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Sphingosine 1-phosphate regulates peritoneal B-cell trafficking for subsequent intestinal IgA production

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Sphingosine 1-phosphate (S1P) is known to play a pivotal role in the regulation of lymphocyte emigration from organized lymphoid tissues such as the peripheral lymph nodes and thymus, but its immunologic role in unorganized and diffused tissues remains to be elucidated. Here we show that the trafficking of peritoneal B cells is principally regulated by S1P. All peritoneal B cells including B1a, B1b, and B2 B cells express comparable levels of the type 1 S1P receptor. Thus, treatment with FTY720, an S1P receptor modulator, caused the rapid disappearance of peritoneal B cells by inhibiting both their emigration from parathymic lymph nodes and their recirculation from the blood into the peritoneal cavity without affecting their progenitor populations. These changes did not affect natural plasma antibody production or phosphorylcholine (PC)–specific antibody production in serum after peritoneal immunization with heat-killed *Streptococcal pneumoniae* (R36A). However, FTY720 dramatically reduced peritoneal B cell-derived natural intestinal secretory IgA production without affecting the expression of J-chain and polyimmunoglobulin receptors. Additionally, FTY720 impaired the generation of PC-specific fecal IgA responses after oral immunization with R36A. These findings point to a pivotal role for S1P in connecting peritoneal B cells with intestinal B-cell immunity. (Blood. 2007;109:3749-3756)

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Introduction

Sphingosine 1-phosphate (S1P) has been identified as an important molecule in the regulation of lymphocyte egress from the organized lymphoid structures including the thymus and secondary lymphoid organs.[1,2] At present, 5 kinds of S1P receptors have been identified, each sharing S1P as its ligand but associating with a different type of G protein, resulting in a distinct signal transduction.[3] Accumulating evidence has demonstrated that type 1 S1P receptor (S1P1) is preferentially expressed on lymphocytes, and their expression is closely regulated by lymphocyte activation or development, which determines lymphocyte emigration from secondary lymph organs as well as the thymus.[4,5] FTY720, 2-amino-2-[2-(4-octylphenyl)-ethyl] propane-1,3-diol hydrochloride, acts as an agonist for S1P receptors, except the type 2 S1P receptor (S1P2).[6-8] FTY720 blocks S1P-mediated signaling by inducing internalization of receptors.[4,9] Therefore, treatment with FTY720 decreased the number of circulating lymphocytes in both blood and lymph, by inhibiting their emigration from the secondary lymphoid organs and thymus and by modulating integrin-dependent lymphocyte homing into peripheral lymph nodes.[10-12]

Several lines of evidence have revealed that S1P also regulates B-cell distribution in the spleen, suggesting that FTY720 can impair plasma antibody production, especially against T-dependent (TD) antigen due to the abolishment of germinal center formation.[13-15] In addition, a recent study revealed that S1P plays an important role in the determination of plasma cell tropism to bone marrow.[16] Despite the substantial evidence pointing to the role of S1P in the regulation of lymphocyte trafficking at the systemic immune compartments, it still remains unclear whether S1P is also involved in lymphocyte trafficking and immune responses in the mucosa-associated unorganized and diffused tissues, such as the intestinal lamina propria and the peritoneal cavity (PerC). The PerC contains numerous B cells, especially B1 B cells that can be distinguished by cell surface markers (eg, B220, IgM, IgD, CD5, and Mac-1) from conventional B2 B cells.[17-19] B1 B cells are thought to play an important role in the protective immunity in PerC by producing antibodies in response to T-independent (TI) antigen, such as phosphorylcholine (PC), a haptenlike antigen associated with many pathogenic bacteria.[20,21] In addition to playing a role in peritoneal immunity, B cells have been shown to be a source of IgA for the formation of secretory IgA antibody (S-IgA) in the intestine.[22-24] S-IgA, a hallmark antibody principally produced at mucosal sites, plays an important role in the creation of immunologic surveillance and homeostasis at mucosa by abolishing pathogenic microbial infections and establishing symbiosis with commensal flora.[25,26] One major source of S-IgA is B2 B cells derived from the common mucosal immune system (CMIS) that links inductive (eg, Peyer patches and isolated lymphoid follicles) and effector tissues (eg, lamina propria region). These B2 B cells have been shown to play a key role in the induction of TD antigen-specific S-IgA.[27,28] In addition to the B cells belonging to the CMIS, peritoneal B cells have also been identified as another source of S-IgA, especially specific for the TI antigen.[23,24] Accumulating evidence suggests that peritoneal B-cell trafficking is biologically regulated, in some cases by chemokines (eg, CXCL12 and CXCL13) and cytokines (eg, IL-10),[29-32] but there is not currently

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enough information to fully understand the involvement of obvious cell trafficking molecules such as S1P in the pathway.

This study first sought to investigate the role of S1P in the regulation of peritoneal B-cell trafficking and then to assess the influence of the S1P-mediated pathway on the production of serum and intestinal antibody production from peritoneal B cells. Our current findings provide new evidence that S1P regulates peritoneal B-cell trafficking and subsequent S-IgA production in the intestine.

Materials and methods

Mice and FTY720 treatment

Female Balb/c and ICR SCID mice (7-9 weeks) were purchased from Japan Clea (Tokyo, Japan). All mice were maintained in horizontal lamina flow cabinets and provided with sterile food and water ad libitum. Mice were injected intraperitoneally with 1 mg/kg/time of FTY720 (Novartis Pharma, Basel, Switzerland).[12] All animals were maintained and experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of the University of Tokyo.

Cell isolation

PerC cells were obtained by flushing the peritoneum with 8 mL ice-cold PBS.[33] Lymphocytes and epithelial cells were isolated from the small intestine by the enzymatic dissociation procedure using collagenase IV (Nitta Gelatin, Osaka, Japan), as previously described.[34] Lymphocytes were collected from the omentum, parathymic lymph nodes, and blood in accordance with a previously established protocol.[29,35,36]

Flow cytometry and cell sorting

A standard protocol, previously described, was used for flow cytometric analysis and cell sorting.[37,38] Cells were first incubated with anti-CD16/32 antibody and then stained with the appropriate fluorescent-conjugated antibodies specific for CD5, CD11b, B220, IgA, and IgM (BD PharMingen, San Diego, CA). Viaprobe (BD PharMingen) was used to discriminate between dead and live cells. Flow cytometric analysis and cell sorting were performed using FACSCalibur and FACSAria (BD Biosciences, Franklin Lakes, NJ), respectively.

Quantitative and conventional RT-PCR

To measure mRNA expression for S1P receptors, quantitative reverse transcription-polymerase chain reaction (RT-PCR) using LightCycler (Roche Diagnostics, Mannheim, Germany) was performed.[37] Briefly, total RNA was collected using a TRIzol reagent (Invitrogen, Carlsbad, CA) and cDNA was synthesized using Powerscript reverse transcriptase (BD Biosciences). The oligonucleotide primers and probes specific for S1P1 (forward primer, TACACTCTGACCAACAAGGA; reverse primer, ATAATGGTCTCT-GGGTTGTC; FITC-probe, TGCTGGCAATTCAAGAGGCCCATCATC; LCRed 640-probe, CAGGCATGGAATTTAGCCGCAGCAAATC), S1P2 (forward primer, CATCGTACTGGGTGTTTTC; reverse primer, CCACG-TATAGATGACAGGA; FITC-probe, AATAGTGGGCTTTGTAGAGGA-CAGGGCAGG; LCRed 640-probe, CCGAACGGGACAGGTGGAG-TCTAAGAGAAG), S1P3 (forward primer, TCCTCTTCCTCATCGA-CGTG; reverse primer, CCTTGCCCTTGACTAGACAG; FITC-probe, TTCATCATGCTGGCTGTCCTCAACTCGG; LCRed 640-probe, CAT-GAACCCTGTCATCTACACGCTGGCC), S1P4 (forward primer, CAT-CTTTAGAGTGGTCCGAG; reverse primer, GCCCAGACATTAGAAC-CAA; FITC-probe, CCGCAGGCTACTCAACACCGTGCTGAT; LCRed 640-probe, ATCTTGGTGGCCTTTGTGGTGTGCTGG), and GAPDH (forward primer, TGAACGGGAAGCTCACTGG; reverse primer, TCCAC-CACCCTGTTGCTGTA; FITC-probe, CTGAGGACCAGGTTGTCTCCT-GCGA; LCRed 640-probe, TTCAACAGCAACTCCCACTCTTCCACC) were designed and synthesized by Nihon Gene Research Laboratory (Sendai, Japan). Conventional RT-PCR was performed to measure pIgR and J-chain, using specific primers (pIgR forward, AGTATTCAGGCAGAGC-CAAC; pIgR reverse, ATTCATCCGGCACAGATATT; J-chain forward, ATGAAGACCCACCTGCTTCTCTGG; J-chain reverse, AGGGTAGCA-AGAATCGGGGGTCAA).

Adoptive cell transfer

For tracing cells in vivo, peritoneal cells (1×10^7 cells) were incubated with 0.25 μM CFSE (Molecular Probes, Eugene, OR) in the dark for 10 minutes at 37°C, and then washed with PBS twice in accordance with a previously described method.[39] The labeled cells were transferred into severe combined immunodeficient (SCID) mice intraperitoneally (4×10^6 cells) or intravenously (1×10^7 cells) and FTY720 was simultaneously administered intraperitoneally. After 12 hours, peritoneal cells were collected for fluorescence-activated cell sorting (FACS) analysis.

For the analysis of antibody production from peritoneal B cells, SCID mice were adoptively transferred with normal peritoneal B cells (5×10^6 cells) via intraperitoneal route and treated with FTY720 every 2 days. As described under "Detection of total immunoglobulin and PC-specific antibody levels in serum and fecal extract by ELISA," 2 weeks after the adoptive transfer, we simultaneously collected serum and fecal extracts for the measurement of total immunoglobulin levels by enzyme-linked immunosorbent assay (ELISA) and isolated mononuclear cells from the intestinal lamina propria for enumeration of antibody-forming cells (AFCs) by enzyme-linked immunospot assay (ELISPOT).

Immunization

Mice were immunized intraperitoneally with 10^7 heat-killed pepsin-treated *Streptococcal pneumoniae* strain R36A (gift of Dr John Kearney, University of Alabama, Birmingham, AL).[20,29] For immunization, mice were orally immunized with 2×10^8 heat-killed pepsin-treated *S pneumoniae* strain R36A together with 10 μg mucosal adjuvant cholera toxin (List Biological Laboratories, Campbell, CA).[40] In the FTY720-treated group, mice were injected intraperitoneally with FTY720 6 hours before the immunization and then again once per day during the experiment. Serum and fecal extracts were prepared for the analysis of PC-specific IgM and IgA production by ELISA, as described in the next section.[34,38]

Detection of total immunoglobulin and PC-specific antibody levels in serum and fecal extract by ELISA

Total immunoglobulin levels in serum and fecal extracts were determined by ELISA as previously described.[37] To measure antibody concentration, purified murine isotype-specific antibodies (BD PharMingen) were used as standards for the quantification. For the detection of PC-specific antibodies, microtiter plates were coated with 5 μg/mL of PC-BSA (Biosearch Technologies, Novato, CA) in bicarbonate buffer (pH 9.6).[29] Following blocking with 5% BSA in PBS, diluted serum or fecal extracts were added and incubated in the coated wells for 2 hours at room temperature. Bound antibodies were then determined using HRP-conjugated anti–mouse IgM or IgA (Southern Biotechnology, Birmingham, AL) and 3,3′,5,5′-tetramethylbenzidine (Moss, Pasadena, CA), as previously described.[29,38]

Enumeration of AFCs by ELISPOT

To measure IgM- or IgA-producing AFCs in the intestinal lamina propria, an ELISPOT assay was used as previously described.[37] Briefly, various concentrations of mononuclear cells were cultured in 96-well nitrocellulose membrane plates (Millititer HA; Millipore, Bedford, MA) coated with 5 μg/mL affinity-purified goat anti-immunoglobulin (Southern Biotechnology) at 37°C for 4 hours. After vigorous washing with PBS and PBS containing 0.05% Tween 20, HRP-conjugated antibodies specific for mouse IgM or IgA (Southern Biotechnology) were added and incubated overnight. The spots of AFCs were developed using 2-amino-9-ethylcarbazole (Polysciences, Warrington, PA) containing hydrogen peroxide.

Statistics

The results were compared using a Student *t* test or a Welch *t* test. $P < .05$ was considered statistically significant.

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Results

Rapid and reversible disappearance of peritoneal B cell by FTY720 treatment

The initial aim of this study was to examine the involvement of S1P in the regulation of peritoneal B-cell trafficking. To accomplish this, we intraperitoneally administered FTY720, a modulator for S1P receptors, and examined cellular population by flow cytometry. The analysis based on forward (FSC) and side scatter (SSC) revealed that FTY720 treatment resulted in the dramatic changes in cellular population with different size and intracellular structure (Figure 1A, top panels). The profile indicated that intraperitoneal administration of FTY720 simultaneously reduced the number of lymphoid cells and induced a remarkable accumulation of granulocytes or monocytes or both (Figure 1A, top panels). Because PerC cells are known to contain both B1 and B2 B cells, we next used the expression of B220 and CD11b to confirm that numbers of both $B220^{+}CD11b^{+}$ B1 and $B220^{+}CD11b^{-}$ B2 B cells were dramatically reduced (Figure 1A, bottom panels). Because the total PerC cell number remained unchanged even after FTY720 treatment (data not shown), the changes in the cellular percentage can be assumed to directly reflect the absolute cell number of each population (Figure 1B). In short, FTY720 treatment significantly decreased B1 and B2 B-cell numbers (Figure 1B).

We next sought to analyze the kinetics of the change and recovery of peritoneal B cells after treatment with FTY720. Marked reductions in both B1 and B2 B-cell levels were found only 3 hours after FTY720 treatment (Figure 1C), and a partial recovery was detected 24 hours after the injection, with full recovery observed 7 days after the administration (Figure 1D). These data suggest that the effect of FTY720 on peritoneal B cells is both rapid and reversible.

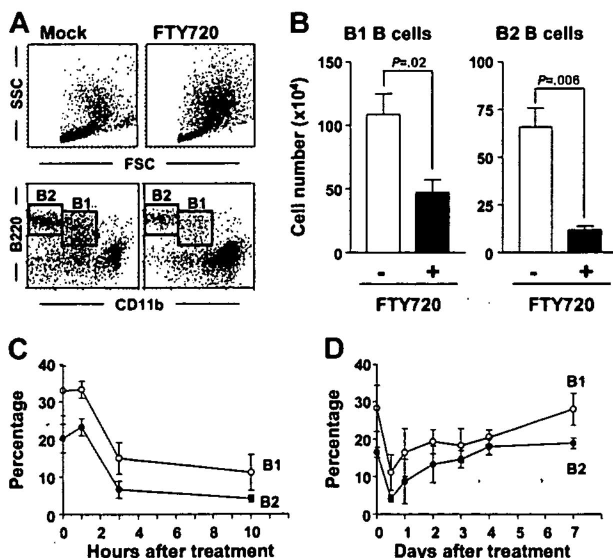

Figure 1. Rapid but reversible disappearance of peritoneal B cells induced by FTY720. (A) PerC cells were isolated 10 hours after injection of FTY720 (right) or mock (left), and cell populations were analyzed using flow cytometry. The data are representative of 5 independent experiments. (B) Cell numbers of $B220^{+}CD11b^{+}$ B1 B cells and $B220^{+}CD11b^{-}$ B2 B cells were calculated by using the total cell number and flow cytometric data. The error bars are ± SEM (n = 5). (C-D) At each time point after FTY720 injection, PerC cells were analyzed by flow cytometry (○, $B220^{+}CD11b^{+}$ B1 B cells; ●, $B220^{+}CD11b^{-}$ B2 B cells). The data represent the mean ± SD (n = 4).

Specific and equal expression of S1P1-encoding mRNA by peritoneal B cells

As might be expected by the comparable effects of FTY720 on the removal of peritoneal B cells, both peritoneal B1 and B2 B cells were revealed by quantitative RT-PCR to express similar levels of mRNA encoding S1P1 with no or only a dim expression of the other subtype of S1P receptors (S1P2-S1P4) (Figure 2A). Because B1 B cells are further divided into B1a and B1b B cells based on CD5 expression,[17-19] we then compared the S1P1 expression of B1a and B1b B cells, finding that the level of S1P1-specific mRNA expression was similar both cell groups (Figure 2B). Consistent with these observations, flow cytometric analysis revealed that FTY720 treatment resulted in roughly equivalent reductions in $B220^{+}CD11b^{+}CD5^{+}$ B1a, $B220^{+}CD11b^{+}CD5^{-}$B1b, and $B220^{+}CD11b^{-}CD5^{-}$B2 B cells, indicating that the S1P-mediated pathway exerted the same degree of influence on the various types of peritoneal B cells (Figure 2C).

No influence of FTY720 on the differentiation and viability of peritoneal B cells

Because it has been previously reported that peritoneal B1 B cells might differentiate into monocytes such as macrophage-like cells,[41] and that high concentrations of FTY720 induced lymphocyte apoptosis,[42] we tested whether FTY720 induced differentiation or apoptosis of PerC cells. To address this issue, we performed in vitro culture of PerC cells with various concentrations of FTY720 or S1P. The cellular population of PerC cells such as B1 B cells, B2 B cells, and $B220^{-}CD11b^{+}$ cells (eg, macrophages) remained unaltered after 3 days of culture with biologic concentrations (1-1000 nM) of FTY720 and S1P (Figure 3A; results at 100 nM were shown). In addition, flow cytometric analysis using annexin V revealed comparable numbers of apoptotic cells in untreated and treated groups (data not shown). Together, these results indicate that FTY720 affected neither differentiation nor apoptosis of PerC cells under these experimental conditions.

FTY720 inhibits B-cell migration into and enhances B-cell emigration out of the PerC

To determine whether FTY720 reduced peritoneal B cells by promoting their emigration from the PerC, or by inhibiting their migration into the PerC, or both, we isolated peritoneal B cells from normal mice, labeled them with 5- (and -6)–carboxyfluorescein diacetate succinimidyl ester (CFSE), and adoptively transferred them into the PerC of SCID mice and compared their emigration in animals receiving FTY720 treatment and in those that did not. We found that FTY720 treatment significantly decreased the numbers of peritoneal $CFSE^{+}$ cells in the PerC of treated mice (Figure 4A). We then addressed whether the transferred B cells were present in the circulation or migrated to other tissues. We barely detected $CFSE^{+}$ cells in the blood of FTY720-treated mice (Figure 4A). Because it has been considered that lymphocytes pass through the omentum and parathymic lymph nodes on their way to the blood from the PerC,[35,36] we next examined the cell population in these tissues. Flow cytometric analysis revealed that the number of $CFSE^{+}$ $B220^{+}$ cells was reduced in the omentum but increased in the parathymic lymph nodes of FTY720-treated mice (Figure 4A). These data indicate that FTY720 treatment induces the accumulation of peritoneal B cells in the parathymic lymph nodes, leading to a reduction of these cells in the PerC and the blood.

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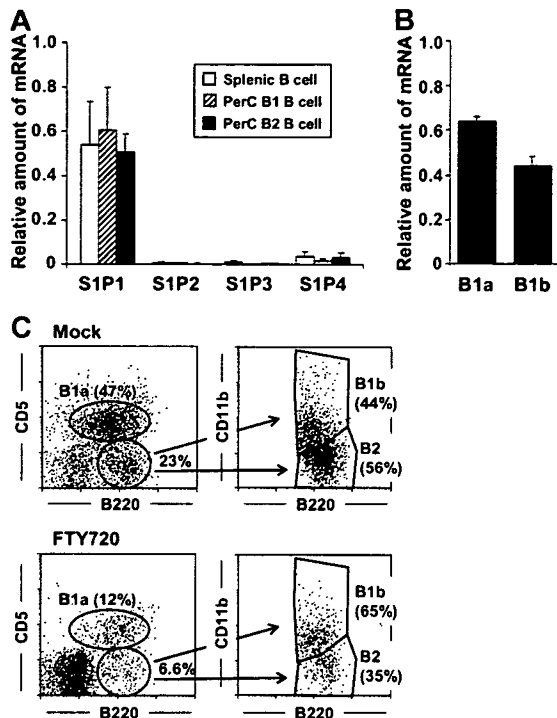

Figure 2. Equal expression of S1P1 by peritoneal B1 and B2 B cells. (A) Quantitative RT-PCR analysis for S1P receptors was performed using RNA isolated from sorted splenic B (□), peritoneal B1 (▨), and B2 (■) B cells. The relative quantity of specific mRNA was expressed as a ratio to GAPDH. The data are expressed as mean ± SD from 4 mice. (B) S1P1 expression in B1a and B1b was determined by quantitative RT-PCR analysis. (C) Flow cytometric analysis was performed to characterize B1a, B1b, and B2 B cells in the PerC of mice treated with FTY720. The data are representative of 3 independent experiments.

Under similar experiments, we also examined the effect of FTY720 on their migration from the blood into the PerC because it was previously reported that mature B cells could home to the PerC from the blood.[29] We transferred CFSE-labeled peritoneal cells to SCID mice via the intravenous route and compared their migration into PerC of mice receiving FTY720 and in those that did not. The number of $CFSE^+$ cells was significantly lower in the PerC when mice were treated with FTY720, indicating that FTY720 inhibited the migration of peritoneal B cells from the blood circulation into the PerC (Figure 4B). In these mice, $CFSE^+$ cell numbers were reduced in the blood, omentum, and parathymic lymph nodes but increased in the bone marrow (Figure 4B and data not shown). These data suggest that FTY720 directs the circulating B cells to migrate to the bone marrow rather than to the PerC. Collectively, these findings indicate that FTY720 removes the peritoneal B cells both by inhibiting their emigration from the parathymic lymph nodes and by changing the tropism of circulating B cells to bone marrow.

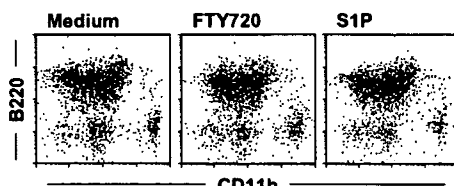

Figure 3. FTY720 does not affect peritoneal B-cell differentiation and viability. PerC cells were cultured with 100 nM FTY720 or S1P for 3 days. Cell populations were examined by flow cytometry using antibodies specific for B220 and CD11b. Results were reproducible, with very similar data obtained from each of 3 independent experiments.

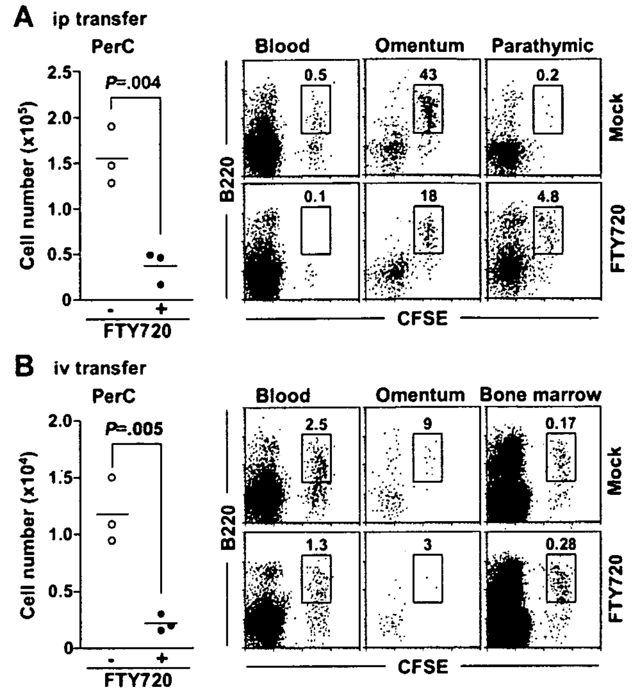

Figure 4. FTY720 simultaneously inhibits their emigration from the parathymic lymph nodes and their entrance from the blood into the PerC. SCID mice were adoptively transferred with CFSE-labeled normal PerC B cells via the intraperitoneal (A) or the intravenous (B) route, and simultaneously treated with (● or bottom panels) or without (○ or top panels) FTY720. After 12 hours, cells were isolated from the PerC, blood, omentum, parathymic lymph nodes, and bone marrow for the analysis of $CFSE^+$ cells. Horizontal bars represent the mean.

No influence of FTY720 treatment on B1 B-cell progenitors in the PerC

Having established that the effects of FTY720 on peritoneal B cells were rapid and reversible (Figure 1), we next sought to determine whether FTY720 influenced peritoneal B-cell development. To address this issue, we investigated the B1 B-cell progenitor population ($CD11b^-IgM^-B220^{low/neg}CD19^+$) recently identified in the bone marrow.[43] In this study, we found the presence of $CD11b^-CD11c^-IgM^-B220^{low/neg}CD19^+$ cells in the PerC at rates of 2.5×10^3 cells/mouse (0.1% in total peritoneal cells; Figure 5A-B). Treatment with FTY720 did not appreciably affect progenitor numbers in either the PerC (Figure 5A-B) or the bone marrow (data not shown). F5

Comparable serum antibody production under natural conditions and after immunization with bacterial antigen

In the next experiment, we set out to determine whether the FTY720-induced disappearance of peritoneal B cells exerted any influence on systemic and mucosal antibody production. Because peritoneal B cells are well characterized as a source of natural antibody production,[17-19] we examined the total serum antibody production in SCID mice following adoptive transfer of normal peritoneal cells and continuous treatment with FTY720. Comparable productions of serum IgG and IgM were detected in mock- and FTY720-treated mice, whereas serum IgA production decreased partially in mice treated with FTY720 (Figure 6A). F6 FTY720 did not affect IgG subclasses, and so TI antigen-associated subclasses of IgG2b and IgG3 were prevalent in both mock- and FTY720-treated mice (Figure 6A). These data indicate that FTY720 induces B1 B-cell alteration in the PerC but does not affect the generation of natural serum antibody production.

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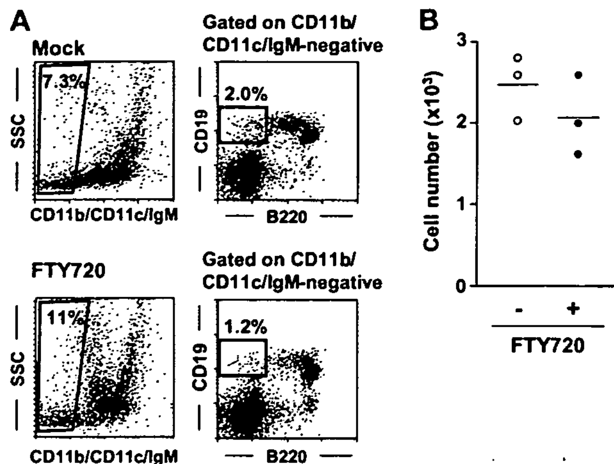

Figure 5. No influence of FTY720 treatment on peritoneal B1 B-cell progenitor. (A) B1 B-cell progenitors were determined as CD11b⁻CD11c⁻IgM⁻B220^int-neg^CD19⁺ cells. These cells were isolated from the PerC 6 hours after mock (top panels) or FTY720 (bottom panels) treatment. Data are representative of 3 independent experiments. (B) The number of peritoneal CD11b⁻CD11c⁻ IgM⁻B220^int-neg^CD19⁺ cells was calculated by using the total cell number and the flow cytometric data. Horizontal bars represent the mean.

To investigate the effects of FTY720 on the induction of bacterial antigen-specific antibody production, we used PC, a main TI antigen on the bacterial wall, as a model antigen, since B1 B cells have been shown to be a major source of PC-specific antibodies.[20,29] Accordingly, we intraperitoneally immunized mock- or FTY720-pretreated mice with R36A, a heat-killed, pepsin-treated *S pneumoniae* strain[20,29] and continued to treat every day for 5 days with mock or FTY720, respectively. Although repeated treatment with FTY720 was meant to maintain the low number of peritoneal B1 B cells during the experiment, in the end similar levels of PC-specific IgM production were detected in both mock- and FTY720-treated mice (Figure 6B). These findings suggest that the alteration of B-cell trafficking induced by FTY720 in the PerC did not affect either natural or bacterial antigen-specific serum antibody production.

Reduction of intestinal IgA production by treatment with FTY720

Finally, we investigated whether the alteration of peritoneal B-cell trafficking affected intestinal antibody production since peritoneal B cells are thought to migrate into intestinal lamina propria and contribute to subsequent IgA production.[22-24] When peritoneal B cells were adoptively transferred into SCID mice, considerable IgA and IgM production was noted in the feces (Figure 7A) and consistent with such antibody production, IgA or IgM AFCs were detected in the lamina propria region (Figure 7B). In contrast, when SCID mice were continuously treated with FTY720 after the adoptive transfer of peritoneal B cells, fecal IgA production was significantly impaired in FTY720-treated mice, whereas fecal IgM production was comparable to that seen in the mock-treated mice (Figure 7A). ELISPOT analysis confirmed this finding, demonstrating that IgA AFCs levels were significantly reduced in the intestinal lamina propria of mice treated with FTY720, whereas IgM AFCs levels were to mock-treated group (Figure 7B). Additionally, RT-PCR analysis revealed that mock- and FTY720-treated mice expressed identical polyimmunoglobulin receptors (pIgRs) in intestinal epithelial cells and J-chain in intestinal B cells. Thus, the reduction of fecal IgA could not be due to a defect in the formation of polymeric IgA or its subsequent transport via epithelial cells into the lumen (Figures 7C-D).

F7

We next examined the contribution, if any, made to S1P-dependent intestinal IgA production by B1 and B2 B cells. To address this issue, we adoptively transferred purified peritoneal B1 or B2 B cells into SCID mice, treated with or without FTY720, and then examined intestinal B cells. Flow cytometric analysis revealed that both B1 and B2 B cells equally developed IgA⁺ and IgM⁺ cells in the intestine (Figure 7E). As might be expected from the observed selective reduction in intestinal IgA production after FTY720 treatment (Figure 7A-B), FTY720 was found to reduce IgA⁺ cells regardless of the subset of B cells from which they originated, suggesting that FTY720 equally affects B1 and B2 B cells. Thus, both B1 and B2 B cell-derived IgA⁺ cells were equally decreased in the intestine of FTY720-treated mice (Figure 7E). These findings were further confirmed by showing that FTY720 treatment reduced both B1- and B2-derived fecal IgA production (data not shown). Additionally, PC-specific fecal IgA production was impaired when mice received FTY720 after oral immunization with R36A (Figure 7F). These results suggest that the cell trafficking of intestinal IgA-committed B1 and B2 B cells from the PerC is under the regulation of S1P.

Discussion

S1P is principally produced by platelets during platelet activation and thrombotic processes.[44] S1P concentrations in serum are stably maintained (100-300 nM concentration) by binding with serum protein (eg, albumin) and enzymatic degradation.[45-47] However,

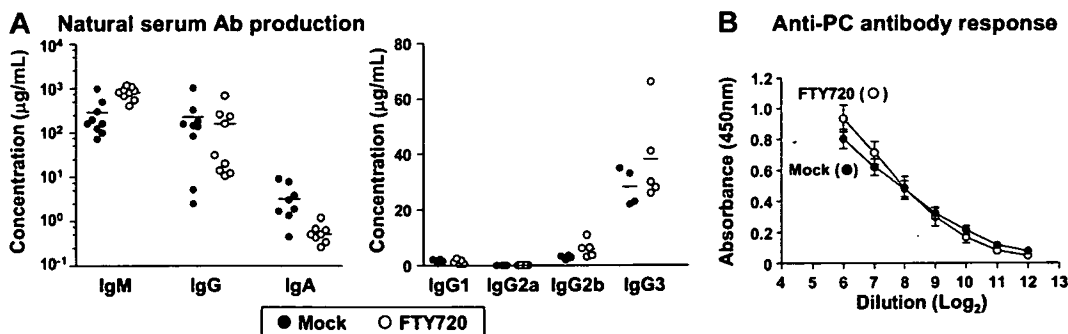

Figure 6. Effects of FTY720 on serum antibody production. (A) SCID mice were adoptively transferred with 5×10^6 normal PerC B cells and were treated with mock (●) or FTY720 (○) every 2 days. Two weeks after the transfer, serum was collected for the measurement of total immunoglobulin levels by ELISA. Horizontal bars represent the mean. (B) Mice pretreated with FTY720 and intraperitoneally immunized with heat-killed, pepsin-treated *S pneumoniae* strain R36A, received daily treatment with FTY720. After 5 days, serum anti-PC IgM was measured by ELISA. The error bars are ± SEM (n = 4) from 2 separate experiments.

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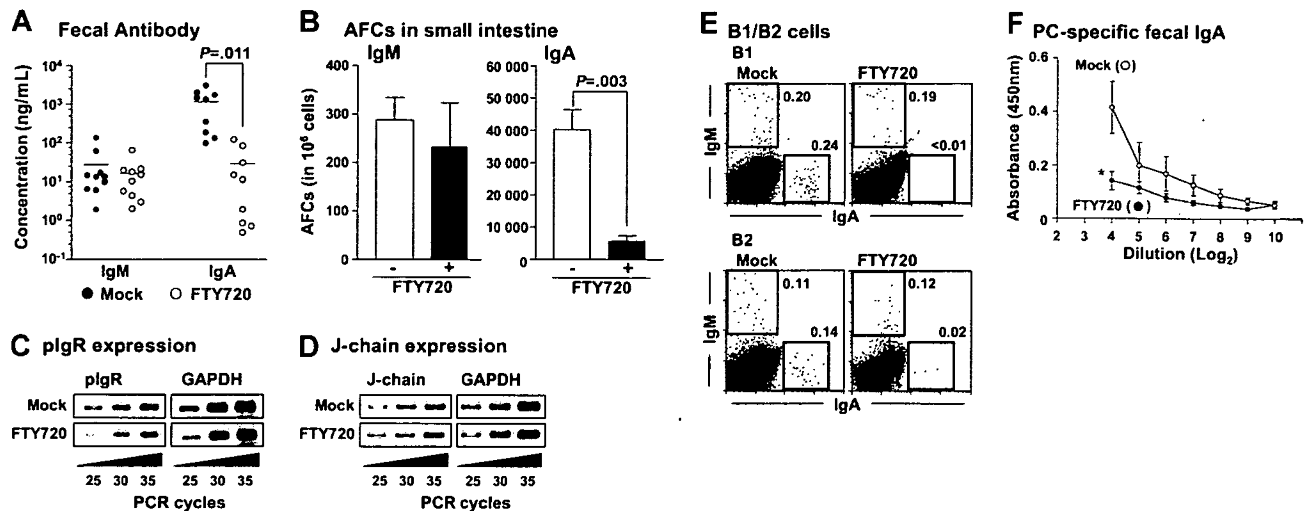

Figure 7. Impaired fecal IgA production after treatment with FTY720. (A) Fecal extracts were collected from the reconstituted SCID mice and analyzed for immunoglobulin production by ELISA as described in Figure 6A. Horizontal bars represent the mean. (B) Similarly, mononuclear cells were isolated from small intestinal lamina propria and used for the ELISPOT assay. The error bars are ± SEM (n = 5). (C-D) pIgR expression in epithelial cells (C) and J-chain expression in lamina propria lymphocytes (D) were examined by RT-PCR. Data are representative of 3 independent experiments. (E) SCID mice were adoptively transferred with purified peritoneal B1 or B2 cells and treated with FTY720 as described in Figure 6A. FACS analysis was performed to detect IgA^+ and IgM^+ cells in the intestinal lamina propria of mice treated with (right) or without (left) FTY720. Data are representative of 3 independent experiments. (F) Mice were orally immunized with R36A together with cholera toxin and treated with (●) or without (O) FTY720. After 3 days, fecal PC-specific IgA levels were measured by ELISA. The error bars are ± SEM (n = 4). $^*P < .05$.

S1P receptors are biologically regulated and so more given to fluctuation.[2,48] It has been well demonstrated that S1P1 is preferentially expressed on the lymphocytes and that its expression is altered during lymphocyte development and activation, a process that plays an important role in the regulation of lymphocyte egress from the organized lymphoid structures including the thymus and secondary lymphoid organs.[4,5] However, its immunologic role in the regulation of lymphocyte trafficking at nonorganized tissues such as the PerC and the intestinal lamina propria region remains less than fully understood. In this study, we demonstrated that the interaction between S1P and its receptor (S1P1) also regulated peritoneal B-cell trafficking, both inbound and outbound. Despite their immunologic differences,[18,21,49] B1a, B1b, and B2 B cells in the PerC expressed similar levels of S1P1 expression and exhibited a comparable dependency on S1P (Figures 1-2). These comparable involvements of S1P-mediated pathway were different from chemokine-mediate pathway. For instance, CXCL13/CXCR5- or CCR7-mediated pathways showed different regulation ability against peritoneal B1 and B2 B cell.[29,30] The difference might instead be explained by the previously demonstrated hierarchy that exists between S1P and chemokines.[13] The study showed that S1P signaling overcame the recruiting activity of CXCL13 in the regulation of marginal zone B-cell localization.[13] Because CXCL13 has been reported to play the major role in the B-cell retention in the PerC,[29] it is likely that S1P simultaneously overcomes the CXCL13-mediated retention of peritoneal B cells and enhances the emigration of B cells out of the peritoneal cavity.

In addition, adoptive transfer experiments also revealed that FTY720 removed peritoneal B cells via at least 2 distinct pathways (Figure 4): (1) inhibition of their egress out of the parathymic lymph nodes on their way to the blood circulation from the PerC and (2) their migration from the blood into the PerC. The first observation accords well with current thinking that lymphocytes can traffic from the peritoneal cavity to the blood through omentum and parathymic lymph nodes[35,36] and that cells accumulate in the lymph nodes after FTY720 treatment.[10-12] Although it has been demonstrated that FTY720 treatment induces an accumulation of naïve B cell in the bone marrow,[10] we demonstrate here for the first time that S1P also regulates the immigration of mature B cells from the blood circulation into the PerC. These results lend support to the recent contention by Butcher's group that lymphocyte egress from structurally nonlymphoid tissues (eg, skin) is not random, but biologically regulated and that CCR7-mediated signaling plays an important role in that regulation.[39] Our current findings point to S1P as another key molecule regulating lymphocyte trafficking in unorganized and diffused tissues (eg, the PerC).

Although the precise developmental pathway for peritoneal B cells remains controversial, it is generally thought that peritoneal B1 B cells originate from fetal liver,[50] fetal omentum,[51] and para-aortic splanchnopleura,[52] whereas B2 cells are preferentially generated from bone marrow.[53] In addition, a recent study has identified B1 B progenitor cells in bone marrow.[43] These progenitor cells are characterized as Lin^- $CD19^+$ $B220^{low\text{-}neg}$ and have the ability to differentiate into either peritoneal B1a or B1b cells.[43] In this study, we found similar $CD11b^-CD11c^-IgM^-CD19^+$-$B220^{low\text{-}neg}$ cells in the PerC (Figure 5). Although it is still unclear whether peritoneal $CD11b^-CD11c^-IgM^-$ $CD19^+B220^{low\text{-}neg}$ cells are derived from the bone marrow or from other sites (eg, fetal liver), it is interesting to note that FTY720 removed a marked number of peritoneal $B220^+$ B cells, but did not affect $CD11b^-CD11c^-IgM^-CD19^+B220^{low\text{-}neg}$ cell numbers (Figure 5). Together with a previous study demonstrating that S1P1 expression is up-regulated during T-cell development in the thymus,[4] our current results raise the possibility that S1P1 expression is similarly up-regulated during B-cell development in PerC, perhaps accounting for the fact that FTY720 affects $B220^+$ PerC cells, but not $CD11b^-CD11c^-$ $IgM^-CD19^+B220^{low\text{-}neg}$ cells (Figures 1-2 and 5).

FTY720 treatment diminishes germinal center formation and so drastically curtails antibody production against the TD antigen, but it does not affect antibody production against TI antigen.[14,15] Consistent with these reports, FTY720 treatment of SCID mice adoptively transferred with normal PerC cells did not influence the natural antibody production in serum. Thus, comparable levels of total plasma IgG production, mainly IgG2b and IgG3, were detected in mock- and FTY720-treated mice (Figure 6A). Since we transferred PerC cells into SCID mice lacking functional T cells,

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the preferential production of IgG2b and IgG3, well-known subclasses dominantly reactive to TI antigen, was expected. We also found that FTY720 did not affect anti-PC IgM production after immunization of normal mice with heat-killed, pepsin-treated *S pneumoniae* strain, R36A (Figure 6B). This observation was consistent with previous reports demonstrating that FTY720 did not influence antibody production against soluble TI antigen (eg, TNP-Ficoll and NP-Ficoll)[14,15] and further suggested that FTY720-mediated alteration of peritoneal B-cell distribution did not affect antibody production against TI antigen regardless of antigen form (eg, soluble or particulate). However, this observation contradicted a previous report demonstrating that peritoneal B-cell paucity in CXCL13-deficient mice resulted in the impaired antibody responses against intraperitoneal immunization of *S pneumoniae*.[29] A variety of scenarios could account for this discrepancy. Our findings showed that the peritoneal B1 and B2 B cells never completely disappeared, even after repeated administration of FTY720 (Figures 1-2 and data not shown). Those remaining cells are nonreactive to FTY720 and might contribute to antibody production against *S pneumoniae*. Using chemokine receptor expression (CXCR5 and CCR7) to distinguish among B-cell populations and to identify the nonreactive population in the PerC, we compared the effect of FTY720 on the cells types and did not find any differences (data not shown). In addition, based on the recent report that CD69 induced the internalization of S1P1 and negatively regulated S1P-mediated signaling,[54] we sought to examine the CD69 expression of peritoneal B cells, demonstrating that peritoneal B cells were exclusively CD69 negative (data not shown). Similarly, no difference was detected in surface immunoglobulin expression (data not shown). Alternatively, though a small dose of bacterial antigen (10^7) was intraperitoneally administered, intact or processed bacterial antigen could be carried into other immunologic sites (eg, spleen, intestine, and bone marrow) by peritoneal macrophages or dendritic cells and activated B cells for the production of PC-specific IgM.

Although the serum antibody production originating peritoneal B cells was unaltered in FTY720-treated mice, peritoneal B1 and B2 B cell–derived intestinal S-IgA production and IgA AFCs in the intestinal lamina propria were markedly reduced after FTY720 treatment and so shown to be under the regulation of S1P. S-IgA production was significantly reduced (150 times less than mock-treated mice), but IgM levels remained largely unaffected (Figure 7A-B). However, FTY720 treatment did not affect pIgR and J-chain expression (Figure 7C-D). Again, several scenarios could account for these findings. First, since previous studies proposed the mutual interaction between S1P- and chemokine-mediated pathway in the lymphocyte trafficking,[13,16,55-57] the cooperative pathway mediated by both S1P and chemokine may determine the selective effects of FTY720 on IgA^+ B cells. These selective effects could be mediated by the differing degree of dependency by IgM^+ B cells and IgA^+ B cells on S1P for the migration of peritoneal B cells into the intestine. In this regard, it was reported that CCR10 expression was prevalent on IgA^+ B cells with a plasmablast and plasma cell phenotype in the blood and the intestine but negligible on IgA^- B cells.[58] We found that intraperitoneally transferred $CFSE^+$ B cells were barely detected in the blood but some $CFSE^+$ B cells were still present in the omentum, which adjoins the gastrointestinal compartment (Figure 4A).[35,36] This finding led us to consider a second scenario, namely, that peritoneal B cells have a unique S1P-independent trafficking pathway from the PerC to the intestine through the omentum. Yet a third possibility is that FTY720 inhibited class switch recombination from IgM to IgA and thereby the subsequent differentiation into IgA plasma cells. Similar phenomena were observed in mice lacking activation-induced cytidine deaminase (AID), an essential molecule for class switch recombination.[59] A previous study demonstrating that sphingosine inhibited IL-5–induced IgA synthesis in LPS-stimulated murine B cells lends credibility to a fourth possibility: that FTY720 induces down-regulation of IgA production.[60] These possibilities may similarly account for the discrepancy that FTY720 reduced PC-specific fecal IgA production but not PC-specific serum IgM after immunization with R36A (Figures 5B and 7F). We are currently engaged in studies to clarify this issue.

In summary, we have demonstrated that S1P plays an important role in the regulation of lymphocyte trafficking not only in the organized lymphoid tissues, but also in the unorganized and diffused tissues. It also plays a pivotal role in linking peritoneal B cells to intestinal IgA production. Collectively, our findings point to a novel immunologic significance for S1P in CMIS-independent mucosal immunity.

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Authorship

Contribution: J.K. designed research, performed research, analyzed data, and wrote the paper; Y.K., M.G., M.H., I.I., F.M., and I.O. performed research and analyzed data; and H.K. designed research and wrote the paper.

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Intraepithelial lymphocytes: their shared and divergent immunological behaviors in the small and large intestine

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Summary: At the front line of the body's immunological defense system, the gastrointestinal tract faces a large number of food-derived antigens, allergens, and nutrients, as well as commensal and pathogenic microorganisms. To maintain intestinal homeostasis, the gut immune system regulates two opposite immunological reactions: immune activation and quiescence. With their versatile immunological features, intraepithelial lymphocytes (IELs) play an important role in this regulation. IELs are mainly composed of T cells, but these T cells are immunologically distinct from peripheral T cells. Not only do IELs differ immunologically from peripheral T cells but they are also comprised of heterogeneous populations showing different phenotypes and immunological functions, as well as trafficking and developmental pathways. Though IELs in the small and large intestine share common features, they have also developed differences as they adjust to the two different environments. This review seeks to shed light on the immunological diversity of small and large intestinal IELs.

Keywords: *intraepithelial T lymphocyte (IEL), small and large intestines, development trafficking, classical and non-classical MHC*

Introduction

Mucosal surfaces of the gastrointestinal and respiratory tracts directly interact with the mucosal lumen, the harshest environment in our body and one that is constantly exposed to many foreign antigens, including food nutrients, allergens, and commensal and pathogenic microorganisms. To protect mucosal sites from these foreign materials and to maintain mucosal homeostasis, the aerodigestive tract is equipped with multiple physical, biological, and immunological barriers.

The acquired-type immunological barrier at the mucosal surface is initiated by the induction of antigen-specific immune responses through mucosa-associated lymphoid tissues (MALT) including the Peyer's patches (PPs), isolated lymphoid follicles (ILFs), and the nasopharynx-associated lymphoid tissues (1, 2). MALT are covered with a follicle-associated epithelium, which

contains antigen-sampling M (microfold) cells that allow for selective transport of antigens from the lumen to underlying antigen-presenting cells such as dendritic cells (DCs) and macrophages (3). These cells present the antigen to T and B cells in MALT, rendering them antigen-primed T and immunoglobulin A (IgA)-committed B cells, respectively. These T and B cells then migrate to effector tissues (e.g. intestinal epithelium, *lamina propria*, and nasal passages) via an immunological intranet known as the common mucosal immune system (CMIS). Because these anatomical and functional characteristics enable MALT to act as inductive tissues for the priming of antigen-specific T- and B-cell responses, they have often been made the target for vaccine delivery (4).

Several physical and biological barriers associated with the innate immune system also protect these sites from microbial invasion and help to maintain their mucosal homeostasis. Closely knit to one another via tight-junction proteins like occludins, claudins, and zonula occludens (5, 6) and characterized by brush-border microvilla as well as a dense mucin layer at the apical site, epithelial cells (ECs) physically bar the entry of pathogenic microorganisms by inhibiting attachment and penetration (7). In addition, they produce antimicrobial peptides such as a β-defensin (7). In addition to these antimicrobial peptides, secretory IgA (S-IgA), the predominant isotype at mucosal sites, is secreted and plays an important role in preventing pathogen invasion (4, 8). S-IgA forms a J-chain-mediated polymeric structure that interacts with the polymeric Ig receptor expressed on mucosal ECs, an interaction that is required for their transport into the lumen (9, 10). S-IgA contributes to both acquired and innate immunity. Acquired immunity, principally mediated by B2 B cells, and innate immunity, mediated by B1 B cells, both play equally important roles in S-IgA production in the murine intestinal lamina propria region (11). The B2 B-cell-mediated S-IgA is mainly derived from the CMIS and thus plays a key role in the recognition of T-dependent antigens, while B1 B cells produce antibodies to T-independent antigen, such as phosphorylcholine, a hapten-like antigen associated with many pathogenic bacteria (1, 2, 12). Based on the undiscriminating reactivity of B1 B-cell-derived S-IgA against commensal and pathogenic microorganisms (1, 2, 12), it has been generally considered that B1 B-cell-derived S-IgA plays a pivotal role in the prevention of attachment of both commensal and pathogenic microorganisms.

Intraepithelial lymphocytes (IELs) play an important role in the maintenance of mucosal homeostasis by actively or negatively regulating mucosal innate and acquired immunity. Residing as single cells among ECs, they monitor for stressed or damaged ECs and express αβ T-cell receptors (TCRs) or γδTCRs, which recognize antigenic peptides presented by conventional major histocompatibility complex (MHC) molecules (13) or by non-classical MHC molecules, respectively (14). In addition to the uniqueness of TCR expression, the developmental pathway and immunological functions of the IELs render them distinctive. Although most previous studies focused on IELs mainly in the small intestine, several lines of evidence have demonstrated that immunological and biological differences between the small and large intestine lead to differences in composition of small and large intestinal IELs as well. This review describes and discusses those features that IELs from the two environments share and those that make them distinct.

IEL subsets in the small and large intestines

Type a and type b IELs in the small and large intestine

IELs are interspersed among ECs in both the small and large intestine, but their frequencies vary, with one IEL for every 4–10 ECs in the small intestine and for every 30–50 ECs in the large intestine (15). In both the small and large intestine, IELs mainly consist of T cells. Two unique characteristics of the IELs allow them to be divided into subsets. First, IELs contain cells expressing the homodimeric form of CD8α (CD8αα), which is only barely detectable in the systemic immune compartments. Second, IELs contain more cells expressing γδTCRs than do peripheral T cells, which almost exclusively express αβTCRs. These unique features allow us to divide IELs into two groups: 'type a', which is also detectable in the blood, lymph, and secondary lymphoid organs including PPs, and 'type b', which is far more prevalent in the mucosal epithelium. Type a mucosal T cells express αβTCRs with CD4 or CD8α, while type b IELs express αβTCRs or γδTCRs with a unique coreceptor, CD8αα, or lack CD8 and CD4 altogether [double negative (DN)]. In addition to the uniqueness of CD8αα and αβ/γδTCR expression, type b IELs can be distinguished from type a IELs because they lack some markers, such as CD2 (16), CD28 (17), cytotoxic T-lymphocyte antigen-4 (18), and Thy-1 (19). Although small and large intestines contain both type a and type b IELs, the ratio between type a and type b IELs differs markedly (*Table 1*). The small intestine is rich in CD8αα IELs, while the large intestine contains very few. For example, 65–75% of the IELs in the small intestine of BALB/c mice are type b IELs (60% are CD8αα, 10% are DN) (*Table 1*). Because about 60–70% of small intestinal type b IELs were γδTCR^{+}, 40% of the total IELs in the small intestine can be assumed to be γδTCR+ (*Table 1*). In contrast, CD8αα IELs represent only a minor

Table 1. Different composition of IELs in the small and large intestine*

	Small intestine (total cell number 5.4 ± 1.4 × 10^6 cells), %		Large intestine (total cell number 4.3 ± 1.8 × 10^5 cells), %	
T cell subsets	Among total IELs	Among the subset	Among total IELs	Among the subset
CD8αα	62.7 ± 2.5		4.7 ± 0.6	
αβTCR		35.7 ± 3.1		67.3 ± 2.5
γδTCR		64.0 ± 6.5		32.7 ± 2.5
No TCR		N.D.		N.D.
CD8αβ	15.6 ± 2.0		7.3 ± 1.2	
αβTCR		84.6 ± 3.1		95.6 ± 0.6
γδTCR		N.D.		N.D.
No TCR		15.3 ± 3.0		4.3 ± 0.6
CD4	9.0 ± 1.7		31.0 ± 5.6	
αβTCR		87.3 ± 2.1		98.6 ± 0.6
γδTCR		N.D.		N.D.
No TCR		12.7 ± 2.1		1.7 ± 0.6
DP	7.3 ± 0.6		<0.1%	
αβTCR		52.0 ± 6.1		N/A
γδTCR		2.0 ± 1.0		N/A
No TCR		46.1 ± 5.3		N/A
DN	5.4 ± 1.2		57.3 ± 5.2	
αβTCR		2.7 ± 0.6		5.3 ± 0.6
γδTCR		20.7 ± 3.1		4.3 ± 0.6
No TCR		76.6 ± 3.2		90.4 ± 1.0

N.D., not detectable; N/A, not applicable; type b IEL in italics.
*The data were obtained from female BALB/C mice (7–10 weeks) and represent means ± SD (n = 5).

population in the large intestine (*Table 1*). Alternatively, the percentage of DN IELs in the large intestine is higher than in the small intestine (60% in the large intestine, 15% in the small intestine) (*Table 1*).

Unique features of type a IELs

Although type a IELs exclusively express αβTCRs and the coreceptors CD4 or CD8αβ, traits typical of peripheral T cells, they also differ from conventional T cells in a number of ways. For instance, the ratio of CD8αβ to CD4 in the small intestinal IELs is much higher than in the spleen, while the ratio in the large intestine is similar to that in the spleen. Additionally, in the small but not the large intestine, CD8αα expression can be coexpressed by CD8αβ (20) and CD4 (21) conventional mucosal T cells as well as by single-positive IELs.

Natural-killer-like cells in the intestinal epithelium

An additional unique feature of IELs is the ability of some, the so-called natural killer T (NKT) IELs (22–24), to express NK receptors. The ligand for one of these NK receptors, NKG2D, is the human non-classical MHC molecule MICA/B (MHC class I-chain-related gene A/B), which is predominantly expressed on damaged or transformed ECs (25). Interestingly, γδTCR recognizes the same MICA/B molecules (26, 27), implying that IELs can use both γδTCR and NKG2D to recognize damaged or stressed ECs through MICA.

Also present in the IEL population are TCR^- NK cells whose characteristics differ from those of splenic NK cells (22, 23, 28). Our group previously demonstrated that the cytotoxic effects of NK IELs were enhanced by interleukin-15 (IL-15) (28). IL-15 has also been reported to regulate NKG2D (29) as well as MICA expression (30). Furthermore, it has recently been reported that IL-15 induces CD94 expression and interferon-γ (IFN-γ) and IL-10 production from IELs, allowing them to show Fas-ligand-mediated killing activity (31). Although little has been reported about NK or NKT IELs in the large intestine (24), it is likely that these NK-like IELs directly and/or indirectly interact with ECs for the maintenance of intestinal homeostasis.

Recent thymic emigrants are a novel subclass of IELs

Staton *et al.* (32) have reported that a naive population in IELs is made up of $CD8^+$ recent thymic emigrants (RTEs). In general, naive T cells migrate into the intestine after activation in the gut-associated lymphoid tissue (GALT) [e.g. PPs and mesenteric lymph nodes (MLNs)], but RTEs are distinguished by the ability to migrate into the small intestine without activation (32) (*Fig. 1*). Unlike naive T cells in the periphery, RTEs exclusively express α4β7 integrin, αE integrin, and CCR9, making them gut-tropic T cells (33) (*Fig. 1*). After migrating directly into the intestinal epithelium from the thymus, they begin to proliferate in response to antigen exclusively present in the gut and to show a phenotype similar to that of resident IELs (32). Based on the

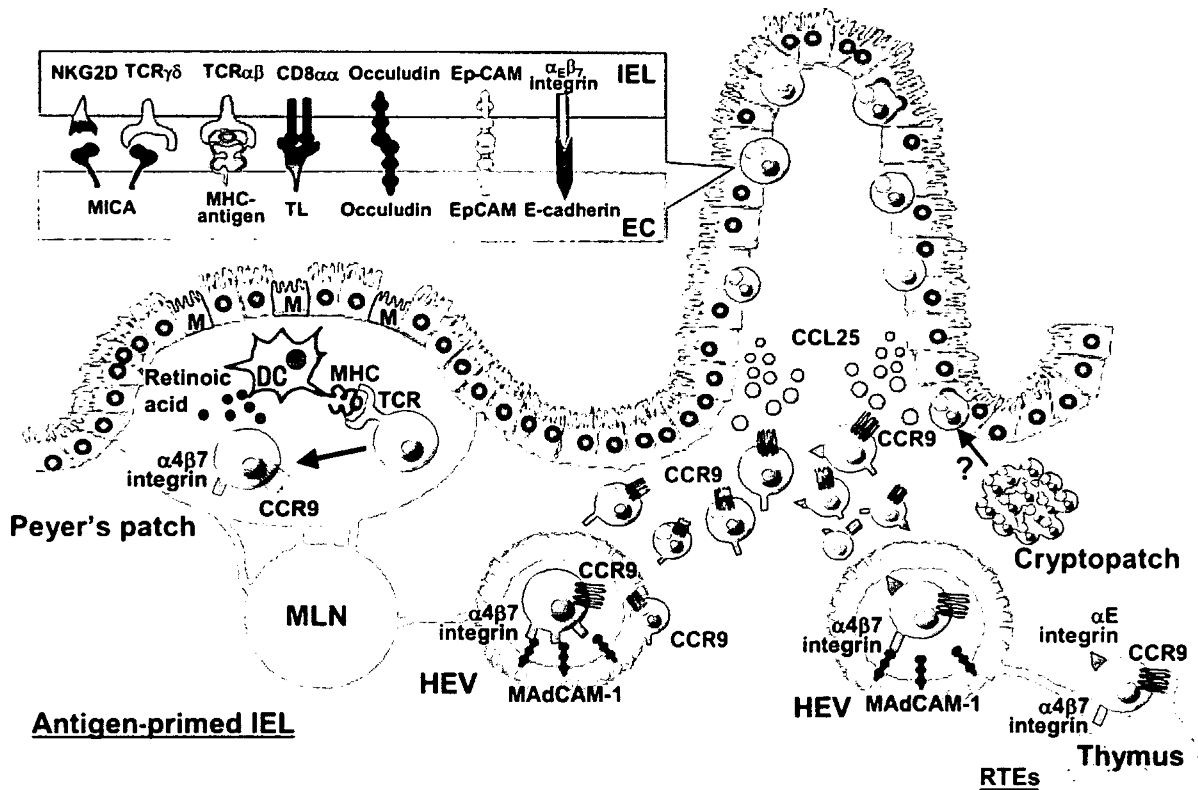

Fig. 1. Unique trafficking pathways for IELs in the intestinal compartments. In the antigen-primed IEL trafficking pathway, DCs in PPs take up antigens transported through M cells and present them to T cells in an MHC-dependent manner. Simultaneously, DCs produce retinoic acid, which causes antigen-primed T cells to express α4β7 integrin and CCR9. α4β7 integrin interacts with intestinal high endothelial venules (HEV) expressing MAdCAM-1, while CCR9 allows specific migration to the CCL25, a chemokine produced by intestinal ECs. In the other pathway, RTEs migrate into the intestinal epithelium without antigen priming at PPs and MLNs. Alternatively, RTEs have already expressed α4β7 integrin, CCR9, and αE integrin in the thymus. The other population of IELs may originate from the CPs, but their trafficking pathway has not been fully elucidated. Upon migration into the intestinal epithelium, IELs express several unique molecules capable of tethering ECs (e.g. αE integrin, Ep-CAM, and occludin) or of recognizing stressed or infected ECs (e.g. αβTCR, γδTCR, NKG2D, and CD8).

results obtained by the Study (32), one can surmise that almost half of the naive IELs in the small intestine are derived from RTEs, while PPs and MLNs contain a naive population of RTEs with much less frequency (less than 3%). With their diverse TCR repertoires, RTEs seem likely to play an important role in maintaining TCR diversity in the intestine. At present, however, it remains unknown whether or not RTEs are present in the large intestinal epithelium.

Differing compositions of naive and activated/memory IELs in the small and large intestine

Not surprisingly given the different environmental circumstances faced in the small and large intestine, IELs in the two environments differ with regard to the ratio between naive and activated/memory IELs. It is well established that small intestinal IELs are functionally characterized as antigen-experienced activated/memory cells expressing CD69 and that they contain few naive cells (less than 5%) (34–36). In contrast, the large intestine contains large numbers of naive-type cells expressing CD62L^{high} and CD44^{int} (more than 40%) but a far less abundant supply of activated/memory cells (34–36).

A correlation may exist between the abundant presence of activated/memory IELs and of CD8αα IELs in the small intestine. As mentioned above, the small intestine contains a large number of type b IELs expressing CD8αα as well as type a IELs expressing CD8αα together with CD4 or CD8αβ (*Table* 1). CD8αα, which can be expressed on activated conventional T cells of the lymph nodes and spleen, plays a key role in mediating the survival of those effector cells that further differentiate into mature memory T cells (37, 38). These surviving conventional T cells acquire the ability to migrate to and reside long-term in non-lymphoid tissues, including the gut, and they readily reinduce CD8αα expression after secondary restimulation. These observations indicate that the expression of CD8αα by the various subsets of mucosal T cells is not a marker to identify their origin or the specificity of their TCRs, but rather this expression reflects their effector/memory phenotype and plays a unique

role in maintaining immune homeostasis within the intestine.

Though both show a memory phenotype, type a and type b IELs represent distinct types of memory cells. Type a IELs resemble conventional memory T cells found in the periphery and *lamina propria* regions (39), and given that TCRβ clonotypes were comparable for type a IELs and lamina propria T cells (39), it is possible that they differentiate via the same pathway. In contrast, CD8αα (αβ IELs) are thought to be natural memory T cells. CD8αα αβ TCR IELs show oligoclonal TCR repertoires that are different from those of type a IELs (40), indicating that CD8αα αβ IELs differentiate via a distinct pathway. The accumulation of autoreactive TCRs in CD8αα αβ IELs (41), along with their early appearance in neonatal mice (42) and human fetal intestine (43), when exposure to exogenous antigen is minimal, provides evidence for the self-specificity of these T cells. In addition, most human fetal IELs already express an effector/memory cell phenotype (43), indicating that a differentiation pathway other than exposure to peripheral exogenous antigen resulted in the acquisition by these cells of an activated memory-like phenotype. However, unlike the CD8αα γδ TCR IELs (γδ IELs), the αβ IELs do not appear in the small intestine of germ-free mice lacking gut flora, although bacterial colonization of these animals restores the population of CD8αα αβ IELs (44). These observations indicate that local stimulation from the lumenal microflora is required for generation and maintenance of the CD8αα αβ IELs.

Antigen recognition by IELs

Type a IELs are reactive to classical MHC-restricted antigen presentation

Similar to peripheral T cells, type a IELs express αβTCRs together with the coreceptor CD8αβ, which recognizes MHC class I, or the coreceptor CD4, which recognizes MHC class II. In general, MHC class I presents peptide generated from cytoplasmic protein (endogenous antigen) (13), while antigens proteolytically processed after endocytosis/phagocytosis are presented by MHC class II molecules (45). Although both MHC class I and class II molecules express self- and non-self-antigen, type a IELs are specific for non-self-antigen because of the positive and negative selection in the thymus.

Antigen recognition by type b CD8αα αβ IELs

The MHC restriction of type b CD8αα αβ IELs is still enigmatic. CD8αα αβ IELs are drastically reduced in number in β2-microglobulin-deficient mice (46–49), but they are present in mice deficient either in the transporter associated with antigen processing (TAP) (49) or in the classical class I molecules ($K^{b-/-}D^{b-/-}$) (50, 51). This finding suggests that type b IELs require a TAP-independent non-classical MHC class Ib molecule.

It has been reported that mice deficient in Qa-2, a non-classical MHC class I molecule that is TAP dependent (52), possessed fewer CD8αα αβ IELs, suggesting that some CD8αα αβ IELs are reactive to Qa-2 (53). However, when they compared BALB/c mice with the Qa-2-null Bailey substrain of BALB/c mice, Cheroutre's group (54) were unable to confirm these findings. Furthermore, Qa-2 is broadly expressed by the IELs themselves (18), and its expression is mostly TAP dependent. Two other non-classical class I molecules, the thymic leukemia (TL) antigen (55) and CD1d (56), have been reported to be expressed by mouse intestinal ECs, although this is controversial in the case of mouse CD1d (57). Both TL and CD1d are TAP independent, but they require β2-microglobulin expression (58–60). However, the TCRs of this IEL subset in mice have never been shown to recognize either of these Ib molecules. Recent structural data indicate that the TL antigen has a very narrow and closed groove that makes interacting with αβTCRs unlikely (61).

Researchers using several MHC class-II-restricted TCR transgenic systems (62, 63) showed that CD8αα IELs were generated when agonist self-peptides were present, just as in the MHC class-I-restricted systems. Despite this finding, peripheral T cells in these same MHC class-II-restricted TCR transgenic mice were $CD4^{+}$. These observations indicate that CD8αα does not function as a TCR coreceptor and that expression of CD8αα on the αβ IELs does not necessarily imply MHC class I restriction. From these results, it can be surmised that CD8αα αβ IELs, unlike type a IELs, are self-reactive but are not selected for self-reactivity to a single antigen and that they may include cells reactive to classical class I molecules, non-classical class I molecules, and MHC class II molecules.

Specificity of γδTCR IELs (γδIELs)

It is still uncertain to what extent the γδTCRs require a selection event for their development, especially in mice. Murine γδ IELs make predominant use of the Vγ5 (also known as Vγ7) gene segment together with several Vδ genes (64). Their CDR3 regions are more diverse than the invariant γδTCRs expressed in the skin and reproductive tracts (65). Although the great majority of these cells express CD8αα, their numbers are not reduced in β2-microglobulin-deficient mice (47, 48). In humans, the γδTCRs expressed by IELs predominantly use Vδ1 (66), and they have an oligoclonal CDR3 repertoire that can be sustained over a period of many months (67). These TCRs recognize MICA and

MICB, class-I-like molecules that are induced by heat shock or stress on ECs (26, 68, 69). Like TL antigen in mice, MIC molecules are not capable of presenting peptides or other ligands (61). As mentioned above, MICA is also recognized by NKG2D. Although mice do not have a functional MIC gene ortholog, they do have NKG2D receptors that recognize class-I-like molecules such as H60 (70) and members of the RAE class-I-like family (71). There are no reports, however, indicating that significant populations of γδ IELs or other mouse γδ T cells recognize these class-I-like molecules.

TL antigen and MICA for CD8αα IELs

Given the MHC class I or class II specificity of the TCRs of CD8αα αβ IELs together with their requirement for β2-microglobulin, an MHC class I-molecule-mediated function other than peptide presentation must be required for CD8αα αβ IEL development and/or maintenance. In this regard, Leishman *et al.* (20) have shown that the mouse TAP-independent, β2-microglobulin-dependent, non-classical MHC class I molecule TL is a unique ligand for CD8αα. TL is constitutively and abundantly expressed by the ECs of the small intestine and so is in close proximity to the CD8αα IELs. The structural analysis of the molecule indicates the absence of an obvious antigen-binding groove (61). The specific interaction of TL with CD8αα on activated CD8αα αβ IELs modifies the TCR-activation signals to allow for regulated immune responses that are compatible with the gut environment (20). Unlike CD8αβ, CD8αα is not internalized with the TCR from the surface of activated T cells (72), and associated $p56^{lck}$ is inefficiently provided to the TCR complex, in part because of the inability of CD8αα to locate effectively into lipid rafts (73). It is, therefore, possible that the interaction of TL with CD8αα might sequester CD8-associated $p56^{lck}$ away from the TCR complex, consequently reducing activation signals while promoting survival. In contrast to the abundant expression of TL in the small intestinal epithelium, TL was only weakly expressed in the large intestinal epithelium (74), which might be consistent with the small numbers of CD8αα IELs in the large intestine.

Our previous study constructed a transgenic mouse with gut-specific MICA expression driven by a T3b promoter, and we surprisingly observed a clonal expansion of $CD4^+$ T cells also possessing CD8αα in the small intestine (75), suggesting that MICA positively regulates the expression of CD8αα (Fig. 2). Furthermore, studies in the inflammatory bowel disease model showed that transgenic MICA was able to attenuate the acute colitis induced by dextran sodium sulfate administration

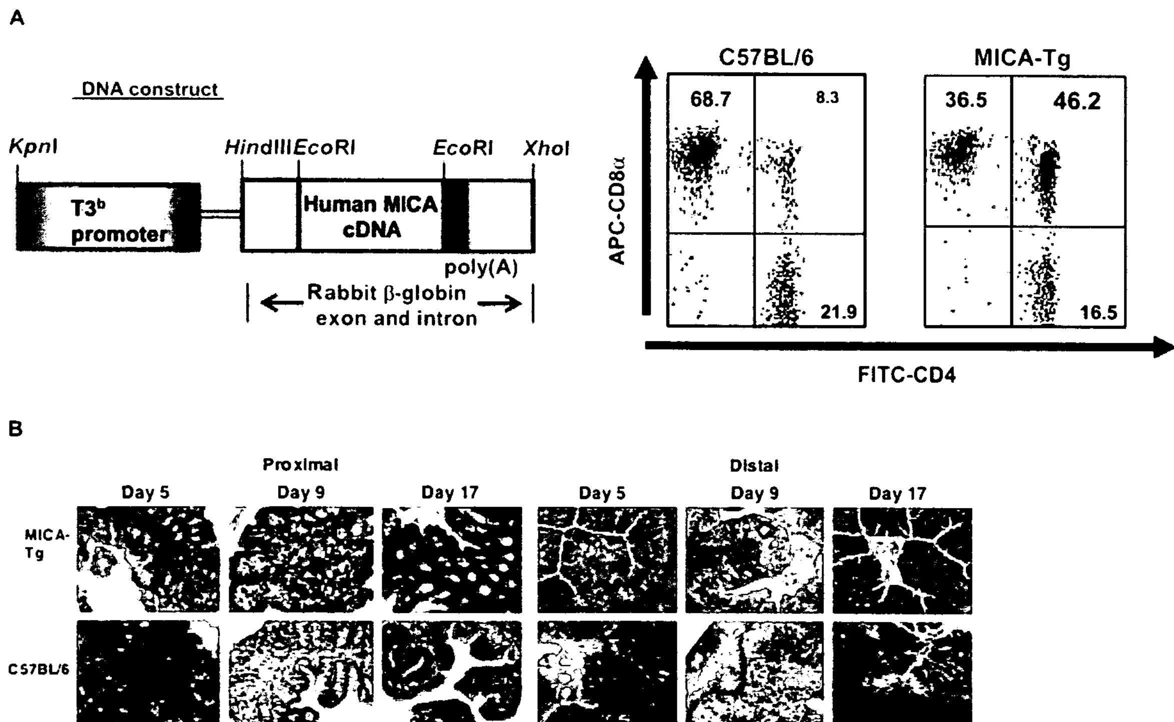

Fig. 2. CD8αα expression by mucosal T cells plays a unique role in maintaining immune homeostasis within the intestine. A transgenic mouse with gut-specific MICA expression driven by a T3b promoter was constructed. Surprisingly, a clonal expansion of $CD4^+$ T cells also possessing CD8αα was observed in the small intestine, suggesting that MICA positively regulates the expression of CD8αα (A). Furthermore, studies in the inflammatory bowel disease model show that transgenic MICA was able to attenuate the acute colitis induced by dextran sodium sulfate administration (B).

(Fig. 2). In this regard, it should be noted that MICA and TL, both of which belong to MHC class Ib molecules, are preferentially expressed in the intestinal ECs (55, 76). Thus, although MICA is both β2-microglobulin and TAP independent, while TL is TAP independent but β2-microglubulin dependent, it is likely that both molecules play a similar role in the development of IELs. Several years ago, Madakamutil *et al.* (38) demonstrated that TCR ligation and activation of CD8αα IELs by TL resulted in increased expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL, suggesting that TL may abrogate antigen-induced cell death.

Unique signal transduction through TCR and FcεRIγ in type b IELs

Type b IELs in both the small and large intestine exclusively express homodimers of FcεRIγ or heterodimers of FcεRIγ with CD3ζ (77–79), while type a IELs and peripheral T cells predominantly express homodimers of CD3ζ, which can form a complex with TCR that is important for signal transduction (77). Thus, most T cells from CD3ζ-deficient mice showed abnormal development (78, 80). However, type b IELs could express TCR in the CD3ζ-deficient mice because FcεRIγ compensated for the CD3ζ function in the TCR–CD3 complex (78–80). Because CD8αα molecules are not able to transfer signaling through TCR (73), selective expression of FcεRIγ on type b IELs might act as an alternative pathway for type b IEL activation.

Uniqueness of IEL developmental pathways

Agonist selection in CD8αα IELs

Although controversy remains as to what extent the γδ IELs require thymic selection (42, 81), much evidence exists in mice indicating that self-specific CD8αα αβ IELs are actively and preferentially selected in the thymus under agonist selection conditions (63, 82). The outcome of thymic selection of conventional αβ T cells is determined by the signal strength received through the TCR when interacting with MHC molecules presenting self-antigens. Intermediate TCR signal strength results in positive selection of conventional αβ T cells, whereas strong TCR-mediated signals delete cells that express those TCR from the conventional T-cell repertoire. By contrast, CD8αα αβ IELs preferentially accumulate under conditions that lead to the deletion of conventional αβ T cells, and, as a consequence, autoreactive TCRs are enriched in the TCR repertoire of CD8αα αβ IELs (41). The existence of an agonist-dependent selection of CD8αα αβ IELs has been shown using mice that express a transgenic TCR and cognate antigen during thymic selection. The H-Y TCR is specific for a Y-chromosome-encoded peptide presented by H-$2D^b$, and H-Y TCR transgenic mice provide a model for the self-based agonist selection of CD8αα αβ IELs (63, 83). When the H-Y TCR transgenic mice were crossed onto a recombination-activating gene $(Rag)^{-/-}$ background, CD8αβ thymocytes matured in the thymus and populated the peripheral tissues of H-Y antigen-negative female mice, whereas few CD8αβ IELs and no CD8αα IELs were detected in these female mice. By contrast, despite deletion of conventional CD8αβ H-Y TCR^+ T cells in $Rag^{-/-}$ male H-Y TCR transgenic mice, large numbers of H-Y TCR^+ IELs largely expressing CD8αα developed (83).

Similar observations have been made in TCR transgenic mice that coexpress the endogenous cognate antigen, such as 2C TCR transgenic mice expressing the H-$2L^d$ alloantigen recognized by the 2C TCR (84) and double-transgenic mice expressing both the TCR and the cognate antigen. Examples of the latter include OT-1 TCR transgenic/RIPmOVA mice [membrane-bound ovalbumin (OVA) antigen transgene under the control of the rat insulin promoter (RIP)] (63), influenza virus nucleoprotein (NP)-specific F5 TCR transgenic mice that express NP peptides (85), and P14 lymphocytic choriomeningitis virus (LCMV)-specific TCR transgenic mice that coexpress the cognate LCMV-specific epitope GP33-41 (86).

The selection of CD8αα αβ IELs is most effective in the presence of high-affinity interactions between the TCR and the agonist self-antigens, as was shown using the clonotypic F5 TCR, which has a high affinity for the influenza virus epitope NP68. NP68/F5 TCR double-transgenic mice preferentially generated CD8αα αβ IELs, whereas double-transgenic F5 TCR mice coexpressing the antagonist peptide NP34 did not (85). Similarly, CD8αα IELs expressing the 2C TCR were readily generated in mice expressing the H-$2L^d$ high-affinity ligand, whereas CD8αβ T cells developed preferentially in the presence of the low-affinity ligand presented by H-$2K^b$ (84). Similarly, H-Y TCR^+ CD8αα IELs were more efficiently generated in H-$2D^{b/b}$ homozygous male mice than in their H-$2D^{b/d}$ heterozygous counterparts (87).

Similar to conventional αβ T cells, CD8αα αβ IELs require the expression of pre-Tα during the initial steps of αβTCR rearrangements (88). The fact that the efficient agonist selection of CD8αα αβ IELs also requires the expression of the TCRα chain connecting peptide domain (α-CPM) further suggests that the agonist and conventional selection processes have similar features and may have evolved simultaneously (63).

Agonistic selection in the thymus

The marked reduction in the numbers of CD8αα αβ IELs in congenic athymic nude mice (89, 90) and in mice that had been neonatally thymectomized (91) indicates that the thymus is

required for the generation of agonist-selected CD8αα αβ IELs. This requirement is further supported by the observation that thymus grafts from male H-Y TCR transgenic mice generated CD8αα H-Y TCR^+ IELs when transplanted into female recipient mice (63), and wildtype thymus grafts efficiently generated CD8αα αβ IELs when transplanted into recipient nude mice (42). In the latter case, it was shown that CD8αα IELs and conventional CD4 and CD8αβ IELs were generated with different kinetics. Fetal thymus grafts or grafts from mice up to weaning age mainly repopulated the intestine of the recipient mice with CD8αα αβ IELs, whereas thymus grafts from older mice were more efficient in generating the conventional αβ T-cell subsets (42). It should not be assumed, however, that the adult thymus had a reduced ability to generate CD8αα αβ IELs as the adult thymus grafts from the male H-Y TCR transgenic $Rag^{-/-}$ mice efficiently did so (63). It is possible that in the absence of conventional positive selection, as in the case of the male H-Y TCR transgenic mice on a $Rag^{-/-}$ background, the generation of CD8αα αβ IELs can continue in adult mice as long as a functional thymus is present.

Is it true that the type b IELs are developed in the thymus? Given that CD8αα αβ IELs continued to be efficiently generated under conditions that caused deletion of CD8αβ T cells in the thymus, some speculated that the precursors of these cells had not been submitted to thymic negative selection (41, 83). Taken together with observations that, in the absence of a thymus, these type b IELs nevertheless develop in lymphopenic mice grafted with normal bone marrow, fetal liver, or intestine (92, 93), this finding suggests that CD8αα αβ IELs are likely the progeny of extrathymic progenitor cells localized within the intestine itself.

Kanamori *et al.* (94) demonstrated that approximately 1500 cryptopatch aggregates (CPs) are dispersed throughout the wall of the small and large intestine in mice (*Fig.* 3). Cells within the CP are composed of mostly lymphoid progenitors expressing stem cell factor receptor, or c-kit, and IL-7Rα, but lacking the lineage markers (CD3, B220, Mac-1, Gr-1, and TER-119). They possess transcripts for germline TCR genes, messenger RNA (mRNA) for CD3ε as well as proteins (i.e. RAG-2 and pre-Tα) involved in TCR gene rearrangement (95), and are able to generate TCR^+ IELs, type b CD8αα αβ IELs, as well as γδ IELs, albeit with a strong bias toward the generation of γδ T cells, in irradiated severe combined immune-deficient mice. These findings demonstrated that c-kit^+ CP cells are committed to the T-cell lineage and are competent for the generation of IELs (93, 96). Both IL-$7R\alpha^{-/-}$ mice (94) and common cytokine receptor γ-chain (γ_c)-deficient mice (95) lack CP structure, but local

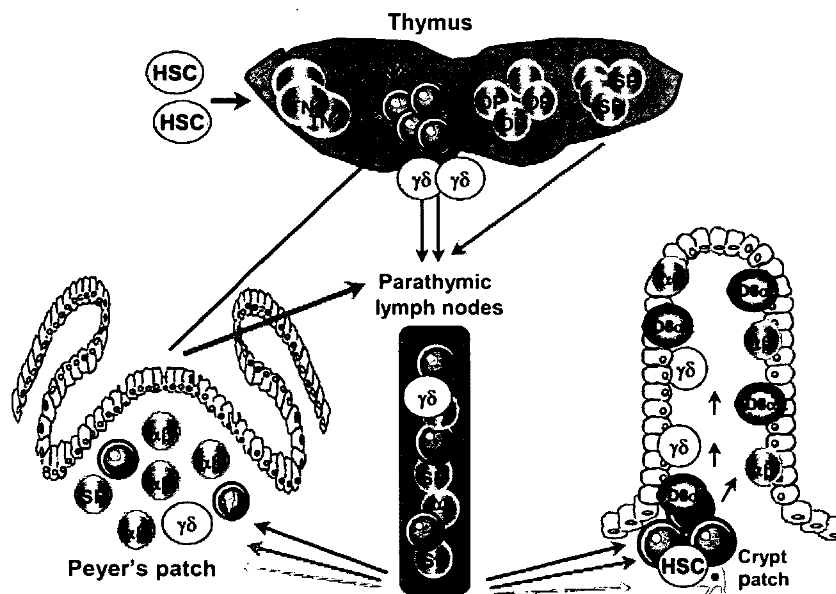

Fig. 3. Thymus-dependent versus thymus-independent IEL development. Bone marrow-derived or fetal-liver-derived hematopoietic stem cells (HSCs) enter the thymus as T-committed cells. Signals from the stroma and from other thymocytes ($CD4^+$ $CD8^+$ DP thymocytes?) induce differentiation toward type b unconventional T cells. Other immature $CD4^-CD8^-CD3^-$ (TN) cells gradually switch off unconventional T-cell genes and differentiate toward conventional $CD4^+$ or $CD8^+$ single-positive (SP) αβ T cells. Both immature and mature cells exit from the thymus. Unconventional T cells and their progenitors (TN2-3 cells) more readily populate the gut epithelium, possibly via CPs, where the progenitors may complete their differentiation into CD8αα IELs. Some TN2-3 progenitors may circulate back to the thymus. The gut epithelium could also be populated by bone marrow-derived HSCs that differentiate into type b γδ T cells in CPs.

(fatty-acid-binding protein promoter) transgene expression of IL-7 by mature enterocytes of IL-$7^{-/-}$ mice restored the development of CPs and γδ IELs, whereas γδ T cells remained absent from all other tissues (97), suggesting an important role for IL-7R signaling in their generation. These observations indicate a correlation between the presence of CP cells and lymphopoiesis in the gut, especially of γδ IELs.

Lambolez *et al.* (88) reported difficulty identifying specific T-lineage-committed precursors among the CP cells. Using multiple lineage markers, they performed a detailed characterization of the progenitor cells isolated from CPs. They also transferred bone marrow from nude mice to irradiated thymectomized γ_c and *RAG-2* double-deficient recipient mice to establish the sequence of appearance of these precursors during lymphoid development in the gut. This analysis provided little evidence that CP lineage $(\text{Lin})^-$ cells are T-cell committed, a finding consistent with data showing that several gene mutations that affect T-cell differentiation, including pre-Tα, TCRα, and CD3ε, had no effect on the appearance or ratio of CP precursor cells (88). However, CP Lin^- cells did express GATA-2, a transcription factor found in pluripotent hematopoietic progenitor cells, indicating that these CP cells might represent precursors of other hematopoietic cells. There is relatively little cell turnover in CPs, and very few CP cells express *RAG-2* mRNA (88). In contrast, potential precursor cells isolated from the epithelium were more committed to the T-cell pathway, and TCRα mutation dramatically altered subtype ratios within the immature Lin^- cells of the epithelium (88).

In seeking to investigate which immune cells synthesize RAG-2, subsequent studies (98) have shown that RAG-2 production is detected exclusively in the thymus in normal mice with an intact thymus (euthymic), but RAG-2 is found in the MLNs of the gut and minimally in the PPs of nude or other athymic mice. Their findings suggest that extrathymic T-cell development is totally repressed in normal euthymic mice and becomes active only in cases of severe T-cell depletion. Whether in normal and athymic mice, neither CP cells nor IELs showed fluorescence-tagged *RAG-2*. To evaluate these findings, Nonaka *et al.* (99) generated athymic nude mice that lacked all lymph nodes and PPs by administering lymphotoxin-β receptor Ig and tumor necrosis factor (TNF)-R55-Ig fusion proteins into pregnant *nu/+* mice and *nu/nu aly/aly* (alymphoplasia) mice that lacked all lymph nodes, PPs, as well as intestinal ILFs. These two kinds of mice, which harbored numerous DN as well as CD8αα γδ IELs possessing TCR Vγ1, Vγ4, and Vγ7 segments in the small intestines, were found to retain CPs. These findings provide compelling evidence that in the *lamina propria* of the intestine, at least some of the DN and CD8αα γδ IELs developed within the tiny clusters, that is, CPs, that are filled with c-kit^+IL-$7\text{R}^+\text{Lin}^-$ lymphopoietic cells.

Podd *et al.* (100) explored whether $\text{CD3}\varepsilon^+$ CP cells are linearly related to γδ IELs using laser capture microdissection analysis. They observed that in-frame TCR Vγ5 rearrangements were detected in the $\text{CD3}\varepsilon^+$ CP cells that are shared with a subset of γδ IELs. However, they did not detect mRNA for *RAG-1* gene expression in the CP aggregates. Thus, their data lend support to the hypothesis that CPs are perhaps reservoirs for some antigen-experienced γδ IELs in mice.

Eberl and Littman (101) performed interesting experiments in mice bearing two transgenes, a Cre recombinase transgene under the control of the retinoid-related orphan receptor γt (RORγt) promoter and a green florescent protein (GFP) transgene controlled by a ubiquitous promoter that is only transcribed when a lox-flanked stop sequence is removed by Cre. RORγt, an orphan nuclear hormone receptor, was detected in immature DP thymocytes, fetal lymphoid-tissue-inducer cells, and c-kit^+Lin^- cells in CPs. In these transgenic mice, all progeny of a Cre-expressing cell become constitutively GFP^+ because the stop sequence of the GFP transgene has been deleted. These experiments, also performed with a CD4–Cre transgene, showed that GFP was expressed by all DP thymocytes and also by all single-positive T cells and αβ IELs, including CD8αα αβ IELs, but not by any other immune cells such as γδ IELs, B cells, or NK cells. This finding elegantly demonstrates that all αβ IELs are of thymic origin and must have been selected by the DP pathway. However, these findings did not exclude an extrathymic origin for γδ and other IELs. They did suggest, however, that the contribution of intestinal precursors including c-kit^+Lin^- cells is minimal in healthy mice but could become more substantial in lymphopenic mice or in mice in which large numbers of T cells have been mobilized in response to intestinal inflammation (101). However, Eberl and Littman (102) mentioned elsewhere that the principal function of the CP cells is to induce the formation of lymphoid follicles in the intestinal *lamina propria* in a manner similar to the induction of lymph nodes and PPs by the RORγt-expressing fetal lymphoid tissue inducer cells.

Resolution of the thymus-dependent versus thymus-independent controversy

Just when researchers seemed to hit an impasse regarding thymus involvement in IEL production, a truly incendiary report by Lambolez *et al.* (103) was published. In this paper, they proposed a previously unknown pathway for T-cell

development that has implications for the thymus every bit as great as for the gut. Reexamining assumptions germane to the basic biology of T-cell development in the thymus, they noticed that neonatal thymus grafts leak specific subsets of early T-committed progenitor cells that will repopulate both the host thymus, in which they rapidly complete normal T-cell development, and the host gut, where they survive for many weeks and give rise via defined intermediates to type b IELs (103). This finding conflicted with previous assumptions that the thymus is a source solely of mature T cells and that it does not have the potential for long-term reconstitution. Furthermore, this report emphasizes that the leaked cells are early $CD4^{-}CD8^{-}CD3^{-}$ triple-negative stage 2 (TN2) and TN3 progenitors that are T-cell committed but that have not yet rearranged their TCR genes. However, CD8αα IELs could not be reconstituted even after adoptive transfer of other more mature DP or single-positive thymocyte populations (*Fig.* 3).

Using an indirect assay, the authors (103) also showed that the leaked TN cells are much more efficient at completing T-cell differentiation in the gut than are cells derived directly from the bone marrow (*Fig.* 3). These results strongly suggest that stroma cells in the thymus and/or other thymocytes could help 'persuade' progenitors to differentiate into unconventional CD8αα IELs. The thymic imprinting might include gene products that favor survival and/or are biased toward unconventional IEL development.

It should be underlined that recent studies using H-Y TCR transgenic mice seem to exclude the possibility that DP thymocytes could repopulate the intestine (104) because H-Y TCRβ and TCRα transgenes in this model are expressed abnormally in DN thymocytes and the mice have no TN2–TN4 cells. Those phenotypes were shown to be artifacts of the transgenic systems. In conditions in which the TCRα chain is physiologically expressed in DP thymocytes of the H-Y mice, the male mice have many DP thymocytes expressing the self-reactive αβ T cells, but no increase in *Smyc* peptide-specific CD8αα IELs is found in the gut. Thus, the accumulation of self-reactive TCR transgenic cells in the gut of conventional H-Y transgenic mice seems to be because of the anomalous expression of the TCRα transgene in early TN thymocytes.

It is not clear whether leaked TN2–TN3 cells home exclusively to the gut or to several mucosal sites. CPs may be special features of the gut that attract immature cells. In this regard, Onai *et al.* (105) reported that $CD11c^{+}$ DCs in CP aggregates express CCL25 and that the signaling via the chemokine receptor CCR9 that is present on $c\text{-}kit^{+}$ CP cells might play a central role in CP and CD8αα IEL development. When the expression of CCR9 on the surface of $c\text{-}kit^{+}$ bone marrow cells was blocked by a viral construct expressing an intracellular form of CCL25, bone-marrow-recipient mice largely failed to develop CP aggregates and CD8αα IELs. By contrast, the cellularity and composition of the thymus and spleen of these mice were well developed. These findings led Onai *et al.* (105) to hypothesize that CCR9–CCL25 interaction is required for CP maturation in the generation of TCR^{-} and TCR^{+} IEL subsets.

The gut itself then seems to retain immature thymic emigrants and to nurture their development. As shown before, this nurturing includes local production of IL-7, which promotes local TCR gene rearrangement (97). A capacity for gene rearrangement might seem unexpected, given a report that a reporter gene expressed from the *Rag-2* promoter is not detected outside the thymus (98). Nonetheless, it has been shown that *Rag-1* is expressed in the gut, albeit in low numbers, from an alternative 5′ exon that may be conserved in the mouse (106). The reporter construct that has been used previously (95) might not register expression by an unorthodox *Rag* exon (98). In summary, the gut is a viable site for T-cell differentiation because it expresses *Rag*, locally produces IL-7, and can accommodate immature T-cell-committed progenitors.

Cytokine requirements for IEL development

IELs require cytokines for their development. As mentioned above, γ_c-deficient mice show impaired development of IELs expressing either αβTCRs or γδTCRs (107, 108). Several cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21) share γ_c as a part of their receptors. Consistent with impaired CP development, IL-7- or IL-7 receptor (IL-7R)-deficient mice did not have γδ IELs but possessed substantial numbers of αβ IELs (109, 110). Local transgene expression of IL-7 by the ECs of $IL\text{-}7^{-/-}$ mice restored the development of γδ IELs associated with CP development (97). Although both αβTCR- and γδTCR-positive CD8αα IELs belong to type b IELs, the developmental pathway of each is distinct, at least in terms of IL-7 requirements. γδ IELs could be preferentially impaired at least in part because IL-7-mediated signaling is essential for the rearrangement of TCRγ genes (111). Additionally, the presence of substantial numbers of αβ IELs in IL-7- or IL-7R-deficient mice implies that other cytokines using γ_c play a pivotal role in the regulation of IEL development, particularly of αβ IELs.

Much attention is currently focused on IL-15, a cytokine that is produced by several kinds of cells including DCs and ECs and that includes an IL-2 receptor β chain (IL-2Rβ) as a part of its receptor. As one might expect from the elimination of αβ and γδ IELs from IL-2Rβ-deficient mice (112), disruption of the IL-15- or IL-15Rα-encoding gene also resulted in a significant

reduction in IELs expressing either αβTCRs or γδTCRs (113, 114). Reciprocally, IL-15 stimulates IEL proliferation and restricts the TCR repertoire of IELs by selectively regulating local Vγ gene chromatin modification (115, 116). In regard to IL-15-induced IEL development, a recent finding has indicated that MyD88 (myeloid differentiation factor 88), an adapter molecule associated with Toll-like receptors, mediates IL-15 production by ECs. As expected, the number of IELs significantly decreased in MyD88-deficient mice (117), suggesting that a MyD88 regulation pathway may help maintain the intestinal homeostasis against commensal microorganisms via the IEL and EC cross-talk system.

IEL trafficking pathway

Determination of IEL tropism by chemokine

It is generally accepted that chemokines play an important role in the regulation of lymphocyte trafficking (118, 119). For the regulation of IEL trafficking into the small intestine, CCR9 has been considered to be a key chemokine (120, 121) (Fig. 1). Its ligand, thymus-expressed chemokine (CCL25), is constitutively expressed on the ECs in the small intestine (122, 123) (Fig. 1). CCL25 has been shown to mediate the localization of type a IELs, especially $CD103^+$-naive cells and, more recently, to activate $CD69^+$ cells in the small intestine (124). CCL25 also regulates precursor differentiation during the generation of type b IELs, but it is less involved in steps that involve IEL retention (125). Mice with defective responses against CCL25 exhibited a significant reduction in CPs and IELs in the small intestine. In contrast to these implications, however, disruption of the CCR9 gene resulted in only a modest decrease in IELs, indicating that other chemokines might be involved in the IEL migration into the small intestine (126, 127). Furthermore, although there are $CCR9^+$ cells in the large intestine, the CCR9/CCL25-mediated pathway is not involved in IEL recruitment into the large intestine because its ligand (CCL25) is poorly expressed on large intestinal ECs (120, 122, 123). It would stand to reason then that chemokines other than $CCR9^+$ are involved in the regulation of IEL trafficking in the large intestine. Indeed, CCL28, which acts as a ligand for $CCR10^+$-IgA-secreting cells (128, 129), is the predominant chemokine expressed on ECs in the large but not the small intestine (130). Although memory-type T cells in the intestine did not express CCR10 (128), it is possible that a CCL28/CCR10-mediated pathway might contribute to the regulation of large intestinal IEL trafficking, especially naive-type IELs preferentially residing in the large intestine. In addition, the various chemokine receptors expressed on IELs, such as CCR3, CCR4, CCR5, and CXCR3 (131–133), may play a pivotal role in the alternative pathway of IEL trafficking. Although CCR6 is not expressed on mature IELs, CCR6-deficient mice showed impaired development of CD4 CD8αα αβ IELs and CD8αα αβ IELs but not CD8αα γδ IELs because of the dysregulated development of CPs (134). These findings suggest that CD8αα αβ IEL and CD8αα γδ IEL developments have different chemokine requirements.

In addition to chemokine-mediated pathways, we recently found that sphingosine-1-phosphate (S1P) plays a role in IEL trafficking, especially in the large intestine (Jun Kunisawa, unpublished data). S1P, a lipid mediator originating from sphingomyelin, has been linked to the regulation of lymphocyte emigration from the secondary lymphoid organs and thymus by an accumulating body of evidence (118). Our new finding suggests that some type a IELs also use S1P in their exit from the GALT (Jun Kunisawa, unpublished data). Because previous studies proposed a mutual interaction between S1P- and chemokine-mediated pathways in lymphocyte trafficking (135, 136), the cooperative pathway mediated by both S1P and chemokines may determine the selective trafficking of IELs into the small and large intestine.

Integrin-mediated IEL trafficking and distribution

In addition to chemokines, adhesion molecules also contribute to the regulation of IEL trafficking (Fig. 1). Among them, β7 integrin has been considered to be an important molecule in determining the localization of intestinal IELs (Fig. 1). Thus, β7-integrin-deficient mice showed critically reduced numbers of IELs in the intestine (137, 138). IEL numbers were equally reduced in the small and large intestine, indicating that a chemokine-mediated rather than an adhesion-molecule-mediated pathway determines whether IELs migrate to the small or large intestine.

Several lines of evidence have demonstrated that DCs derived from the GALT (PPs and MLNs) but not those derived from the spleen are capable of determining the gut tropism of intestinal T cells by the induction of high levels of α4β7 integrin and CCR9 expression (139–141). A recent study showed that retinoic acid, which induces the α4β7 integrin and CCR9 expression on T cells (142), is dominantly expressed by GALT DCs (Fig. 1). Thus, mucosal T cells activated by orally administered antigens presented by GALT DCs tend to migrate into distant intestinal effector sites, including the intestinal epithelium, by obtaining mucosal trafficking molecules (e.g. α4β7 integrin and CCR9). Regarding the integrated integrin- and chemokine-mediated induction pathway, CCL25, a ligand for CCR9, is expressed in the crypt region of the small intestine, which is close to the mucosal addressin cell adhesion molecule 1 $(MAdCAM\text{-}1)^+$ vessels (123, 127). These findings further indicate that α4β7

integrin–MAdCAM-1 and CCL25–CCR9 interactions synergistically recruit gut-tropic IELs into the small intestine.

The unique migration pathway of RTE into the small intestine

Because the migration of RTE into the small intestinal epithelium was detected in the lymphotoxin-α-deficient mice lacking organized secondary lymphoid organs such as PPs and MLNs, their migration must have been independent of the GALT structure (32). In contrast, the migration of RTEs was abolished in CCR9-deficient mice or mice treated with anti-CCL25 antibody or anti-α4β7 integrin antibody, suggesting that the migration was dependent on a CCL25/CCR9-mediated and α4β7-integrin-mediated pathway (32) (*Fig.* 1). As one might expect given the preferential expression of CCR9 on RTEs, RTEs homed to the small intestinal epithelium more efficiently than did non-RTE-naive T cells (32). Although RTEs share with type a IELs a similar dependency on CCL25/CCR9 and α4β7 integrin, RTEs do not require antigen-mediated activation. Thus, much unlike type a IELs, RTEs isolated from OT-I^{+}*Rag1*$^{-/-}$ mice can migrate into the small intestinal epithelium without antigen stimulation (32, 143).

Retention molecules on IELs

The intimate integrin-mediated interaction between ECs and IELs plays a pivotal role not just in the infiltration of the IELs into the intestine but also in the retention of IELs at the epithelium. Many attempts have been made at identifying the molecules that tether IELs and ECs together in the small intestine. CD103 (αE integrin), which interacts with the E-cadherin expressed on ECs, is expressed on IELs (144) (*Fig.* 1). It was previously reported that transforming growth factor-β (TGF-β) induced downregulation of α4β7 integrin and simultaneously upregulated CD103 (145). Thus, α4β7 integrin expression was reduced following IEL entry into the small intestinal epithelium, a reduction that coincided with an increase in CD103 expression (146). In addition to the TGF-β-mediated pathway, a recent study (146) proposed that CCR9-mediated signaling promoted the induction of CD103. In CCR9-deficient CD8^{+} T cells, upregulation of CD103 after migration into the small intestinal epithelium was markedly delayed (146). Additionally, the interaction between CD103 and E-cadherin was involved in the destruction of the intestinal epithelium during graft-versus-host disease (147), indicating that CD103 is a pivotal molecule regulating lymphocyte trafficking not only under natural conditions but also during pathological situations. Further, although CD103 was not required for the entry into the intestine (138), CD103-deficient mice exhibited a decreased number of IELs (148). However, the reduction was modest, suggesting that other molecules contribute to the retention of IELs at the epithelium. In this regard, several lines of evidence have demonstrated that IEL retention may be mediated by their expression of some integrins (e.g. α1β1 integrin, α4β1 integrin, and β2 integrin) (149–151). As in CD103-deficient mice, no or only a partial reduction in IELs was seen following the disruption of each of these integrins, suggesting that other integrins and/or molecules may compensate for their lack (151, 152). Additionally, we previously reported that IELs expressed the EC adhesion molecule (Ep-CAM) (153) (*Fig.* 1). Because Ep-CAM is also expressed on ECs and mediates homophilic cell-to-cell adhesion (154), interaction between IELs and ECs via Ep-CAM may play a pivotal role in IEL retention. Further, a recent study (155) indicates that IELs express tight-junction molecules such as occludins and zonula occludens-1 (*Fig.* 1). Taken together, the intimate biological interactions between ECs and IELs provide physiological and immunological barriers that act as a first line of defense at the intestine.

Physiological and biological functions of IELs

CD8αα αβ IELs in immune protection and mucosal homeostasis

Despite their high frequency, potentially autoreactive type b CD8αα αβ IELs are not self-destructive under normal conditions. Rather, their presence in the gut epithelium correlates with immune regulation and immune quiescence. Because endogenous, natural, specific self-antigens for these CD8αα αβ IELs have not yet been identified, studies analyzing their functional ability are limited to the use of TCR transgenic mice, which express a cognate antigen for this TCR either naturally or through expression of a second transgene.

These transgenic mice, which have few conventional αβ T cells expressing the transgenic TCR in the thymus, spleen, and lymph nodes, generate enhanced numbers of transgenic CD8αα αβ IELs specific for the self-antigen (63) that are not anergic (83, 86, 156). Upon a systemic LCMV infection in double-transgenic mice expressing both an LCMV epitope (GP33-41)-specific TCR transgene and the cognate antigen transgene (GP33-41) driven by an MHC class I promoter, CD8αα αβ IELs showed signs of virus-induced activation (86). This activation was antigen specific as the CD8αα αβ IELs from double-transgenic mice expressing an OVA-peptide-specific TCR and the OVA antigen did not show activation following LCMV infection. Unlike conventional (GP33-41-specific) CD8αβ T cells, which lose self-tolerance upon LCMV infection, the activation of these self-specific CD8αα αβ IELs did not result in