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MyD88-Dependent Pathway in T Cells Directly Modulates the Expansion of Colitogenic CD4+ T Cells in Chronic Colitis

Takayuki Tomita,* Takanori Kanai,† Toshimitsu Fujii,* Yasuhiro Nemoto,* Ryuichi Okamoto,* Kiichiro Tsuchiya,* Teruji Totsuka,* Naoya Sakamoto,* Shizuo Akira, † and Mamoru Watanabe*  

TLRs that mediate the recognition of pathogen-associated molecular patterns are widely expressed on/in cells of the innate immune system. However, recent findings demonstrate that certain TLRs are also expressed in conventional TCRαβ+ T cells that are critically involved in the acquired immune system, suggesting that TLR ligands can directly modulate T cell function in addition to various innate immune cells. In this study, we report that in a murine model of chronic colitis induced in RAG-2−/− mice by adoptive transfer of CD4+CD45RBhigh T cells, both CD4+CD45RBhigh donor cells and the expanding colitogenic lamina propria CD4+CD44high memory cells expresses a wide variety of TLRs along with MyD88, a key adaptor molecule required for signal transduction through TLRs. Although RAG-2−/− mice transferred with MyD88−/−CD4+CD45RBhigh cells developed colitis, the severity was reduced with the delayed kinetics of clinical course, and the expansion of colitogenic CD4+ T cells was significantly impaired as compared with control mice transferred with MyD88+/+CD4+CD45RBhigh cells. When RAG-2−/− mice were transferred with the same number of MyD88+/− (Ly5.1+) and MyD88−/− (Ly5.2+) CD4+CD45RBhigh cells, MyD88+/−/CD4+ T cells showed significantly lower proliferative responses assessed by in vivo CFSE division assay, and also lower expression of antiapoptotic Bcl-2/Bcl-xL molecules and less production of IFN-γ and IL-17, compared with the paired MyD88+/+CD4+ T cells. Collectively, the MyD88-dependent pathway that controls TLR signaling in T cells may directly promote the proliferation and survival of colitogenic CD4+ T cells to sustain chronic colitis.  


Inflammatory bowel diseases (IBD)1 are caused by excessive tissue damaging by chronic inflammatory responses in the gut wall, and commonly take persistent courses (1, 2). According to the present understanding, the diseases are caused by infiltrated colitogenic effector/memory CD4+ T cells within the inflamed mucosa, which are presumably primed by commensal Ag-loading dendritic cells (DCs) in lymphoid tissues (3). However, the nature of colitogenic CD4+ T cells over time during chronic colitis under the persistent presence of commensal bacteria remains largely unknown. Importantly, it is well-known that experimental colitis does not develop when mice are kept in a germfree condition (4–6), suggesting that intestinal microflora are essential to initiate and main-
Purification of T cell subsets

For isolation of peripheral blood (PB) lymphocytes, 600 μl of PB was collected from each mouse and was diluted 1/1 with PBS. The diluted PB was layered over Lymphoprep II (IBL) and centrifuged at 400 x g for 30 min at room temperature. Lymphocytes were then isolated from the plasma-ficoll interface. Spleen (SP) and mesenteric lymph nodes (MLN) were mechanically disrupted into single-cell suspensions. Bone marrow (BM) was collected from the femur by flushing with sterile PBS.

CD4⁺ T cells were isolated from SP cells of MyD88⁻/⁻ mice and littermate MyD88⁺/⁺ mice using the anti-CD4 (L3T4) MACS system (Miltenyi Biotec) according to the manufacturer’s instruction. Enriched CD4⁺ T cells (94–96% pure, as estimated by FACS Calibur; BD Biosciences) were then labeled with PE-conjugated anti-mouse CD4 (RM4-5; BD Pharmingen) and FITC-conjugated anti-CD45RB (16A; BD Pharmingen). The subpopulation of CD4⁺CD45RB⁺⁺ cells was collected by two-color sorting on a FACS Aria (BD Biosciences), and was >98.0% pure on reanalysis.

To obtain LP CD4⁺ T cells, colitis was induced in RAG-2⁻/⁻ mice by adoptive transfer of CD4⁺CD45RB⁺⁺ T cells either from MyD88⁻/⁻ or from littermate wild-type (WT) MyD88⁺/⁺ mice as described previously (15). Colitic CD4⁺CD45RB⁺⁺ T cell-transferred RAG-2⁻/⁻ mice were sacrificed at 5–10 wk after transfer. The entire colon was opened longitudinally, washed with PBS, and cut into small pieces. The dissected mucosa was incubated with Ca²⁺- and Mg²⁺-free HBSS containing 1 mM DTT (Sigma-Aldrich) for 45 min to remove mucus and then treated with 2.0 mg/ml collagenase (Roche) and 0.01% DNase (Worthington Biomedical) for 2 h. The cells were pelleted twice through a 40% isotonic Percoll solution, and then resuspended in 5% Ficoll-Hypaque density gradient centrifugation (40%–75%). Enriched lamina propria (LP) CD4⁺ T cells were obtained by positive selection using anti-CD4 (L3T4) MACS magnetic beads. The resultant cells contained >95% CD4⁺ cells when analyzed by FACS Calibur. To assess the expression of TLRs and MyD88 in CD4⁺ T cells using RT-PCR, every cell population was isolated by FACS Aria (BD Biosciences) to gain >98% CD4⁺ purity.

RT-PCR

Total RNA was isolated by using Isogen reagent (Nippon Gene). Aliquots of total RNA (0.5 μg) were used for cDNA synthesis in a 20-μl reaction volume using random primers. One microliter of reverse transcriptase product was measured with 0.25 U of rTaq DNA polymerase (Toyobo) in 50 μl at room temperature. Lymphocytes were then isolated from the plasma-ficoll-hypaque density gradient centrifugation (40–75%). Enriched lamina propria (LP) CD4⁺ T cells were obtained by positive selection using anti-CD4 (L3T4) MACS magnetic beads. The resultant cells contained >95% CD4⁺ cells when analyzed by FACS Calibur. To assess the expression of TLRs and MyD88 in CD4⁺ T cells using RT-PCR, every cell population was isolated by FACS Aria (BD Biosciences) to gain >98% CD4⁺ purity.

Amplification data were analyzed with an Applied Biosystems Sequence Detection Software version 1.3. The relative expression of the gene of interest was normalized by the expression of β-actin.

In vivo experimental design

We performed a series of in vivo transfer experiments to investigate the role of TLR signaling in CD4⁺CD45RB⁺⁺ T cells or in colitogenic LP CD4⁺ T cells in the development and persistence of murine chronic colitis.

Experiment 1. To assess the requirement of MyD88-dependent signaling in the development of colitis including the processes for T cell priming and activation, along with the persistence of colitogenic effector or memory CD4⁺ T cells, we performed a cell transfer experiment using MyD88⁻/⁻ and littermate WT MyD88⁺/⁺ mice as donors. CD4⁺CD45RB⁺⁺ T cells from MyD88⁻/⁻ (n = 6) or MyD88⁺/⁺ (n = 6) donors were injected i.p. into RAG-2⁻/⁻ mice and the recipients were monitored for 4–6 wk after transfer. In another set of experiment using the present protocol, we monitored the groups of mice (each n = 5) to 10 wk after transfer to assess the kinetics of the development.

Experiment 2. To further assess the necessity of MyD88-dependent signaling in the development of colitis, we performed in vivo competition experiments. The same number (2.5 x 10⁵ cells/mouse) of CD4⁺CD45RB⁺⁺ T cells from MyD88⁻/⁻ (Ly5.1⁺) or MyD88⁻/⁻ (Ly5.2⁺) mice were coinjected i.p. into RAG-2⁻/⁻ mice (n = 6), and the recipients were monitored for 6 wk after transfer.

Experiment 3. To assess the requirement of MyD88-dependent signaling for the persistence of colitogenic memory CD4⁺ T cells in this CD4⁺CD45RB⁺⁺ T cell-transferred colitis model, independently from the impact of naïve T cell priming, activation, and differentiation, we performed the adoptive retransfer of colitogenic LP memory CD4⁺ T cells derived from colitic mice that were separated with CD4⁺CD45RB⁺⁺ T cells of either MyD88⁻/⁻ (n = 6) or MyD88⁺/⁺ (n = 6) after 10 wk from transfer (17).

Experiment 4. To further assess the requirement of MyD88-dependent signaling for the persistence of colitogenic memory CD4⁺ T cells, we...
performed in vivo competition experiments. The same number (2.0 × 10^6 cells/mouse) of colitogenic LP memory CD4+ T cells obtained from colitic mice that were transferred with either MyD88-/- (Ly5.1) or MyD88+/+ (Ly5.2) CD4+CD45RBhigh T cells from either MyD88-/- mice (Ly5.1) or MyD88-/- mice (Ly5.2) after 10 wk from transfer were coinjected i.p. into new RAG-2-/- mice (n = 6), and the mice were monitored for 6 wk after the retransfer.

In experiments 1–4, all mice were assessed for a clinical score (18) that is the sum of four parameters listed as follows: hunching and wasting, 0 or 1; colon thickening, 0–3 (0, normal colon; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); stool consistency, 0–3 (0, formed; 1, soft stool; 2, diarrhea; 3, bloody stool) (18). To monitor the clinical sign during the observed period over time, the ongoing disease activity index is defined as the sum (0–5 points) of the above-mentioned parameters except the colon thickening.

**Experiment 5.** To assess the requirement of MyD88-dependent signaling for the lymphopoein-driven rapid proliferation (19) of colitogenic memory CD4+ T cells, we performed a short-term observation of in vivo competition experiments in combination with the CFSE-labeling method. The same number (2.0 × 10^6 cells/mouse) of CFSE-labeled LP memory CD4+ T cells from colitic mice that were initially transferred with MyD88-/- (Ly5.1) or MyD88+/+ (Ly5.2) CD4+CD45RBhigh T cells at 10 wk after transfer were injected i.p. into new RAG-2-/- mice (n = 6). In experiment 5, mice were sacrificed 10 days after retransfer, and assessed for cell divisions by CFSE dilution.
Histological examination

Tissue samples were fixed by 10% neutral-buffered formalin, and paraffin-embedded sections (5 μm) were stained with H&E. Tissue samples from the proximal, middle, and distal parts of the colon were prepared and subjected for analysis. The sections were analyzed without prior knowledge of the type of T cell reconstitution. The most affected area was graded by the severity of lesions. The degree of colonic inflammation was calculated using a previous scoring system (20): mucosal damage, 0; normal, 1; 3–10 intraepithelial cells (IEL)/high power field (HPF), focal damage, 2; >10 IEL/HPF and rare crypt abscesses, 3; >10 IEL/HPF, multiple crypt abscesses and erosion/ulceration, submucosa damage, 4; normal or widely scattered leukocytes, 1; focal aggregates of leukocytes, 2; diffuse leukocyte infiltration with expansion of submucosa, 3; diffuse leukocyte infiltration, muscularis damage, 0; normal or widely scattered leukocytes, 1; widely scattered leukocyte aggregates between muscle layers, 2; leukocyte infiltration with focal effacement of the muscularis, 3; extensive leukocyte effacement with transmural effacement of the muscularis.

Cytokine ELISA

To measure cytokine production, 1 × 10⁵ LP CD4⁺ T cells were cultured in 200 μl of culture medium at 37°C in a humidified atmosphere containing 5% CO₂, using 96-well plates (Costar) which were precoated with 5 g/ml hamster anti-mouse CD3e mAb (145-2C11; BD Pharmingen) and hamster 2 μg/ml anti-mouse CD28 mAb (37.51; BD Pharmingen) in PBS overnight at 4°C. Culture supernatants were removed after 48 h and assayed for cytokine production. Cytokine concentrations were determined by specific ELISA following the manufacturer’s recommendation (R&D Systems).

Flow cytometry

To detect the surface expression of molecules, isolated splenocytes, MLN, PB, BM, or LP mononuclear cells were preincubated with an Fc R-blocking mAb (H1.2F3). Biotinylated Abs were detected with PE-streptavidin. mAb (16A), anti-CD62L (MEL-14), anti-CD44 mAb (IM7), anti-CD69 mAb (145-2C11), anti-CD4 mAb (RM4-5), and a panel of 15 FITC-conjugated mAbs. Each percentage value indicates the frequency of each Vβ from the proximal, middle, and distal parts of the colon were prepared and subjected for analysis. The sections were analyzed without prior knowledge of the type of T cell reconstitution. The most affected area was graded by the severity of lesions. The degree of colonic inflammation was calculated using a previous scoring system (20): mucosal damage, 0; normal, 1; 3–10 intraepithelial cells (IEL)/high power field (HPF), focal damage, 2; >10 IEL/HPF and rare crypt abscesses, 3; >10 IEL/HPF, multiple crypt abscesses and erosion/ulceration, submucosa damage, 4; normal or widely scattered leukocytes, 1; focal aggregates of leukocytes, 2; diffuse leukocyte infiltration with expansion of submucosa, 3; diffuse leukocyte infiltration, muscularis damage, 0; normal or widely scattered leukocytes, 1; widely scattered leukocyte aggregates between muscle layers, 2; leukocyte infiltration with focal effacement of the muscularis, 3; extensive leukocyte effacement with transmural effacement of the muscularis.

CFSE labeling of T cells

T cell division in vivo was assessed by flow cytometry of CFSE-labeled cells. Isolated LP CD4⁺ T cells were cultured in vitro with the cytolytic dye CFSE (Molecular Probes) before reconstitution by incubation for 10 min at 37°C with 5 μM CFSE. The labeling reaction was quenched by washing in ice-cold RPMI 1640 supplemented with 10% FCS.

Statistical analysis

The results are expressed as mean ± SEM. Groups of data were compared by Mann-Whitney U test. Differences in data were considered to be statistically significant when p < 0.05.

Results

TLRs are expressed in CD4⁻CD45RB⁺⁺ donor cells and colitic LP CD4⁺ cells

To assess the direct involvement of TLR signaling in regulating cell function of CD4⁺ T cells composing chronic colitis under the presence of commensal bacteria, we examined whether mRNAs of TLR1–9 and their adaptor molecule, MyD88, are expressed in donor T cells or the LP CD4⁺ T cells in colitic RAG-2⁻⁻ mice transferred with CD4⁺CD45RB⁺⁺ T cells. To do so, we isolated...
mice transferred with WT or MyD88
expression all members of TLR1–9
avoid contamination of cells, such as macro-
within the recipient mice (Fig. 2A). When WT MyD88
CD45RBhigh T cells were transferred into RAG-2−/− mice, the recipients rapidly developed severe wasting disease associated with clinical signs of severe colitis. Particularly, weight loss (Fig. 2A), persistent diarrhea and also occasionally bloody stool or anal prolapse was observed by tracking the clinical score up to 6 wk after transfer (Fig. 2C). However, when MyD88−/− CD45RBhigh T cells were transferred into RAG-2−/− mice, the recipients also developed wasting disease and colitis despite the delayed onset (see the following result in Fig. 5), but the clinical score at 6 wk after transfer was significantly lower as compared with that of mice transferred with control MyD88+/+ CD4+ CD45RBhigh T cells (Fig. 2C). Thus, the delayed onset and milder clinical score of mice transferred with MyD88−/− CD4+ CD45RBhigh cells would easily be explained by the lack of a MyD88-dependent pathway in donor CD4+ T cells, but not in other innate immune cells of the recipient mice.

At 6 wk after transfer, the colon from of mice transferred with MyD88+/+ donor cells, but not that from mice transferred with MyD88−/− donor cells, was enlarged and had a greatly thickened wall (data not shown). In addition, the enlargement of the SP and MLN was also present in mice transferred with MyD88+/+ donor cells as compared with mice transferred with MyD88−/− donor cells (data not shown). Histological examination revealed that mice transferred with MyD88+/+ donor cells developed severe colitis showing prominent epithelial hyperplasia and erosion with a massive infiltration of mononuclear cells in LP of the colon (Fig. 2D). In contrast, mice transferred with MyD88−/− donor cells developed milder colitis as compared with mice transferred with MyD88+/+ donor cells. This difference was statistically confirmed by histological scoring of multiple colon sections, which was mice transferred with MyD88+/+ donor cells, 17.0 ± 1.0; and mice transferred with MyD88−/− donor cells, 6.2 ± 2.42 (p < 0.01) (Fig. 2E). Importantly, flow cytometry analysis revealed that the LP CD4+ T cells isolated from recipients transferred with either MyD88−/− or MyD88+/+ CD4+ CD45RBhigh T cells were CD44highCD62L−/− CD44highCD62L−/− donor cells (Fig. 2F), indicating that the transferred CD4+ CD45RBhigh T cells could differentiate into effector-memory T cells even in the absence of the MyD88-dependent pathway within colitic CD4+ T cells.

A further quantitative evaluation of CD4+ T cell infiltration was made by isolating LP, MLN, and SP CD3+ CD4+ T cells. As shown in Fig. 2G, significantly lower numbers of CD4+ T cells were recovered from LP, MLN, and SP of mice transferred with each CD4+ population under highly stringent gate definitions using FACSaria to avoid contamination of cells, such as macrophages, DCs, and B cells. As shown by RT-PCR in Fig. 1, whole splenocytes including T cells, B cells, macrophages, and DCs were used as the positive control, and expressed all members of TLR1–9 and MyD88. Under this condition, CD4+ CD45RBhigh donor cells expressed MyD88 and TLRs except TLR-4, 5, and 9 along with MyD88, while colitic LP CD4+ T cells expressed all members of TLRs and MyD88, indicating that TLR signaling via MyD88 may be directly involved in the priming, activation, proliferation, and survival of CD4+ T cells in the present transfer model. The data were further by completely no detection of PCR products from a template prepared without the addition of reverse transcriptase, excluding a possibility of signals derived from contaminating genomic DNA rather than mRNA (data not shown).

RAG-2−/− mice transferred with MyD88−/− CD4+ CD45RBhigh T cells developed milder colitis

To explore whether the MyD88-signaling pathway in T cells is involved in the development of chronic colitis, we transferred MyD88−/− or MyD88+/+ CD4+ CD45RBhigh T cells into RAG-2−/− (MyD88−/+ ) recipient mice maintaining an intact MyD88-dependent pathway of the innate immune system, meaning that only the transferred CD4+ T cells lack the MyD88-dependent pathway within the recipient mice (Fig. 2A). When WT MyD88+/+ CD4+ CD45RBhigh cells were transferred into RAG-2−/− mice, the recipients rapidly developed severe wasting disease associated with clinical signs of severe colitis. Particularly, weight loss (Fig. 2B), persistent diarrhea and also occasionally bloody stool or anal prolapse was observed by tracking the clinical score up to 6 wk after transfer (Fig. 2C). However, when MyD88−/− CD4+ CD45RBhigh T cells were transferred into RAG-2−/− mice, the recipients also developed wasting disease and colitis despite the delayed onset (see the following result in Fig. 5), but the clinical activity index was monitored during the course. Data are indicated as mean ± SE of five mice in each group. *p < 0.05. WT, MyD88+/+; B, Ongoing disease activity index was monitored during the course. Data are indicated as mean ± SE of five mice in each group. *p < 0.05. C, Histological examination of the colon from WT (upper) or MyD88−/− (lower) CD4+ CD45RBhigh T cells at 10 wk after transfer. Original magnification, ×100. D, Histological scoring of mice transferred with WT or MyD88−/− CD4+ CD45RBhigh T cells at 10 wk after transfer. Data are indicated as the mean ± SEM of five mice in each group. E, LP CD4+ T cells were isolated from mice transferred with WT or MyD88−/− CD4+ CD45RBhigh T cells at 10 wk after transfer, and the number of CD4+ cells was determined by flow cytometry. Data are indicated as mean ± SEM of five mice in each group. F, Phenotypic characterization of LP CD4+ T cells isolated from mice transferred with WT or MyD88−/− CD4+ CD45RBhigh T cells at 10 wk after transfer. The percentage of positive cells per total CD4+ T cells (CD69+/CD44+, IL-7Ra+/CD44+, CD44/high CD62L−/CD44+) was determined using flow cytometry.
MyD88−/− donor cells as compared with mice transferred with MyD88+/− donor cells. To further address the survival of CD4+ T cells, we next assessed whether regulation of Bcl-2 and Bcl-xL expression requires the MyD88-dependent signaling pathway using a quantitative RT-PCR. As expected, the SP CD4+ T cells from mice transferred with MyD88−/− donor cells expressed a significantly lower level of Bcl-2 and Bcl-xL compared with those from mice transferred with MyD88+/− donor cells (Fig. 2F). We also examined the cytokine production by isolated LP CD4+ T cells from recipient mice transferred with MyD88+/− or MyD88−/− donor cells along with LP CD4+ T cells from healthy MyD88+/+ or MyD88−/− mice. As shown in Fig. 2F, LP CD4+ T cells from mice transferred with MyD88−/− donor cells produced significantly less IFN-γ and IL-17 as compared with those from mice transferred with MyD88+/+ donor cells upon in vitro stimulation by anti-CD3/anti-CD28 mAbs. LP CD4+ T cells from both healthy WT and MyD88−/− mice produced only a small amount of these cytokines, showing no significant difference under the same condition (Fig. 2F).

Vβ repertoire is almost constant regardless of WT or MyD88−/− donor cells

Although we found that mice transferred with MyD88−/− donor cells develop milder colitis compared with mice transferred with MyD88+/− donor cells, possibly due to the lack of a MyD88 pathway within CD4+ T cells, it remained unclear whether expanded CD4+ T cells in the recipient mice recognize the same antigenic epitopes of CD4+ T cells. To clarify this issue, SP CD4+ T cells from both groups of mice were analyzed for their TCR Vβ repertoire by flow cytometry. As shown in Fig. 3, the polyclonal dominant TCR Vβ repertoire with the dominancy of Vβ8.1/8.2 and Vβ8.3 was almost constant regardless of MyD88+/+ or MyD88−/− donor cells. Only the frequency of Vβ5.1/5.2 in mice transferred with MyD88−/− donor cells was significantly increased as compared with that in mice transferred with WT donor cells, indicating that colitogenic CD4+ T cells recognizing the same or similar Ag epitopes could develop independently from the TLR-MyD88 signaling pathway in CD4+ T cells.

Expansive activity of WT donor cells predominates over that of MyD88−/− donor cells in vivo competition assay

To further assess the requirement of TLR-MyD88 signaling for the expansion of CD4+ donor cells, we performed in vivo competition experiments. The same number (2.5 × 105 cells/mouse) of CD4+CD45RBlow donor cells from Ly5.1-background (Ly5.1+) MyD88+/+ and Ly5.2-background (Ly5.2−) MyD88−/− mice were coinjected i.p. into the identical RAG-2−/− mice (Fig. 4A). As expected, recipient mice developed severe colitis at 6 wk after cotransfer (data not shown), and a significantly lower proportion of Ly5.2+ MyD88−/− CD4+ T cells was observed not only in the inflamed LP, but also in SP,
MLN, PB, and BM, as compared with the paired Ly5.1+/MyD88−/− CD4+ T cells (Fig. 4B). Furthermore, the ratio of IFN-γ-expressing cells within total MyD88−/− LP CD4+ T cells was significantly lower compared with that in total MyD88−/− LP CD4+ T cells (Fig. 4C). Consistent with the lower expression of IFN-γ in MyD88−/− LP CD4+ T cells, expression of the activation marker CD69 on MyD88−/− LP or SP CD4+ cells was significantly lower than on MyD88−/− LP or SP CD4+ T cells, respectively (Fig. 4D).

RAG-2−/− mice transferred with MyD88−/− cologenic LP CD4+ donor cells develop milder colitis

To next assess the role of MyD88-dependent pathway in persistent colitis, we next examined cologenic LP CD4+ T cell-mediated colitis model (17), which lacks the impact of naive T cell priming, activation, and differentiation phase required in the former CD4+CD45RBhigh T cell-transferred colitis model. We first confirmed that RAG-2−/− mice transferred with MyD88−/−CD4+CD45RBhigh T cells do develop colitis to a similar extent to mice transferred with MyD88−/−CD4+CD45RBhigh T cells at the late stage of 10 wk after transfer as confirmed by the weight curve (Fig. 5A), albeit the ongoing disease activity index (Fig. 5B) and histological assessment (Fig. 5, C and D) delayed onset and kinetics. Consistent with these findings, the recovered cell number was equivalent between mice transferred with MyD88−/− or MyD88−/− CD4+CD45RBhigh T cells (Fig. 5E). Furthermore, the expression of activation (CD69)/differentiation (IL-7Ra, CD44, and CD62L) on LP CD4+ T cells showed no difference between two groups of mice (Fig. 5F), indicating that MyD88 deficiency solely contributes to the delayed kinetics of the development of colitis.

We thus isolated the LP CD4+ T cells from colitic recipient mice transferred with either MyD88−/− or MyD88−/− CD4+CD45RBhigh T cells at 10 wk after transfer, to use for the subsequent memory T cell transfer. We transferred the isolated colitic LP CD4+ T cells into new RAG-2−/− mice to focus on the persistence of colitogenic CD4+ memory T cells (Fig. 6A). Similar with the results using CD4+CD45RBhigh T cell-mediated colitis model in Fig. 2, the recipient mice transferred with colitic MyD88−/− LP CD4+ T cells showed milder wasting disease (Fig. 6B) with milder clinical signs of colitis at 4 wk after retransfer, as compared with mice transferred with colitic MyD88−/− LP CD4+ T cells (Fig. 6C). Histological examination also revealed that mice transferred with MyD88−/− LP CD4+ T cells developed milder colitis at 4 wk after retransfer as compared with mice transferred with MyD88−/− LP CD4+ T cells (Fig. 6D). The difference was statistically confirmed by histological scoring of colon sections, which showed as follows: mice transferred with MyD88−/− LP CD4+ T cells, 17.8 ± 0.86 and mice transferred with MyD88−/− LP CD4+ T cells, 9.4 ± 1.86 (p < 0.01) (Fig. 6E). Furthermore, a significantly lower number of CD4+ T cells was recovered from SP, LP, and MLN of mice transferred with MyD88−/− donor cells as compared with mice transferred with MyD88−/− donor cells (Fig. 6F). As shown in Fig. 6G, LP CD4+ T cells from mice transferred with MyD88−/− LP donor cells produced significantly less IFN-γ and IL-17 as compared with those from mice transferred with MyD88−/− LP donor cells.

To further assess the expansive activity of colitic LP CD4+ memory T cells, we again performed in vivo competition experiments. The same number (2.0 × 106 cells/mouse) of colitic Ly5.1+ MyD88−/− and Ly5.2+ MyD88−/− LP donor cells obtained from colitic mice transferred with Ly5.1+ MyD88−/− or Ly5.2+ MyD88−/− CD4+CD45RBhigh T cells at 10 wk after transfer was coinjected i.p. into identical RAG-2−/− mice (Fig. 7A). Six wk after cotransfer, a significantly lower proportion of Ly5.2+ MyD88−/− CD4+ T cells was recovered from the inflamed LP, SP, and MLN, as compared with the paired Ly5.1+ MyD88−/−CD4+ T cells (Fig. 7B). Furthermore, the ratio of IFN-γ-expressing CD4+ T cells within total MyD88−/− LP CD4+ T cells was significantly decreased as compared with that within total MyD88−/− LP CD4+ T cells (Fig. 7C).

MyD88 signaling contributes to the lymphopenia-driven rapid proliferation of colitogenic CD4+ T cells

To finally examine the effect of MyD88 signaling on the lymphopenia-driven rapid proliferation (18) of the colitogenic CD4+ memory T cells, we used the in vivo CFSE dilution method to examine cells undergoing proliferation after a short period from transfer. First, the LP CD4+ T cells obtained from colitic RAG-2−/− mice transferred with either MyD88−/− or MyD88−/− CD4+CD45RBhigh T cells at 10 wk after transfer were labeled with CFSE and adoptively cotransferred into new RAG-2−/− mice. Cell divisions were determined 10 days after cotransfer by assessing the CFSE dilution (Fig. 8A). As depicted in Fig. 8B, the markedly delayed division pattern of CD4+ T cells from mice transferred with MyD88−/− donor cells was observed as compared with that in mice transferred with MyD88−/− donor cells. This difference was statistically confirmed by comparing the CFSE− cells between Ly5.1+ and Ly5.2+ cells (Fig. 8C), indicating that the MyD88-dependent signaling pathway in T cells promotes the rapid proliferation of colitogenic CD4+ memory T cells in a lymphopenic condition.
of colitis? In other words, from where do colitogenic CD4⁺ T cells receive proliferative and/or survival signals to sustain chronic colitis? First, it is well-known that commensal bacteria are essentially required for the development and the persistence of colitis, because 1) almost all models of T cell-mediated colitis do not develop colitis under the germfree condition (4–6), and 2) several groups elegantly demonstrated the requirement of specific Ags for the development and persistence of colitis by showing that colitis is induced and sustained by administration of OVA peptide-expressing *Escherichia coli* into OVA-specific TCR-transgenic mice in an Ag-specific manner (25, 26). These results indicated that TCR signaling through Ags, especially Ags derived from commensals, are needed for the development and persistence of colitis. Second, in addition to Ags derived from commensal bacteria, we here showed that the MyD88-dependent signaling pathway directly bolsters up the proliferation and survival of colitogenic CD4⁺ T cells. However, it is of note that RAG-2⁻/⁻ mice transferred with MyD88⁻/⁻ CD4⁺CD45RBhigh T cells did develop colitis with CD4⁺ T cell infiltration in the inflamed mucosa albeit the onset was delayed as compared with the control, indicating that the direct MyD88-dependent signaling pathway in colitogenic CD4⁺ T cells may act as a costimulator to tune the essential TCR signaling for the maintenance of these cells. However, at the molecular level, it still remains unknown how the identical CD4⁺ T cells coordinate TCR and TLR signaling initiated from the commensal bacteria for activation, proliferation, and survival. Further studies will be required to address this important issue.

So far, most studies regarding TLRs have focused on cells of the innate immune system, such as DCs, macrophages, and epithelial cells, and now it is recognized that members of TLRs play an essential role in the innate immune recognition allowing the detection of commensal bacteria, followed by the second activation of T cells (9–11). However, recent works showed that conventional TCR αβ⁺ CD4⁺ T cells also express TLRs (12), suggesting that PAMPs may directly modulate the function of CD4⁺ T cells. Importantly, Gelman et al. (27) recently reported that TLR signaling in primary CD4⁺ T cells directly enhances proliferation through MyD88 and PI3K-dependent pathway, in response to a T cell-dependent Ag. Thus, the present study may add the identification of the role of TLR signaling in the activation/function of the pathogenic memory CD4⁺ T cells. Although we showed that the MyD88-dependent signaling pathway positively reinforces the proliferation and survival of colitogenic CD4⁺ T cells in colitic mice, it has been previously reported that TLR-4 is predominantly expressed on regulatory CD4⁺CD25⁺ T cells rather than CD4⁺CD45RBhigh naive cells, and TLR-4-specific signaling by LPS increases the regulatory CD4⁺CD25⁺ T cell activity, resulting in suppression of inflammatory responses in vivo (16). We slightly, but substantially, detected TLR-4 mRNA as well as other TLRs in colitic LP CD4⁺ T cells, thus it is interesting to know how the stimulatory and inhibitory TLR-signaling pathway in T cells orchestrates the complicated immune responses in chronic colitis.

Such characteristics of TLR/MyD88-expressing colitogenic CD4⁺ T cells raise another important question of whether the colitogenic CD4⁺CD45RBhighCD62L⁻IL-7Ra⁺ T cells (Fig. 2) can be defined as effector-memory T cells rather than just effector T cells under the persistent presence of commensal Ags and/or self Ags, because it is accepted that memory T cells are generated after Ag clearance for the first time, but not under persistent presence of Ags, shown in models of chronic viral infections to CD8⁺ T cells, using lymphochytic choriomeningitis virus or influenza A virus infections (28). Because the candidate Ags for colitogenic CD4⁺ T cells are thought to be derived
from the intestinal bacterial Ags that are never eliminated from the body, it is doubtful whether colitogenic CD4⁺ memory T cells can be generated under such a situation. However, as recent studies have suggested that persistent presence of Ags is required for the long-term maintenance of CD4⁺ memory T cells, it is possible that the nature of CD4⁺ memory T cells is quite different from that of CD8⁺ memory T cells (29, 30). Thus, the present results may support another idea that the persistent presence of both commensal bacteria-derived PAMPs and specific Ags is required for the maintenance of long-term colitogenic CD4⁺ memory T cells, and the subsequent progressive, disabling disease course.

It is also possible that nonpathogenic commensals stimulate TLR signaling of colitogenic CD4⁺ memory T cells to sustain the disease without providing specific Ags for such cells. In other words, it should be verified whether specific Ags or PAMPs from the commensal bacteria are essential for the priming or memory phase. Consistently, the current study also provides an explanation of why a common recurrence of IBD is observed during complications of microbial infection, such as acute Salmonella enterocolitis, which may possibly supply large amounts of “bystander” PAMPs (31).

Finally, an important point should also be discussed: whether the present experimental design solely assesses the role of direct TLR signaling in various stages of CD4⁺ T cells during the development of chronic colitis, because MyD88 is also involved in signaling downstream of endogenous cytokines, IL-1 and IL-18, in addition to TLR signaling (13, 14). Further studies will be required to address this issue by assessing which TLR is the most important for the stimulation of colitogenic CD4⁺ T cells, followed by in vivo experiment using the corresponding TLRnull mice.

In summary, we here demonstrate that the MyD88-dependent pathway that mediates downstream signals of TLRs is crucially involved in the proliferative and survival responses of colitogenic CD4⁺ T cells, which is required for the perpetuation of chronic colitis. Thus, in addition to the specific commensal Ags, homeostatic cytokines, and costimulatory molecules, therapeutic approaches targeting PAMPs may be feasible in the treatment of IBD.

Disclosures
The authors have no financial conflict of interest.

References