Identification of Novel Lymphoid Tissues in Murine Intestinal Mucosa Where Clusters of c-kit$^+$ IL-7R$^+$ Thy1$^+$ Lympho-hemopoietic Progenitors Develop

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Summary

We have revealed that about one and a half thousand tiny clusters, filled with one thousand closely packed lymphocytes, can be found throughout the murine small and large intestinal mucosa. They are located in crypt lamina propria (cryptopatches; CP) and can be first detected at 14–17 d after birth. A large fraction of lymphocytes in CP expresses c-kit, IL-7R, Thy1 and a lymphocyte function-associated antigen, LFA-1, whereas most of them remain CD3$^-$, TCR $\alpha\beta^-$, TCR $\gamma\delta^-$, sIgM$^-$, and B220$^-$. The population size of IL-2Rα^+, HSA$^+$ and Pgp-1$^+$ subsets is variable (20–50%) and the composition of CD8$^+$, Ly-1$^+$, and CD4$^+$ subsets is smaller but also variable (3–20%). In the small intestine, CP do not contain cells undergoing apoptosis nor cells bearing RAG-1 molecules, but do contain dendritic stromal cells bearing CD11c/CD18 molecules. The frequency of DNA replicating cells in CP is higher than that in Peyer's patches (PP), is lower than that in the thymic cortex and is almost comparable with that in the thymic medulla. The numbers of CP remain the same in aged mice (>114 wk) but double after estrogen treatment even though the thymi are attenuated sharply in both conditions. Thus, with respect to histogenesis, lymphocyte composition and tissue level of cellular behavior, neither PP, isolated lymphoid follicles, peripheral LNs, nor thymus are identical with CP. Finally, CP are virtually absent in lamina propria of IL-7R-deficient mice that display a profound reduction in thymic and peripheral lymphoid cellularity. By contrast, CP are present in germ-free mice and in athymic (nu/nu), SCID, TCR $\beta \times \delta^{-/-}$, RAG-2$^{-/-}$, PP-deficient (aly/aly), stem cell factor (Sl/Sl^d) and c-kit (W/W^v) mutant mice. Taking all of these results together, CP are the first identification of gut-associated murine lymphoid tissues where the generation of IL-7-dependent lympho-hematopoietic progenitors for T and/or B cell descendants may start to take place at the age of commencement of weaning.

In the past decade, a substantial number of murine T cells have been shown to develop without passing through the thymus (1–11). Among them, intestinal intraepithelial T lymphocytes (IELs)[1] constitute the largest group of peripheral extrathymic T cells (1–4, 7, 8). Although considerable insights into IELs have been made during the past few years (7, 8, 12–16), much remains to be learned about their physiological significance and the precise anatomical nursery where the differentiation of IELs is allowed to proceed. In fact, the long-lasting issue of exactly how and where IELs develop, and the role of the thymus in this process, continue to be subjects of importance (17). Specifically, it has remained an open question whether bone marrow-derived precursor IELs that reached the epithelium start to rearrange their TCR genes and differentiate further into mature IELs or complete all these processes somewhere else, most likely in the intestinal mucosa and then migrate into the epithelial layer. In an effort to investigate this issue, we carried out immunohistochemical analysis of the murine gastrointestinal tract, and noticed that numerous tiny clusters (~1,650 clusters/intestine) filled with closely packed lymphoid cells (~1,000 cells/cluster) occur throughout the small and large intestinal mucosa except for the stomach.

[1]*Abbreviations used in this paper:* BrdU, bromodeoxyuridine; CP, cryptopatches; GALT, gut-associated lymphoid tissue; IEL, intestinal intraepithelial T lymphocytes; PP, Peyer's patches; RAG, recombination activating gene; SCF, stem cell factor.

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Anatomically, gut-associated lymphoid tissue (GALT) consists of organized structures and diffusely distributed populations of cells. Organized GALT is comprised of aggregated lymphoid follicles (Peyer's patches [PP]), isolated lymphoid follicles, and mesenteric LNs although the number and location of PP and isolated lymphoid follicles vary among species. PP are usually visible from the serosa or mucosal surface, whereas isolated lymphoid follicles are not grossly visible and are located along the length of gastrointestinal tract, with increasing frequency in the human colon and rectum (18, 19). However, isolated lymphoid follicles, like PP, have a T cell–rich parafollicular region, a dark $sIgM^+$ B cell–rich peripheral zone and a lighter germinal center consisted mainly of B lymphoblasts (20). Although literature on the development and cellular composition of isolated lymphoid follicles are still limited, they are histologically and functionally similar in other respects to PP (19–21). In sharp contrast, the newly identified clusters that settle in crypt lamina propria do not exhibit the organized lymphoid tissue but are instead filled with closely packed lymphoid cells, of which only less than 2% express either cell surface TCR or IgM. Since we could not find any previous descriptions of such small lymphoid colonies in murine intestinal mucosa, we named the clusters cryptopatches (CP). In any event, we believe the existence of CP in the intestinal mucosa has not been previously evaluated and reported simply because they are very small and constituting only a thinly scattered population. In the present study, we have explored the development of CP and have characterized in detail the cells that sojourn in there. The results show that CP are not only distinct from PP, isolated lymphoid follicles and peripheral LNs in terms of their histogenesis but also distinguished by a conspicuous colonization with lymphoid cells expressing c-kit tyrosine kinase receptors (c-kit), interleukin 7 receptors (IL-7R) and Thy1.

The significance of these observations is discussed from the viewpoint that CP are the newly identified gut-associated lymphoid tissues where the development of T and/or B lympho-hemopoietic progenitors takes place. In this context, our findings may shed light on such a missing link between bone marrow-derived lymphoid lineage-committed precursor cells (22, 23) and T cells, which have developed outside the thymus, e.g., murine $CD8\alpha\alpha^+$ IELs bearing $\gamma\delta$ T cell antigen receptors (1–3, 8, 13).

Materials and Methods

Mice. BALB/cA Jcl nu/nu (nu/nu), C.B-17/Icr Jcl scid/scid (scid/scid) and aly/aly Jcl mutant mice (24, 25), their corresponding wild-type BALB/cA Jcl nu/+ (nu/+), C.B-17/Icr Jcl scid/+ (scid/+) and aly/+ Jcl littermates, and C57BL/6J Jcl (B6) mice were purchased from Clea Japan, Inc. (Tokyo, Japan). Stem cell factor (SCF) mutant $WBB6F_1$-Sl/Sl^d (Sl/Sl^d) mice and c-kit mutant $WBB6F_1$-W/W^v (W/W^v) mice, and their corresponding wild-type $WBB6F_1$-+/+ (Sl/Sl^+) and $WBB6F_1$-+/+ (W/W^+) littermates were purchased from Japan SLC, Inc. (Shizuoka, Japan). TCR β mutant ($\beta^{-/-}$) mice and TCR δ mutant ($\delta^{-/-}$) mice have been described (26, 27) and we obtained TCR $\beta \times \delta$ double mutant ($\beta \times \delta^{-/-}$) mice from the F_2 generation of an intercross between $\beta^{-/-}$ and $\delta^{-/-}$ mice (16). Recombination activating gene (RAG)-2 mutant mice (28) were a generous gift from Dr. Y. Shinkai (Nippon Roche Research Center, Kamakura, Japan). Details of the generation of IL-$7R^{-/-}$ mice have recently been described (29). B6 mice of 114 wk of age were a generous gift from Dr. K. Hirokawa (Tokyo Medical and Dental University, Tokyo, Japan). Germ-free BALB/cA mice have been described elsewhere (30).

Abs. The following mAbs were used: Anti-CD3 mAb (145-2C11) (31), anti-$\alpha\beta$ mAb (H57-597) (32), anti-$\gamma\delta$ mAb (3A10) (27), anti-CD4 mAb (GK1.5) (33), anti-CD8α mAb (53.6.7) (34), anti-Ly-1 mAb (53.7.3) (34), anti-IL-2Rα mAb (PC-61) (35) and anti-LFA-1 mAb (FD441.8) (36). Either the culture supernatants of hybridoma clones listed in the parentheses or purified antibodies were used. Anti-μ chain mAb (LO-MM; Caltag Laboratories Inc., South San Francisco, CA), anti-κ chain mAb (R5-240; PharMingen, San Diego, CA), anti-B220 mAb (RA6-B2; PharMingen), anti-Thy1.2 mAb (30H12; Becton Dickinson, Sunnyvale, CA), anti-bromodeoxyuridine (BrdU) mAb (BU1/75, ICR 1; BiOSIS, Compiegne, France) and anti-RAG-1 mAb (G109-256.2; PharMingen) were also used. Anti-c-kit mAb (ACK-2) (37) was a gift from Dr. S. Nishikawa (Kyoto University, Kyoto, Japan), anti-IL-7R mAb (A7R34) (38) was a gift from Dr. T. Sudo (Toray Industries, Inc., Kamakura, Japan), anti-Pgp-1 mAb (KM-201) (39) and anti-NK1.1 mAb (PK136) (40) were gifts from Dr. T. Abo (Niigata University, Niigata, Japan), anti-HSA mAb (M1-69) (41) was a gift from Dr. T. Uede (Hokkaido University, Sapporo, Japan). Anti-stroma cell mAb (Th-3) (42) was a gift from Dr. K. Hirokawa (Tokyo Medical and Dental University, Tokyo, Japan) and anti-CD11c mAb (N418) (43) and anti-interdigitating and/or -dendritic cell mAb (NLDC-145) (44) were gifts from Dr. K. Inaba (Kyoto University, Kyoto, Japan).

Immunohistochemical Procedure. Thymus and mesenteric LNs, and ~10 mm in length of the longitudinally opened small and large intestines were 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 applied to poly-L-lysine-coated slide glasses (Matsunami Glass IND., LTD., Japan). The tissue sections that had been air-dried and fixed in acetone for 10 min at room temperature were preincubated with Block-ace (Dainippon Pharmaceutical Co., LTD., Osaka, Japan) for 10 min at 37°C to block nonspecific binding of the primary mAbs. The sections were then incubated with appropriately diluted primary rat or hamster mAbs for 30 min at 37°C or overnight at 4°C, and rinsed three times with PBS, followed by incubation with biotin-conjugated goat anti–rat IgG (Cedarlane Laboratories Limited, Ontario, Canada) or with biotin-conjugated goat anti–hamster IgG (Vector Laboratories, Inc., Burlingame, CA). Subsequently, the sections were washed three times with PBS and then incubated with avidin-biotin peroxidase complexes (Vectastatin ABC kit; Vector Laboratories, Inc.). The 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 non-immune hamster serum showed only a minimal background staining.

At least five independent immunohistochemical stainings of tissue sections per mAb were performed, and the data were also

presented as geometric range of positive cell fractions determined under microscopy.

Immunofluorescence Procedure. 6-μm-thick cryostat tissue sections were fixed in acetone for 10 min at room temperature, washed three times with PBS, and then pretreated with Block-ace. The sections were then incubated with anti-c-kit mAb for 30 min at 37°C, followed by incubation with PE-conjugated goat anti–rat IgG(Fab′)$_2$ (GIBCO BRL, Gaithersburg, MD). Subsequently, the sections were incubated with anti-CD11c mAb and then counterstained with FITC-conjugated goat anti–hamster IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). For other stainings, the sections that had been incubated with anti-c-kit or IL-7R mAb for 30 min at 37°C, followed by incubation with PE-conjugated goat anti–rat IgG(Fab′)$_2$ were counterstained with FITC-conjugated anti-thy1.2 mAb (Becton Dickinson). Finally, the sections were analyzed by a laser scanning microscope, LSM-GB200 (Olympus Optical Co., LTD., Tokyo, Japan).

In Vivo Labeling and In Situ Immunocytochemical Visualization of Proliferating Cells. DNA replicating cells were determined as described by Gratzner (45). Mice were injected i.p. with bromodeoxyuridine (BrdU) (20 mg/kg of body weight) five times at 6-h intervals. 1 h after the last injection, the thymi and small intestines were removed and embedded in O.C.T. compound at −80°C. Six μm-thick cryostat tissue sections were fixed in 4% paraformaldehyde for 15 min at 4°C, washed three times with PBS and treated with 2 M HCl for 30 min at 37°C, followed by neutralization with 0.1 M sodium tetraborate. After the treatment of Block-ace, the sections were incubated overnight with rat anti-BrdU mAb at 4°C, washed three times with PBS and then incubated with biotinylated goat ant–rat IgG. The subsequent blocking of endogenous peroxidase activity and histochemical visualization of the BrdU incorporated cells were carried out according to the method described above.

TdT-Mediated dUTP-Biotin Nick End Labeling (TUNEL). DNA nick end labeling of tissue sections by TUNEL method was carried out by using MEBSTAIN Apoptosis kit (Medical and Biological Laboratories Co., LTD., Nagoya, Japan) according to the manufacturer's instructions. In brief, 6-μm-thick cryostat tissue sections were fixed in 4% paraformaldehyde for 20 min at room temperature, rinsed three times with PBS containing 0.5% Tween 20. Nuclei of tissue sections were stripped from proteins by incubation with 40 μg/ml proteinase K for 30 min at 37°C and the sections were then pretreated with 2% H_2O_2 to block endogenous peroxidase activity. After preincubation of tissue sections with TdT buffer, DNA nick end labeling of the sections was performed by incubation with 0.15 U/μl TdT and 12.5 μM biotinylated dUTP in 100 μl TdT buffer for 60 min at 37°C. The reaction was terminated by transferring the slides to TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min at room temperature. The sections were then rinsed three times with distilled water, incubated with the blocking buffer for 10 min at room temperature and washed three times with PBS. Treatment of the sections with avidin-biotin peroxidase complexes and the subsequent color development were carried out according to the method described above. For positive control, the sections were treated with 0.07 U/ml DNase 1 (Takara Shuzo Co., LTD., Shiga, Japan) before the DNA nick end labeling and, for negative control, TdT was omitted from the DNA nick end labeling solution.

In Situ Immunocytochemical Detection of Recombination Activating Gene-1 (RAG-1) Products. 6-μm-thick cryostat tissue sections were fixed in 4% paraformaldehyde for 15 min at room temperature, washed three times with PBS and preincubated with Block-ace. The sections were then incubated overnight with anti-RAG-1 mAb at 4°C, followed by incubation with biotinylated goat anti–mouse IgG2b (Southern Biotechnology Associates, Inc., Birmingham, AL). The subsequent blocking of endogenous peroxidase activity and histochemical visualization were carried out according to the method described above, but the counterstaining was not performed for microscopy.

Estrogen Administration. Treatment of mice with estrogen and analysis of lymphoid tissues from the treated animals were carried out as described by Okuyama et al. (46). In brief, 1 mg of estrogen (Ovahormone depo; Teikoku Zoki, Inc., Tokyo, Japan)/mouse was injected subcutaneously to B6 mice and, 10–14 d after the injection, thymi and small intestines were isolated and snap frozen in O.C.T. compound for histological and immunohistochemical studies.

Results

Small Lymphoid-Cell Clusters Are Distributed Throughout the Murine Intestinal Mucosa Along Duodenal-to-Colonic Axis. We examined hematoxylin/eosin-stained tissue sections of the gastrointestinal tract prepared from B6 mice, and noticed that numerous tiny clusters filled with closely packed lymphocytes (see below) occurred throughout the small and large intestines except for the stomach (Fig. 1). In the small intestine, the clusters are located in the crypt lamina

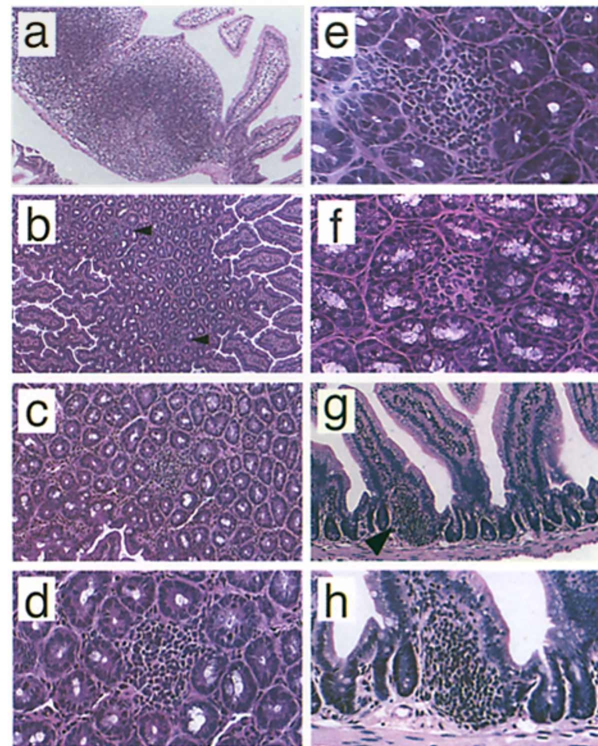

Figure 1. Localization of cryptopatches (CP) in the hematoxylin/eosin-stained tissue sections of small and large intestines from 12-wk-old B6 mice. (*a*) PP, ×100; (*b*) Jejunum, ×100. Arrowheads indicate CP. (*c*) Jejunum, ×200; (*d*) Jejunum, ×400; (*e*) Ileum, ×400; (*f*) Colon, ×400; (*g*) Jejunum, ×200. Arrowhead indicates CP. (*h*) Jejunum, ×400.

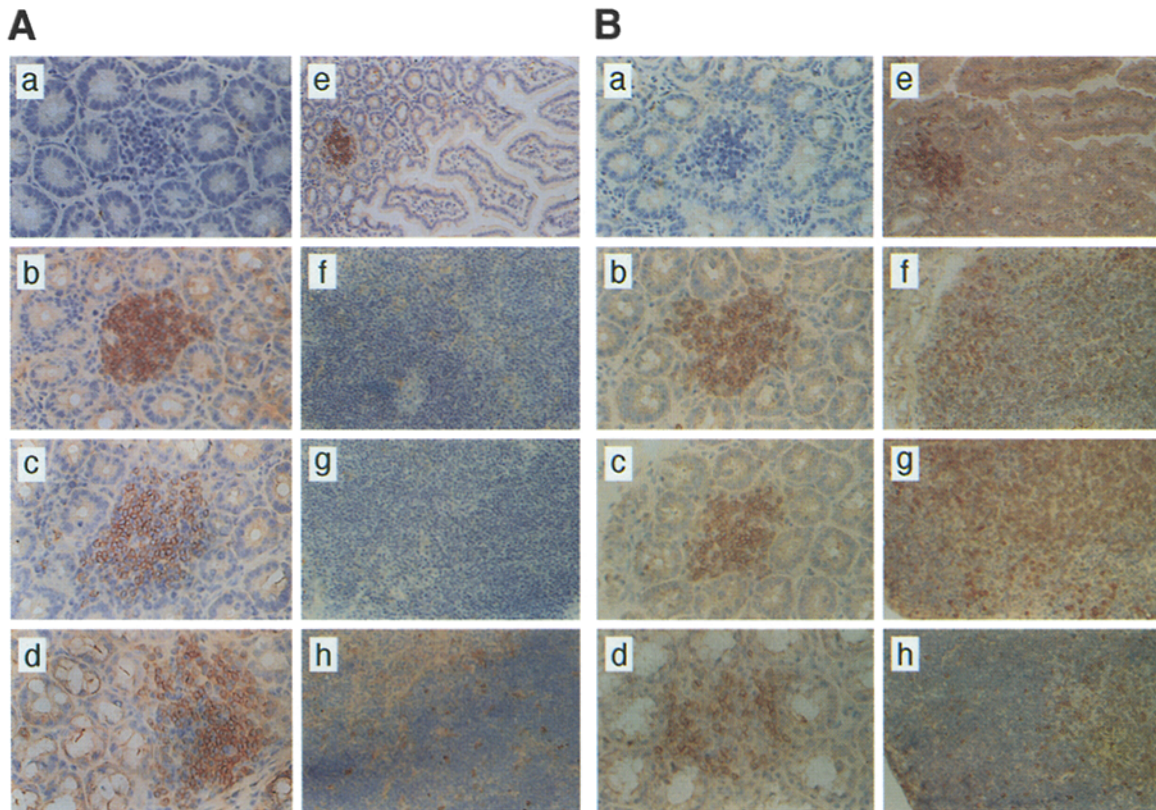

Figure 2. Immunohistochemical visualization of (*A*) c-kit$^+$ and (*B*) IL-7R$^+$ lymphocytes. (*a*) Jejunal CP, ×400. The first Ab used was isotype-matched normal rat IgG (negative control). (*b*) Jejunal CP, ×400; (*c*) Ileal CP, ×400; (*d*) Colonic CP, ×400; (*e*) Jejunal CP and villus lamina propria, ×200; (*f*) PP, ×200; (*g*) mesenteric LN, ×200; (*h*) thymus, ×200.

propria (Fig. 1, *b–e, g,* and *h*) and do not appear to be subdivided into specific areas based on their cellular composition (Fig. 1, *d, e,* and *h*). Since we could not find out any previous descriptions dealing with such small lymphoid cell accumulations in murine intestinal mucosa, we named the clusters cryptopatches (CP).

We microscopically enumerated the total number of CP in the small intestine with the aid of 10 mm × 10 mm grid attached to an ocularpiece which gave us 0.624 mm^2 per grid on the tissue section. For this purpose, the intestines from 12-wk-old female B6 mice were opened longitudinally, cut laterally into 10-mm pieces, pasted on a filter paper, snap frozen, sectioned horizontally with a cryostat at 6 μm, and then stained with HE. The number of CP in 100 arbitrary grids was 58, i.e., 58 CP per 62.4 mm^2 tissue section. The average length and width of the longitudinally-opened small intestine were 300 mm and 5.5 mm, respectively. Thus, ~1,500 CP per small intestine are present in adult B6 mice: 58 × (5.5 × 300/62.4). The same calculation formula gave us ~150 CP in the large intestine (Fig. 1 *f*). Next, we determined an average number of lymphoid cells colonizing a small intestinal CP. With the aid of 10 mm ruler attached to an ocular piece, we measured diameters of 100 arbitrarily chosen CP (Fig. 1, *c–e*) and the mean diameter turned out to be 86.8 ± 29.2 μm. We have extensively examined the HE-stained tissue sections, and, on the basis of vertical (Fig. 1, *g* and *h*) and transverse (Fig. 1, *d* and *e*) profiles of CP, we assumed that the CP were barrel-shaped (diameter/height = 1:1.5). Thus, 770 × 10^3 μm^3 is the approximate mass of an average CP. If we assume that the mean diameter of cells in CP is 10 μm and take 70% of them as lymphocytes (see below), one can estimate roughly the total number of lymphocytes to be ~1,000/CP and then absolute numbers of CP lymphocytes to be ~1.5 × 10^6/small intestine. Based on the same calculation formula, absolute numbers of CP lymphocytes in the large intestine were estimated to be ~1.5 × 10^5.

A Large Fraction of Cells in CP Expresses c-kit, IL-7R, Thy1 and LFA-1 Molecules. In an attempt to characterize the cells colonizing in CP, immunohistochemical analysis was carried out by using various kinds of mAbs that were reactive with different but well-defined cell surface molecules of murine lymphocytes. Surprisingly, it was found that the majority of CP cells express c-kit (~70%; Fig. 2 *A*), IL-7R (~70%; Fig. 2 *B*), Thy1 (~70%; Fig. 3, *a–c*) and a lymphocytes function-associated antigen, LFA-1 (~80%; Fig. 3 *d*). Thus, c-kit and IL-7R are expressed by a large fraction of cells in jejunal, ileal, and colonic CP (c-kit; Fig. 2 *A, b–e*) (IL-7R; Fig. 2 *B, b–e*). A large fraction of cells in jejunal (Fig. 3 *b*), ileal (data not shown) and colonic (Fig. 3 *c*) CP also expresses Thy1. IL-7R$^+$ cells are present in jejunal lamina propria (Fig. 2 *B, e*), PP (Fig. 2 *B, f*), LNs (Fig. 2 *B,*

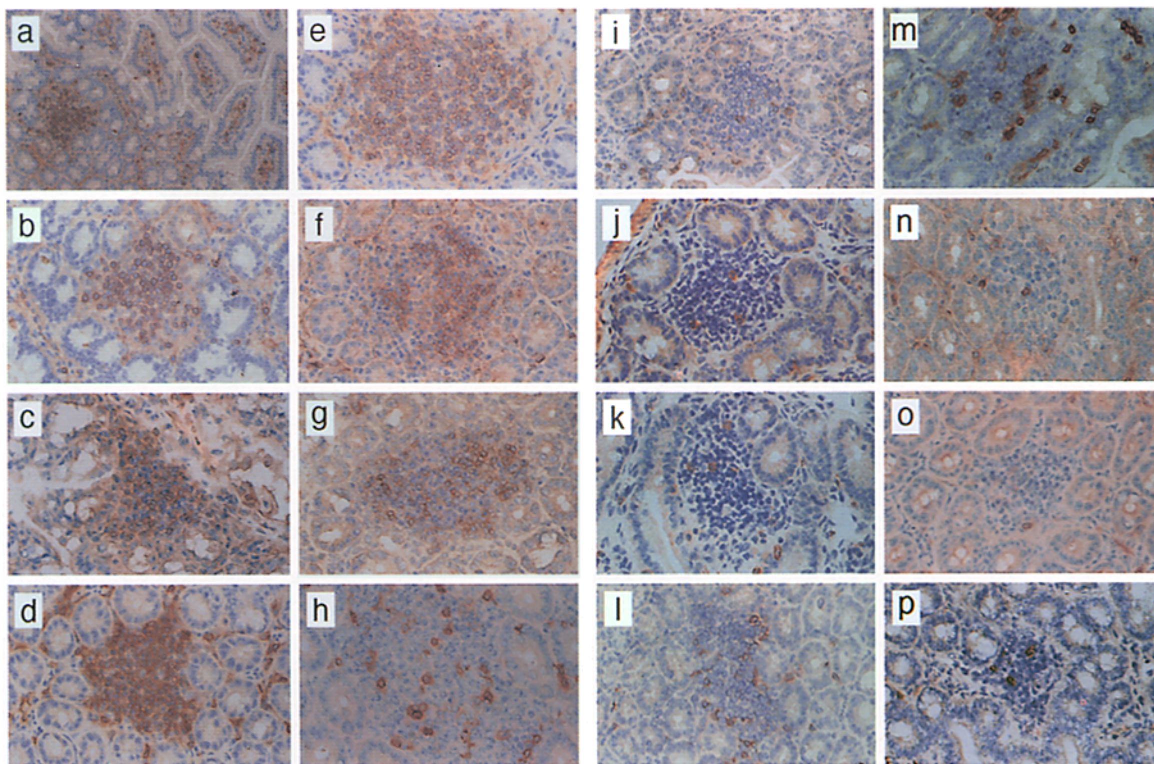

Figure 3. Immunohistochemical visualization of cells in jejunal CP expressing various cell surface molecules of murine lymphocytes. (*a*) Thy1-expressing lymphoid cells in CP, villus epithelium and villus lamina propria are seen; (*b*) Thy1$^+$ cells; (*c*) Thy1$^+$ cells in colonic CP; (*d*) LFA-1$^+$ cells; (*e*) Pgp-1$^+$ cells; (*f*) HSA$^+$ cells; (*g*) IL-2Rα^+ cells; (*h*) Ly-1$^+$ cells; (*i*) CD3$^+$ cells; (*j*) TCR$\alpha\beta^+$ cells; (*k*) TCR$\gamma\delta^+$ cells; (*l*) CD4$^+$ cells; (*m*) CD8α^+ cells; (*n*) μ-chain$^+$ cells; (*o*) κ-chain$^+$ cells; (*p*) B220$^+$ cells. Original magnifications are $\times$200 in (*a*) and $\times$400 in (*b*–*p*).

g) and thymus (Fig. 2 *B*, *h*). Thy1$^+$ cells are abundant in jejunal villus epithelium and lamina propria (Fig. 3 *a*), PP, LNs and thymus (data not shown). By contrast, c-kit$^+$ cells are almost undetectable in jejunal villus epithelium and lamina propria (Fig. 2 *A*, *e*), PP (Fig. 2 *A*, *f*) and LNs (Fig. 2 *A*, *g*), and are sparse in thymus (Fig. 2 *A*, *h*). Further, besides jejunal CP (Fig. 3 *d*), a large fraction of cells in ileal and colonic CP also expresses LFA-1 (data not shown).

CP Are Anatomical Sites Where Lympho-Hemopoietic Progenitors Accumulate. Since the majority of CP cells (~70%) express c-kit, IL-7R and Thy1, we examined such lymphocytes in the light of phenotypic transition of thymocytes which takes place during the progression of TN thymocytes toward single positive mature T cells (47, 48), along with cell surface markers specific for mature B cells, and the results were presented in Fig. 3. As shown in Fig. 3, among jejunal CP cells of adult B6 mice, ~50% are Pgp-1$^+$ (*e*), ~30% are HSA$^+$ (*f*), ~20% are IL-2Rα^+ (*g*), ~10% are Ly-1$^+$ (*h*) Fig. 3 also shows that the population size of CD3 (*i*), TCR$\alpha\beta$ (*j*), TCR$\gamma\delta$ (*k*), μ-chain (*n*), κ-chain (*o*), and B220 (*p*) expressing lymphocyte subsets is minimal and less than 2% of cells colonizing in jejunal CP, whereas that CD4 (*l*) and CD8 (*m*) expressing lymphocyte subsets are ~20% and ~5%, respectively. Notably, the population size of each lymphocyte subset described above remained almost the same in ileal and colonic CP (data not shown).

CP Contain Stroma Components and Cells at S Phase of the Cell Cycles but not Cells Undergoing Apoptosis nor Cells Bearing RAG-1 Molecules. To further characterize CP, we tried to examine the following properties of their resident cells by immunohistochemistry. First, do CP contain non-lymphoid stromal cell components indispensable for lymphocyte development? Th-3 mAb (42) and NLDC-145 mAb (44) were able to reveal the meshwork structure of cortical stromal cells in the thymus as depicted elsewhere (42, 44) but failed to stain any cells in CP (data not shown). By contrast, N418 mAb which identifies CD11c/CD18 molecules (43) visualized dendritic profiles in CP (Fig. 4 *A*, *b*), T cell areas of PP (Fig. 4 *A*, *c*) and thymic medulla (Fig. 4 *A*, *d*). More importantly, c-kit$^+$ lymphocytes and CD11c/CD18$^+$ stromal cells in CP were found to constitute two discrete nonoverlapping populations (Fig. 5 *A*). Second, are CP lymphocytes replicating in situ? We injected BrdU into mice to visualize the cells that had passed through the S phase of the cell cycle. As a result, a significant fraction of cells in CP (~7%) and thymus (~11%) was found to be in the S phase of the cell cycle when enumerated 1 h after the single i.p. infusion of BrdU. The net accumulations of BrdU-incorporated cells after the multiple infusions of BrdU (see Materials and Methods) in CP (~19%), PP (~5%), and thymic cortex (~35%) as well as medulla (~15%) are depicted in Fig. 4 *B*, *b*–*d*, respectively. Note that intestinal

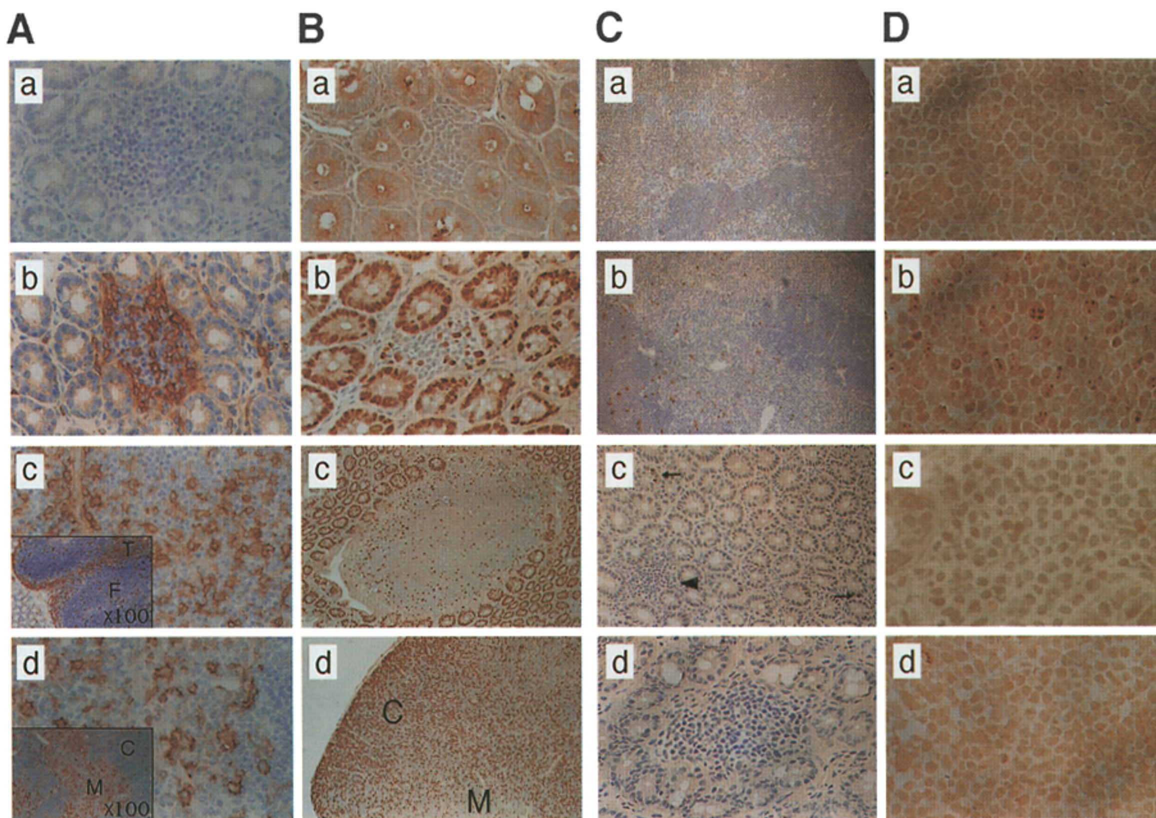

Figure 4. Immunohistochemical visualization of (*A*) CD11c$^+$ dendritic cells, (*B*) DNA replicating cells, (*C*) cells undergoing apoptosis, and (*D*) cells containing RAG-1 molecules. (*A*) ×400; (*a*) Jejunal CP. The tissue section was incubated first with non-immune hamster serum (negative control). (*b*) Jejunal CP. (*c*) PP. *T*, T cell area; *F*, follicle. (*d*) Thymus. *C*, cortex; *M*, medulla. (*B*) (*a*) Jejunal CP, ×400. The first Ab used was isotype-matched normal rat IgG (negative control). (*b*) Jejunal CP and epithelial cells in the crypt, ×400. (*c*) PP and epithelial cells in the crypt, ×100. (*d*) Thymus, ×100. *C*, cortex; *M*, medulla. (*C*) (*a*) Thymus, ×100. TdT was omitted from the DNA nick end labeling solution (negative control). (*b*) Thymus, ×100 (positive control). (*c*) Jejunal CP and epithelial cells in the crypt, ×200. Arrowhead indicates CP and small arrows indicate epithelial cells undergoing apoptosis. (*d*) Jejunal CP, ×400. (*D*) ×1,000; (*a*) Thymus. The first Ab used was isotype-matched normal mouse IgG (negative control). (*b*) Thymus (positive control). (*c*) Jejunal CP. (*d*) Mesenteric LN.

epithelial cells are proliferating vigorously at the crypt base (Fig. 4 *B, b and c*). Third, do CP contain cells undergoing apoptosis and cells bearing RAG-1 molecules? The answer appears to be no. In fact, the thymus contains cells undergoing apoptosis (Fig. 4 *C, b*) and bearing RAG-1 molecules (Fig. 4 *D, b*), but on the other hand CP do not contain apoptotic cells (Fig. 4 *C, c and d*) and like mesenteric LNs (Fig. 4 *D, d*), RAG-1-bearing cells (Fig. 4 *D, c*). Note that several intestinal epithelial cells undergoing apoptosis are seen in the crypt base (Fig. 4 *C, c, small arrow*).

Finally, most lymphocytes in CP express simultaneously c-kit, IL-7R and Thy1 molecules (triple positive), as determined by double immunofluorescence analysis (Fig. 5, *B and C*), and are also LFA-1$^+$. The data so far obtained by immunohistochemical study on cell surface molecules expressed by CP cells are consolidated in Table 1.

CP in Young, Aged and Estrogen-treated Mice. We next examined the intestines obtained from mice of all ages (Table 2). Fetal intestines lack CP. CP are in fact absent until day 14 of postnatal life, whereas PP become detectable early in life at 2–3 d after birth. In mice at the 17th postnatal day, however, CP populated with c-kit$^+$ IL-7R$^+$ Thy1$^+$ lymphocytes are detected without exception. Surprisingly, the numbers and cellularity of CP are maintained until at least 114 wk of age, although the thymi are attenuated drastically in the aged condition. A representative CP of the aged mice containing c-kit$^+$ lymphocytes is shown in Fig. 6 *a*. It seemed also of interest to evaluate the effect of estrogen administration on CP because it has recently been reported that estrogen activates extrathymic T cell differentiation in the liver (46), although the same sex hormone is a negative regulator of B lymphopoiesis and thymopoiesis (49). 10–14 d after the subcutaneous administration of estrogen (1 mg/mouse), absolute numbers of small intestinal CP containing c-kit$^+$ IL-7R$^+$ Thy1$^+$ lymphocytes increased about twofold as compared with those in control mice, whereas absolute numbers of thymocytes decreased more than 10-fold in the treated condition (Table 2). This observation was reproducible and multiple infusions of estrogen could not increase the number of CP beyond the twofold (data not shown).

CP in Germ-free Mice and Various Mutant Mice. We evaluated the effect of microbial deprivation in germ-free mice on the development of CP and found that the numbers and

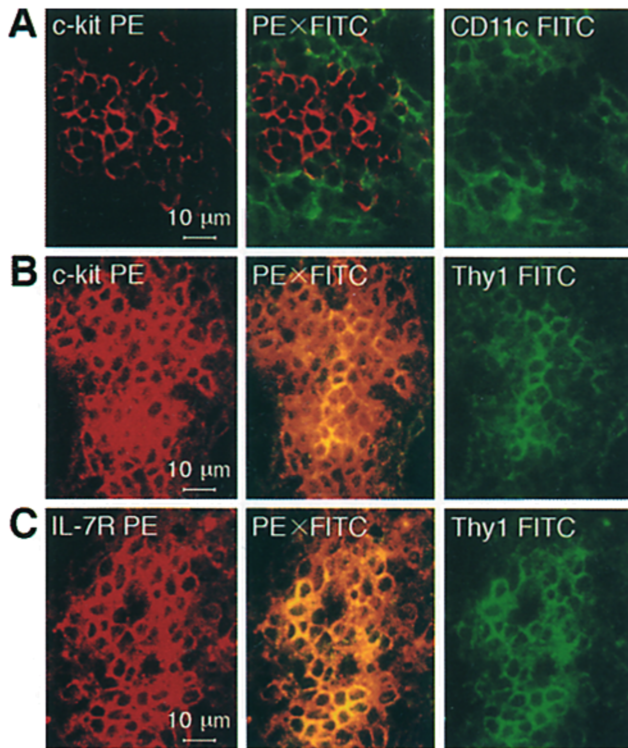

Figure 5. Double immunofluorescence analysis of cells in the jejunal CP from 12-wk-old B6 mice. (*A*) Anti-c-kit/anti-CD11c double labeling of jejunal CP. Lymphoid cells are positively stained with anti-c-kit mAb (*PE*), but not with anti-CD11c mAb (*FITC*), resulting in a red color. Conversely, dendritic cells are positively stained with anti-CD11c mAb (*FITC*), but not with anti-c-kit mAb (*PE*), resulting in a green color. (*B*) Anti-c-kit/anti-Thy1 double labeling of jejunal CP. Lymphoid cells are positively stained with anti-c-kit mAb (*PE*) followed by anti-Thy1 mAb (*FITC*), resulting in a yellow color. (*C*) Anti-IL-7R/anti-Thy1 double labeling of jejunal CP. Lymphoid cells are positively stained with anti-IL-7R mAb (*PE*) followed by anti-Thy1 mAb (*FITC*), resulting in a yellow color.

cellularity of CP remained the same in the germ-free condition. Anti-c-kit mAb staining of a jejunal CP in germ-free mice is presented in Fig. 6 *b*. To confirm whether CP are newly identified lymphoid tissues distinct from PP, LNs and thymus or not, we analyzed CP of various mutant mice, i.e., athymic (nu/nu), SCID, TCR $\beta \times \delta^{-/-}$, RAG-$2^{-/-}$, PP-deficient (aly/aly), IL-$7R^{-/-}$, c-kit (W/W^v), and SCF (Sl/Sl^d) mutant mice. In every mutant except IL-$7R^{-/-}$ mice, the development of CP populated prominently with c-kit^+ IL-$7R^+$ $Thy1^+$ lymphocytes is unaltered and is almost comparable with that of normal B6 mice. In Fig. 6, jejunal CP containing c-kit^+ lymphocytes in nu/nu (*c*), SCID (*d*), $\beta \times \delta^{-/-}$ (*e*), RAG-$2^{-/-}$ (*f*), and aly/aly (*g*) mutants are shown. The numbers and cellularity of CP containing IL-$7R^+$ $Thy1^+$ lymphocytes are maintained in W/W^v mice (data not shown) although the expression of c-kit by the mutant lymphocytes is very weak as anticipated (Fig. 6 *h*). Importantly, CP are hardly detectable in IL-$7R^{-/-}$ mice (Table 2), whereas CP are maintained intact in aly/aly mice that show a generalized lack of LNs and PP (24, 25). These results indicate that the development of

Table 1. *Expression of Cell Surface Molecules by Resident Cells in the Small Intestinal Cryptopatches*

Molecules	% Positive cells*
LFA-1	70–80
c-kit	60–70
IL-7R	60–70
Thy1	60–70
Pgp-1	40–50
HSA	20–30
CD4	15–20
IL-2Rα	10–20
Ly-1	5–10
CD8α	3–5
CD3	0–2
TCRαβ	0–2
TCRγδ	0–2
μ-chain	0–2
κ-chain	0–2
B220	0–2
CD11c	20–30

*Geometric range of positive cell fractions obtained by more than five independent immunohistochemical examinations of the small intestinal cryptopatches from 8–20-wk-old B6 mice.

CP is dependent on IL-7-mediated signals but not dependent on intestinal flora, thymus, PP, LN, and TCR as well as immunoglobulin gene rearrangements.

Discussion

CP are not detectable until the second week of postnatal life, the time when the commencement of weaning starts and the morphogenesis of mouse intestine is nearly completed (50), whereas PP are already microscopically well-developed by postnatal day 3 (51). Cells in CP exhibited a considerable DNA-replicating capacity (Fig. 4 *B, b*) and hematopoietic stem cell origin of the major population (∼80%) was verified by the expression of LFA-1 molecules (52). In fact, five months after lethal irradiation and reconstitution with bone marrow cells, it was found that >60% CP cells were donor bone-marrow cell derived by MHC class I typing (unpublished observation). An outstanding feature of resident CP cells revealed in the present study was the conspicuous concentration (up to 70% cells) of c-kit^+ IL-$7R^+$ $Thy1^+$ lymphocytes, indicating strongly their lymphoid commitment (22, 23, 29, 53–60). Thus, CP are composed of small collection of lymphocytes (1,000 per CP) and, from the point of view of their postnatal histogenesis (Table 2 and Fig. 6 *g*) and cellular composition (Figs. 2 and 3), CP are not only distinct from PP but also dissimilar in many respects to isolated lymphoid follicles. Importantly, CP containing c-kit^+ IL-$7R^+$ $Thy1^+$ lymphocytes

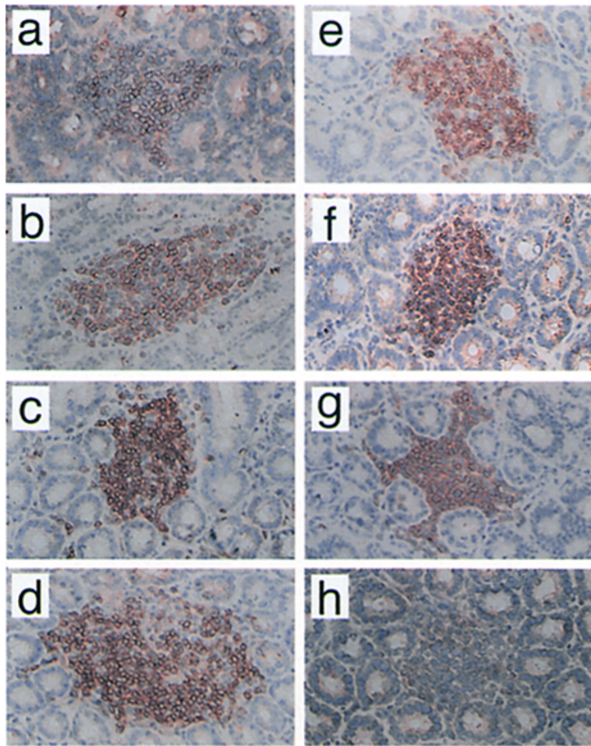

Figure 6. Representative immunohistochemical visualization of c-kit$^+$ lymphocytes in jejunal CP from (*a*) 114-wk-old B6 mouse, (*b*) Germ-free mouse, (*c*) Athymic (nu/nu) mouse, (*d*) SCID mutant mouse, (*e*) TCR $\beta \times \delta^{-/-}$ double mutant mouse, (*f*) RAG-$2^{-/-}$ mutant mouse, (*g*) aly/aly mutant mouse, and (*h*) c-kit (W/W^v) mutant mouse. Original magnification is ×400.

are maintained in nu/nu, SCID and RAG-$2^{-/-}$ mice which, in conjunction with the minimal colonization of TCR$^+$ and sIgM$^+$ cells in CP of normal mice, strongly support the notion of lymphoid tissues consisted primarily of precursor T and/or B lymphocytes. Consistent with our present observations, a somewhat similar, yet in many ways distinct, CP-like lymphoid tissues have recently been identified in rat intestinal mucosa (no. 172 in Abstract Book of 9th International Congress of Immunology. 1995. San Francisco, CA).

The interactions between lymphocytes and stromal compartments of lymphoid tissue have an essential role in lymphocyte migration, positioning, and development (61). The N418 mAb was reported to recognize CD11c/CD18 molecules, a member of the β2-integrin family (43), expressed by dendritic cells in the T-dependent areas of mouse splenic white pulp (43). In this regard, N418-reactive CP cells (~30% CP cells) display the profiles similar to anti-CD11c stain of dendritic stromal cells (Fig. 4 *A, c* and *d*). It has been established that the treatment of mice with estrogen suppress not only B lymphopoiesis in bone-marrow but also T lymphopoiesis in thymus (49). Interestingly, however, the same estrogen administration has recently been shown to stimulate extrathymic T cell production in the liver (46). In the present study, we verified that CP respond to estrogen administration and double in number during 10–14 d after the treatment (Table 2).

The c-kit$^+$ IL-7R$^+$ Thy1$^+$ phenotype of CP lymphocytes is reminiscent of the c-kit^{low} IL-7R$^+$ Thy1$^+$ phenotype as determined for the murine fetal blood pro-thymocyte population which is T lineage-committed before thymus colonization (23). The earliest T cell precursors in the adult murine thymus have been identified within CD3$^-$ CD4^{low} CD8$^-$ population (62). In fact, of all CP cells in normal B6 mice, ~20% are CD4$^+$, whereas ~5% are CD8$^+$ and ~2% are CD3$^+$. Thus, CP contain apparently CD3$^-$ CD4$^+$ CD8$^-$ lymphocytes (~15%). In addition to activated T and B cells, IL-2Rα chain is expressed on TN thymocytes (48, 63) and precursor B220$^+$ B cells in the bone marrow (64). In this context, most IL-2Rα$^+$ cells in CP (~20%) are B220$^-$, suggesting T lineage- rather than B lineage-commitment of the resident IL-2Rα$^+$ B220$^-$ lymphocytes. TN thymocytes can be subdivided into four subsets based upon expression of Pgp-1 and IL-2Rα (48). Besides Pgp-1 and IL-2Rα, Thy-1, HSA and Ly-1 (63), and more recently, c-kit (22, 48) have been shown to be useful cell surface markers for further dissection of TN thymocyte subsets. Importantly, we confirmed the existence of Pgp-1 (~50%), HSA (~30%), IL-2Rα

Table 2. *Identification of the Small Intestinal Cryptopatches in Various Mice**

	3–14-d-old mice	17-d-old mice	8–20-wk-old adult mice	114-wk-old aged mice	Estrogen-treated adult mice‡	IL-7R-deficient mice
Cryptopatches	−	+	+	+	+	−
(Number/small intestine)		(~150)	(~1,500)	(~1,700)	(~2,900)	
Thymus	+	+	+	±	±	±
(Cell number)		(~150 × 10^6)	(~100 × 10^6)	(~5 × 10^6)	(~10 × 10^6)	(~5 × 10^6)
Peyer's patches	+	+	+	+	+	±

*Young, adult, aged, and estrogen-treated animals were B6 strain of mice, and IL-7R-deficient mice had a genetic background of (129/Ola × B6)F_2 mice: +, detectable; ±, sharply attenuated; −, not detectable.
‡14 d after s.c. administration with estrogen (1 mg/mouse).

(~20%), and Ly-1 (~10%) expressing subsets in c-kit$^+$ IL-7R$^+$ Thy1$^+$ CP lymphocyte pool. Thus, on the basis of cellular, structural and functional properties described above, CP appear to fulfill the criteria of lymphoid tissues where T rather than B lineage-committed precursors consisting of cells at different maturational stages are developing. Nevertheless, with respect to the phenotypes illuminated by the present immunohistochemical study, it should be pointed out that the questions of whether CP-colonizing cells also contain B lympho-hemopoietic and/or mast cell lineage-committed progenitors have remained unanswered. It has recently been reported that the murine fetal blood contains Thy1^{low} c-kit^{high} progenitor mastocytes which are able to reconstitute the mast cell compartment of genetically mast cell-deficient W/W^v mice (65). However, the possibility that CP contain a substantial fraction of mast cell progenitors is remote since CP heavily populated with c-kit^{low} IL-7R$^+$ Thy1$^+$ cells in W/W^v mice (Fig. 6 *h*) and with c-kit$^+$ IL-7R$^+$ Thy1$^+$ cells in genetically mast cell-deficient Sl/Sl^d mice (data not shown) were observed.

IL-7R$^{-/-}$ mice that lack CP (Table 2) and γδ T cells (29) had normal number of functional NK cells expressing NK1.1 molecules (29), and NK1.1$^+$ cells were not detectable in CP of normal B6 mice (data not shown). These findings indicate that NK-cell development does not depend on the signal from IL-7R nor on the presence of CP. By contrast, the signal transmitting through an interaction of IL-7 with IL-7R rather than that of SCF with c-kit is indispensable for the postnatal development of CP (Table 2 and Fig. 6 *h*). Finally, although microbial deprivation in germ-free mice has a marked influence on organized and diffusely distributed GALT (30, 66), it was corroborated that the development of CP is essentially nondependent on the microorganism-induced antigenic load in the gut lumen.

Taken together, it is conceivable that CP are newly identified gut-associated lymphoid tissues where lymphoid lineage-committed precursors are developing. For further expansion, receptor gene rearrangement and differentiation into T and/or B cells, anatomical site other than CP are, however, indispensable. Better understanding of these novel lymphoid tissues will not only provide us with an additional basis for the cellular dissection of mucosal immunity but may also shed light on the extrathymic development of, at least some, T cells such as γδ IELs (1–3, 8, 13). In conclusion, isolation and subsequent purification of CP lymphocytes, characterization of such lymphocytes, and in vitro as well as in vivo progeny studies on the purified population are all mandatory before we evaluate the physiological significance of CP and their lymphoid residents. These procedures were found to suffer from several obstacles imposed by anatomical and numerical restraints, but we are currently trying to overcome all those difficulties.

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