indeed important to establish GALT, such as Payer's patches and cryptopatches, and also to maintain IELs (30), but not needed to develop and sustain colitis, since many Ags, such as intestinal bacterial Ags, may be sufficient to stimulate colitogenic $CD4^+$ T cells in the intestinal LP without stimuli from IL-7. To prove it, we performed a combinational experiment using adoptive transfer and parabiosis systems in the present study. Although the parabiosis system seems to be somewhat artificial and problematic on some level as two mice, host and donor, are forced to have a surgical stress and behavioral limitation (Groups 3 and 4), mice laboring colitogenic $CD4^+$ T cells are surgically joined, resulting in prompt development of anastomoses of blood vessels within a few days. Even in the present setting, it is noteworthy that IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice joined with colitic IL-$7^{+/+}$ × RAG-$1^{-/-}$ donor mice developed a wasting disease and colitis to the similar level of colitic IL-$7^{+/+}$ × RAG-$1^{-/-}$ donor mice over time.

In this parabiosis system, however, it was also possible that certain stem cells that are committed to differentiate into IL-7-producing mesenchymal cells or epithelial cells homed to the intestine, and might have been involved in the development and persistence of colitis in IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice joined with colitic IL-$7^{+/+}$ × RAG-$1^{-/-}$ donor mice (Group 3). To rule out this possibility, we also demonstrated that IL-7 expression was not detected in the colon of the IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice both at the protein and mRNA levels (Fig. 6). Consistent with the present result, another group demonstrated that restoring intestinal IL-7 expression to IL-$7^{-/-}$ mice did not result in the development of colitis (31). Collectively, the current results clearly indicate that intestinal IL-7 is not essential, but systemic IL-7 from extraintestinal sites is essential, for the development and sustainment of colitis.

It is also very important to know why IL-7 is decreased in the inflamed mucosa of colitis in terms of pathogenesis of chronic colitis. In other words, it is possible that the lack or decrease of IL-7 production in inflamed mucosa of colitis is pathologically needed to maintain chronic colitis. Consistent with this hypothesis, we previously demonstrated that although IL-7 promoted proliferation of human LP IL-7Rα-expressing $CD4^+$ T cells, double stimuli by IL-7 and anti-CD3 mAb conversely suppressed it (21). In addition, Fluur and colleagues (32) very recently reported that IL-7 induces Fas-mediated T cell apoptosis by inducing Fas expression on $CD4^+$ T cells. Thus, it appears that intestinal IL-7 physiologically plays a key role in the elimination of pathological LP $CD4^+$ T cells activated by intestinal bacteria. Further studies will be needed to address this issue.

Interestingly, the recovered cell number of LP $CD4^+$ T cells was equivalent between host and donor mice both in Group 2 and 3, although it was likely that total production of IL-7 in Group 3 parabionts between one IL-$7^{+/+}$ mouse and one IL-$7^{-/-}$ mouse was approximately half compared with that in Group 2 parabionts between two IL-$7^{+/+}$ mice. Because it seems that the production of IL-7 is maintained at a constant rate and is uninfluenced by extrinsic stimuli (33, 34), this result indicates that factors other than IL-7, such as stimulation by commensal bacteria might control the homeostasis of cell number in the LP, but not in the BM and SP. Further studies will be needed to address this issue.

BM is a major source of IL-7 in the body (26). In contrast to the LP, it is noteworthy that the number of $CD4^+$ T cells recovered from the BM and SP of the colitic IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice (Group 3) was significantly decreased compared with that of the IL-$7^{+/+}$ × RAG-$1^{-/-}$ host mice. Regarding this result, we recently demonstrated that $CD4^+$ effector-memory-like T (T_{EM}-like) cells reside in the BM of colitic SCID and RAG-$1/2^{-/-}$ mice induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells (20). Importantly, these resident BM $CD4^+$ T_{EM}-like cells are closely attached to IL-7-producing stromal cells in the colitic BM. Most importantly, the accumulation of BM $CD4^+$ T_{EM}-like cells was significantly decreased in IL-7-deficient recipients reconstituted with the colitogenic LP $CD4^+$ T_{EM}-like cells. Together with the present study, these findings suggest that the BM $CD4^+$ T_{EM}-like cells residing in mice with chronic colitis play a critical role as a reservoir for lifelong persisting colitis in an IL-7-dependent manner. However, it is still possible that IL-7 produced by sites other than intestine or BM, such as skin, liver, eye, lymph nodes (LN), and SP, also contribute to the development and perpetuation of colitis. In this regard, we very recently demonstrated that splenectomized LN-null lymphotoxin $\alpha^{-/-}$ × RAG-$2^{-/-}$ mice transferred with colitogenic LP $CD4^+$ T cells develop colitis (35), suggesting that IL-7 production at least by LN and SP does not appear to be essential. To further clarify the role of IL-7 produced by BM mesenchymal cells in the pathogenesis of chronic colitis, BM chimera of IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice, which are lethally irradiated and transplanted with the BM cells from IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice, may be quite beneficial. Interestingly, however, it is also well known that extraintestinal complications of IBD patients such as skin, liver, and mucocutaneous manifestations (36) appears to be closely associated with sites of local IL-7 production by keratinocytes, hepatocytes, and uvea cells. Although no inflammation was not observed at least in liver and skin in the present model of colitis (data not shown), further studies will be needed to address this issue.

Clinicopathologically, IBD is characterized by chronic intestinal inflammation. Surgery does not cure IBD, especially Crohn's disease, as relapse is a rule after remission, suggesting that IBD is not a circumscribed disease, but rather a systemic disease mediated by colitogenic memory $CD4^+$ T cells distributing throughout the body via the bloodstream, which may hide in their reservoir, such as BM. Consistent with this hypothesis, recent findings showing usefulness of leukocytoapheresis, which removes peripheral blood cells for the treatment of refractory IBD patients (37, 38), suggests that recirculation of colitogenic memory $CD4^+$ T cells from the gut to some reservoir and vice versa, may play a role in the perpetuation of chronic colitis. Furthermore, we have recently demonstrated that FTY720 that has an ability to inhibit circulation of lymphocytes prevents the development of SCID/RAG-$1/2^{-/-}$ colitis induced by adoptive transfer of LP colitogenic $CD4^+$ T_{EM}-like cells (39). Together with the current results, it would be possible that the circulation of colitogenic $CD4^+$ T_{EM}-like cells is quite active in IBD, making them continue to circulate in the blood and migrate to IL-7-producing reservoir from the IL-7-depleted LP.

In summary, in this study, we demonstrated that systemic IL-7, but not intestinal IL-7, is essential for the development and perpetuation of colitis, suggesting that therapeutic approaches targeting systemic IL-7 using the biologics against IL-7 may be feasible in the treatment of IBD.

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Disclosures

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MyD88-Dependent Pathway in T Cells Directly Modulates the Expansion of Colitogenic CD4$^+$ T Cells in Chronic Colitis[1]

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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 TCR$\alpha\beta^+$ 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$^+$CD45RB^{high} T cells, both CD4$^+$CD45RB^{high} donor cells and the expanding colitogenic lamina propria CD4$^+$CD44^{high} 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$^+$CD45RB^{high} 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$^+$CD45RB^{high} cells. When RAG-2$^{-/-}$ mice were transferred with the same number of MyD88$^{+/+}$ (Ly5.1$^+$) and MyD88$^{-/-}$ (Ly5.2$^+$) CD4$^+$CD45RB^{high} 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-x$_L$ 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. ***The Journal of Immunology*****, 2008, 180: 5291–5299.**

Inflammatory bowel diseases (IBD)[3] 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 maintain colitogenic CD4$^+$ T cells by stimuli through 1) TCR signaling by one or more commensal Ags (signal 1) and 2) TLR signaling by pathogen-associated molecular patterns (PAMPs) in addition to cytokines (signal 3) and costimulatory signaling (signal 2) (7, 8). However, there are no reports showing that TLR signaling directly stimulates colitogenic CD4$^+$ T cells for their proliferation and/or survival.

It is widely recognized that TLRs are expressed in/on the innate immune cells (9, 10), such as macrophages, DCs, and epithelial cells, and are crucially important primarily to activate these professional and nonprofessional APCs and secondarily promote T cell responses (11). However, accumulating evidence has shown that certain TLRs are also expressed on/in TCR$\alpha\beta^+$ T cells that are major acquired immune cell populations (12), suggesting that TLR signaling may possibly have some direct function on adaptive immunity. Hence, to assess the direct role of TLR signaling initiated by PAMPs of commensal bacteria in modulation of colitogenic CD4$^+$ T cell expansion during the development and the persistence of chronic colitis, we performed a series of adoptive transfer colitis experiments by transfer of CD4$^+$CD45RB^{high} T cells that are deficient for MyD88, a key adaptor molecule of TLR signaling (13, 14), into RAG-2$^{-/-}$ recipient mice whose TLR pathways remained intact throughout the innate immune system.

Materials and Methods

Animals

Six- to 10-wk-old Ly5.2-background (Ly5.2$^+$) MyD88$^{-/-}$ mice (13) were used. Ly5.2$^+$ C57BL/6 mice were purchased from Japan Clea. Ly5.1-background C57BL/6 mice and Ly5.2$^+$ C57BL/6 RAG-2$^{-/-}$ mice were obtained from Taconic Farms and Central Laboratories for Experimental Animals (Kawasaki, Japan). Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of Tokyo Medical and Dental University (Tokyo, Japan). All experiments were approved by the

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[3] Abbreviations used in this paper: IBD, inflammatory bowel disease; DC, dendritic cell; PAMP, pathogen-associated molecular pattern; PB, peripheral blood; SP, spleen; MLN, mesenteric lymph node; BM, bone marrow; WT, wild type; LP, lamina propria; IEL, intraepithelial cell; HPF, high-power field.

regional animal study committees and were done according to institutional guidelines and Home Office regulations.

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 Lymphosepar II (IBL) and centrifuged at 400 × *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 $MyD88^{-/-}$ 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 FACSCalibur; 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 $CD4^+CD45RB^{high}$ 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 $CD4^+CD45RB^{high}$ T cells either from $MyD88^{-/-}$ or from littermate wild-type (WT) $MyD88^{+/+}$ mice as described previously (15). Colitic $CD4^+CD45RB^{high}$ T cell-transferred RAG-$2^{-/-}$ mice were sacrificed at 6–10 wk after transfer. The entire colon was opened longitudinally, washed with PBS, and cut into small pieces. The dissected mucosa was incubated with Ca^{2+}, Mg^{2+}-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 subjected to 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 FACSCalibur. 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 transcription product was amplified with 0.25 U of rTaq DNA polymerase (Toyobo) in a 25-μl reaction. Sense and antisense primers and the cycle numbers for the amplification of each gene (16) were as follows: TLR1, sense 5′-TCTCT GAAGGCTTTGTCGATACA-3′ and antisense 5′-GACAGAGCCTGTA AGCATATTCG-3′ (35 cycles); TLR2, sense 5′-TCTAAAGTCGATC CGCGACAT-3′ and antisense 5′-TACCCAGCTCGCTCACTACGT-3′ (35 cycles); TLR3, sense 5′-TTGTCTTCTGCACGAACCTG-3′ and antisense 5′-CGCAACGCAAGGATTTTATT-3′ (35 cycles); TLR4, sense 5′-CAAGAACATAGATCTGAGCTTCAACCC-3′ and antisense 5′-GCT GTCCAATAGGGAAGCTTTCTAGAG-3′ (35 cycles); TLR5, sense 5′-ACTGAATTCCTTAAGCGACGTA-3′ and antisense 5′-AGAAGATAA AGCCGTGCGAAA-3′ (35 cycles); TLR6, sense 5′-AACAGGATAC GGAGCCTTGA-3′ and antisense 5′-CCAGGAAAGTCAGCTTCGTC-3′ (35 cycles); TLR7, sense 5′-TTCCGATACGATGAATATGCACG-3′ and antisense 5′-TGAGTTTGTCCAGAAGCCGTAAT-3′ (35 cycles); TLR8, sense 5′-GGCACAACTCCCTTGTGATT-3′ and antisense 5′-CATTTG GGTGCTGTTGTTTG-3′ (35 cycles); TLR9, sense 5′-CCGCAAGA CTCTATTTGTGCTGG-3′ and antisense 5′-TGTCCCTAGTCAGG GCTGTACTCAG-3′ (35 cycles); MyD88, sense 5′-GGCCTTGTTAGA CCGTGAGG-3′ and antisense 5′-TCATCTTCCCCTCTGCCCTA-3′ for (35 cycles); β-actin, sense 5′-GTGGGCCGCTCTAGGCACCAA-3′ and antisense 5′-CTCTTTGATGTCACGCACGATTTC-3′ (30 cycles). PCR products were separated on 1.8% agarose gels, stained with ethidium bromide, and visualized with a Lumi-Imager F1 (Roche Diagnostics).

Quantitative PCR

To validate gene expression changes, quantitative RT-PCR analysis was performed by Applied Biosystems 7500 using validated TaqMan Gene Expression Assays (Applied Biosystems). The TaqMan probes and primers for mouse Bcl-2 (assay identification number Mm00477631_m1) and mouse Bcl-x_L (assay identification number Mm00437783_m1) were Assay-on-Demand gene expression products (Applied Biosystems). The mouse β-actin gene was used as endogenous control (catalog number 4352933E; Applied Biosystems). The thermal cycler conditions were as follows: hold for 10 min at 95°C, followed a cycle of 95°C for 15 s and 60°C for 1 min for 45 cycles. All samples were performed in triplicate.

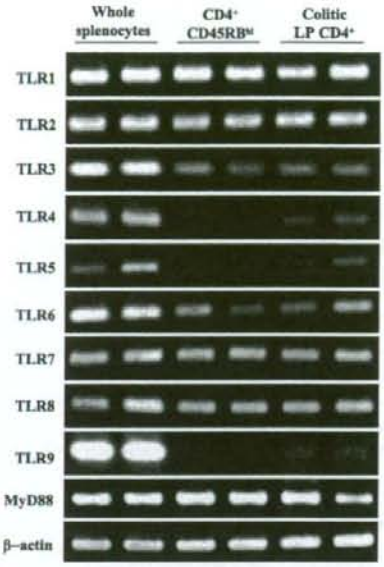

FIGURE 1. $CD4^+CD45RB^{high}$ donor cells and colitic LP $CD4^+$ T cells express mRNAs for TLR and MyD88. Splenocytes and $CD4^+CD45RB^{high}$ T cells from normal C57BL/6 mice, or LP $CD4^+$ T cells from colitic RAG-$2^{-/-}$ mice transferred with $CD4^+CD45RB^{high}$ T cells, were strictly isolated using FACSAria (purity, >98.0%) and subjected for total RNA extraction. The RNA was reverse transcripted and amplified with gene for specific primers. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. All the RT-PCR experiments were performed at least three times on independent samples.

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 $CD4^+CD45RB^{high}$ 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. $CD4^+CD45RB^{high}$ 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 (2.5×10^5 cells/mouse) of $CD4^+CD45RB^{high}$ 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 $CD4^+CD45RB^{high}$ T cell-transferred colitis model, independently from the impact of naive T cell priming, activation, and differentiation, we performed the adoptive retransfer of colitogenic LP memory $CD4^+$ T cells derived from colitic mice that were transferred with $CD4^+CD45RB^{high}$ T cells of either $MyD88^{+/+}$ ($n = 6$) or $MyD88^{-/-}$ mice ($n = 6$) 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

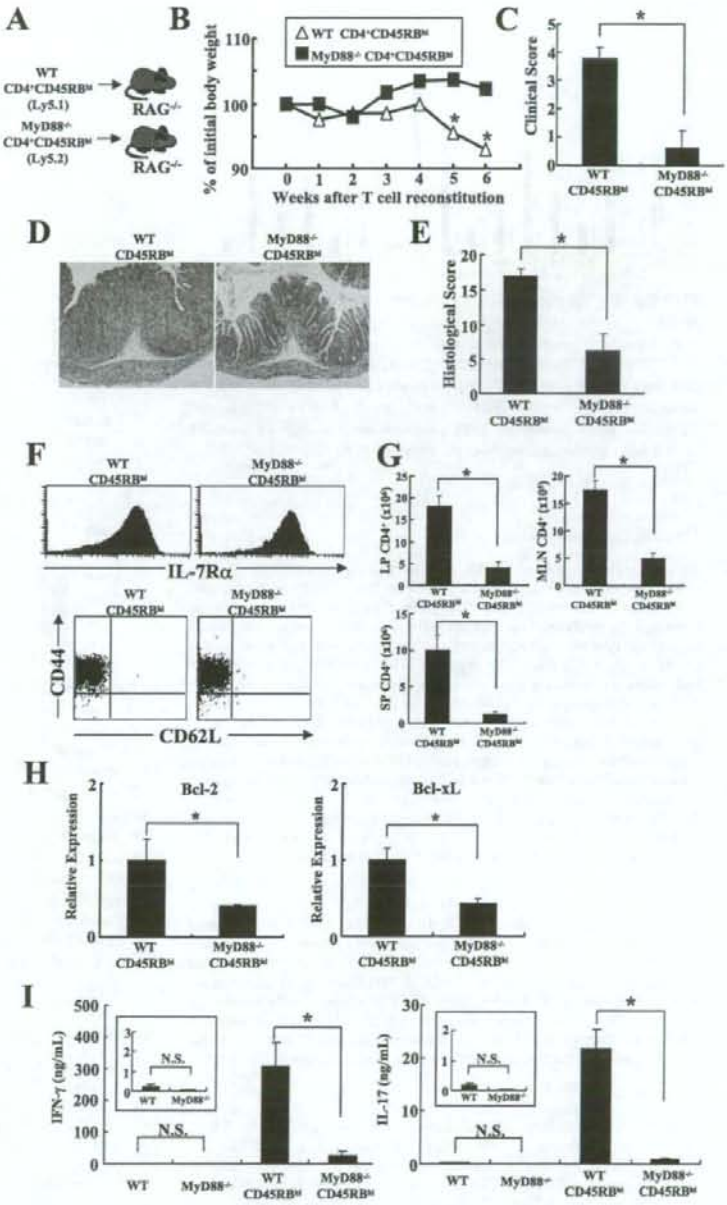

FIGURE 2. RAG-2$^{-/-}$ mice transferred with MyD88$^{-/-}$CD4$^+$CD45RB^{high} T cells develop milder colitis. *A*, RAG-2$^{-/-}$ mice were transferred with splenic MyD88$^{+/+}$ (WT) ($n = 6$) or MyD88$^{-/-}$ ($n = 6$) CD4$^+$CD45RB^{high} T cells (3×10^5 cells/mouse). *B*, Change in body weight over time is expressed as percent of the original weight. Data are represented as the mean ± SEM of six mice in each group. *, $p < 0.05$. *C*, Clinical scores were determined at 6 wk after transfer as described in *Materials and Methods*. Data are indicated as mean ± SEM of six mice in each group. *, $p < 0.01$. *D*, Histological examination of the colon from WT (*left*) or MyD88$^{-/-}$ (*right*) CD4$^+$CD45RB^{high} T cells at 6 wk after transfer. Original magnification, ×100. *E*, Histological scoring of mice transferred with WT or MyD88$^{-/-}$CD4$^+$CD45RB^{high} T cells at 6 wk after transfer. Data are indicated as mean ± SEM of six mice in each group. *, $p < 0.01$. *F*, Phenotypic characterization of LP CD4$^+$ T cells isolated from mice transferred with WT or MyD88$^{-/-}$CD4$^+$CD45RB^{high} T cells at 6 wk after transfer. *G*, LP, MLN, and SP CD4$^+$ T cells were isolated from mice transferred with WT or MyD88$^{-/-}$CD4$^+$CD45RB^{high} T cells at 6 wk after transfer, and the number of CD4$^+$ cells was determined by flow cytometry. Data are indicated as mean ± SEM of six mice in each group. *, $p < 0.01$. *H*, Expression of Bcl-2 and Bcl-x$_L$ mRNAs in SP cells was determined by quantitative RT-PCR, and are shown as relative amount of indicated mRNA normalized by expression of β-actin. Data are represented as the mean ± SEM of six samples. *, $p < 0.05$. *I*, Cytokine production by LP CD4$^+$ T cells. LP CD4$^+$ T cells were isolated at 6 wk after transfer and stimulated with anti-CD3 and anti-CD28 mAbs for 48 h. IFN-γ and IL-17 concentrations in culture supernatants were measured by ELISA. Data are indicated as mean ± SEM of six mice in each group. *, $p < 0.01$. WT; healthy WT mice, MyD88$^{-/-}$; healthy MyD88$^{-/-}$ mice, WT CD45RB^{high}; RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells, MyD88$^{-/-}$CD45RB^{high}; RAG-2$^{-/-}$ mice transferred with MyD88$^{-/-}$CD4$^+$ CD45RB^{high} T cells.

performed in vivo competition experiments. The same number (2.0×10^5 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$^+$CD45RB^{high} 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, no colon thickening; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); and stool consistency, 0–3 (0, normal beaded stool; 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 lymphopenia-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$^+$CD45RB^{high} 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.

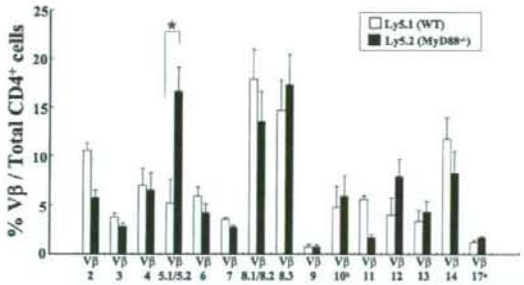

FIGURE 3. Vβ repertoire shows little difference between MyD88$^{+/+}$ or MyD88$^{-/-}$ donor cells. To analyze the TCR Vβ family repertoire, SP cells were isolated from mice transferred with Ly5.1^{+}MyD88$^{+/+}$ (WT) or Ly5.2^{+}MyD88$^{-/-}$CD4^{+}CD45RB^{high} T cells at 6 wk after transfer, and then triple-stained with PerCP-conjugated ant-CD3ε mAb (145-2C11), PE-conjugated anti-CD4 mAb (RM4-5), and a panel of 15 FITC-conjugated Vβ mAbs. Each percentage value indicates the frequency of each Vβ pooled from three independent experiments ($n = 6$). *, $p < 0.05$.

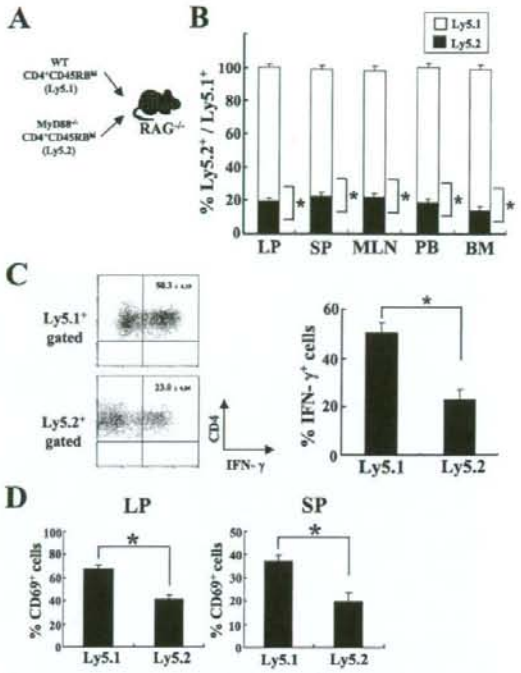

FIGURE 4. Expansive activity of MyD88$^{+/+}$ donor cells predominates over that of MyD88$^{-/-}$ donor cells in an in vivo competition assay. *A*, The same number (2.5×10^5 cells/mouse) of CD4^{+}CD45RB^{high} T cells from Ly5.1^{+}MyD88$^{+/+}$ (WT) mice and Ly5.2^{+}MyD88$^{-/-}$ mice was coinjected i.p. into RAG-2$^{-/-}$ mice ($n = 6$). *B*, Six weeks after transfer, LP, SP, MLN, PB, and BM CD4^{+} T cells were isolated, and the ratio of Ly5.1^{+} and Ly5.2^{+} CD4^{+} cells was determined by flow cytometry. *, $p < 0.01$. *C*, The frequencies of IFN-γ-producing LP CD4^{+} T cells per total Ly5.1^{+} or Ly5.2^{+} cells were analyzed in the indicated subpopulations by flow cytometry. Data are represented as mean ± SEM of three independent experiments. *, $p < 0.01$. *D*, Phenotypic characterization of LP and SP CD4^{+} T cells after transfer of CD4^{+}CD45RB^{high} T cells. % CD69^{+}, Percentages of CD4^{+}CD69^{+} cells per total CD4^{+} cells. Data are represented as mean ± SEM of six mice per group. *, $p < 0.05$.

Histological examination

Tissue samples were fixed by 10% neutral-buffered formalin, and paraffin-embedded sections (5 μm) were stained with H&E. Three 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): mucosa damage, 0; normal, 1; 3–10 intraepithelial cells (IEL)/high power field (HPF) and focal damage, 2; >10 IEL/HPF and rare crypt abscesses, 3; >10 IEL/HPF, multiple crypt abscesses and erosion/ulceration, submucosa damage, 0; 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 infiltration with transmural effacement of the muscularis.

Cytokine ELISA

To measure cytokine production, 1×10^5 LP CD4^{+} T cells were cultured in 200 μl of culture medium at 37°C in a humidified atmosphere containing 5% CO_2, using 96-well plates (Costar) which were precoated with 5 μg/ml hamster anti-mouse CD3ε 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 FcγR-blocking mAb (CD16/32; 2.4G2; BD Pharmingen) for 15 min followed by incubation with specific FITC-, PE-, PerCP-, allophycocyanin- or biotin-labeled Abs for 20 min on ice. The following mAbs except biotin-conjugated anti-mouse IL-7Rα (A7R34; eBioscience) were obtained from BD Pharmingen; anti-CD3ε mAb (145-2C11), anti-CD4 mAb (RM4-5), anti-CD45RB mAb (16A), anti-CD62L (MEL-14), anti-CD44 mAb (IM7), anti-CD69 mAb (H1.2F3). Biotinylated Abs were detected with PE-streptavidin. Standard three- or four-color flow cytometric analysis was performed by the FACSCalibur equipped with CellQuest software. Background fluorescence was assessed by the staining of control irrelevant isotype. To analyze the TCR Vβ family repertoire, splenic cells were triple-stained with PerCP-conjugated anti-CD3ε mAb (145C-11), PE-conjugated anti-CD4 mAb (RM4-5), and either of the following FITC-conjugated mAbs: Vβ2; KJ25, Vβ3; KT4, Vβ4; MR9-4, Vβ5.1/5.2; RR4-7, Vβ6; TR310, Vβ7; MR5-2, Vβ8.1/2; B21.14, Vβ8.3; MR10-2, Vβ9; B21.5, Vβ10^{b}; RR3-15, Vβ11; MR11-1, Vβ12; IN12.3, Vβ13; 14.2, Vβ14; and KJ23, Vβ17^{a}. All the Abs were purchased from BD Pharmingen.

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 stained in vitro with the cytoplasmic 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^{high} 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^{high} T cells. To do so, we isolated

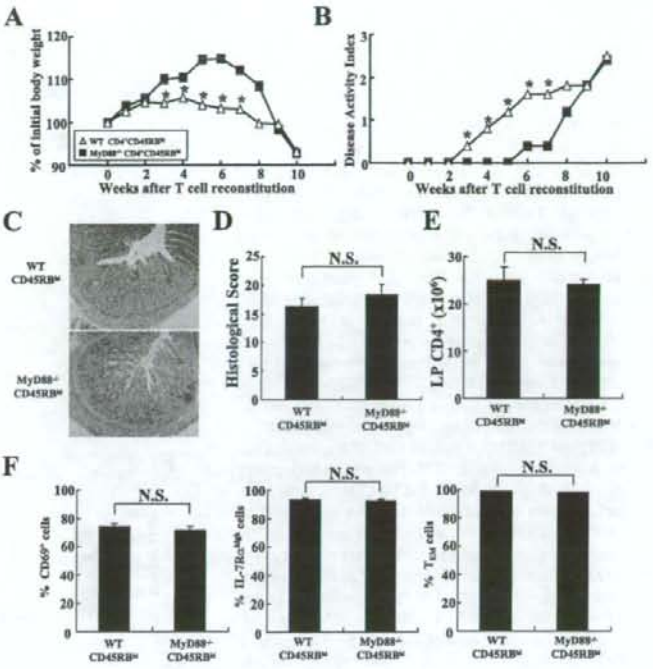

FIGURE 5. RAG-$2^{-/-}$ mice transferred with MyD88$^{-/-}$CD4$^+$CD45RB^{high} T cells develop colitis with the delayed kinetics, but reach to a similar level of mice transferred with MyD88$^{+/+}$CD4$^+$CD45RB^{high} T cells at 10 wk after transfer. *A*, Change in body weight is expressed as the percent of the original weight. Data are represented as mean ± SEM 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 ± SEM of five mice in each group. *, $p < 0.05$. *C*, Histological examination of the colon from WT (*upper*) or MyD88$^{-/-}$ (*lower*) CD4$^+$CD45RB^{high} T cells at 10 wk after transfer. Original magnification, ×100. *D*, Histological scoring of mice transferred with WT or MyD88$^{-/-}$CD4$^+$CD45RB^{high} 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$^+$CD45RB^{high} 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$^+$CD45RB^{high} T cells at 10 wk after transfer. The percentage of positive cells per total CD4$^+$ T cells (CD69$^+$/CD4$^+$, IL-7Rα^+/CD4$^+$, CD4$^+$CD44^{high}CD62L$^-$/CD4$^+$) was determined using flow cytometry.

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$^+$CD45RB^{high} 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$^+$ CD45RB^{high} 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$^+$CD45RB^{high} 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. 2*A*). When WT MyD88$^{+/+}$ CD4$^+$CD45RB^{high} 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. 2*B*), persistent diarrhea and also occasionally bloody stool or anal prolapse was observed by tracking the clinical score up to 6 wk after transfer (Fig. 2*C*). However, when MyD88$^{-/-}$CD4$^+$ CD45RB^{high} 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$^+$CD45RB^{high} T cells (Fig. 2*C*). Thus, the delayed onset and milder clinical score of mice transferred with MyD88$^{-/-}$CD4$^+$ CD45RB^{high} 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. 2*D*). 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. 2*E*). Importantly, flow cytometry analysis revealed that the LP CD4$^+$ T cells isolated from recipients transferred with either MyD88$^{-/-}$ or MyD88$^{+/+}$ CD4$^+$CD45RB^{high} T cells were CD44^{high}CD62L$^-$IL-7Rα^{high} (Fig. 2*F*), indicating that the transferred CD4$^+$CD45RB^{high} 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. 2*G*, significantly lower numbers of CD4$^+$ T cells were recovered from LP, MLN, and SP of mice transferred with

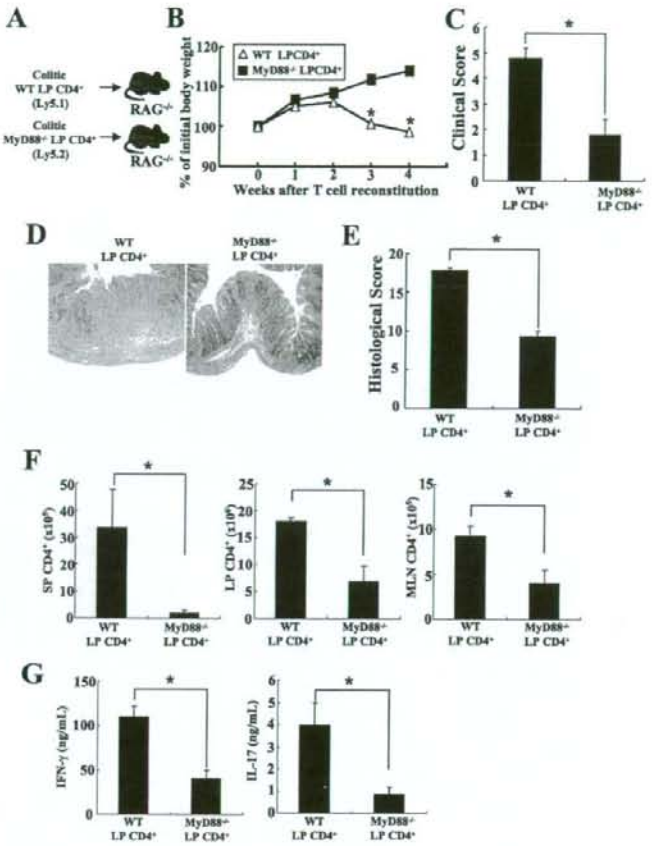

FIGURE 6. RAG-$2^{-/-}$ mice transferred with colitogenic MyD88$^{-/-}$ LP CD4$^+$ T cells develop milder colitis. *A*, RAG-$2^{-/-}$ mice were transferred with colitogenic MyD88$^{+/+}$ (WT) ($n = 6$) or MyD88$^{-/-}$ ($n = 6$) LP CD4$^+$ T cells (4×10^5 cells/mouse). *B*, Change in body weight is expressed as percent of the original weight. Data are represented as mean ± SEM of six mice in each group. *, $p < 0.05$. *C*, Clinical scores were determined at 4 wk after transfer as described in *Materials and Methods*. Data are indicated as mean ± SEM of six mice in each group. *, $p < 0.01$. *D*, Histological examination of the colon from mice transferred with colitic WT (*left*) or MyD88$^{-/-}$ (*right*) CD4$^+$ T cells at 4 wk after transfer. Original magnification, ×100. *E*, Histological scoring of mice transferred with colitic WT or MyD88$^{-/-}$CD4$^+$ T cells at 4 wk after transfer. Data are indicated as mean ± SEM of six mice in each group. *, $p < 0.05$. *F*, LP, MLN, and SP CD4$^+$ T cells were isolated from of mice transferred with colitic WT or MyD88$^{-/-}$CD4$^+$ T cells at 4 wk after transfer, and the number of CD4$^+$ cells was determined by flow cytometry. Data are indicated as mean ± SEM of six mice in each group. *, $p < 0.05$. *G*, Cytokine production by LP CD4$^+$ T cells. LP CD4$^+$ T cells were isolated at 4 wk after transfer and stimulated with anti-CD3 and -CD28 mAbs for 48 h. IFN-γ and IL-17 concentrations in culture supernatants were measured by ELISA. Data are indicated as mean ± SEM of six mice in each group. *, $p < 0.05$.

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-x_L 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-x_L compared with those from mice transferred with MyD88$^{+/+}$ donor cells (Fig. 2*H*). 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. 2*I*, 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. 2*I*).

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 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 (2.5×10^5 cells/mouse) of CD4$^+$CD45RB^{high} 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. 4*A*). 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. 4*B*). 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. 4*C*). 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. 4*D*).

RAG-2$^{-/-}$ mice transferred with MyD88$^{-/-}$ colitogenic LP CD4$^+$ donor cells develop milder colitis

To next assess the role of MyD88-dependent pathway in persistent colitis, we next examined colitogenic 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$^+$CD45RB^{high} T cell-transferred colitis model. We first confirmed that RAG-2$^{-/-}$ mice transferred with MyD88$^{-/-}$CD4$^+$CD45RB^{high} T cells do develop colitis to a similar extent to mice transferred with MyD88$^{+/+}$CD4$^+$ CD45RB^{high} T cells at the late stage of 10 wk after transfer as confirmed by the weight curve (Fig. 5*A*), albeit the ongoing disease activity index (Fig. 5*B*) 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$^+$CD45RB^{high} T cells (Fig. 5*E*). Furthermore, the expression of activation (CD69)/differentiation (IL-7Rα, CD44, and CD62L) on LP CD4$^+$ T cells showed no difference between two groups of mice (Fig. 5*F*), 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$^+$CD45RB^{high} 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. 6*A*). Similar with the results using CD4$^+$CD45RB^{high} T cell-mediated colitis model in Fig. 2, the recipient mice transferred with colitic MyD88$^{-/-}$ LP CD4$^+$ T cells showed milder wasting disease (Fig. 6*B*) with milder clinical signs of colitis at 4 wk after retransfer, as compared with mice transferred with colitic MyD88$^{+/+}$ LP CD4$^+$ T cells (Fig. 6*C*). 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. 6*D*). 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. 6*E*). 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. 6*F*). As shown in Fig. 6*G*, 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 (2.0×10^5 cells/mouse) of colitic Ly5.1$^+$MyD88$^{+/+}$ and Ly5.2$^+$MyD88$^{-/-}$ LP donor cells obtained from colitic mice transferred with Ly5.1$^+$MyD88$^{+/+}$ or

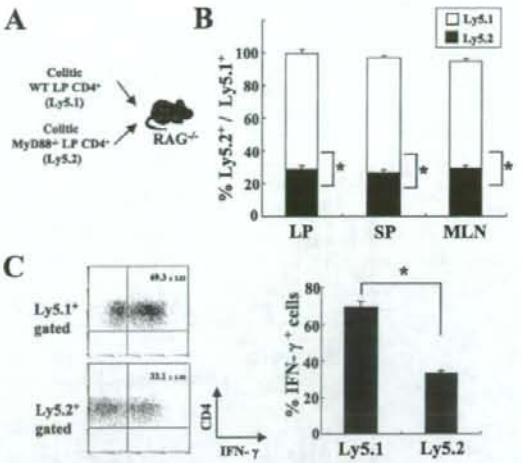

FIGURE 7. Expansion activity of colitic MyD88$^{+/+}$ LP donor cells predominates over that of MyD88$^{-/-}$ donor cells in an in vivo competition assay. *A*, The same number (2.0×10^5 cells/mouse) of colitic LP MyD88$^{+/+}$ (WT) (Ly5.1$^+$) and MyD88$^{-/-}$ (Ly5.2$^+$) CD4$^+$ T cells mice was injected i.p. into RAG-2$^{-/-}$ mice ($n = 6$). *B*, Six weeks after transfer, LP, SP, and MLN CD4$^+$ T cells were isolated from mice, and the ratio of Ly5.1$^+$ and Ly5.2$^+$ CD4$^+$ cells was determined by flow cytometry. *, $p < 0.01$. *C*, The frequencies of IFN-γ-producing cells per the total Ly5.1$^+$ or Ly5.2$^+$ cells were analyzed in the indicated subpopulations by flow cytometry. Data are represented as mean ± SEM of three independent experiments. *, $p < 0.01$.

Ly5.2$^+$MyD88$^{-/-}$ CD4$^+$CD45RB^{high} T cells at 10 wk after transfer was coinjected i.p. into identical RAG-2$^{-/-}$ mice (Fig. 7*A*). 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. 7*B*). 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. 7*C*).

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$^+$CD45RB^{high} 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. 8*A*). As depicted in Fig. 8*B*, 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. 8*C*), indicating that the MyD88-dependent signaling pathway in T cells promotes the rapid proliferation of colitogenic CD4$^+$ memory T cells in a lymphopenic condition.

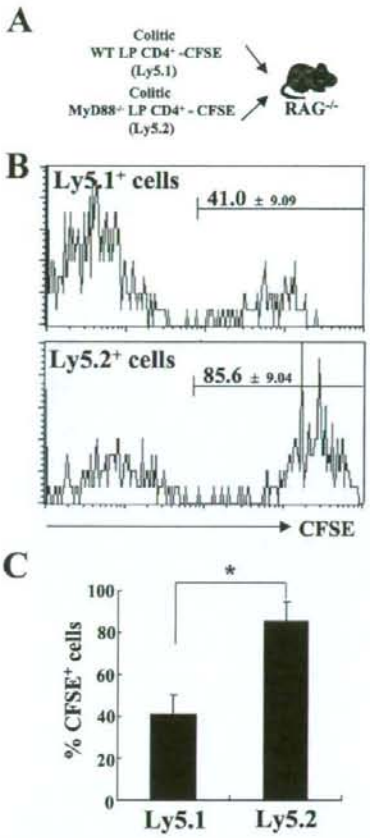

FIGURE 8. MyD88 pathway contributes to the lymphopenia-driven rapid proliferation of colitogenic $CD4^+$ T cells. *A*, The same number (2.0×10^6 cells/mouse) of CFSE-labeled colitic LP $MyD88^{+/+}$ (WT) ($Ly5.1^+$) and $MyD88^{-/-}$ ($Ly5.2^+$) $CD4^+$ T cells were coinjected i.p. into new RAG-$2^{-/-}$ mice ($n = 6$). *B*, The donor cells in the host spleen were analyzed 10 days after transfer by staining CD4, Ly5.1, and Ly5.2. Histograms show CFSE profiles of the two donor cell types in the host spleen. Data are representative of five independent experiments. *C*, Percentages of positive CFSE staining per total $Ly5.1^+$ or $Ly5.2^+$ cells were analyzed in the indicated subpopulations by flow cytometry. Data are represented as mean ± SEM of three independent experiments. *, $p < 0.05$.

Discussion

In the present study, we demonstrated that the MyD88-dependent signaling pathway in T cells directly modulates the proliferation and survival of TLRs/MyD88-expressing colitogenic $CD4^+$ T cells during the development and persistence of colitis. So far, it has been believed that T cell activation and expansion is induced and maintained by TCR signaling through the interaction with Ag-loading DCs that are primarily activated by PAMP/TLR-induced maturation (21). However, this study provides a new pathway by which the MyD88-dependent signaling pathway within $CD4^+$ T cells may directly play a pivotal role in the acquired immune components of chronic colitis by enhancing PAMP-specific immune responses collaborating with Ag-specific TCR signaling and homeostatic cytokines, such as IL-7 and IL-15 (18, 22–24).

How do commensal bacteria-derived PAMPs contribute to the maintenance of colitogenic $CD4^+$ T cells during the perpetuation 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 $MyD88^{-/-}$ $CD4^+CD45RB^{high}$ 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 $\alpha\beta^+CD4^+$ 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 $CD4^+CD45RB^{high}$ 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 $CD4^+CD44^{high}CD62L^-$IL-$7R\alpha^{high}$ 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 lymphocytic 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 rather 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 complication 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 TLR^{null} 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.

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Blockade of CXCL12/CXCR4 Axis Ameliorates Murine Experimental Colitis

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ABSTRACT

Recent studies indicate that the CXCL12/CXCR4 interaction is involved in several inflammatory conditions. However, it is unclear whether this interaction has a role in the pathophysiology of inflammatory bowel disease (IBD). We investigated the significance of this interaction in patients with IBD and in mice with dextran sulfate sodium (DSS)-induced colitis and the effect of a CXCR4 antagonist on experimental colitis. First, we measured CXCR4 expression on peripheral T cells in patients with IBD. Furthermore, we investigated CXCR4 expression on leukocytes and CXCL12 expression in the colonic tissue of mice with DSS-induced colitis, and we evaluated the effects of a CXCR4 antagonist on DSS-induced colitis and colonic inflammation of interleukin (IL)-10 knockout (KO) mice. Colonic inflammation was assessed both clinically and histologically. Cytokine production from mesenteric lymph node cells was also examined. CXCR4 expression on peripheral T cells was significantly higher in patients with active ulcerative colitis (UC) compared with normal controls, and CXCR4 expression levels of UC patients correlated with disease activity. Both CXCR4 expression on leukocytes and CXCL12 expression in colonic tissue were significantly increased in mice with DSS-induced colitis. Administration of a CXCR4 antagonist ameliorated colonic inflammation in DSS-induced colitis and IL-10 KO mice. CXCR4 antagonist reduced tumor necrosis factor-α and interferon-γ production from mesenteric lymph node cells, whereas it did not affect IL-10 production. The percentage of mesenteric $Foxp3^+CD25^+$ T cells in DSS-induced colitis was not affected by CXCR4 antagonist. These results suggest that blockade of this chemokine axis might have potential as a therapeutic target for the treatment of IBD.

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a chronic, relapsing, and remitting condition with unknown etiology that exhibits various features of immunologic abnormality (Fiocchi, 1998; Blumberg et al., 1999). The pathogenesis of IBD involves the interplay of environmental, genetic, microbial, and immune factors, which result in chronic intestinal inflammation. Among these factors, immune cells, including $CD4^+$ T cells, have crucial roles in the pathophysiology of IBD (Sartor, 1995). It is important to note that the expression of chemoattractive proteins (chemokines) and adhesion molecules expressed on various cells of the intestinal tissues regulate the recruitment of such inflammatory cells. Therefore, regulation of the migration of inflammatory leukocytes into the intestinal tissues is considered to be a therapeutic option for patients with IBD.

Chemokines are small cytokines exhibiting selective chemoattractive properties for targeting leukocytes. Based on

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ABBREVIATIONS: IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; DSS, dextran sulfate sodium; IL, interleukin; KO, knockout; Abs, antibodies; FACS, fluorescence-activated cell sorter; MTWSI, modified Truelove Witts severity index; GFP, green fluorescent protein; TF14016, 4-fluorobenzoyl*H*-Arg-Arg-Nal-Cys-Tyr-Cit-Lys-D-Lys-Pro-Tyr-Arg-Cit-Cys-Arg-NH_2, (S-S bridged, Nal = L-2-naphthylalanine; Cit = L-citrulline); PBS, phosphate-buffered saline; MLN, mesenteric lymph node; Gr-1, granulocyte-differentiation antigen-1; Mac-1, macrophage adhesion molecule-1; α-SMA, α-smooth muscle actin; PECAM-1, platelet endothelial cell adhesion molecule-1; rRNA, ribosomal RNA; TNF, tumor necrosis factor; IFN, interferon; Th, T helper.

the motif of the first two cysteines, chemokines are categorized into four major subfamilies: CC, CXC, C, and CX3C chemokines. The most important function of chemokines is their ability to regulate leukocyte recruitment, retention, and trafficking in inflamed tissues (Homey et al., 2002; Ogawa et al., 2004). The expression of several chemokines is increased in the colonic tissue of murine experimental colitis models (Andres et al., 2000; Ajuebor et al., 2004) as well as in colonic biopsy specimens from patients with IBD (Gijsbers et al., 2006). Therefore, much attention has been directed to such chemokines as one of the therapeutic targets for patients with IBD.

CXCL12 was first characterized as pre-B cell growth stimulating factor (Nagasawa et al., 1996a) and is constitutively expressed in stromal cells within the bone marrow (Tokoyoda et al., 2004). Its primary physiologic receptor is CXCR4, which also functions as an entry receptor for strains of human immunodeficiency virus (Bleul et al., 1996). The CXCL12/CXCR4 chemokine axis has an important role in the migration of progenitors during embryonic development of the cardiovascular, hematopoietic, and central nervous systems (Nagasawa et al., 1996b; Tachibana et al., 1998; Zou et al., 1998). Thus, this chemokine axis is considered to serve as a homing beacon during differentiation.

Recent studies showed that this chemokine axis is also involved in several inflammatory diseases, including rheumatoid arthritis (Nanki et al., 2000; Tamamura et al., 2004; Haas et al., 2005), inflammatory liver diseases (Terada et al., 2003; Wald et al., 2004), uveitis (Curnow et al., 2004), and pulmonary fibrosis (Phillips et al., 2004). Nanki et al. (2000) reported that memory T cells highly express CXCR4, and the CXCL12 concentration is extremely high in the synovial fluid of patients with rheumatoid arthritis. Furthermore, Wald et al. (2004) reported that CXCL12 is up-regulated in the endothelium of neoblood vessels of chronically inflamed liver tissues, and $CXCR4^+$ lymphocytes are increased in hepatitis C virus-infected liver tissues with chronic hepatitis. These findings suggest that the CXCL12/CXCR4 axis has an important role in cell trafficking not only in the homeostatic state but also under inflammatory conditions. However, it is not clear whether this chemokine axis is involved in the pathophysiology of IBD.

To elucidate the role of the CXCL12/CXCR4 interaction in colonic inflammation, we first investigated CXCR4 expression on peripheral T cells in patients with IBD. Next, we investigated the expression of both CXCR4 in peripheral T cells and its ligand CXCL12 in the colonic tissue in a dextran sulfate sodium (DSS)-induced colitis mouse model, and then we examined the effect of a CXCR4 antagonist on DSS-induced colitis and interleukin (IL)-10 knockout (KO) mice.

Materials and Methods

Human Samples of Peripheral Blood Cells. Peripheral blood cells were obtained from the following: 17 patients (7 men, 10 women) with active UC; 9 patients (2 men, 7 women) with inactive UC; 8 patients (6 men, 2 women) with active CD; 16 patients (13 men, 3 women) with inactive CD; 6 patients (4 men, 2 women) with infectious colitis; and 5 patients (5 men) as normal controls. Lymphocytes of blood samples were separated by Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). Cells were stained with appropriate fluorochrome-conjugated antibodies (Abs) and were incubated for 30 min at 4°C. Monoclonal antibody against human CD3 (UCHT1) was obtained from eBioscience (San Diego, CA), and anti-human CXCR4 (12G5) and IgG isotype control were obtained from Dako Denmark A/S (Glostrup, Denmark). Stained cells were analyzed with a fluorescence-activated cell sorter (FACS) (Beckman Coulter, Fullerton, CA). To determine disease activity, modified Truelove Witts severity index (MTWSI) was used for patients with UC and CD activity index was used for patients with CD. UC patients who scored >4 on the MTWSI and CD patients who scored >150 on CD activity index were classified to have active disease. Informed consent was obtained from each patient, and the experimental designs of these studies were approved by the Kyoto University Hospital Ethics Committee.

Animals. Female C57BL/6 mice (8–10 weeks of age, weighing 17–20 g) obtained from Japan SLC Inc. (Shizuoka, Japan) and CXCL12/green fluorescent protein (GFP) knockin mice (Tokoyoda et al., 2004) were used for the experiments. They were fed with standard laboratory chow and tap water ad libitum. All mice were housed in specific pathogen-free conditions in the animal facility of Kyoto University. The studies were approved by the animal protection committee of our institution.

Experimental Design of DSS-Induced Colitis. For the induction of colitis, C57BL/6 mice (wild-type and CXCL12/GFP knockin mice) were given 2.5% DSS (molecular mass, 36–50 kDa; MP Biomedicals, Irvine, CA) in their drinking water for 5 days (from day 0 to 4). On day 5, they were switched to regular drinking water. Normal control mice received regular drinking water throughout the experiment.

CXCR4 antagonist TF14016 was obtained from Prof. N. Fuji (Kyoto University, Kyoto, Japan) (Tamamura et al., 2003, 2004). One hundred micrograms of TF14016 dissolved with 200 μl of phosphate-buffered saline (PBS) or 200 μl of PBS alone was administered intraperitoneally once a day during the study period (from day 0 to day 10). Body weight was measured daily throughout the experiment, and mice were killed by cervical dislocation at 10 days after the start of DSS administration. The colonic tissues and mesenteric lymph nodes (MLNs) were removed from each mouse and examined as described below. At necropsy, the length from the ileocecal junction to the anal verge was measured as the colonic length.

Microscopic Assessment of Colonic Damage. The distal third of the colon was evaluated because this segment is most severely affected in DSS-induced colitis (Okayasu et al., 1990). The entire colon was removed, opened longitudinally, and washed with PBS. The distal third of the colon was dissected and then the longitudinal section (1.5 cm from the anal verge) was prepared. For section staining, samples were fixed in acetone and stained with hematoxylin and eosin, and histologically was analyzed in a blind manner. Histological damage was quantified by the histological scoring system described by Williams et al. (2001). In brief, the sections were graded to access inflammation severity, inflammation extent, and crypt damage. The grading index for inflammation severity was as follows: 0, none; 1, mild; 2, moderate; and 3, severe. The grading index for inflammation extent was as follows: 0, none; 1, mucosa; 2, mucosa and submucosa; and 3, transmural. The grading index for crypt damage was as follows: 0, none; 1, basal one-third damaged; 2, basal two-thirds damaged; 3, crypts lost but surface epithelium present; and 4, crypts and surface epithelium lost. Each of these grades was also scored according to the percentage of involvement (0, 0%; 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, 76–100%). Each subscore (inflammation severity score, inflammation extent score, and crypt damage score) was the product of the grade multiplied by the percentage of involvement. The total colitis score was the sum of the three subscores.

Flow Cytometry Analysis. For analyzing changes of CXCR4 expression on leukocytes, peripheral blood was taken by tail cutting at day 0, 3, 7, and 10 after start of DSS administration. Erythrocytes were removed using lysis buffer (0.16 M NH_4Cl and 0.017 M Tris, adjusted to pH 7.2), and leukocyte population was resuspended in Dulbecco's modified Eagle's medium containing 2% fetal calf serum. Cells (1×10^6) were stained with the appropriate fluorochrome-

conjugated Abs and were incubated for 30 min at 4°C. Monoclonal Abs against granulocyte-differentiation antigen-1 (Gr-1) (RB6-8C5), macrophage adhesion molecule-1 (Mac-1) (M1/70), CXCR4, CD4 (L3T4), CD8 (Ly-2), CD25 (7D4), and rat IgG isotype control were obtained from BD Pharmingen (San Diego, CA). Regulatory T cells ($Foxp3^+$ cells) were stained with the Mouse Regulatory T cell Staining Kit (eBioscience). Stained cells were analyzed with FACSCalibur (BD Biosciences, San Jose, CA). Dead cells were excluded by propidium iodide staining. The data are presented as relative fluorescence intensity or geometric mean fluorescence intensity depicting the degree of the expression of the surface molecule on the cell.

Immunohistochemical Staining and Confocal Microscopy. For section staining, samples were fixed in 4% paraformaldehyde and equilibrated in 30% sucrose/PBS or fixed with acetone for 2 min. Cryostat sections of colonic tissues were stained and mounted with Permafluor (Beckman Coulter). All confocal microscopy was carried out on a LSM 510 META (Carl Zeiss Inc., Thornwood, NY). Monoclonal Abs against α-smooth muscle actin (α-SMA), platelet endothelial cell adhesion molecule-1 (PECAM-1), CD4, CD8, Gr-1, Mac-1 (BD Pharmingen), and mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) were used. For secondary antibodies, Alexa Fluor 546 goat anti-rat or rabbit IgG and Cy5 donkey anti-rat IgG (Jackson ImmunoResearch Laboratories Inc.) were used. Biotinylated antibodies were visualized with streptavidin-Alexa Fluor 546 (Invitrogen, Carlsbad, CA) or streptavidin-Cy5 (Jackson ImmunoResearch Laboratories Inc.).

Isolation and Stimulation of Mesenteric Lymph Node Cells. Mesenteric lymph nodes were collected under sterile conditions in ice-cold medium, and lymph nodes were mechanically disrupted and filtered through a cell strainer (70 μm). Cells (2×10^6/well) were incubated with immobilized anti-CD3 (5 μg/ml, anti-mouse CD3e; BD Pharmingen) in 200 μl of culture medium for 72 h at 37°C in 5% CO_2 air. Cytokine levels in the supernatant of the culture medium were measured by enzyme-linked immunosorbent assay kit (eBioscience).

Quantitative Analysis of RNA Expressions. Samples of colonic tissue for mRNA isolation were removed from the distal third of the colon at 10 days after the start of DSS administration. Total RNA was extracted using the guanidium isothiocyanate-phenol-chloroform method. RNA (1 μg) was reverse transcribed with MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA), and the resulting complementary DNAs (50 ng/reaction mixture) were analyzed for CXCL12 mRNA expression by real-time polymerase chain reaction using an ABI Prism 7700 sequence detection system (Applied Biosystems). The reaction mixtures were incubated for 2 min at 50°C, denatured for 10 min at 95°C, and subjected to 45 amplification cycles consisting of annealing and extension at 60°C for 1 min followed by denaturation at 95°C for 15 s. The primers and probes used for this experiment were obtained from Applied Biosystems. To quantify isolated RNA and to measure cDNA synthesis efficiency, target cDNAs were normalized to the expression levels of the endogenous reference housekeeping gene, 18S ribosomal RNA (rRNA). The oligonucleotide primers used for CXCL12 rRNA amplification and detection were 5′-CCA GAG CCA ACG TCA AGC AT-3′ (forward) and 5′-CAG CCG TGC AAC AAT CTG AA-3′ (reverse). The oligonucleotide primers used for 18S rRNA amplification and detection were 5′-TAGAGTGTTCAAAGCAGGCCC-3′ (forward) and 5′-CCAACAAATAGAACCGCGGT-3′ (reverse). For simplicity, the expression level of the target gene was expressed as values relative to the control in the experiment.

Migration Assay. Fresh bone marrow cells and MLN cells from C57BL/6 mice were preincubated with or without 1 μM of TF14016 for 30 min at 37°C. Then they were transferred to the upper layer of 3- or 5-μm pore polycarbonate membrane (Transwell; Corning Inc., Corning, NY), which overlaid the lower chamber containing 100 ng/ml CXCL12. After 2 h, a fraction of the cells that migrated to the lower chamber was stained and analyzed by flow cytometry.

Experimental Design of IL-10 KO Mice. Fifty milligrams/mice per day of CXCR4 antagonist, TF14016, or PBS alone was administered intraperitoneally to IL-10 KO mice at 6 weeks of age. Twenty-eight days (4 weeks) after the start of treatment, TF14016 or PBS-treated mice were killed for histological analysis of colonic tissue.

Microscopic Assessment of Chronic Damage in IL-10 KO Mice. Mice were monitored for clinical signs of colitis, including diarrhea and weight loss. At necropsy, samples of the colon (transverse, distal, and proximal) and the rectum were collected and histopathologically examined as described previously. For each section, inflammation (macrophage, lymphocyte, and neutrophil infiltration in the lamina propria or submucosa) was scored for severity according to the following criteria: normal, 0; minimal, 1; mild, 2; moderate, 3; marked, 4; and severe, 5. Gland loss and epithelial hyperplasia were scored by percentage of area involved: none, 0; 1, 1–10% of the mucosa affected; 2, 11–25% affected; 3, 26–50% affected; 4, 51–75% affected; and 5, 76–100% affected. The summed scores for inflammation (lamina propria or submucosa), gland loss, and gland hyperplasia were then determined for each animal. One pathologist without knowledge of this study scored the sections according to standard criteria.

Statistical Analysis. All normalized data were represented as mean ± S.D. In human experiments assessing CXCR4 expression on T cells, the Kruskal-Wallis test with Bonferroni/Dunn analysis was used. A linear regression analysis was used to access the quantitative relation between the intensity of CXCR4 expression on T cells and disease activity. In animal experiments, for two-group comparisons, the Student's unpaired t and Mann-Whitney U tests were used. For multiple comparisons, the Kruskal-Wallis test with Bonferroni/Dunn analysis for nonparametric analysis or two-way analysis of variance with Bonferroni/Dunn ad hoc analyses for parametric analysis was performed. A repeated analysis of variance was performed assess the effect of TF14016 treatment on changes in body weight after an induction of DSS-induced colitis. In experiments using TF14016 treatments, because control mice showed a negligible level of inflammatory cytokine in tissue, one comparison using the unpaired t test was performed. Any significant interaction was detected in multiple comparisons (data not shown). A two-tailed p value of <0.05 was considered statistically significant. The SPSS software package for Windows (version 10; SPSS, Tokyo, Japan) was used.

Results

CXCR4 Expression on Peripheral T Cells Was Increased in Patients with Active UC. First, we examined whether this chemokine axis is involved in human IBD. We investigated CXCR4 expression on peripheral T cells from patients with IBD and with acute infectious colitis compared with those from healthy controls. CXCR4 expression on peripheral T cells in patients with active UC was significantly higher than that in inactive UC and controls, whereas this expression in patients with active CD, inactive CD, inactive UC, and infectious colitis was not different from that in controls (Fig. 1a). Furthermore, there was a significant correlation between CXCR4 expression on peripheral T cells and disease activity (MTWSI) in patients with active UC (Fig. 1b).

Cxcl12 Expression Was Increased in the Colonic Tissue of DSS-Induced Colitis Mice. Next, we investigated the changes of CXCL12 expression in colonic tissue before and after DSS administration using CXCL12/GFP knockin mice. Confocal microscopic analysis was performed before and at 10 days after the start of DSS administration. CXCL12-expressing cells were mainly observed in the perivascular sites of the normal colonic mucosa (Fig. 2, a–d).

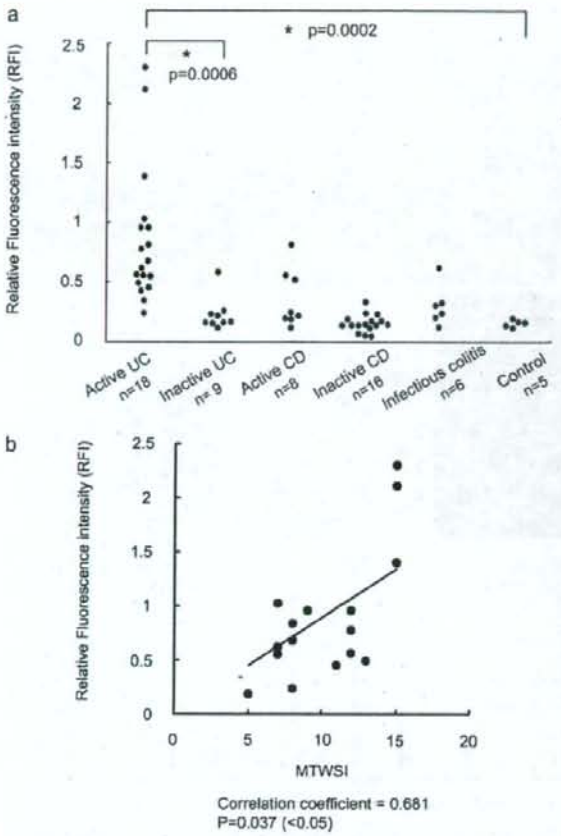

Fig. 1. CXCR4 expressions on peripheral T cells in patients with IBD. a, CXCR4 expression on peripheral T cells from patients with IBD and acute infectious colitis and healthy controls. Eighteen patients with active UC, 9 patients with inactive UC, 8 patients with active CD, 16 patients with inactive CD, 6 patients with infectious colitis, and 5 patients as normal controls. *, $p < 0.05$ compared with inactive UC and normal controls. b, correlation between CXCR4 expression of peripheral T cells and disease activity (MTWSI) in patients with UC.

The CXCL12-expressing cells were morphologically considered to be reticular cells adjacent to the endothelial (PECAM-1$^+$ and/or α-SMA$^+$) cells because they were negative for PECAM-1, α-SMA, and other blood cell marker's staining.

At 10 days after the start of DSS administration, the number of CXCL12-expressing cells was increased in the inflamed colonic mucosa compared with normal colonic tissues (Fig. 2, e and f). Expression of CXCL12 mRNA was also significantly higher in the colonic tissue of mice with DSS-induced colitis (at 10 days after DSS administration) than that of normal mice (Fig. 2g). These results suggest that enhanced CXCL12 expression in the colonic mucosa might induce the migration of inflammatory cells into the inflamed colonic tissues of mice with DSS-induced colitis.

CXCR4 Expression on Peripheral T Cells Is Increased in Mice with DSS-Induced Colitis. To investigate whether the CXCL12/CXCR4 chemotactic axis is involved in DSS-induced colonic inflammation, we analyzed CXCR4 expression on peripheral blood cells from mice with DSS-induced colitis. Serial changes in CXCR4 expression in the peripheral blood cells of these mice were evaluated by flow cytometry. FACS analysis revealed that CXCR4 expression on peripheral granulocytes (Gr-1$^+$ cells) was significantly increased at 7 days and normalized at 10 days after the start of DSS administration. CXCR4 expression on both peripheral CD4$^+$ and CD8$^+$ cells from mice with DSS-induced colitis was also significantly increased at 7 and 10 days (CD4$^+$), and at 3, 7, and 10 days (CD8$^+$) after the start of DSS administration, compared with the levels before DSS administration (day 0) (Fig. 2h).

A CXCR4 Antagonist Efficiently Inhibits Leukocyte Migration to CXCL12 in Vitro. To evaluate whether a CXCR4 antagonist, TF14016, efficiently blocks leukocyte migration toward CXCL12, we performed an in vitro leukocyte chemotaxis assay. Migration analysis showed that the CXCL12-induced chemotactic responses of bone marrow granulocytes and mesenteric CD4$^+$ as well as CD8$^+$ T cells were significantly inhibited by TF14016 (Fig. 3).

Effect of a CXCR4 Antagonist on DSS-Induced Colitis. To investigate whether the blockade of the CXCL12/CXCR4 chemotactic axis attenuates inflammation in DSS-induced colitis, we intraperitoneally administered a CXCR4 antagonist, TF14016, to mice with DSS-induced colitis. During DSS administration, the percentage of body weight in control mice (DSS alone) decreased. The body weight loss of mice with DSS-induced colitis treated with TF14016, however, was significantly lower than that of nontreated mice from 7 to 10 days after the start of DSS administration (Fig. 4a). There was also a significant difference in colonic length between nontreated and TF14016-treated mice with DSS-induced colitis (Fig. 4b).

In mice with DSS-induced colitis, the histologic findings demonstrated epithelial destruction, remarkable inflammatory cell infiltration with lymphoid aggregation, and submucosal edema (Fig. 5a). In contrast, in TF14016-treated mice with DSS-induced colitis, epithelial destruction and inflammatory cell infiltration, including lymphoid aggregation, were obviously reduced compared with nontreated mice (Fig. 5b). As a result, the total colitis score in TF14016-treated mice with DSS-induced colitis was significantly lower than that in the nontreated mice with DSS-induced colitis (Fig. 5c). Immunohistochemical analysis revealed that the number of CD4$^+$ and CD8$^+$ T cells and lymphocyte aggregation in the lamina propria were remarkably decreased in TF14016-treated mice with DSS-induced colitis compared with the nontreated mice with DSS-induced colitis (Fig. 6, a and b).

Blockade of the CXCL12/CXCR4 Axis Reduces the Production of Proinflammatory Cytokines, but Not IL-10 Production, from MLN Cells in Mice with DSS-Induced Colitis. We also measured the cytokine production from unseparated MLN cells from mice with DSS-induced colitis treated with TF14016. The production of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and IL-10 from unseparated MLN cells was significantly increased in mice with DSS-induced colitis. However, TF14016 treatment significantly reduced the increased production of TNF-α and IFN-γ. In contrast, TF14016 treatment had no effect on the production of IL-10 in mice with DSS-induced colitis (Fig. 7).

Blockade of the CXCL12/CXCR4 Axis Did Not Block the Migration of Foxp3$^+$ Regulatory T Cells to MLN. To elucidate why IL-10 production in MLN cells was not

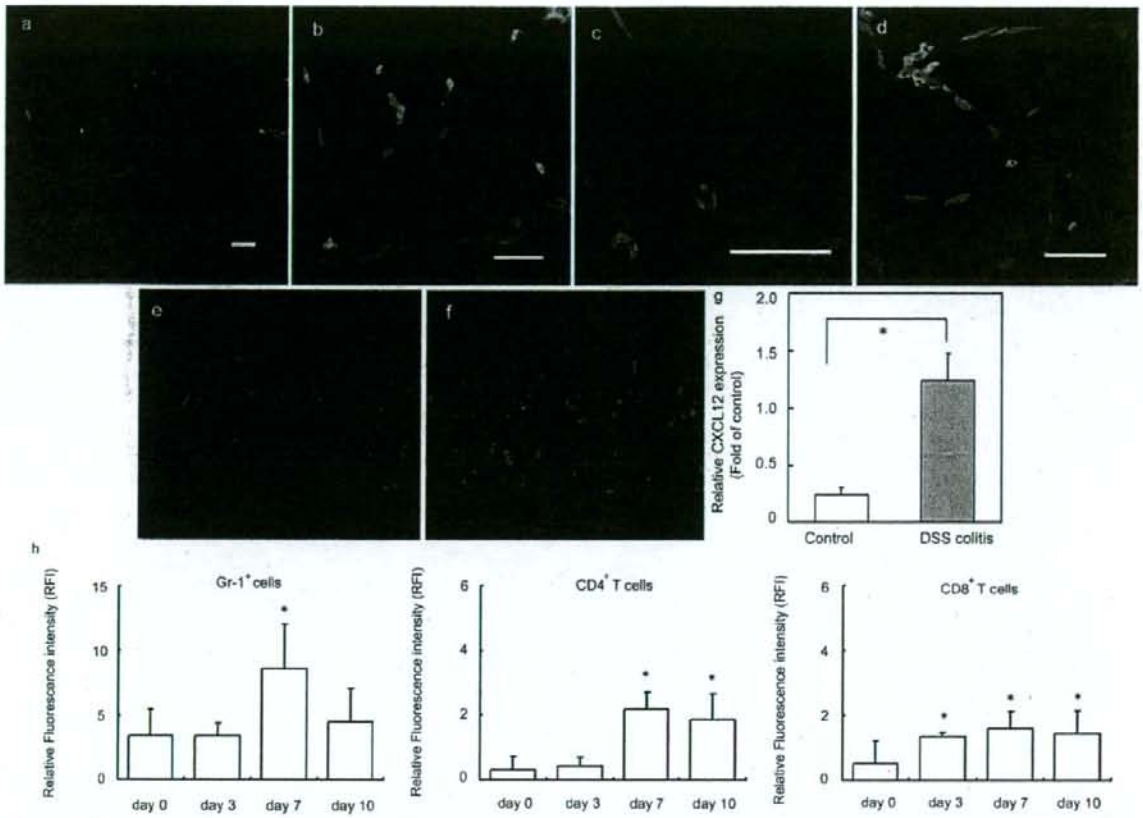

Fig. 2. CXCL12 expression in the colonic tissue and serial changes in CXCR4 expression on peripheral blood cells of mice with DSS-induced colitis. a to d, colonic tissue sections from CXCL12/GFP knockin mice were stained with antibodies against PECAM-1 (blue) and α-SMA (red) (b–d, magnified; b and c, vertical section; d, horizontal section). White bars indicate 20 μm (a) and 10 μm (b–d). e to g, the change of CXCL12 expression was analyzed in CXCL12/GFP knockin mice before (e) and 10 days after the start of DSS administration (f). g, CXCL12mRNA expression in colonic tissues was quantified by quantitative real-time polymerase chain reaction. Results are presented as means ± S.D. (n = 7 in each group). *, $p < 0.05$ compared with control mice without DSS. h, Peripheral blood cells were obtained from mice with DSS-induced colitis before and 3, 7, and 10 days after the start of DSS administration. Serial changes of CXCR4 expression on peripheral granulocytes (Gr-1$^+$ cells) and T cells (CD4 and CD8 cells) were evaluated by FACS. Results are presented as means ± S.D. (n = 5 in each group). *, $p < 0.05$ compared with normal mice (day 0).

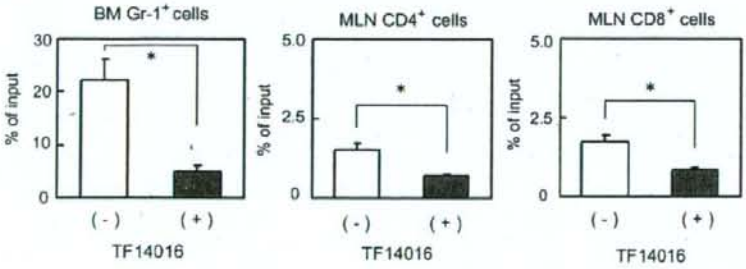

Fig. 3. TF14016 inhibits chemotactic responses to CXCL12 in vitro. CXCL12-induced chemotactic responses of bone marrow granulocytes (Gr-1$^+$ cells) and mesenteric T cells (CD4$^+$ and CD8$^+$ cells) were assessed in the presence (□) or absence (■) of TF14016. Data are expressed as percentage (%) of input of the loaded cells to the lower chamber. Results are presented as means ± S.D. (n = 4 in each group). *, $p < 0.05$ compared with leukocytes in the absence of TF14016.

changed by TF14016 treatment despite the decrease in the severity of colitis, we focused on the effect of TF14016 on regulatory T cells, one of MLN's IL-10-producing cells. First, we analyzed CXCR4 expression on regulatory T cells in MLN. In mice with DSS-induced colitis, CXCR4 expression on mesenteric CD4$^+$CD25$^-$ T cells (nonregulatory T cells) was significantly elevated compared with that of normal mice. In contrast, there was no significant difference in CXCR4 expression on mesenteric CD4$^+$CD25$^+$ regulatory T cells between DSS-induced colitis mice and normal mice (Fig. 8a).

We then analyzed the population of Foxp3$^+$ regulatory T cells in MLN. FACS analysis revealed that although the percentage of Foxp3$^+$CD25$^+$ regulatory T cells in MLN was significantly increased in mice with DSS-induced colitis com-

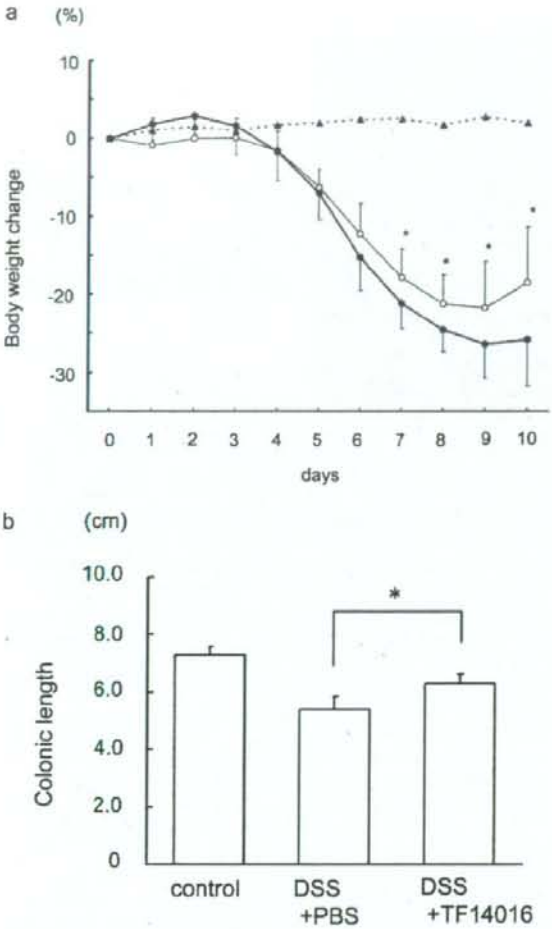

Fig. 4. Therapeutic effect of a CXCR4 antagonist, TF14016, on body weight changes and colonic length in mice with DSS-induced colitis. a, serial changes in body weight were measured daily throughout the experiment. Data are expressed as the mean percentage change from starting body weight. b, colonic length was measured from the ileocecal junction to the anal verge at 10 days after the start of DSS administration. ●, nontreated mice with DSS-induced colitis; ○, mice with DSS-induced colitis treated with TF14016; and ▲, normal mice without DSS. Results are presented as means ± S.D. (n = 10–13 in each group). *, $p < 0.05$ compared with the nontreated mice with DSS-induced colitis.

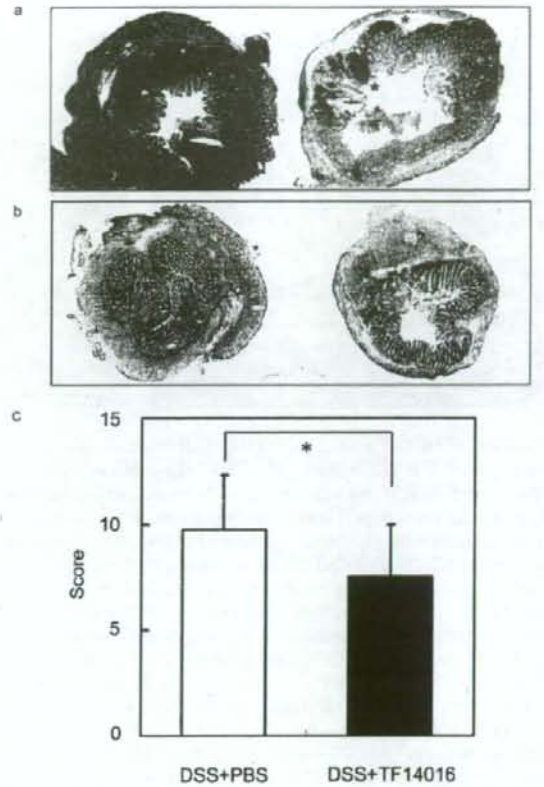

Fig. 5. Histologic changes in colonic tissues of mice with DSS-induced colitis treated with TF14016. a and b, the representative histologic findings of colonic tissues of DSS-induced colitis mice treated with PBS (a) and TF14016 (b) are shown. c, histologic scores of mice with DSS-induced colitis treated with and without TF14016. Results are presented as means ± S.D. (n = 8 in each group). ★, lymphoid aggregation. *, $p < 0.05$ compared with the nontreated mice with DSS-induced colitis.

pared with normal mice, TF14016 treatment did not affect the percentage of $Foxp3^+CD25^+$ regulatory T cells in mice with DSS-induced colitis (Fig. 8b).

Effect of a CXCR4 Antagonist on IL-10 KO Mice. All IL-10 KO mice treated with TF14016 or PBS survived throughout the study period. Histologic examination of colonic tissue from PBS-treated IL-10 KO mice demonstrated epithelial hyperplasia, crypt abscess, and severe acute and chronic cellular infiltration and lymphoid aggregation in lamina propria (Fig. 9a). In contrast, colonic inflammation and amount of lymphocyte aggregation were significantly decreased in IL-10 KO mice treated with a CXCR4 antagonist (Fig. 9b). As shown in Fig. 9c, colonic histological scores in IL-10 KO mice treated with TF14016 were significantly lower than in those treated with PBS alone.

Discussion

The present study demonstrated that CXCR4 expressions on peripheral T cells of active UC patients were significantly higher than those of normal controls, and they were significantly correlated with disease activity. Furthermore, the expression of both CXCR4 on peripheral T cells and CXCL12 in the colonic tissue of mice with DSS-induced colitis were significantly increased compared with normal mice. More importantly, the administration of a CXCR4 antagonist decreased the severity of DSS-induced colitis and colonic inflammation of IL-10 KO mice, as assessed by clinical, histologic, and immunologic parameters. These results strongly suggest that the CXCL12/CXCR4 chemokine axis has an important role in the pathophysiology of IBD, particularly in UC, and that antagonist has a therapeutic effect on experimental colitis.

Previous reports demonstrated that CXCL12 mRNA expression ratio in biopsy specimens from the colonic mucosa of

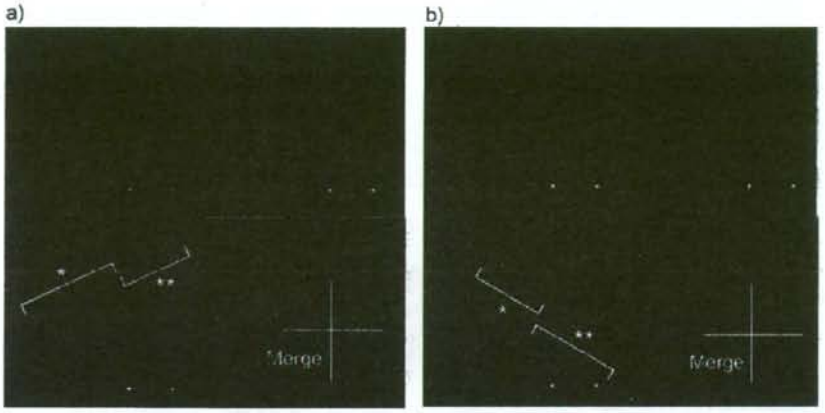

Fig. 6. Confocal microscopic findings of colonic tissues in DSS-induced colitis mice treated with TF14016. The colon sections from mice with DSS-induced colitis treated with PBS (a) or TF14016 (b) were stained with antibody against CD4 (green) and CD8 (red). White bars indicate 100 μm. A representative figure of five mice is shown. *, mucosa-submucosa; **, muscularis propria-serosa.

patients with UC was significantly higher than that in patients with CD (Katsuta et al., 2000). In addition, IL-4 induces surface CXCR4 expression on human T cells, suggesting that this receptor might be associated with T helper (Th)2 cells (Jourdan et al., 1998; Annunziato et al., 1999). On the contrary, CXCR4/CXCL12 axis is associated with several inflammatory diseases such as rheumatoid arthritis, in which IL-6 and TNF-α are mainly involved. Our data also showed that CXCR4 expression in peripheral T cells is likely to be increased in patients with active CD compared with inactive CD, although there was no significant difference. These data may suggest that Th1 immune response is involved in CXCR4 expression. Fuss et al. (1996) reported that lamina propria $CD4^+$ T lymphocytes from UC patients produce both Th1 cytokine IFN-γ and Th2 cytokine IL-5. Several reports showed that anti-TNF-α antibody administration reduced intestinal inflammation in patients with UC (Rutgeerts et al., 2005). Taken together, the immune response in acute flare of UC is very complicated because both Th1 and Th2 immune response and several proinflammatory cytokines are involved. Considering these data, Th2 immune response (IL-4 and IL-5) might augment CXCR4 expression on peripheral T cells under the condition of enhanced Th1 immune response. Accordingly, CXCR4 expression on peripheral T cells was strongly increased in active UC patients and that its expression level was proportional to the disease activity. In this regard, CXCR4 expression on peripheral T cells of patients with UC could be a good marker of their disease activity. However, further investigation will be needed to elucidate the exact mechanism of significant up-regulation of CXCR4 in UC patients compared with CD patients.

In animal models, we investigated CXCL12 expression in colonic tissues using CXCL12/GFP knockin mice because its expression was hardly observed by immunohistochemistry. In the steady state, CXCL12 expression was observed in submucosal lesions, mainly adjacent to the vascular endothelial cells. Based on their location, the majority of CXCL12-expressing cells are considered to be pericytes, mesenchymal-like cells located close to small blood vessel walls. Previous studies (Imai et al., 1999a,b; Peled et al., 1999a; Ponomaryov et al., 2000) reported that human and murine endothelial cell lines express CXCL12 mRNA and produce CXCL12 protein. CXCL12 is expressed on neoblood vessel endothelial cells in the portal tracts and on active lymphoid follicles, suggesting the involvement of CXCL12 in the initial entry of cells into the liver during chronic hepatitis B and hepatitis C virus infection (Wald et al., 2004). In contrast to those previous reports, we found that endothelial cells did not express CXCL12 in colonic tissues, although the reason for the discrepant results is not known. We also revealed that CXCL12 expression in the inflamed colonic tissue of mice with DSS-induced colitis was significantly increased compared with normal mice. These data might suggest that circulating $CXCR4^+$ leukocytes are attracted to inflamed colonic tissues by the increased number of CXCL12-expressing cells at the perivascular sites.

To further clarify the role of the CXCL12/CXCR4 chemokine axis, we next observed CXCR4 expression on peripheral leukocytes in mice with DSS-induced colitis and found that CXCR4 expression on granulocytes and $CD4^+$ and $CD8^+$ lymphocytes is increased in mice with DSS-induced colitis compared with wild-type mice. Thus, it may be reasonable to speculate that those CXCR4-expressing T cells are recruited to the inflamed mucosa of DSS-induced colitis by enhanced expression of CXCL12. The fact that the CXCR4 antagonist ameliorates DSS-induced colitis may further support such possibility. Recent studies have indicated that various cytokines and growth factors, including IL-2, IL-4 (Jourdan et al., 1998), IL-6, stem cell factor (Peled et al., 1999b), vascular endothelial growth factor, basic fibroblastic growth factor, and transforming growth factor-β (Buckley et al., 2000), can enhance CXCR4 expression on a number of cell types. In DSS-induced colitis, the production of various cytokines and growth factors are increased (Dieleman et al., 1994; Matsuura et al., 2005). Accordingly, these cytokines or growth factors might be involved in the induction of CXCR4 expression on granulocytes and T cells in DSS-induced colitis.

CXCR4 expression on T cells was significantly higher at 7 and 10 days after DSS administration than before DSS administration, whereas CXCR4 expression on granulocytes was increased only at day 7. In general, colonic inflammation induced by DSS administration is considered to be mainly caused by direct chemical injury to colonic epithelial cells and activation of resident macrophages and neutrophils (Okayasu et al., 1990; Cooper et al., 1993; Dieleman et al., 1994). However, we found little difference in the infiltration of mononuclear cells or neutrophils in colonic mucosa between mice with DSS-induced colitis and normal mice at 10 days

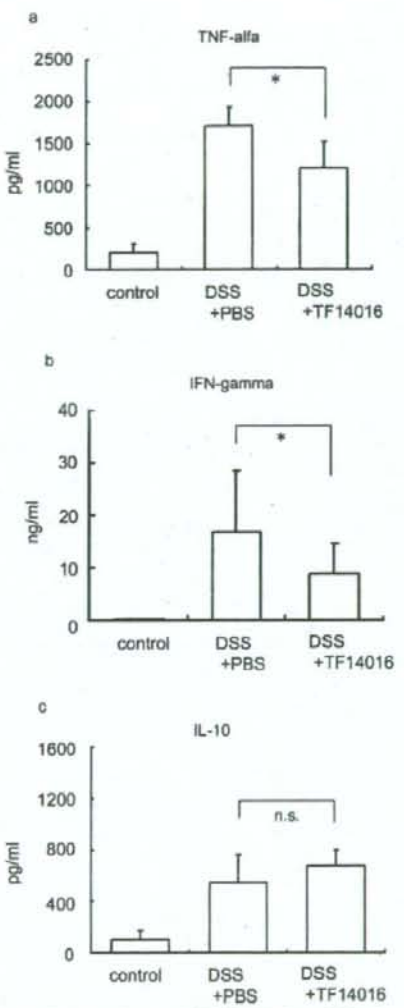

Fig. 7. The cytokine production of MLN cells from DSS-induced colitis mice treated with or without TF14016. MLN cells from mice 10 days after the start of DSS administration were cultured with immobilized anti-CD3. Supernatants were collected after 72 h, and TNF-α (a), IFN-γ (b), and IL-10 (c) were tested by enzyme-linked immunosorbent assay. Results are presented as means ± S.D. ($n = 8$ in each group). *, $p < 0.05$ compared with DSS-induced colitis mice without TF14016 treatment. n.s., not significant.

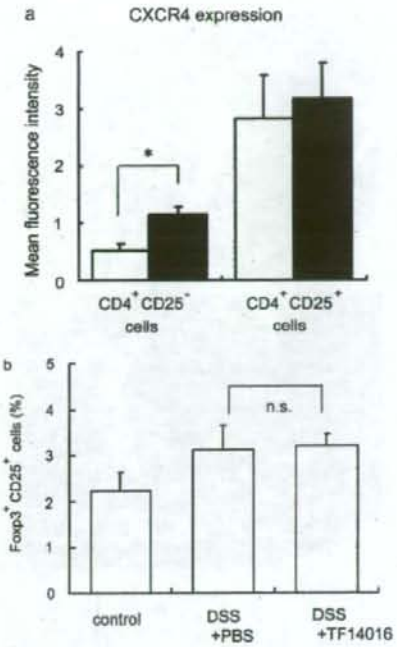

Fig. 8. CXCR4 expression on mesenteric regulatory T cells and the population of mesenteric Foxp3+ regulatory T cells in mice with DSS-induced colitis. a, CXCR4 expression on mesenteric $CD4^+CD25^-$ and $CD4^+CD25^+$ cells was analyzed before (white bar) and 10 days after starting DSS administration (black bar). b, the population of mesenteric Foxp3+ regulatory T cells from TF14016-treated and the nontreated mice with DSS-induced colitis were analyzed by FACS. Results are presented as means ± S.D. ($n = 5$ in each group). *, $p < 0.05$ compared with normal mice. n.s., not significant.

after starting DSS administration (data not shown). These data suggest that granulocytes are attracted to the inflamed colonic tissue soon after DSS administration by other chemokines like CXCL8/IL-8 and CXCL10/inducible protein-10, the expressions of which are increased during the early phase of DSS-induced colitis (Murano et al., 2000; Melgar et al., 2006). On the other hand, the sustained increase of CXCR4 expression on peripheral T cells at the late phase of DSS-induced colitis, as observed in the present study, suggests that T cells are involved in sustained colonic inflammation after DSS administration in C57BL/6 mice. Melgar et al. (2006) reported that only 5-day administration of DSS induces chronic inflammation of the colon in C57BL/6 mice. Taken together, the increased expression of CXCR4 on T cells might mainly contribute to the development of chronic colitis induced by DSS administration.

An important finding of our study is that blocking the CXCL12/CXCR4 chemotaxis axis significantly ameliorated DSS-induced colitis and colonic inflammation of IL-10 KO mice. Indeed, administration of a CXCR4 antagonist, TF14016, significantly reduced body weight loss of mice with DSS-induced colitis. Moreover, histologic findings revealed that the number of infiltrated lymphocytes and amount of lymphocyte aggregation in both DSS-induced colitis mice and IL-10 KO mice were significantly decreased when treated by the administration of a CXCR4 antagonist. These data strongly support an idea that the CXCL12/CXCR4 chemokine interaction has an important role in the development of experimental colitis, probably through the recruitment of CXCR4-positive lymphocytes to the inflamed colonic mucosa.

It should be noted that although the CXCR4 antagonist significantly reduced the expressions of TNF-α and IFN-γ, it did not alter IL-10 production from MLN cells in mice with DSS-induced colitis, despite the amelioration of colonic inflammation. To clarify the reason for the lack of the effect of the CXCR4 antagonist on IL-10 production, we observed the effect on regulatory T cells in MLN. We first found that although CXCR4 expression on mesenteric $CD4^+CD25^-$ cells

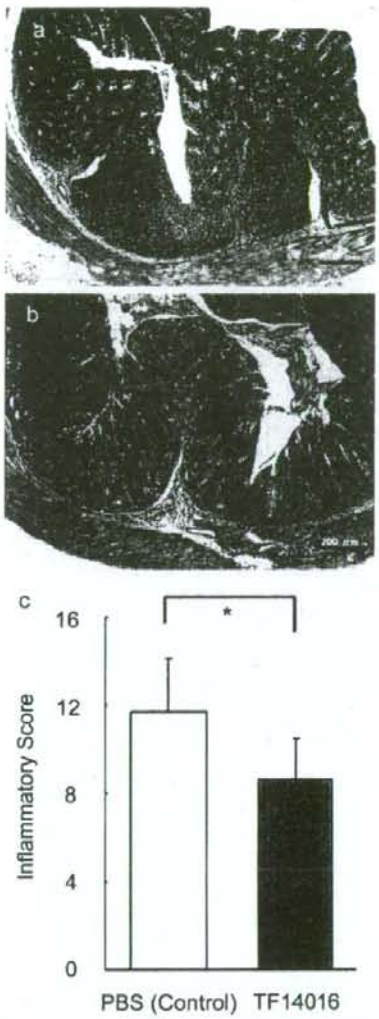

Fig. 9. Administration of a CXCR4 antagonist, TF14016, ameliorated histopathologic features in IL-10 KO mice. a and b, the representative histologic findings of proximal colon sections of IL-10 KO mice treated with PBS (a) and TN14016 (b) are shown. c, histologic scores of IL-10 KO mice treated with and without TF14016. Results are presented as means ± S.D. (n = 11 in each group). Black bars indicate 200 μm. *, $p <$ 0.05 compared with IL-10 KO mice treated with PBS.

was increased in DSS-induced colitis, there was no significant change in CXCR4 expression on mesenteric $CD4^+CD25^+$ cells. Furthermore, although the percentage of $Foxp3^+CD25^+$ cells in mice with DSS-colitis was significantly higher than normal mice, administration of the CXCR4 antagonist did not affect the percentage of $Foxp3^+CD25^+$ cells. These data indicated that the lack of the effect of CXCR4 antagonist on IL-10 production seems to result from both no increase of CXCR4 expression on regulatory T cells in mice with DSS-induced colitis and no change of the percentage of regulatory T cells by the CXCR4 antagonist administration. Moreover, our data showed that the increased CXCR4 expression on $CD4^+$ T cells in mice with colonic inflammation is mainly attributed to its increased expression on $CD4^+CD25^-$ T cells. Taken together, the present data suggested that the ameliorating action of the CXCR4 antagonist on DSS-induced colitis is mainly due to its inhibitory effect on migration of $CD4^+CD25^-$ T cells with increased CXCR4 expression that seem to be involved in exacerbation of intestinal inflammation in IL-10 KO mice that lack the function of regulatory T cells.

In conclusion, taken together with human and mouse studies, CXCL12/CXCR4 chemokine axis seems to be involved in the pathophysiology of IBD, especially ulcerative colitis. Considering the potent anti-inflammatory effect of the CXCR4 antagonist on experimental colitis, the CXCR4 antagonist might be one of the therapeutic options for patients with IBD. However, further clinical trials will be needed to assess this possibility.

Acknowledgments

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