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Homeostatic Proliferation of Naive CD4+ T Cells in Mesenteric Lymph Nodes Generates Gut-Tropic Th17 Cells

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Homeostatic proliferation of naive T cells in the spleen and cutaneous lymph nodes supplies memory–phenotype T cells. The “systemic” proliferative responses divide distinctly into fast or slow cell division rates. The fast proliferation is critical for generation of effector memory T cells. Because effector memory T cells are abundant in the lamina propria of the intestinal tissue, “gut-specific” homeostatic proliferation of naive T cells may be important for generation of intestinal effector memory T cells. However, such organ-specific homeostatic proliferation of naive T cells has not yet been addressed. In this study, we examined the gut-specific homeostatic proliferation by transferring CFSE-labeled naive CD4+ T cells into sublethally irradiated mice and separately evaluating donor cell division and differentiation in the intestine, mesenteric lymph nodes (MLNs), and other lymphoid organs. We found that the fast-proliferating cell population in the intestine and MLNs had a gut-tropic αβ7+ Th17 phenotype and that their production was dependent on the presence of commensal bacteria and OX40 costimulation. Mesenteric lymphadenectomy significantly reduced the Th17 cell population in the host intestine. Furthermore, FTY720 treatment induced the accumulation of αβ7+/IL-17A+ fast-dividing cells in MLNs and eliminated donor cells in the intestine, suggesting that MLNs rather than intestinal tissues are essential for generating intestinal Th17 cells. These results reveal that MLNs play a central role in inducing gut-tropic Th17 cells and in maintaining CD4+ T cell homeostasis in the small intestine. The Journal of Immunology, 2013, 190: 5788–5798.

Mature T cells circulate through the peripheral lymphoid and extralymphoid organs, forming peripheral T cell compartments (1). These compartments are homeostatically regulated (2–4) and consist of three groups of T cells that are phenotypically distinguished by surface Ags: naive T (T naïve) cells (CD44lowCD62Lhigh), central memory T (T central memory) cells (CD44highCD62Lhigh), and effector memory T (T effector memory) cells (CD44highCD62Llow) (1, 5, 6). T naïve and T central memory cells migrate across high endothelial venules into secondary lymphoid tissues such as lymph nodes, Peyer’s patches (PPs), and the spleen (1, 6). In contrast, T effector memory cells preferentially accumulate in extralymphoid organs, notably in the mucosal tissues of the intestine, lungs, and genital surfaces (6–8). Because most pathogens infect mammalian hosts through mucosal surfaces, T effector memory cells provide a first line of defense against reinfection (6, 9).

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Abbreviations used in this article: GVHD, graft-versus-host disease; IBD, inflammatory bowel disease; ILN, inguinal lymph node; MLN, mesenteric lymph node; MLX, mesenteric lymphadenectomy; OX40L, OX40 ligand; PP, Peyer’s patch; PPs, Peyer’s patch–deficient; SFB, segmented filamentous bacteria; T central memory T; T effector memory T; T naïve T.

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T cells in MLNs differentiate into Th17 cells after being primed by migrating dendritic cells.

Homeostatic proliferation is a proliferative T cell response elicited by lymphopenia (2, 4). This systemic proliferative response contributes not only to maintenance of T cell homeostasis (2, 4) but also to pathogenesis for inflammatory diseases including IBDs and GVHD (32–35). The systemic homeostatic proliferation has been studied by transferring T cells into lymphopenic hosts, such as sublethally irradiated or Rag-deficient mice, and examining division and differentiation of donor cells in the spleen and/or cutaneous lymph nodes (36–39). Because the homeostatic proliferation of donor Ts2c cells supplies a memory–phenotype T cell pool (40–45), this system can be used to determine the origin of intestinal Th17 cells, including Th17 cells. It is important to note that in the systemic homeostatic proliferation under lymphopenic conditions, cell populations can be divided into two groups by their division rate—slow, in which cells divide only once or twice per week, and fast, in which cells divide more than seven times within 7 d (46, 47). Slow proliferation depends on IL-7, occurs in secondary lymphoid organs, and produces a cell population that retains the CD44highCD62Lhigh naive phenotype and has limited differentiation potential (2). In contrast, fast proliferation produces cells that robustly differentiate into the CD44highCD62Llow T EM phenotype cells (46, 47). However, it is unclear whether the two distinct types of homeostatic proliferations may also occur in an organ-specific fashion such as in the intestine and MLNs. And if so, the question of where and how fast proliferation occurs, and which organs accumulate the fast-dividing cells, has not yet been resolved.

In this study, we examined the gut-specific homeostatic proliferation in MLNs and intestinal tissue and demonstrated that the fast-dividing population derived from donor CD4+ Ts2c cells accumulates in the host’s small intestine. Fast proliferation occurs in the MLNs dependently of OX40 costimulation and requires the presence of commensal gut flora, which leads to production of gut-tropic T cells. Although PPs affect T cell accumulation in the intestine, they are dispensable for generating αβ+ T cells. MLNs, however, play a critical role in priming intestinal Th17 cells. This study identifies MLNs as an essential site of fast homeostatic proliferation and shows that MLNs are essential in regulating Th17 cell homeostasis in the small intestine.

Materials and Methods

Mice

Ly5.2+ C57BL/6 mice between 6 and 8 wk of age were purchased from Japan SLC (Hamamatsu, Japan); Ly5.1+ C57BL/6 mice have been described previously (35). Aly/aIy mice were purchased from CLEA Japan (Tokyo, Japan). All mice were maintained under specific pathogen-free conditions at the Institute for Animal Experimentation, Tohoku University Graduate School of Medicine. All procedures were performed according to protocols approved by the Institutional Committee for the Use and Care of Laboratory Animals of Tohoku University.

Generation of PP-deficient mice

PP-deficient (PPx) mice were generated following the protocol described by Nishikawa and colleagues (48). Briefly, 2 mg A7R34, a mAb against the IL-7R α-chain, was administered i.v. to pregnant C57BL/6 mice at 14.5 d postcoitus. Control rat IgG (Life Technologies Japan) or control rat IgG (BioXcell) was injected i.p. into recipient mice every 2 d, beginning 1 d before the transfer. The purity was >99%. CD4+ Ts2c cells were labeled with CFSE and used as antigen-presenting cells. MSNs and MLNs were removed by sublethal irradiation (5 Gy) 1 d before the transfer. In some experiments, 100 μg blocking anti-OX40 ligand (OX40L) mAb (50) or control rat IgG was injected i.p. into recipient mice every 2 d, beginning 1 d before the transfer. FTY720 (1.0 mg/kg body weight; Cayman Chemical, Ann Arbor, MI) dissolved in PBS, or control PBS alone, was administered i.p. to recipient mice each day, beginning 1 d after the transfer.

Isolation of lymphocytes

Single-cell suspensions were prepared from the spleen, PPs, and peripheral lymph nodes. Lymphocytes from the lamina propria of the small intestine were isolated following the method described by Honda and colleagues (51). Briefly, the small intestine was opened lengthwise, washed to remove fecal content, and cut into small pieces. The pieces were stirred in RPMI 1640 medium containing 2% FCS and 2 mM EDTA for 20 min at 37°C. After epithelial cells and intraepithelial lymphocytes were removed, the tissues were cut into much smaller pieces. These pieces were stirred in RPMI 1640 medium containing 2% FCS, 400 U/ml collagenase type IV, and 1.5 × 105 cells were transferred intravenously into Ly5.2+ recipient C57BL/6 mice that had been subjected to sublethal irradiation (5 Gy) 1 d before the transfer. In some experiments, 300 μg blocking anti-OX40 ligand (OX40L) mAb (50) or control rat IgG was injected i.p. into recipient mice every 2 d, beginning 1 d before the transfer. FTY720 (1.0 mg/kg body weight; Cayman Chemical, Ann Arbor, MI) dissolved in PBS, or control PBS alone, was administered i.p. to recipient mice each day, beginning 1 d after the transfer.

Flow cytometry

Cells from the small intestine, peripheral lymph nodes, spleen, and PPs were prepared and suspended in PBS containing 2% FCS and 0.02% NaN3. Cells were incubated with a CD16/32 mAb (2.4G2) and stained for 30 min on ice with Abs against cell surface markers. CD4-PE (RM4-5), CD4-Pacific Blue (RM4-5), CD62L-FITC (MEL14), CD44-allophycocyanin (IM7), CD45.1 (Ly5.1)-PE (A20), anti–IFN-γ-allophycocyanin (XMG1.2), and anti–IL-17A Brilliant Violet421 (TC11-18H10.1), anti–CCR9-AlexaFluor647 (29-2L17), and anti–CCR6-Bright Violet421 (29-2L17) mAbs were purchased from BioLegend (San Diego, CA). CD4-PE-Cy7 (IM7) and anti–α4β7- allophycocyanin (DATK32) were obtained from eBioscience (San Diego, CA). Anti-OX40-biotin (OX86) was obtained from BD Biosciences. Anti-OX40L antibody was produced by the Center for Developmental Biology, RIKEN, Kobe, Japan. PP deficient was a gift from S. Nishikawa and colleagues (49). In brief, a median incision was made in the abdomen, exposing the MLNs, vessels, and intestine from the end of the duodenum to the colon, and mesenteric lymphadenectomy was performed by microsurgical isolation along the length of the superior mesenteric artery to the aortic root. Branch vessels supplying the nodes were cauterized with a Gemini Cautery System (Roboz Surgical Instrument, Gaithersburg, MD), protecting the superior mesenteric artery, vein, and branch vessels supplying the intestine. The abdomen was closed with a 6-0 silk suture (Natsume Seisakucho, Tokyo, Japan). For sham operations, the same procedure was performed without removing the MLNs. These mice were used for each transfer experiment 10 d after the surgery. When these mice were sacrificed, MLN deficiency was confirmed by careful macroscopic inspection.

Antibiotic treatment and 16S rRNA gene quantitative PCR analysis

Mice were treated with ampicillin (1 g/l), neomycin (1 g/l), vancomycin (0.5 g/l), and metronidazole (1 g/l) (Sigma-Aldrich, St. Louis, MO) in drinking water for 3 wk. For the determination of gut microflora, bacterial genomic DNA was isolated from feces using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), and real-time PCR was done using SYBR Premix Ex Taq (TaKaRa Bio, Otsu, Japan). Quantitative PCR analysis was carried out using a 7500 real-time PCR system (Life Technologies Japan). The real-time PCR program started at an initial step at 95°C for 10 s, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Data were acquired at the final step at 72°C. Relative quantity was calculated by a ΔΔCT method and normalized to the amount of GAPDH, the amount of which was not affected by antibiotic treatment (data not shown), and presented as relative fold change to an external sample. The following primer sets were used: total bacteria, 5′-GGTGAATACGTTCCCGG-3′ and 5′-TACGCTACTTGTTGACGACT-3′; Clostridium leptum, 5′-CCTTCCGGTCCCGAGTGA-3′ and 5′-GAAATTACACAATCTCACCCTGCT-3′; segmented filamentous bacteria (SFBD), 5′-ACGGAGAATCTCGCACAATTA-3′ and 5′-GCCATCTTTACGCCCGATTC-3′; Bacteroides, 5′-CCAGCAAGCGGGTATAA-3′ and 5′-GCGATTCCGCAACTTCTC-3′; and GAPDH, 5′-CCAGGTGTGTCCTCGGACGACT-3′ and 5′-CCTGTTGTCGTAGCCGTATTC-3′.

Adoptive transfer

CD4+ Ts2c cells (CD44highCD62Lhigh) were purified from the spleen of Ly5.1+ C57BL/6 mice by cell sorting using a FACSAria II (BD Biosciences, San Jose, CA). The purity was >99%. CD4+ Ts2c cells were labeled with CFSE and used as antigen-presenting cells. MSNs and MLNs were removed by sublethal irradiation (5 Gy) 1 d before the transfer. In some experiments, 300 μg blocking anti-OX40 ligand (OX40L) mAb (50) or control rat IgG was injected i.p. into recipient mice every 2 d, beginning 1 d before the transfer. FTY720 (1.0 mg/kg body weight; Cayman Chemical, Ann Arbor, MI) dissolved in PBS, or control PBS alone, was administered i.p. to recipient mice each day, beginning 1 d after the transfer.

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(MGP34) was as described previously (50). Streptavidin–allophycocyanin (BD Biosciences) was used to visualize biotin-labeled Abs. To detect intracellular IFN-γ and IL-17A, lymphocytes were stimulated with 20 ng/ml PMA and 1 μg/ml ionomycin (Sigma-Aldrich) for 5 h. Cells were then stained with surface markers, fixed, permeabilized using a Cytofix/Cytoperm kit (BD Biosciences), and stained with fluorochrome-conjugated anti–IFN-γ and anti–IL-17A mAbs. Flow cytometry was performed with a FACSCanto II (BD Biosciences), and the data were analyzed with FACS Diva (BD Biosciences) and FlowJo (Tree Star, Ashland, OR) software.

Statistical analysis

Statistical analysis was performed by Student $t$ test. The $p$ values $<0.05$ were considered significant.

**Results**

The small intestine harbors numerous CD4+ TEM cells under steady-state conditions

T_N and T_CM cells are reported to circulate through secondary lymphoid tissues, whereas T_EM cells accumulate in extralymphoid organs, including the small intestine (1, 6). To confirm this, we looked for naive and memory CD4+ T cell phenotypes, identified by CD44 and CD62L, in various organs under steady-state conditions. Of the CD4+ T cells found in secondary lymphoid tissues including the spleen, inguinal lymph nodes (ILNs), and MLNs, ~70% had a T_N phenotype and 20–30% had a T_EM phenotype (Fig. 1). Only a small proportion of T_CM cells were detected in these tissues. In

![FIGURE 1](image1.png)

**FIGURE 1.** The small intestine harbors numerous CD4+ TEM cells in the lamina propria. Lymphocytes were harvested from organs of 10-wk-old unimmunized wild-type mice, and the T_N, T_CM, and T_EM populations in CD4+ T cells in the indicated organs were determined by flow cytometry. Numbers in the FACS plots show the percent frequency of the T_EM population in CD4+ cells for each organ. Representative results are shown for four mice in each group. Bar graph: the percent frequency (mean ± SD) of T_N, T_CM, and T_EM populations in CD4+ T cells in the indicated organs ($n = 4$ mice per group). T_EM frequency in the spleen was statistically examined by comparison with the frequency in other organs. Similar results were obtained in four independent experiments. ***$p < 0.001$.

![FIGURE 2](image2.png)

**FIGURE 2.** Fast-proliferating donor cells preferentially accumulate in the small intestine during homeostatic proliferation. Purified donor Ly5.1+CD4+ T_N cells were labeled with CFSE and transferred into sublethally irradiated Ly5.2+ recipient mice. CFSE intensity in donor cells from the indicated organs was analyzed on the indicated days after transfer. (A) Numbers in FACS panels show the percent frequency of fast-proliferating (CFSE−) cells among the donor cells in each organ. Representative results from five mice are shown. Graph: the percent frequency (mean ± SD) of the fast-proliferating population among donor cells in the indicated organs from five mice on the days indicated. The frequency of the fast-proliferating population among donor cells in the intestine was statistically examined by comparison with the frequency in other organs. (B) The number of fast-proliferating (dotted line) and slow-proliferating (solid line) cells (mean ± SD) among donor cells in the indicated organs from five mice on the indicated days. Similar results were obtained in five independent experiments. *$p < 0.05$, ***$p < 0.001$. 

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PPs, the frequencies of T_N and T_EM populations were slightly lower and higher, respectively, than those in the other secondary lymphoid tissues (Fig. 1). By contrast, in the lung and in the lamina propria of the small intestine, >60% of CD4^+ T cells had a T_EM phenotype (Fig. 1). These data confirm that T_EM cells preferentially accumulate in extralymphoid organs, including the small intestine.

**Fast-dividing cells accumulate in the small intestine through homeostatic proliferation**

Newly generated peripheral CD4^+ T cells emerging from the thymus are naive. This suggests that the T_EM–phenotype CD4^+ T cells found in the small intestine might be activated elsewhere and that the intestine may have a mechanism to preferentially collect and maintain these CD4^+ T_EM cells. To investigate how T_EM–phenotype CD4^+ T cells accumulate in the intestine, we organ-specifically examined homeostatic CD4^+ T cell proliferation in several lymphoid tissues and the small intestine.

Donor Ly5.1^+CD4^+ T_N cells were sorted by FACS, labeled with CFSE, and adoptively transferred into syngeneic Ly5.2^+ recipient mice that had been sublethally irradiated. We separately harvested cells from the intestine, MLNs, ILNs, PPs, and the spleen at 3, 6, 9, and 12 d after transfer and analyzed the population and CFSE dilution of donor cells in the respective tissues. Two distinct populations of donor cells even in the intestine and MLNs were distinguished by CFSE dilution—a slow-dividing, CD62L^high population with one or two cell division(s) per week, and a fast-dividing, CD62L^low population with more than seven cell divisions per week (Fig. 2A; data not shown), as was previously shown in the spleen and/or cutaneous lymph nodes (46, 47). The donor cell population was hardly detected in PPs (data not shown). Both slow- and fast-dividing populations increased during homeostatic proliferation in the intestine, MLNs, ILNs, and the spleen (Fig. 2A, 2B). Slow-dividing cells predominated in lymphoid tissues but were greatly outnumbered by fast-dividing cells in the intestine (Fig. 2A, 2B). These results indicate that fast-proliferating, T_EM–phenotype cells preferentially accumulate in the small intestine during homeostatic proliferation.

**The fast-dividing cell population in the small intestine depends on PPs and MLNs**

We next examined whether the homeostatic proliferation of the donor T cells specifically found in the gut requires lymphoid organs such as PPs and MLNs or whether it occurs in the small intestine itself. We transferred donor T_N cells into irradiated aly/aly mice, in which PPs and peripheral lymph nodes (including MLNs) are defective (52, 53), and monitored the donor cell population in the lamina propria of the small intestine. Although the homeostatic proliferation of donor cells was almost intact in the spleen of aly/aly mice, very few donor T cells could be recovered from the intestine (Fig. 3A, 3B). This suggests that the accumulation of homeostatically proliferating T cells in the small intestine depends on PPs and/or MLNs.

To identify differences in the roles of PPs and MLNs in accumulating donor T cells in the intestine, we generated PPX mice by

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**FIGURE 3.** Gut-specific homeostatic proliferation of donor T cells requires secondary lymphoid organs. (A and B) Defective accumulation of donor cells in the intestine of aly/aly mice. Purified CFSE-labeled Ly5.1^+ CD4^+ T_N cells were transferred into sublethally irradiated Ly5.2^+ wild-type (A, left panel; B, left panel) or aly/aly (A, right panel; B, right panel) mice. CFSE intensity was analyzed in CD4^+Ly5.1^+ donor cells harvested from the intestine and the spleen 9 d after the transfer. (A) Numbers in the FACS panels show percent frequency of fast-proliferating populations in CD4^+Ly5.1^+ donor cells; representative results for three mice are shown. (B) Absolute numbers (mean ± SD) of fast- and slow-dividing populations in each organ indicated (n = 3 mice/group). Similar results were obtained in three independent experiments. (C and D) Reduced accumulation of both fast- and slow-dividing donor cells in the intestine of PPX mice. PPX or wild-type (control) recipient mice were used as described in (A) and (B). (C) Numbers in FACS plots show percent frequency of fast-dividing cells among donor cells; results shown are representative of six mice. (D) The number of fast- and slow-proliferating cells (mean ± SD) in each organ (n = 6 mice/group). Similar results were obtained in six independent experiments. (E and F) Defective accumulation of fast- but not slow-dividing donor cells in the MLX mouse intestine. Recipient MLX or sham-operated (control) mice were used as described in (A) and (B). (E) Numbers in FACS plots show percent frequency of fast-dividing cells among donor cells; results are representative of seven mice. (F) Counts of fast- and slow-proliferating cells (mean ± SD) in each organ indicated (n = 7 mice/group). Similar results were obtained in seven independent experiments. *p < 0.05.
injecting a mAb against the IL-7R α-chain into pregnant mice (48). PPs were absent in the offspring at 8 wk of age, but T cell populations in other lymphoid organs were unchanged (data not shown). Slow-dividing donor cells were absent in the small intestine of PPX mice, whereas the fast-dividing population was only marginally smaller than that in control mice (Fig. 3C, 3D). In contrast, donor cell proliferation in the spleen was comparable in PPX and control mice. These results indicate that the slow-dividing population in the small intestine is strongly dependent on PPs, whereas the fast-dividing population is only partly regulated by PPs.

To evaluate the role of MLNs in the homeostatic T cell response in the intestine, donor T\(\text{N}\) cells were adoptively transferred into mesenteric lymphadenectomized (MLX) mice in which MLNs had been surgically resected along the superior mesenteric artery to the aortic root. Although the fast-dividing T cell population in the small intestine was significantly smaller in MLX mice than in mice that underwent a sham operation, the slow-dividing population was unchanged (Fig. 3E, 3F). Homeostatic proliferation in the spleen was comparable in MLX and sham-operated mice.

Collectively, these results suggest that the slow-proliferating T cell population in the small intestine depends on PPs but not on MLNs, whereas the fast-proliferating population is mediated by both PPs and MLNs.

**MLNs but not PPs are essential for generating gut-homing T cells**

The integrin α\(\text{4}\)β\(\text{7}\) and chemokine receptors CCR9 and CCR6 are required for the T cell migration into the intestine (12, 54).

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**FIGURE 4.** Fast homeostatic proliferation generates gut-tropic T cells in MLNs. (A) Flow cytometry measurement of α\(\text{4}\)β\(\text{7}\), CCR9, and CCR6 on splenic CD\(\text{4}\)\^CD\(\text{4}\)\^CD\(\text{6}\)\^low T\(\text{N}\) and CD\(\text{4}\)\^CD\(\text{4}\)\^CD\(\text{6}\)\^low T\(\text{EM}\) cells in unimmunized wild-type mice under steady-state conditions. Filled histograms: negative control staining. Similar results were obtained in three independent experiments. (B) Purified, CFSE-labeled Ly5.1\^CD\(\text{4}\)\^ T\(\text{N}\) cells were transferred into sublethally irradiated Ly5.2\^ donor cells in the indicated organs on day 9 were examined by flow cytometry. Numbers in FACS panels show percent frequency of α\(\text{4}\)β\(\text{7}\)^+, CCR9^+, or CCR6^+ cells among fast-proliferating (CFSE\(^2\)) donor cells. Representative results from three mice are shown. Bar graph: the percent frequency (mean ± SD) of α\(\text{4}\)β\(\text{7}\)^+ cells among fast-proliferating (CFSE\(^2\)) donor cells (mean ± SD) from three recipient mice. The frequency of α\(\text{4}\)β\(\text{7}\)^+ cells in the indicated organs was statistically examined by comparison with the frequency in MLNs. Similar results were obtained in three independent experiments. (C and D) MLNs are essential for the optimal generation of α\(\text{4}\)β\(\text{7}\)^+ cells. Purified Ly5.1\^CD\(\text{4}\)\^ T\(\text{N}\) cells were labeled with CFSE and transferred into sublethally irradiated Ly5.2\^ PPX (C) or MLX (D) mice. The α\(\text{4}\)β\(\text{7}\)^+ donor cell population in the fast-dividing cells was analyzed as shown in (B). The numbers in FACS panels show the percent frequency of α\(\text{4}\)β\(\text{7}\)^+ cells among fast-proliferating (CFSE\(^2\)) donor cells. Representative results for six mice are shown. Bar graph: the percent frequency (mean ± SD) of α\(\text{4}\)β\(\text{7}\)^+ donor cells in CFSE\(^2\) donor cells (\(n = 6\) mice/group). Similar results were obtained in six independent experiments. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\).
thus examined $\alpha_4\beta_7$, CCR9, and CCR6 levels on donor cells undergoing homeostatic proliferation. Before transfer, splenic CD4$^+$ T$_N$ cells expressed intermediate levels of $\alpha_4\beta_7$ but undetectable levels of CCR9 and CCR6 (Fig. 4A). After transfer, some donor T cells acquired significant levels of $\alpha_4\beta_7$, CCR9, and CCR6 through fast division (Fig. 4B). In particular, fast-dividing cell populations from the small intestine and MLNs contained larger proportions of the gut-homing receptor-expressing donor cells than did those recovered from the spleen or ILNs (Fig. 4B). In contrast, the slow-dividing populations in these organs retained their initial levels of these receptors (data not shown). These results suggest that the gut-tropic T cells may develop in the small intestine or GALTs during the homeostatic proliferation.

To examine whether PPs and MLNs are required for generating the gut-homing T cells, we determined the frequencies of $\alpha_4\beta_7^+$ donor cells in PPX and MLX mice. The $\alpha_4\beta_7^+$ proportion was unchanged in fast-dividing cells recovered from the small intestine or spleen of PPX mice (Fig. 4C), but the proportion was significantly reduced in MLX mice (Fig. 4D). These findings suggest that MLNs but not PPs are essential for generating $\alpha_4\beta_7^+$ cells through fast cell division.

**Donor T cells in the small intestine derive from $\alpha_4\beta_7^+$ cells generated in MLNs via fast proliferation**

Having found that MLNs are important for generating fast-proliferating $\alpha_4\beta_7^+$ cells, we postulated that donor CD4$^+$ T$_N$ cells that settle in MLNs rapidly proliferate, acquire the $\alpha_4\beta_7^+$ phenotype, and migrate to the small intestine via recirculation. We tested this by examining homeostatic proliferation in mice treated with the immunomodulatory drug FTY720, which depletes S1P$_1$ on lymphocytes, thus preventing their emigration from secondary lymphoid organs and inducing their accumulation in lymph nodes (55–57). FTY720 treatment significantly increased the number of $\alpha_4\beta_7^+$ cells and decreased the number of $\alpha_4\beta_7^-$ cells in fast-dividing cells recovered from MLNs (Fig. 5A, 5B). In contrast, the number of $\alpha_4\beta_7^-$ cells was markedly reduced in the spleen, whereas the $\alpha_4\beta_7^-$ cell population was unaffected (Fig. 5A, 5B). Therefore, fast proliferation in MLNs may critically contribute to the generation of circulating $\alpha_4\beta_7^+$ cells that are found in the spleen. Furthermore, FTY720 treatment strongly reduced the number of donor cells, including $\alpha_4\beta_7^+$ cells, in the small intestine (Fig. 5C, 5D). These results suggest that $\alpha_4\beta_7^+$ cells may arise from fast-proliferating cells in MLNs and then migrate into the small intestine through recirculation.

**Intestinal bacteria promote fast proliferation in MLNs**

Fast division during systemic homeostatic proliferation is driven by foreign Ags such as commensal bacteria (46). To determine whether the fast population in MLNs may be induced by commensal bacteria, we examined gut-specific homeostatic proliferation in antibiotic-treated hosts. The removal of commensal bacteria in the hosts’ feces by antibiotic treatment was confirmed by quantitative real-time PCR (Fig. 6A). As expected, antibiotic treatment significantly suppressed the generation of fast-proliferating $\alpha_4\beta_7^+$ but not $\alpha_4\beta_7^-$ cells in MLNs (Fig. 6B, 6C). Furthermore, administration of antibiotics strikingly reduced the number of donor cells, including $\alpha_4\beta_7^+$, fast-dividing cells, in the small intestine (Fig. 6D, 6E). Therefore, the commensal microflora is critically involved in the fast proliferation in MLNs.

**OX40 signaling is critical for fast proliferation in MLNs**

The in vivo blockade of T cell costimulatory signals mediated through OX40, which belongs to the TNF receptor superfamily, improves experimental colitis by suppressing the homeostatic proliferation of pathogenic CD4$^+$ T cells (34, 35). Therefore, we examined OX40 expression on donor T cells in MLNs during homeostatic proliferation and found OX40 on fast- but not slow-proliferating cells (Fig. 7A). To clarify the role of OX40 in fast proliferation, we treated recipient mice with an inhibitory anti-OX40L mAb and monitored donor cell proliferation in MLNs. Treatment with the anti-OX40L mAb significantly inhibited the generation of fast-proliferating $\alpha_4\beta_7^+$ cells in MLNs, without affecting slow cell division (Fig. 7B, 7C). Treatment with the anti-OX40L mAb also strongly reduced the total, fast-proliferating, and $\alpha_4\beta_7^+$ donor T cell populations in the small intestine (Fig. 7D). This indicates that OX40 costimulation is required for generating gut-tropic, fast-dividing cells in MLNs.
Intestinal commensal bacteria are required for fast proliferation in MLNs and the accumulation of donor cells in the intestine. (A) Antibiotic treatment deleted commensal gut flora. Bacterial DNA was extracted from feces of antibiotics-treated or non-treated mice, and quantitative PCR analysis for 16S rRNA-coding DNA was carried out. The quantities of total bacteria, Clostridium, SFB, and Bacteroides (mean ± SD) are indicated (n = 4 mice/group). Similar results were obtained in two independent experiments. (B-E) Antibiotics significantly reduced αβ^+ fast-dividing donor cells in MLNs and diminished donor cells in the intestine. Nine days after adoptive transfer of CFSE-labeled CD4^+ T cells to antibiotic-treated or control mice, donor cells in MLNs and the intestine were examined by flow cytometry. (B) The number in each FACS panel indicates the percent frequency of αβ^+ population among donor cells in MLNs. Results shown are representative data of three mice per group. Graph: the percent frequency (mean ± SD) of αβ^+ or αβ^− population among donor cells in MLNs (n = 3 mice/group). (C) Graph: the number (mean ± SD) of αβ^+ cells among CFSE^− fast-proliferating donor cells in MLNs (n = 3 mice/group). (D) The number in each FACS plot indicates percent frequency of donor cells in the intestine. Results shown are representative data from three mice in each group. (E) Graph: numbers of total, fast proliferating, and αβ^+ donor cells (mean ± SD) in the intestine (n = 3 mice/group). Similar results were obtained in two independent experiments. *p < 0.05, **p < 0.01.

Gut-tropic Th17 cells arise in MLNs during homeostatic proliferation

The CD4^+ T cell population in the small intestine includes a significant number of Th17 cells (27). Therefore, we measured IFN-γ and IL-17A cytokine levels in donor cells recovered from the small intestine, spleen, and MLNs. Neither cytokine was produced by the slow-dividing population, whereas the fast-dividing population expressed both (Fig. 8A; data not shown). When gating on the fast-dividing population, an IFN-γ^+ profile was dominant in the spleen (Fig. 8A, spleen), but in the intestine, IL-17A^+ cells greatly outnumbered IFN-γ^+ ones (Fig. 8A, intestine). In MLNs, IFN-γ and IL-17A were detected at equal levels (Fig. 8A, MLN). These results indicate that Th17 cells are generated through fast proliferation in an organ-specific manner and that gut-specific Th17 cells may arise in MLNs or the small intestine.

FTY720 treatment caused αβ^+ cells to accumulate in MLNs and strongly depleted donor cells in the intestine (Fig. 5). Therefore, if fast homeostatic proliferation in MLNs is responsible for the differentiation of gut-associated Th17 cells, FTY720 treatment should induce donor Th17 cells to accumulate in MLNs. As expected, FTY720 treatment increased the IL-17A^+ population and decreased the IFN-γ^− population in MLNs (Fig. 8B, MLN). However, FTY720 increased the IFN-γ^+ population but did not affect the minor IL-17A^+ population found in the spleen (Fig. 8B, spleen). These results suggest that CD4^+ T cells that produce IL-17A are almost exclusively generated by MLNs and those producing IFN-γ by the spleen. This result is supported by our finding that MLN-derived αβ^+ and spleen-derived αβ^− populations included IL-17A^+ and IFN-γ^− cells, respectively (data not shown). Furthermore, removing MLNs from recipient mice significantly reduced the IL-17A^+ but not the IFN-γ^− population in the small intestine (Fig. 8C). Collectively, these results demonstrate that MLNs are essential for the generation of gut-tropic Th17 cells through fast homeostatic proliferation.

Discussion

In this study, we examined the homeostatic proliferation of CD4^+ T_{N} cells in an organ-specific manner and found that the two distinct types of homeostatic proliferation, fast and slow, which were discovered in the spleen and cutaneous lymph nodes (46, 47), also occur in MLNs and intestinal tissue. Furthermore, we demonstrated that the fast homeostatic proliferation of CD4^+ T_{N} cells in MLNs critically contributes to the generation of gut-homing T_{EM} phenotype Th17 cells, indicating an important role of the fast gut-specific proliferation. CD4^+ T_{EM} cells, including Th17 cells, present a significant portion of the intestinal T cell population (27), and control bacterial infections in the gut (28, 29). Therefore, the present results may further our understanding of gut-homing T_{EM} cell differentiation in vivo.

Homeostatic proliferation is a physiological proliferative T cell response induced by an emergent immunological status such as lymphopenia (2). For example, in elderly people, patients thymectomized during early childhood, or patients infected with HIV-1, who are all situated under lymphopenic conditions, homeostatic proliferation contributes to maintaining the T cell number (58). Besides, neonatal mice, which do not yet have sufficient T cells, show the homeostatic proliferation to increase the T cell number up to the physiological status (59, 60). Although we induced lymphopenia by irradiation, the gut-specific homeostatic proliferation may contribute to generation of intestinal Th17 cells under a certain physiological condition.

Several studies have reported that slow homeostatic proliferation is mediated by self peptides/MHC and IL-7 in secondary lymphoid organs (2, 46, 47). Consistent with these studies, we found the slowly proliferating population to be present mainly in secondary lymphoid organs, including the spleen, MLNs, and ILNs (Fig. 2).
Studies in several animal models have demonstrated that OX40–
inducing fast proliferation. In particular, both commensal bacteria
addition to commensal bacteria, OX40 costimulation is crucial for
organ-specific proliferation may
occurs. Our results show that the
number of fast-proliferating cells among donor cells in MLNs. Results
after transfer. (B) cells in MLNs and the intestine were examined by flow cytometry 9 d
treated with blocking anti-OX40L mAb or with control rat IgG. Donor
cells in MLNs and the intestine were examined by flow cytometry.
Filled histograms show negative control staining. Similar results were
populations in donor cells in MLNs was examined by flow cytometry.

However, less has been known about where fast proliferation
occurs. Our results show that the α4β7+ fast-proliferating popu-
lation is generated mainly in MLNs and that it may contribute to
gut-tropic Th17 generation. The organ-specific proliferation may contribute to generation of organ-specific TEm cells.

To our knowledge, this study reveals for the first time that, in
addition to commensal bacteria, OX40 costimulation is crucial for
inducing fast proliferation. In particular, both commensal bacteria
and OX40 are required for fast proliferation in MLNs and for gut-
tropic T cell accumulation in the lamina propria of the intestine.
Studies in several animal models have demonstrated that OX40–
OX40L interactions mediate the development of IBDs and GVHD,
both of which are pathogenically associated with the homeostatic
proliferation of CD4+ TNA cells (34, 35, 61). Therefore, it is likely that α4β7+ Th17 cells generated through fast homeostatic prolif-
eration in MLNs are also involved in the pathogenesis of these
diseases in an OX40-dependent manner. This possibility led us to
examine the OX40L expression on several types of CD11c+MHC
II+ dendritic cells, which may probably present Ags of intestinal
commensal bacteria to T cells, in the MLNs of recipient mice,
because OX40L is thought to be expressed on APCs such as
dendritic cells and B cells (62–64). Because blocking OX40L
signaling in inducing gut-homing Th17 cells requires further study.

Several studies have demonstrated that MLNs are important for generating gut-tropic T cells, which express specific
markers such as α4β7, CCR9, and CCR6 (12, 54). Previous studies showed that MLN-derived T lymphoblasts preferentially
accumulate in the intestine (65, 66). A recent study using a T cell
transfer method revealed that donor T cells acquire α4β7+ in the
MLNs upon in vivo Ag stimulation (24). Other reports found that
dendritic cells from MLNs and PPs are capable of inducing α4β7+ T cells in vitro (17, 25, 67, 68). However, a study using a virus-
specific TCR-transgenic T cell system showed that a sufficient number of α4β7+ T cells were generated in the spleen in response
to viral infection, even in mice treated with FTY720, suggesting
that MLNs and PPs are dispensable (69). Despite the controversy
on the roles of MLNs and PPs, our present study clearly shows that
MLNs, but not the spleen or PPs, are essential for generating
gut-tropic α4β7+ effector T cells, because α4β7+ donor cells in the
intestine were markedly reduced in MLN-deficient PP-intact MLX
mice (Figs. 3E, 3F, 4D), and a fast-proliferating population was
found in the intestine even in MLN-intact PP-deficient PPX mice—but not in MLN-deficient PP-deficient aly/aly mice (Fig. 3A–D).
Indeed, the donor cell number in PPs was less than 1/50th of that
in MLNs 9 d after transfer (data not shown). Therefore, MLNs, but
not PPs, may be the main place for gut-tropic T cell proliferation
in our model, although the presence of PPs crucially contributed to
distribution of slow proliferating donor cells in the intestine (Fig.
3C, 3D). Furthermore, FTY720 treatment induced α4β7+ donor
cells to accumulate in MLNs, consequently depleting them in the
intestine and spleen (Fig. 5); this finding also suggests that MLNs are critical for inducing gut-homing T cells. It is still unclear,
however, whether Ag stimulation is required for the homeostatic
proliferation-induced generation of gut-tropic T cells despite
the indispensability of intestinal flora. The contribution of MLNs to the
generation of gut-homing T cells may differ in the context of
homeostatic proliferation or Ag-specific activation.

The generation of Th17 cells has been thought to occur in the
lamina propria of the intestine (31) in part because the frequency of
these cells is much higher in the intestinal lamina propria than in
PPs or MLNs (70, 71). Indeed, the lamina propria of the colon is
rich in CX3CR1+CD103−CD70brightCD11c− cells, which have the
capacity to generate Th17 cells in vitro (70). It was also recently
reported that CD11c+E-cadherin+ cells in the colonic
lamina propria and in inflamed MLNs promote Th17 differenta-
tion in vivo (72). However, it is unlikely that the donor T cells
directly entered the lamina propria of the intestine to be primed for
Th17 differentiation by intestinal APCs in our experimental set-
tings, because donor T cells were not found in the intestinal
lamina propria of aly/aly mice (Fig. 3A, 3B), and because FTY720

FIGURE 7. Blockade of OX40L–OX40 interactions inhibits fast pro-
liferation in MLNs and the accumulation of donor cells in the small
intestine. (A) Nine days after adoptive transfer of CFSE-labeled CD4+
TNA cells, OX40 expression on the fast (CFSE−) and slow (CFSEhigh)
populations in donor cells in MLNs was examined by flow cytometry.
Filled histograms show negative control staining. Similar results were
obtained in two independent experiments. (B–D) Recipient mice were
 treated with blocking anti-OX40L mAb or with control rat IgG. Donor
cells in MLNs and the intestine were examined by flow cytometry 9 d
after transfer. (B) The number in each FACS panel indicates the percent
frequency of fast-proliferating cells among donor cells in MLNs. Results
shown are representative data of six mice per group. (C) Graph: the
number (mean ± SD) of fast-proliferating cells among donor cells in
MLNs (n = 6 mice/group). (D) Graph: the numbers of total, fast prolif-
erating, and α4β7+ donor cells (mean ± SD) in the intestine (n = 6 mice/
group). Similar results were obtained in six independent experiments.
*p < 0.05, **p < 0.01.
treatment or mesenteric lymphadenectomy depleted donor T cells, including Th17 cells, from the intestine (Figs. 5C, 5D, 8C). Th17 cells may be generated in MLNs, after which they may migrate and accumulate in the lamina propria of the small intestine. Intestinal Th17 cells are induced by SFB, an intestinal commensal bacterium (27). Consistently, the gut-tropic αβ+ population, which were rich in Th17 cells (data not shown), was markedly reduced by antibiotic treatment that removed almost all commensal bacteria including SFB (Fig. 6A–C). Therefore, gut-homing Th17 cells are generated dependently of commensal bacteria. By contrast, mechanisms for generating Th1 cells, which were at least in part generated in the spleen (Fig. 8B), are unclear. Because the αβ+ fast population, which was rich in the Th1 cells (data not shown), was not affected by antibiotic treatment (Fig. 6B), the Th1 cells may be induced by a different mechanism from Th17 cells, which require intestinal commensal bacteria. Further studies on the distinct mechanisms between Th1 and Th17 generations will be needed.

In summary, our results clearly demonstrate that gut-specific fast homeostatic proliferation plays a critical role in inducing gut-homing αβ+ IL-17A+ T cells, which is dependent on OX40 signals. Although the systemic inhibition of OX40 signals, either in OX40L-knockout mice or by treatment with an anti-OX40L mAb, drastically ameliorates several types of IBDs and GVHD (34, 35, 61), our study revealed that the OX40–OX40L interactions necessary for generating intestinal Th17 cells may occur in the MLNs. Therefore, it may be possible to develop therapeutic strategies for IBDs and GVHD by controlling the OX40–OX40L interactions in MLNs. In addition, the finding that the role of MLNs in developing gut-homing T EM cells, including Th17 cells, is distinct from that of the PPs or spleen may contribute to a deeper understanding of intestinal mucosal immunity.

FIGURE 8. Gut-tropic IL-17A+ donor cells arise during fast homeostatic proliferation in MLNs. Nine days after transfer, donor cells were collected from the indicated organs of recipient mice and were stimulated ex vivo with PMA and ionomycin. Intracellular expression of IFN-γ and IL-17A in fast-proliferating donor cell populations was examined by flow cytometry. (A) Frequency of IFN-γ– or IL-17A–producing cells among fast-proliferating donor cells in each of the indicated organs. Graph: the mean ± SD from five mice per group. Similar results were obtained in five independent experiments. (B) FTY720 treatment, as described in Fig. 5; FACS results shown (left panel) are representative of three mice per group. Numbers in FACS histograms indicate percent frequency of IFN-γ– or IL-17A–producing cells among the fast proliferating donor cells in each organ. Graph (right panel): the percent frequency of IFN-γ– or IL-17A–producing cells (mean ± SD) among fast-proliferating donor cells in each organ (n = 3 mice/group). Similar results were obtained in two independent experiments. (C) MLX or sham-operated control recipient mice. Numbers in FACS histograms indicate percent frequency of cells producing IFN-γ or IL-17A among the fast proliferating donor cells in the lamina propria of the small intestine. Results shown are representative of five mice per group. Graph (right panel) represents the percent frequency (mean ± SD) of IFN-γ– or IL-17A–producing cells among fast-proliferating donor cells in the intestinal lamina propria (n = 5 mice/group). Similar results were obtained in five independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
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Disclosures

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