		14.9		2.48		52.5		1.29	
	2	+	22	97	107	1.28		25.7		2.03		64.2		0.96	
	3	+	22	99	119	2.62		34.5		2.62		84.5		1.07	
	4	+	35	98	157	7.22		58.1		1.57		92.6		1.26	
	5	+	35	98	98.0	1.67		40.2		2.94		55.9		1.96	
	6	+	60	99	139	16.7		54.2		11.8		80.6		2.92	
	7	+	60	98	172	9.98		96.3		6.19		119		2.41	
nu/nu	8	+	83	99	168	20.2	23.3 ± 16.0	104	97.4 ± 33.3	12.9	11.7 ± 4.06	116	125 ± 42.6	7.56	4.94 ± 2.65
CR$\gamma^{-/Y}$	9	+	83	99	221	46.4		135		15.9		183		6.85	
Ly5.2	10	−	25	62	62.0	4.15		14.3		1.36		31.6		0.50	
	11	−	25	53	74.2	8.16		11.1		2.23		28.2		0.59	
	12	−	35	73	80.3	12.8		45.0		2.57		62.6		0.64	
	13	−	46	86	73.1	17.5		30.0		3.14		54.8		1.46	
	14	−	46	87	120	36.0		56.4		3.00		92.4		1.44	
	15	−	60	90	126	17.6		84.4		8.06		111		0.88	
	16	−	60	97	233	17.5		163		5.83		186		5.13	
	17	−	77	89	134	20.1	22.3 ± 7.89	83.1	114 ± 38.2	6.97	7.32 ± 1.16	96.5	139 ± 42.5	1.47	3.07 ± 2.21
	18	−	83	95	200	34.0		126		8.40		164		4.80	
	1	+	35	99	218	63.2		78.5		6.54		129		26.2	
nu/+	2	+	60	99	351	91.3		214		23.2		228		45.6	
CR$\gamma^{-/Y}$	3	+	60	98	258	41.3		165		13.7		181		28.4	
Ly5.2	4	+	83	98	245	71.1	78.7 ± 29.8	169	202 ± 45.0	19.6	19.1 ± 3.95	176	220 ± 54.3	36.8	39.6 ± 8.79
	5	+	83	99	396	111		261		19.8		293		47.5	

[a] Two-color flow cytometric analyses were performed on IEL isolated from various BM chimeric mice. IEL were incubated first with biotinylated anti-Ly5.1 mAb and then with streptavidin-PE and FITC-conjugated anti-TCR-$\alpha\beta$, anti-TCR-$\gamma\delta$, anti-CD4, anti-CD8α, or anti-CD8β mAb. Absolute numbers of Ly5.1$^+$ IEL were calculated on the basis of total numbers of IEL recovered, and those of BM-derived $\alpha\beta$, $\gamma\delta$, CD4, and CD8$\alpha\beta$ IEL subsets were calculated on the basis of total numbers of Ly5.1$^+$ IEL. Absolute numbers of CD8$\alpha\alpha$ IEL were calculated from (numbers of Ly5.1$^+$CD8α^+ IEL − numbers of Ly5.1$^+$CD8β^+ IEL). Dead cells were excluded by PI gating.

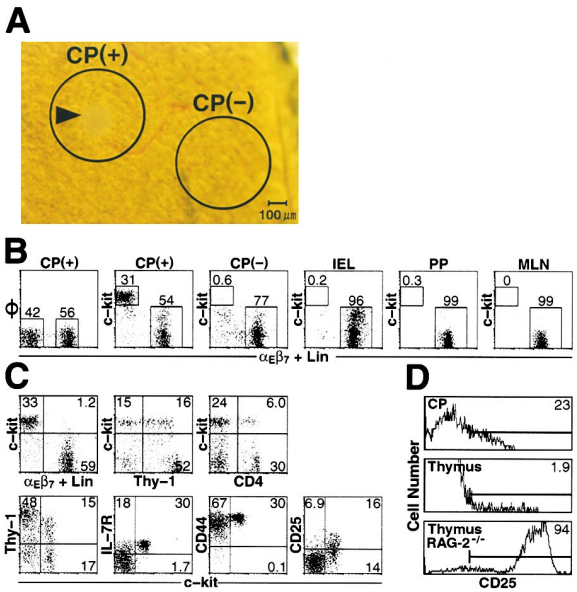

Figure 5. Isolation of Lymphoid Cells from the Small Intestinal Tissue Fragments Containing (CP^+) and Not Containing CP (CP^-), and Flow Cytometric Analysis of Lymphocytes that Localize in Various GALT of Adult B/c Mice

(A) Stereomicroscopic view of the small intestinal mucosa. Circles indicate the CP^+ and CP^- regions to be extracted with the aid of an amputated and tapered 21 needle (inner diameter, 570 μm).

(B) Flow cytometric profiles of CP^+ cells (735 ± 175 per fragment), CP^- cells (80.1 ± 29.5 per fragment), IEL, PP cells, and MLN cells. Lin is the abbreviation of lineage markers (lineage markers; CD3, B220, Mac-1, Gr-1, and TER119). Note that only CP^+ cells contain a large population of c-$kit^{high}\alpha_E\beta_7^-Lin^-$ (c-kit^{high}) lymphocytes.

(C) c-kit^{high} CP cells are IL-$7R^+CD44^+Thy\text{-}1^{+/-}CD4^{+/-}CD25^{low/-}$.

(D) Expression of CD25 by CP cells (B/c mice), thymocytes (B/c mice), and thymocytes from *RAG-2*$^{-/-}$ mice. Note that a quarter of CP cells express low levels of CD25.

the white gave us a lymphocyte preparation in which the population of c-kit^{high} subset reached ~60%.

To determine whether T lineage–committed precursors are present in c-kit^{high} CP cell population, we purified c-kit^{high} CP cells (purity, 99.6%) and c-$kit^{low/-}$ and $\alpha_E\beta_7^+$ and/or Lin^+ (c-$kit^{low/-}$) CP cells (purity, 99.7%) by flow cytometry (Figure 6A) and examined whether these cell populations express CD3ϵ- and pre-Tα-specific mRNA. CD3ϵ transcripts were found in c-kit^{high} CP cells, although at levels 5-fold below those found in c-$kit^{low/-}$ CP cells, IEL, MLN cells, and thymocytes, and in contrast to IEL and sorted c-$kit^{low/-}$ CP cells, sorted c-kit^{high} CP cells had no or drastically reduced pre-Tα transcripts (Figure 6B).

The fact that c-kit^{high} CP cells are $CD44^+CD25^{low/-}$ and have CD3ϵ but no detectable pre-Tα transcripts offers a very close analogy with triple negative (TN; $CD3^-4^-8^-$) c-$kit^+CD44^+CD25^-$ thymocytes and/or TN thymocytes at an early stage of c-$kit^+CD44^+CD25^-$ to c-$kit^+CD44^+CD25^+$ transition. In this context, it is important to examine the status of TCR genes in the c-kit^{high} CP cells, since TCR genes retain their germline configuration in c-$kit^+CD44^+CD25^-$ and c-$kit^+CD44^+CD25^+$ thymocytes (Godfrey and Zlotnik, 1993; Godfrey et al., 1994; Hozumi et al., 1996; Zuniga-Pflucker and Lenardo, 1996; Shortman et al., 1998). For this purpose, we determined germline as well as rearranged TCRγ and -β gene transcripts in sorted c-kit^{high} CP cells by semiquantitative RT–PCR analysis (Figure 6B). First, the germline Jγ1-Cγ1 and/or Jγ2-Cγ2 bands were detected in c-kit^{high} CP cells and thymocytes but were not detected in c-$kit^{low/-}$ CP cells, IEL, and MLN cells. In accordance with previous findings (Takagaki et al., 1989), rearranged Vγ7-Cγ1 transcripts were abundant in cells from c-$kit^{low/-}$ CP and IEL compartments, whereas the same transcripts were undetectable or very few in cells from c-kit^{high} CP, MLN, and thymus compartments. These results indicate that c-kit^{high} CP cells include cells expressing germline TCRγ transcripts that do not rearrange the genes encoding TCRγ chain. Second, a considerable amount of TCR-Cβ2-specific mRNA was similarly expressed among cells from all compartments. In contrast, mRNA from c-kit^{high} CP cells failed to display any detectable signals for rearranged Vβ8.1/2-Cβ1/2 transcripts, whereas mRNA from c-$kit^{low/-}$ CP cells, IEL, MLN cells, and thymocytes displayed a comparable level of strong signals. During the development of TN thymocytes, TCRγ and TCRβ rearrangements occur simultaneously (Godfrey and Zlotnik, 1993; Godfrey et al., 1994; Zuniga-Pflucker and Lenardo, 1996; Shortman et al., 1998), or rearrangement at the TCRγ and -δ loci may precede that at the TCRβ locus (Haars et al., 1986; Shortman et al., 1998), and a high level of c-kit expression defines TN thymocytes that have not yet rearranged their TCR genes (Godfrey et al., 1994). Thus, the present findings, in conjunction with the progression of TCR gene rearrangements in TN thymocytes, lead us to conclude that, like TCRγ transcripts, TCR-Cβ2 mRNA detected in c-kit^{high} CP cells are also germline TCRβ transcripts.

Discussion

We studied CP histogenesis and appearance of IEL with time by transplantation of T cell–depleted BM cells into athymic $CR\gamma^{-/Y}$ mice that lack thymus, PP, CP, and TCR^+ IEL. The results showed that BM-derived $CD11c^+$ DC aggregates, namely CP anlage, were first detected in the crypt LP of 6 Gy-irradiated athymic $CR\gamma^{-/Y}$ mice, but at this stage, very few c-kit^+ cells were present in these DC aggregates. After BM-derived c-kit^+ cells accumulated in the CP anlage, BM-derived TCR^- IEL were detected in the epithelial cell layers of villi around the regenerated CP. Soon thereafter, these TCR^- IEL repopulated all of the epithelial cell layers, and with the rapid increase in this population, BM-derived αβ and γδ IEL developed.

CD11c/CD18 molecule is a member of the β2 integrin family (Metlay et al., 1990) expressed by DC in the mouse lymphoid tissues such as splenic white pulp (Metlay et al., 1990), but it appears not to be DC-specific integrin (Huleatt and Lefrancois, 1995). In mice, at least two $CD8^+$ lymphoid and $CD8^-$ myeloid DC are present (Vremec et al., 1992; Suss and Shortman, 1996). Since the development of erythromyeloid lineages has been reported to proceed normally in mice carrying null mutation at CRγ gene (Cao et al., 1995; DiSanto et al., 1995) and our immunohistochemical analysis failed to detect $CD8\alpha$ on $CD11c^+$ CP cells (Kanamori et al., 1996),

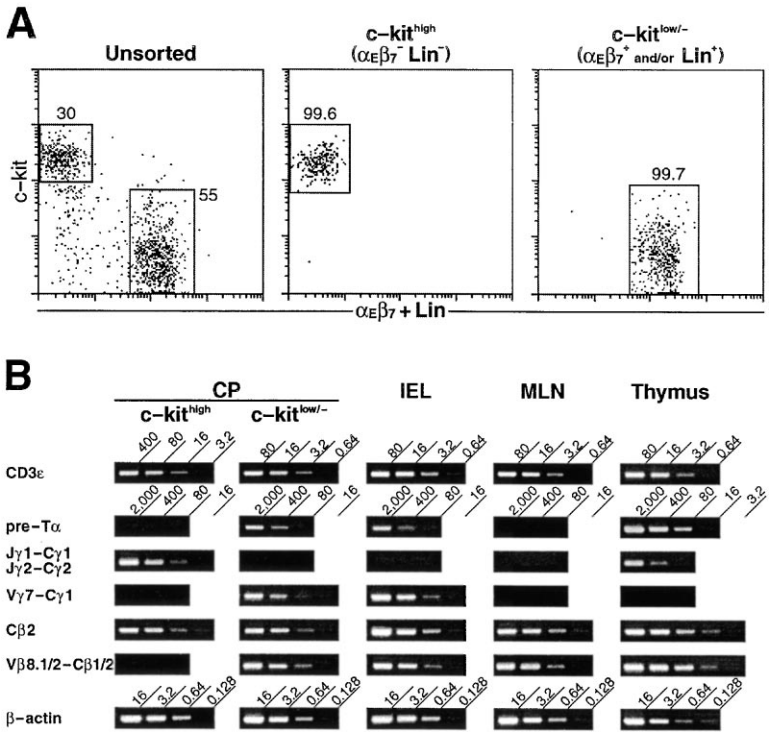

Figure 6. Semiquantitative RT–PCR Analysis of CD3ε, pre-Tα, Germline TCRγ, Rearranged TCR-Vγ7-Cγ1, TCR-Cβ, and Rearranged TCR-Vβ8.1/2-Cβ1/2 mRNA Levels in Sorted c-kit$^{high}\alpha_E\beta_7^-$Lin$^-$ (c-kit^{high}) CP Cells, Sorted c-kit$^{low/-}$ and $\alpha_E\beta_7^+$ and/or Lin$^+$ (c-kit$^{low/-}$) CP Cells, IEL, MLN Cells, and Thymocytes from B/c Mice

(A) Flow cytometric profiles of CP$^+$ cells before and after purification by FACS Vantage. (B) Serial 5-fold dilutions of RNAs equivalent to RNAs extracted from the indicated numbers of cells were reverse transcribed and the cDNA products PCR amplified. Although the signal is 5-fold reduced in c-kit^{high} CP cells, all cells express substantial levels of CD3ε gene. In contrast, while the signal for pre-Tα is detected in c-kit$^{low/-}$ CP cells, IEL, and thymus cells, the same signal is almost undetectable in c-kit^{high} CP and MLN cells. Germline Jγ1-Cγ1- and Jγ2-Cγ2-specific mRNA are detectable only in c-kit^{high} CP and thymus cells, whereas rearranged Vγ7-Cγ1-specific mRNA are detectable only in c-kit$^{low/-}$ CP cells and IEL. All cells express comparable levels of Cβ gene, but in contrast to the other cells, c-kit^{high} CP fail to express any detectable Vβ8.1/2-Cβ1/2-specific signal, indicating that the Cβ signal in c-kit^{high} CP cells is most likely from germline transcripts of TCRβ gene.

CD11c$^+$ DC in CP are most likely myeloid DC. In any case, the dysfunction of CD11c$^+$ DC is not the reason for the lack of CP in CRγ$^{-/Y}$ mice, because, in sharp contrast to c-kit$^+$ cells, CD11c$^+$ DC in the reconstituted CP were derived entirely from the mutant CRγ$^{-/Y}$ recipient mice in the case of unirradiated BM chimeric mice (Figure 1).

In contrast to the restoration of CP, PP remained defective in the present BM chimeric animals. Organogenesis of mouse PP involves three distinct steps in the late embryonic stage (Yoshida et al., 1999), whereas CP can still not be detected on day 10 after birth but gradually increase in number and size thereafter (Kanamori et al., 1996). All these things taken together, the formation of CP is basically different from that of PP, although they constitute organized murine GALT. Two additional and related points that merit consideration in the context of these findings are that PP and all peripheral LN are missing from mice with alymphopenia (*aly/aly*) mutation (Nanno et al., 1994) and also from mice deficient in lymphotoxin (*LT*$^{-/-}$) (De Togni et al., 1994). In fact, LT-mediated signal has been shown to be indispensable for the early phase of PP organogenesis (Yoshida et al., 1999). In sharp contrast, both *aly/aly* (Nanno et al., 1994; Kanamori et al., 1996) and *LT*$^{-/-}$ mice (unpublished observation) possess well-developed CP and αβ and γδ IEL, indicating that neither *aly/aly* mutation nor LT–receptor interactions are crucial for CP formation.

Immunohistochemical and flow cytometric analyses revealed that the maturation of TCR$^-$ IEL by conversion to CD8αα$^+$ and/or TCR$^+$ IEL was essentially a slow event. Many reports in the literature have proven that a few TCR$^-$ IEL are present in the IEL compartment of WT mice and that they differentiate to form TCR$^+$ IEL (Guy-Grand et al., 1991, 1992; Lin et al., 1994; Hamad et al., 1997; Mowat and Viney, 1997; Page et al., 1998). Moreover, in young mice (<5 weeks old), up to 25% of IEL are TCR$^-$ (Poussier and Julius, 1994), and as the number of IEL increases with age, the frequency of these cells decreases, but their absolute number remains constant throughout life. Thus, regeneration of IEL in the present BM chimeric animals recapitulated the ontogenic development of IEL in young mice (Guy-Grand et al., 1992; Lin et al., 1994; Poussier and Julius, 1994).

There are two noteworthy observations that we made in the present study using chimeric mice. First, after 60 days post cell transfer, a large fraction of BM-derived Ly5.1$^+$ IEL expressed CD8αα molecules in irradiated (~71%) and unirradiated (~80%) athymic CRγ$^{-/Y}$ mice as well as in irradiated (~70%) euthymic CRγ$^{-/Y}$ mice, whereas the composition of BM-derived CD8αβ$^+$ αβ IEL in irradiated (~2.8%) and unirradiated (~1.8%) athymic CRγ$^{-/Y}$ mice was significantly smaller than that in irradiated (~12.7%) euthymic CRγ$^{-/Y}$ mice (Table 1). These findings indicate that thymus and/or thymus-derived T cells are required for the generation of a major CD8αβ$^+$ αβ IEL and that a much reduced but consistent generation of CD8αβ$^+$ αβ IEL in the intestines of athymic CRγ$^{-/Y}$mice is not related to sublethal irradiation. Thus, although our data reinforce the thymus dependence of CD8αβ$^+$ αβ IEL generation (Rocha et al., 1994; Lefrancois and Olson, 1997), they are inconsistent with the results of the hematopoietic cell transplantation study in which marked TI CD8αα$^+$ IEL but virtually null TD CD8αβ$^+$ αβ IEL development in unirradiated thymectomized hosts was described (Lefrancois and Olson, 1997). The fact that a significant number of BM-derived CD4$^+$ αβ IEL was invariably recovered from athymic CRγ$^{-/Y}$ recipient mice with or without irradiation (Table 1) is also inconsistent with what is observed in the recon-

stitution models employing thymectomized and 4 Gy-irradiated *RAG*$^{-/-}$ mice (Rocha et al., 1994) and thymectomized and unirradiated c-kit mutant *W/W*^{v} mice (Lefrancois and Olson, 1997). In these model experiments, it has been demonstrated that the thymus is obligatory for the development of CD4$^+$, CD8$\alpha\beta^+$, and CD4$^+$CD8$\alpha\alpha^+$ $\alpha\beta$ IEL. The second point was the highly efficient reconstitution of IEL: as early as 35 DAR, almost 100% of IEL in irradiated athymic and euthymic CR$\gamma^{-/Y}$ mice and, during 60 to 83 DAR, 89% to 97% of IEL in unirradiated athymic CR$\gamma^{-/Y}$ mice were replaced with donor Ly5.1$^+$ IEL (Table 1). In an experimental system with no irradiation (Lefrancois and Olson, 1997) in which thymectomized c-kit (*W/W*^{v}) mutant mice were transplanted with fetal liver cells, the efficiency of fetal liver–derived IEL reconstitution at 6 months post cell transfer was not only incomplete but also variable. In this regard, the disruption of multiple cytokine signaling pathways, every one of which is important for lymphoid development and the absence of NK cell activity in the CRγ mutant condition (Cao et al., 1995; DiSanto et al., 1995) imparts WT precursor IEL with an outstanding growth advantage, and in consequence, nearly complete IEL replacement can be achieved in these CR$\gamma^{-/Y}$ recipient mice.

Finally, our results showed that a large population of c-kit^{high}IL-7R$^+$CD44$^+$Thy-1$^{+/-}$CD4$^{+/-}$CD25$^{low/-}$$\alpha_E\beta_7^-Lin^-$ lymphocytes was detected only in CP but not in the other GALT of B/c mice. It is evident that the characteristics of cell surface phenotype of c-kit^{high} CP cells are literally the same as those of most immature thymocytes (Godfrey and Zlotnik, 1993; Godfrey et al., 1994; Zuniga-Pflucker and Lenardo, 1996; Shortman et al., 1998). Compartmentalization of T lineage–committed precursors in the gut CP was also verified in the present study by showing that germline TCRγ and -β but not rearranged TCRγ and -β gene-specific mRNA were detected in c-kit^{high} CP cells. Also, although pre-Tα transcripts were hardly detectable, a substantial amount of CD3ϵ-specific mRNA was present in c-kit^{high} CP cells. It is well known that germline transcription of the TCR and immunoglobulin genes occurs immediately before V(D)J recombination of these genes, and this means that various DNA recombination enzymes are ready to approach these chromosomal segments to commence the region-specific recombinations (Alt et al., 1987; Hempel et al., 1998; Ye et al., 1999). The presence of both TCRγ and -β germline transcripts in the c-kit^{high} CP cell population, in conjunction with other observations made in the present and previous (Saito et al., 1998; Oida et al., 2000) studies, lends strong support to the notion that immature T cell progenitors that match the developmental stage of TN c-kit^{high}CD44$^+$CD25$^{low/-}$ thymocytes before pre-Tα gene transcription (Saint-Ruf et al., 1994; Wilson and MacDonald, 1995; Koyasu et al., 1997) but after expression of CD3ϵ-specific mRNA (Wilson and MacDonald, 1995; Wang et al., 1999) are present in gut CP. In conclusion, it is now evident that CP is a novel member of murine GALT that promotes the development of TI IEL progenitors, and exploration of hematopoietic progenitors for lympho-myeloid descendants other than TI IEL that might reside in gut CP is an important goal for future experiments.

Experimental Procedures

Mice

C57BL/6J Jcl (B6 Ly5.2, H-2^b), BALB/cA Jcl (B/c Ly5.2, H-2^d), and athymic (nu/nu) BALB/cA Jcl (nu/nu B/c Ly5.2, H-2^d) mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). C57BL/6-Ly5.1(B6 Ly5.1) mice were described elsewhere (Suzuki et al., 1998), and *RAG-2*$^{-/-}$ mice and heterozygous female mice carrying a truncated mutation of the common cytokine receptor γ gene (CR$\gamma^{-/X}$ B6 Ly5.2 mice) were also described previously (Oida et al., 2000). CR$\gamma^{-/X}$ mice were crossed with B6 mice, and male CRγ-deficient (CR$\gamma^{-/Y}$) offspring were typed by PCR analysis of tail DNA with a set of primers to the neomycin-resistance gene (Oida et al., 2000). B6 CR$\gamma^{-/X}$ mice were also crossed with athymic B/c mice, and their heterozygous CR$\gamma^{-/X}$ progeny were backcrossed to athymic B/c mice to produce euthymic (nu/+) mutant CR$\gamma^{-/Y}$ and athymic (nu/nu) mutant CR$\gamma^{-/Y}$ littermates. Although the genetic composition differed among individual littermate mice, it was confirmed that the difference was irrelevant to the distinctive cellular and phenotypic properties of lymphoid cells from the corresponding euthymic and athymic CR$\gamma^{-/Y}$ mice. To obtain Ly5 heterozygous donor BM cells, B6 Ly5.1 mice were crossed with athymic B/c Ly5.2 mice to produce nu/+ (B6 $\times$ B/c)F1 (Ly5.1/2, H-2$^{b/d}$) animals. These F1 mice were also backcrossed to athymic B/c Ly5.2 mice to produce control athymic (nu/nu) mice carrying heterozygous Ly5 (Ly5.1/2) alloantigens. All mice used for experiments were between 7 and 16 weeks of age, and absence of the thymus in various athymic mice was checked at necropsy.

Production of Chimeric Mice

We made BM chimeras according to the method described elsewhere (Fujiura et al., 1996). To eliminate T cells, 2 $\times$ 10^7 BM cells were incubated with anti-Thy-1.2 mAb HO-13 (Kubota et al., 1981) (final dilution, 1/250) and nontoxic guinea pig serum (final dilution, 1/10) as the C source in a total volume of 1 ml Hanks' balanced salt solution for 45 min at 37°C. This treatment lysed more than 99% thymocytes (data not shown). Fifteen to twenty million of the treated BM cells from male B6 Ly5.1 mice and male nu/+ (B6 $\times$ B/c)F1 (Ly5.1/2, H-2$^{b/d}$) mice were injected intraveneously into 6 Gy-irradiated/unirradiated CR$\gamma^{-/Y}$ B6 Ly5.2 mice and 6 Gy-irradiated/unirradiated athymic CR$\gamma^{-/Y}$ Ly5.2 or 6 Gy-irradiated euthymic CR$\gamma^{-/Y}$ Ly5.2 mice, respectively. Fifteen to twenty million of day 14 nu/+ (B6 $\times$ B/c)F1 (Ly5.1/2, H-2$^{b/d}$) fetal liver cells were also injected intraveneously into 6 Gy-irradiated athymic CR$\gamma^{-/Y}$ Ly5.2 mice.

Antibodies

The following mAbs, described elsewhere (Kanamori et al., 1996; Saito et al., 1998; Oida et al., 2000), were used. For immunohistochemical staining: anti-c-kit (ACK-2), anti-CD3 (145-2C11), anti-IL-7R (A7R34), and anti-CD11c (N418) mAbs and biotinylated anti-Ly5.2 (104) mAb. Biotinylated anti-Ly5.1 mAb (A20; PharMingen, San Diego, CA) was also employed in this study. For flow cytometric analysis: FITC-conjugated anti-CD3 (145-2C11), anti-TCR$\alpha\beta$ (H57-597), anti-TCR$\gamma\delta$ (GL3), anti-IgM (II/41), anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-CD8β (53-5.8), anti-B220 (RA3-6B2), anti-CD103 ($\alpha_E\beta_7$) (2E7), anti-Thy-1.2 (30-H12), and anti-c-kit (ACK-4) mAbs, and biotinylated anti-TCR$\alpha\beta$ (H57-597), anti-TCR$\gamma\delta$ (GL3), anti-CD44 (1M7), anti-Thy-1.2 (30-H12), anti-c-kit (ACK-4), and anti-IL-7R (A7R34) mAbs. FITC-conjugated anti-Mac-1 (M1/70; PharMingen), anti-Gr-1 (RB6-8C5; PharMingen), and anti-TER-119 (TER-119; a gift from Dr. Y. Katsura, Kyoto University, Kyoto, Japan) mAbs, and biotinylated anti-CD25 (7D4; PharMingen) and anti-Ly5.1 (A20; PharMingen) mAbs were also employed in this study.

Immunohistochemical Procedure

Longitudinally opened small intestine ~10 mm in length was either pasted on a filter paper for the horizontal section or rolled for the vertical section and then embedded in O. C. T. compound (Tissue-Tek, Miles Inc., Elkhart, IN) at −80°C. The tissue segments were sectioned with a cryostat at 6 μm and sections preincubated with Block-ace (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) to block nonspecific binding of mAbs. The sections were then incubated with rat or hamster mAb for 30 min at 37°C and rinsed three

times with PBS, followed by incubation with biotin-conjugated goat anti-rat IgG Ab (Cedarlane Laboratories Limited, Ontario, Canada) or with biotin-conjugated goat anti-hamster IgG (Vector Laboratories, Inc., Burlingame, CA). In staining with biotinylated anti-Ly5.1 and -Ly5.2 mAbs, the second biotin-conjugated anti-IgG Ab was not used. Subsequently, the sections were washed three times with PBS and then incubated with avidin-biotin peroxidase complexes (Vectastatin ABC kit, Vector Laboratories, Inc.). Histochemical color development was achieved by Vectastatin DAB (3,3′-diaminobenzidine) substrate kit (Vector Laboratories, Inc.) according to the manufacturer's instructions. Finally, the sections were counterstained with hematoxylin for microscopy. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 and 0.1% NaN_3 in distilled water for 10 min at room temperature. Tissue sections incubated either with isotype-matched normal rat IgG or with nonimmune hamster serum showed only minimal background staining.

Immunofluorescence Procedure

Cryostat tissue sections 6 μm thick were fixed in acetone for 10 min at room temperature, washed three times with PBS, and then pretreated with Block-ace. Subsequently, the sections were incubated with biotinylated anti-Ly5.1 mAb (A20; PharMingen) for 60 min at 4°C, followed by incubation with rhodamine-conjugated streptavidin (Molecular Probes Inc., Eugene, OR). The sections were then incubated with anti-CD11c mAb (N418) and counterstained with FITC-conjugated goat anti-hamster IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Finally, the sections were examined under a Fluorescence Microscope Axiovert 100 (Carl Zeiss, Inc., Jena, Germany) equipped with an image analysis system (Signal Analytics Co., Vienna, VA).

Flow Cytometry and Cell Sorting

IEL and CP cells were isolated according to the methods described elsewhere (Oida et al., 2000). Lymphoid cells were incubated first with biotinylated mAb and then with streptavidin-PE (Becton Dickinson, Sunnyvale, CA) and FITC-conjugated second mAb. Stained cells were suspended in Hanks' solution without phenol red containing 0.02% NaN_3, 2% heat-inactivated fetal bovine serum, and 0.5 μg/ml propidium iodide (PI) and analyzed using FACScan with LYSYSII software (Becton Dickinson). Dead cells were excluded by PI gating. Lymphoid cells were incubated with anti-Fcγ II/III mAb (2.4G2; PharMingen) before staining to block nonspecific binding of labeled mAbs to FcR. c-kit$^{high}\alpha_E\beta_7{}^-Lin^-$ (c-kit^{high}) CP cells and c-kit$^{low/-}$ and $\alpha_E\beta_7{}^+$ and/or Lin$^+$ (c-kit$^{low/-}$) CP cells were sorted by FACS Vantage (Becton Dickinson).

Semiquantitative Reverse Transcriptase PCR Analysis of mRNA Levels

Total RNA was prepared from various lymphocytes with an RNeasy Mini Kit (QIAGEN). RNA samples were treated with DNase (RT grade) (NIPPON GENE, Toyama, Japan) to remove contaminating 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 M-MLV reverse transcriptase (GIBCO–BRL, Life Technologies). PCR was carried out in a volume of 100 μl containing RT products, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 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 fragment length of PCR products were Vγ7-Cγ1, 5′-AAGCTAGAGGGGTCCTCTGC-3′ and 5′-CTTAT GGAGATTTGTTTCAGC-3′, 380 bp (Takagaki et al., 1989) and Vβ8.1/2-Cβ1/2, 5′-ATGTACTGGTATCGGCAGGACACGG-3′ and 5′-GAG ACCTTGGGTGGAGTCAC-3′, 267 bp (Koyasu et al., 1997). Those of CD3ε, pre-Tα, TCR-Cβ, and β-actin transcripts have been described previously (Oida et al., 2000). To detect germline TCRγ transcripts, PCR primers were designed to be positioned 96 bp upstream from Jγ1 gene segment (5′-CAGAAGATCGGTTTGTCAGG-3′) and at the first exon of Cγ1 gene (5′-GGGGAAATGTCTGCATCAAG-3′) (184 bp), and these primers also amplify the germline Jγ2-Cγ2 transcripts (174 bp).

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