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

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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 α_β^+ 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 T N and T CM 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 EM 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 EM cells provide a first line of defense against reinfection (6, 9).

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_N) cells (CD4^+CD62L^high), central memory T (T_CM) cells (CD4^+CD62L^high), and effector memory T (T_EM) cells (CD4^+CD62L^low) (1, 5, 6). T_N and T_CM 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_EM 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_EM cells provide a first line of defense against reinfection (6, 9).

In the small intestine, CD4^+ T_EM cells are primarily found in the lamina propria and CD8^+ T_EM cells in the epithelium (10). The T cell population in the intestine induces pathogen-specific immunity and inhibits inappropriate responses to self and harmless Ags, ensuring intestinal homeostasis (10, 11). The effector and memory memory T cells that populate the intestine are reported to be produced in PPs and mesenteric lymph nodes (MLNs) (6, 11, 12). In PPs, M cells in the epithelium transport luminal Ags to the apical side (13), where dendritic cells wait for them (14–16). These dendritic cells prime and activate T_N cells that enter the PP and induce the expression of α_β^+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: 000–000.
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 T cells supplies a memory–phenotype T cell pool (40–45), this system can be used to determine the origin of intestinal TEM 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 CD44hiCD62Llow naive phenotype and has limited differentiation potential (2). In contrast, fast proliferation produces cells that robustly differentiate into the CD44hiCD62Llow-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+ T cells accumulates in the host’s small intestine. Fast proliferation occurs in the MLNs dependently on 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/aly 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 previously (35). Aly/aly 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.

Adoptive transfer

CD4+ Tc cells (CD44hiCD62Llow) were purified from the spleen of Ly5.1+ C57BL/6 mice by cell sorting using a FACS Aria II (BD Biosciences, San Jose, CA). The purity was >99%. CD4+ Tc cells were labeled with CFSE (Life Technologies Japan) and 1.5 × 10^5 cells were transferred intravenously into Ly5.2-RFP 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. to recipient mice every 2 d, beginning 1 d before the transfer.

Analysis

All quantitative data are presented as means ± SE. Data were analyzed using two-tailed Student’s t tests or one-way ANOVA with Tukey’s post hoc test, as indicated. The level of statistical significance was set at p < 0.05. All statistical analyses were performed using GraphPad Prism.

Flow cytometry

Cells from the small intestine, peripheral lymph nodes, spleen, and peripheral lymph nodes were prepared and analyzed using flow cytometry. 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-FTTC (MEL14), CD44-allophycocyanin (IM7), CD45.1 (Ly5.1)-PE (A20), anti-IFN-γ-allophycocyanin (XMG1.2), anti-I-IL-17A-BrilliantViolet421 (TC11-18H10.1), anti-CCR9-AlexaFluor647 (CW-1.2), and anti-CCR6-BrilliantViolet421 (29-2L17) were purchased from BioLegend (San Diego, CA). CD44-PE-Cy7 (IM7) and anti-αβ+-allophycocyanin (DATK32) were obtained from ebioscience (San Diego, CA). Anti-OX40-biotin (OX86) was obtained from BD Biosciences.

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 36 s at 72°C. Data were acquired at the final step at 72°C. Relative quantity was calculated by a line 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′-GGTGAATAGTCGTCGCG-3′ and 5′-TACGGCTACCTTGTTACGACT-3′; Clostridium leptum, 5′-CCTTCGC- GTGCGSAGTTA-3′ and 5′-GAATTTACACTACATCCACTGCTT-3′; segmented filamentous bacteria (SPB), 5′-AOGAGGACGTCTCOCGACA- TTACC-3′ and 5′-GGCATCCCTTTACCCGGCATTTC-3′; Bacteroides, 5′-CCAGCAGCCGCTGGATAA-3′ and 5′-CGGATCCGCTACTTCTC-3′; and GAPDH, 5′-CCAGGTTCCTCTCGAGACTCTT-3′ and 5′-CCTGTT- TGCTTAGCCGATTTCA-3′.
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) in the presence of monensin (BD Biosciences) 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+ T<sub>EM</sub> cells under steady-state conditions

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

FIGURE 1. The small intestine harbors numerous CD4<sup>+</sup> T<sub>EM</sub> cells in the lamina propria. Lymphocytes were harvested from organs of 10-wk-old unimmunized wild-type mice, and the T<sub>N</sub>, T<sub>CM</sub>, and T<sub>EM</sub> populations in CD4<sup>+</sup> T cells in the indicated organs were determined by flow cytometry. Numbers in the FACS plots show the percent frequency of the T<sub>EM</sub> population in CD4<sup>+</sup> cells for each organ. Representative results are shown for four mice in each group. Bar graph: the percent frequency (mean ± SD) of T<sub>N</sub>, T<sub>CM</sub>, and T<sub>EM</sub> populations in CD4<sup>+</sup> T cells in the indicated organs (n = 4 mice per group). T<sub>EM</sub> 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. Fast-proliferating donor cells preferentially accumulate in the small intestine during homeostatic proliferation. Purified donor Ly5.1<sup>+</sup>CD4<sup>+</sup> T<sub>N</sub> cells were labeled with CFSE and transferred into sublethally irradiated Ly5.2<sup>+</sup> 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<sup>−</sup>) 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.
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, and 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, ■) or aly/aly (A, right panel; B, □) mice. CFSE intensity was analyzed in CD4\(^+\)Ly5.1\(^+\) donor cells harvested from the intestine and the spleen 9 d after 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<sub>N</sub> 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<sub>4</sub>β<sub>7</sub> and chemokine receptors CCR9 and CCR6 are required for the T cell migration into the intestine (12, 54).

**FIGURE 4.** Fast homeostatic proliferation generates gut-tropic T cells in MLNs. (A) Flow cytometry measurement of α<sub>4</sub>β<sub>7</sub>, CCR9, and CCR6 on splenic CD4<sup>+</sup>CD44<sup>lo</sup>CD62L<sup>hi</sup> T<sub>N</sub> and CD4<sup>+</sup>CD44<sup>hi</sup>CD62L<sup>low</sup> T<sub>EM</sub> 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<sup>+</sup>CD4<sup>+</sup> T<sub>N</sub> cells were transferred into sublethally irradiated Ly5.2<sup>+</sup> mice, and a<sub>4</sub>β<sub>7</sub> (top panel), CCR9 (middle panel), and CCR6 (bottom panel) expressions on CD4<sup>+</sup>Ly5.1<sup>+</sup>CFSE<sup>2</sup> donor cells in the indicated organs on day 9 were examined by flow cytometry. Numbers in FACS panels show percent frequency of a<sub>4</sub>β<sub>7</sub><sup>+</sup>, CCR9<sup>+</sup>, or CCR6<sup>+</sup> cells among fast-proliferating (CFSE<sup>2</sup>) donor cells. Representative results from three mice are shown. Bar graph: the percent frequency (mean ± SD) of a<sub>4</sub>β<sub>7</sub><sup>+</sup>, CCR9<sup>+</sup>, or CCR6<sup>+</sup> 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<sub>4</sub>β<sub>7</sub><sup>+</sup> cells. Purified Ly5.1<sup>+</sup>CD4<sup>+</sup> T<sub>N</sub> cells were labeled with CFSE and transferred into sublethally irradiated Ly5.2<sup>+</sup>PPX (C) or MLX (D) mice. The α<sub>4</sub>β<sub>7</sub><sup>+</sup> 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<sub>4</sub>β<sub>7</sub><sup>+</sup> cells among fast-proliferating (CFSE<sup>+</sup>) donor cells. Representative results for six mice are shown. Bar graph: the percent frequency (mean ± SD) of α<sub>4</sub>β<sub>7</sub><sup>+</sup> donor cells in in CFSE<sup>+</sup> 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 αβ7+, CCR9, and CCR6 levels on donor cells undergoing homeostatic proliferation. Before transfer, splenic CD4+ T N cells expressed intermediate levels of αβ7 but undetectable levels of CCR9 and CCR6 (Fig. 4A). After transfer, some donor T cells acquired significant levels of αβ7, 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 MLNs but not PPs are essential for generating αβ7+ cells through fast cell division.

**Donor T cells in the small intestine derive from αβ7+ cells generated in MLNs via fast proliferation**

Having found that MLNs are important for generating fast-proliferating αβ7+ cells, we postulated that donor CD4+ T N cells that settle in MLNs rapidly proliferate, acquire the αβ7+ 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 S1P1 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 αβ7+ cells and decreased the number of αβ7− cells in fast-dividing cells recovered from MLNs (Fig. 5A, 5B). In contrast, the number of αβ7+ cells was markedly reduced in the spleen, whereas the αβ7− cell population was unaffected (Fig. 5A, 5B). Therefore, fast proliferation in MLNs may critically contribute to the generation of circulating αβ7+ cells that are found in the spleen. Furthermore, FTY720 treatment strongly reduced the number of donor cells, including αβ7+ cells, in the small intestine (Fig. 5C, 5D). These results suggest that αβ7+ 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 αβ7+ but not αβ7− cells in MLNs (Fig. 6B, 6C). Furthermore, administration of antibiotics strikingly reduced the number of donor cells, including αβ7+ 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 expressed 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 αβ7+ 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 αβ7+ 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.
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 gaging on the fast-dividing population, an IFN-γ+ population was present mainly in the spleen (Fig. 8A, spleen), but in the intestine, IL-17A+ producing cells greatly outnumbered IFN-γ+ producing 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⁺ Tₘ 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⁺ Tₘ cells in MLNs critically contributes to the generation of gut-homing Tₑₘ phenotype Th17 cells, indicating an important role of the fast gut-specific proliferation. CD4⁺ Tₑₘ cells, including Th17 cells, represent 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 Tₑₘ 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).
However, less has been known about where fast proliferation occurs. Our results show that the αβ7+ fast-proliferating population 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 co-stimulation 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, therefore it is likely that αβ7+ Th17 cells generated through fast homeostatic proliferation 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 commensals 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 significantly reduced the number of αβ7+ cells in the MLNs (Fig. 7C), we speculated that CD103+ dendritic cells, which are important for inducing αβ7+ T cells (26), might express OX40L. Unexpectedly, we did not find OX40L-expressing dendritic cells in the MLNs of recipient mice, even when the mice were subjected to sublethal irradiation (data not shown). Therefore, a cell population other than CD11c+ dendritic cells may express OX40L and mediate fast proliferation in MLNs. The precise role of OX40 signaling in inducing gut-homing Th17 cells requires further study.

Several studies have demonstrated that MLNs and PPs 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 αβ7 in the MLNs upon in vivo Ag stimulation (24). Other reports found that dendritic cells from MLNs and PPs are capable of inducing αβ7+ 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 αβ7+ 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 αβ7+ effector T cells, because αβ7+ 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 αβ7+ 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 dispensability 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+CD70highCD11c+ 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 differentiation 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 settings, 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 proliferation in MLNs and the accumulation of donor cells in the small intestine. (A) Nine days after adoptive transfer of CFSE-labeled CD4+ Tn 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 proliferating, and αβ7+ 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 \( \alpha_\beta_7^{+} \) 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 \( \alpha_\beta_7^{+} \) 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 \( \alpha_\beta_7^{+} \) 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-\( \gamma \) and IL-17A in fast-proliferating donor cell populations was examined by flow cytometry. (A) Frequency of IFN-\( \gamma \)– 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-\( \gamma \)– or IL-17A–producing cells among the fast proliferating donor cells in each organ. Graph (right panel): the percent frequency of IFN-\( \gamma \)– 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-\( \gamma \) 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-\( \gamma \)– 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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References


