It is well established that several aspects of manifestation, including clinical features and genetic alteration characteristics, differ markedly between sporadic- and colitis-associated colorectal cancers.[5,6,41] Adenomatous polyps are considered to be the major precursor of sporadic colorectal cancers,[42] and inactivation of the *APC* gene is believed to be the initial event in sporadic colorectal cancer development, followed by changes in the *K-ras, DCC,* and *TP53* genes.[43] In contrast to sporadic cancer, an alteration in the *TP53* gene is usually an early event in the molecular pathogenesis of IBD-related colorectal cancer, whereas the mutation frequency of *APC* and *K-ras* is substantially lower than that in sporadic cancers. It is noteworthy that genetic alterations in the *TP53* gene likely precede dysplasia, the precancerous lesions in the inflammatory colon.[44] Moreover, there is an increased frequency of *TP53* mutations in noncancerous UC colon tissues.[45] The molecular mechanisms underlying the contribution of inflammatory conditions to the accumulation of *TP53* mutations are not well-defined. Here, we show that AID activation induced significant levels of *TP53* mutations in colonic mucosal cells. Thus, aberrant expression of AID might be involved in the generation of genetic alterations in the *TP53* gene in inflamed colonic epithelial cells.

An interesting point in our study is that the *APC, K-ras,* and *c-myc* genes were less frequently mutated by AID activation than the *TP53* gene in human colonic cells. These findings are consistent with previous observations that target gene selection of AID for somatic hypermutation varies among target cells.[8,9] Similar to its effect in colonic cells, aberrant AID expression in gastric epithelial cells induces nucleotide alterations in the *TP53* gene, whereas no mutations are induced in *c-myc*.[9] In contrast, AID expression in cultured hepatoma-derived cells results in the appearance of nucleotide alterations in the *c-myc* gene.[8] It is not clear why the *TP53* gene is more sensitive to AID activation in colonic epithelial cells. One possibility is that transcription levels of the genes targeted by AID are higher than other genes because AID-induced hypermutation depends on target gene transcription levels.[46,47] Consistent with this hypothesis, semiquantitative RT-PCR analysis revealed that *TP53* mRNA expression was higher than the *APC, K-ras,* and *c-myc* genes in LoVo cells. Further analysis is required to identify the specific target genes of AID-mediated mutagenesis in human colonic mucosal cells.

A previous study demonstrated that the development of hyperplasia of isolated lymphoid follicles in AID-deficient mice is associated with a 100-fold expansion of anaerobic flora in the small intestine.[48] On the other hand, oral administration of antigens from anaerobic bacterial flora of the intestine reduces the severity of experimental acute colitis.[49] Thus, further analyses are required to examine whether the AID expression level in the colon epithelium might influence the pattern of intestinal bacterial flora and the colonic inflammation in IBD patients.

In conclusion, we found that proinflammatory cytokines induced the aberrant expression of AID in human colonic cells, leading to the generation of somatic mutations in the host genome, including the *TP53* gene. Our findings provide a novel linkage between chronic inflammation and enhanced susceptibility to somatic mutations and an increased risk of colorectal cancers.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.06.091.

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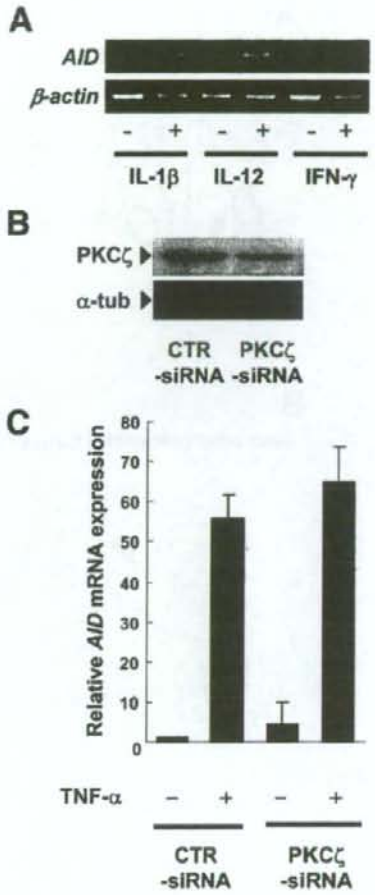
Conflicts of interest: No conflicts of interest exist.

Supplemental Table 1. Oligonucleotides Used for Subcloning in the Current Study

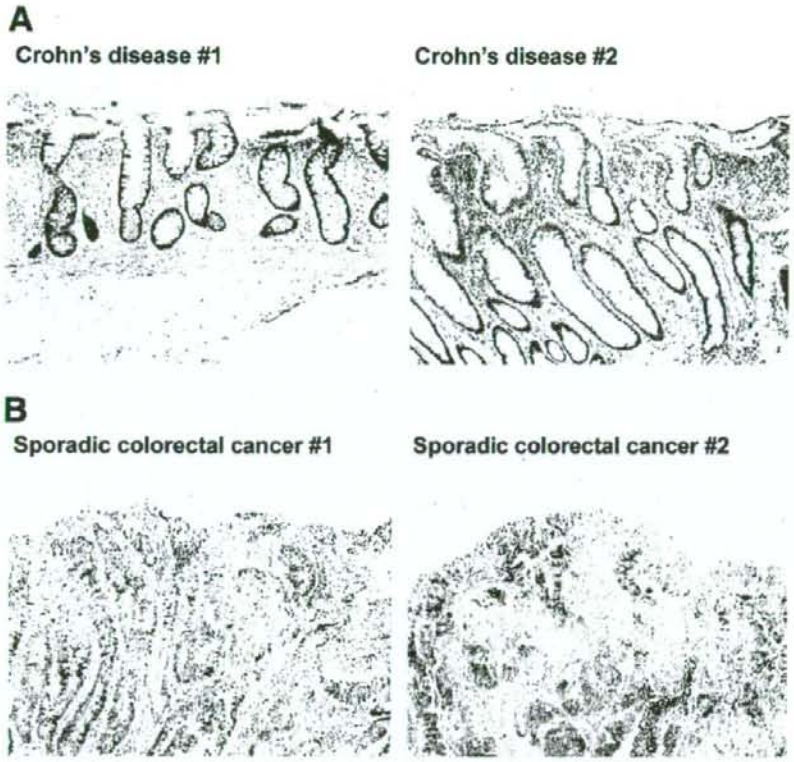
Primer	Nucleotide sequence
TP53-S (exons 2-6)	5'-GCCGAATTCATTGGCAGCCAGACTGCCTTC-3'
TP53-AS (exons 2-6)	5'-CCGCTCGAGAAATTTCCTTCCACTCGGATA-3'
TP53-S (exons 6-11)	5'-CCGGAATTCAGTGGAAGGAAATTTGCGTGT-3'
TP53-AS (exons 6-11)	5'-ATCCTCGAGTCAGTGGGGAACAAGAAGTG-3'
APC-S	5'-GCCGAATTCTTCTGCTAATACCCTGCAA-3'
APC-AS	5'-ATCCTCGAGCAGCATCTGGAAGAACCT-3'
K-ras-S	5'-CGCGGATCCAACTTGTGGTAGTTGG-3'
K-ras-AS	5'-CCGCTCGAGACCATCTTTGCTCATC-3'
c-myc-S	5'-GCCGAATTCGTAGTGGAAAACCAGCAGCC-3'
c-myc-AS	5'- ATCCTCGAGTGCTGATGTGTGGAGACGTG-3'



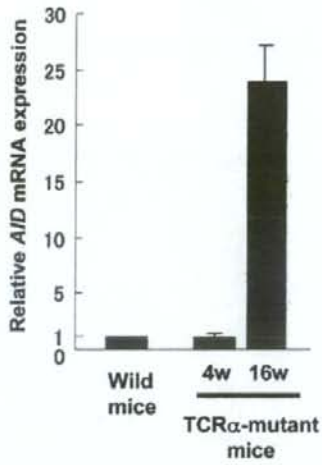
Supplementary Figure 1. Regulation of AID expression by cytokine stimulation in human colonic cells. (*A*) Semi-quantitative RT-PCR analyses for *AID* expression in LoVo cells treated with IL-1-β, IL-12 or IFN-γ treatment. Total RNA was extracted from LoVo cells after 12 h of treatment with IL-1β (25ng/ml), IL-12 (100ng/ml) or IFN-γ (100ng/ml) treatment. Semi-quantitative RT-PCR was performed using oligonucleotide primer sets specific for human *AID* (upper panel) and *β-actin* (lower panel). (*B*) LoVo cells were transfected with siRNA targeting *PKCζ* (Invitrogen)for 48 h. Whole cell lysates were probed by anti-PKCζ antibody (Cell Signaling Technology; upper panel) or anti-α-tubulin antibody (lower panel). (*C*) Effects of PKCζ-siRNA on TNF-α-induced AID gene expression. LoVo cells were transfected with siRNA targeting *PKCζ* or control siRNA and lysates were prepared from the siRNA-treated cells after the stimulation with TNF-α (100ng/ml) for 12 h. Total RNA was extracted from each specimen and subjected to quantitative real-time RT-PCR analyses for *AID* expression. The data present the means of *AID* mRNA expression relative to the internal control *18S rRNA* (mean ± SD; n=3).

Supplemental Table 2. Mutation Frequencies in the *TP53* Gene of LoVo Cells With the Wild or Mutant AID Expression

	Duration of AID activation	Mutated clones (n/total)	Mutation number (n/total bases)
AID (wild)	6 weeks	3/40	4/24200
AID (R35E)	6 weeks	2/42	2/25500
AID (R35E/R36D)	6 weeks	1/39	1/23600



Supplementary Figure 2. Expression of AID protein in various human colonic tissue specimens. Representative immunostaining for AID in the inflamed colonic epithelial mucosa of patients with Crohn's disease (*A* #1 and #2) and sporadic colorectal cancers (*B* #1 and #2). (Original magnification: *A&B*, ×100).



Supplementary Figure 3. Expression of AID in TCRα-mutant mice. *Total RNA was collected from the colonic mucosa of 16-weeks-old of wild type and* 4-weeks-old or 16-weeks-old TCRα-mutant mice. Expression levels of AID transcripts were determined by quantitative real-time RT-PCR analyses. Histological examination revealed that severe colitis was observed only in the colon of the 16-weeks-old TCRα-mutant mice.

Tetomilast Suppressed Production of Proinflammatory Cytokines from Human Monocytes and Ameliorated Chronic Colitis in IL-10-Deficient Mice

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Background: Tetomilast (OPC-6535) was originally developed as a compound inhibiting superoxide production in neutrophils. Although its mechanism of action is not completely understood, phosphodiesterase type 4 inhibitory function has been postulated. The therapeutic effect of PDE4 inhibitors has been reported for chronic inflammatory disorders such as chronic obstructive pulmonary diseases. In this study we aimed to examine whether tetomilast could be a novel drug for inflammatory bowel diseases by further clarifying its antiinflammatory effects.

Methods: Cytokines from human peripheral blood mononuclear cells were measured by enzyme-linked immunosorbent assay (ELISA) and Cytokine Beads Array. The transcripts were quantified by reverse-transcriptase polymerase chain reaction (RT-PCR). Phosphorylation of transcription factors was examined by phosflow. To examine its in vivo effect, a once-daily oral dose of tetomilast was tested in murine IL-$10^{-/-}$ chronic colitis.

Results: Tetomilast suppressed TNF-α and IL-12 but not IL-10 production from lipopolysaccharide (LPS)-stimulated human monocytes. It suppressed TNF-α, IFN-γ, and IL-10 from CD4 lymphocytes. Tetomilast suppressed cytokine production at the transcriptional level but did not alter phosphorylation of p65, ERK, p38, and STAT3. HT-89, a protein kinase A inhibitor, did not abolish the effect of tetomilast, suggesting that it was independent from the classical cAMP/PKA pathway. IL-10 was not essential to the inhibitory effect of tetomilast on TNF-α and IL-12. Tetomilast ameliorated IL-$10^{-/-}$ chronic colitis with reduced clinical symptoms, serum amyloid A, and histological scores with decreased TNF-α mRNA expression.

Conclusions: Tetomilast exerts its antiinflammatory effects on human monocytes and CD4 cells. Combined with in vivo data these findings support the feasibility of tetomilast as a novel drug for inflammatory bowel diseases.

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Key Words: tetomilast, PDE4 inhibitor, monocyte

Inflammatory bowel disease (IBD) is represented by 2 major forms, ulcerative colitis (UC) and Crohn's disease (CD). Although the etiology of IBD remains unclear, accumulating evidence suggests that dysfunction of the mucosal immune system plays an important role.[1,2] Among a variety of immune cells in the gut, CD4 lymphocytes have been intensely investigated in terms of abnormal adaptive immunity. TNF-α and IFN-γ have been considered as the key cytokines in IBD and there have been many reports showing their involvement in intestinal inflammation.[3–5] However, recently innate immunity has also drawn much attention since *NOD2* was identified as a susceptibility gene in CD.[6,7] In this context we and other groups have revealed that intestinal macrophages assume unique characters and they become defective in IBD.[8,9] The current concept is that intestinal macrophages do not overreact with luminal antigens such as commensal bacteria in order to keep intestinal mucosa from unnecessary inflammation. However, IBD macrophages switch their nature from antiinflammatory/tolerant to a proinflammatory phenotype and produce excessive amounts of TNF-α and IL-12, leading to continuous mucosal inflammation.[8,10] The essential role of these cytokines in IBD was clinically corroborated by the dramatic therapeutic effect of infliximab, anti-TNF-α antibody, for CD[11,12] as well as recently for UC.[13] Moreover, clinical trials of human anti-IL-12 antibody and anti-IFN-γ antibody also showed promising efficacy in CD.[14,15] These findings clearly showed the effectiveness of an anticytokine strategy as potent therapies for IBD.[16] However, biologicals have disadvantages compared with small mole-

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cules, including restriction to nonoral routes of administration, immunogenicity, and high cost. Tetomilast (OPC-6535) was identified through an in vitro screening of thiazole-derivatives inhibiting superoxide production in neutrophils.[17] Preclinical studies have demonstrated that tetomilast inhibits several specific proinflammatory functions of activated leukocytes including the release of superoxide anions by neutrophils and the adhesion of neutrophils to endothelial cells. Although its mechanism of action has not been fully elucidated, tetomilast is known to inhibit phosphodiesterase-4 (PDE4). The inhibition of PDE4 increases intracellular second messenger cAMP, which is involved in many pathways of cell function such as transcription regulations, ion channels, and signaling proteins.[18] PDE4 inhibition has become a promising strategy for the development of antiinflammatory drugs in various indications, especially in chronic obstructive pulmonary diseases (COPD).[19] Clinical trials of the newly developed PDE4 inhibitors cilomilast and roflumilast are now ongoing for COPD.[20,21] For IBD, 3 separate groups reported that rolipram, a prototypical PDE4 inhibitor, showed efficacy in dextran sulfate sodium (DSS) colitis[22,23] or trinitrobenzene sulfonic acid (TNBS) colitis.[24] Tetomilast also has been reported to show therapeutic effects in IBD animal models as well as in other inflammatory diseases.[25] However, all these animal models were acute diseases and the efficacy of PDE4 inhibition in chronic inflammation as well as the detailed mode of action needs to be clarified. The present article describes that tetomilast suppressed in vitro production of TNF-α and IL-12 from human monocytes. Downregulation of proinflammatory cytokine production occurred at the transcriptional level and was independent of endogenous IL-10. The effect was also independent of the classical cAMP/protein kinase A (PKA) pathway. Moreover, tetomilast showed its in vivo antiinflammatory effect in an IL-$10^{-/-}$ chronic colitis model with a decrease of intestinal TNF-α expression. These data underscore the promising profile of tetomilast as a novel therapy for IBD and bolster its clinical implication.

MATERIALS AND METHODS

Reagents, Stimulations, and Cytokines

Tetomilast was obtained from Otsuka Pharmaceutical Company (Tokyo, Japan). Tetomilast was dissolved in DMSO (Sigma, St. Louis, MO) immediately before use. DMSO was included as vehicle control at the appropriate dilution (1:200 at least) for in vitro experiments. 8-Bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP; Sigma) was kept as a 50 mM stock solution at $-20°C$ and diluted into complete medium immediately before use. Gel filtration grade lipopolysaccharide (LPS) (*Escherichia coli* O111: B4) was obtained from Sigma-Aldrich (Milwaukee, WI). Recombinant human IFN-γ (sp. act. 1×10^7 U/mg) and mouse M-CSF was purchased from R&D Systems (Minneapolis, MN). Immobilized anti-CD3 and anti-CD28 antibodies were purchased from BD Pharmingen (San Diego, CA). The PKA inhibitor H-89 [N-[2-(p-Bromo-cinnamyl-amino) ethyl]-5-isoquinolinesulfonamide. 2 HCl] was obtained from Alexis Biochemicals (San Diego, CA).

Isolation of Human Monocytes and CD4 Cells from PBMC

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood samples by density gradient centrifugation using Lymphoprep (Nycomed Pharma, Oslo, Norway). The cells were aspirated from the interface, washed in phosphate-buffered saline (PBS), and resuspended at 1×10^6 cells/mL in RPMI 1640 medium (Sigma-Aldrich) containing 10% heat-inactivated fetal bovine serum (BioSource, Camarillo, CA), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen, La Jolla, CA). Monocytes and CD4 cells were purified using a magnetic cell separation system (MACS; Miltenyi Biotec, Auburn, CA) with antihuman CD14, CD4 microbeads, respectively.

Induction of Cytokine Secretion in the Presence or Absence of Tetomilast

Monocytes were stimulated with either LPS alone (100 ng/mL) or a combination of LPS (1 μg/mL) and recombinant human IFN-γ (100 ng/mL) at a final concentration 5×10^5 cell/mL in 96-well flat-bottom culture plates (Costar, Cambridge, MA). Supernatants were harvested after 24 hours and stored at $-20°C$ until determination of cytokine levels. For stimulation of CD4 cells, 10 μg/mL of immobilized anti-CD3 and 5 mg/mL of anti-CD28 antibodies were used.

Cytokine Assay

To measure the concentrations of cytokines, the BD Cytometirc Beads Array (CBA) human Inflammation Kit, Th1/Th2 Cytokine Kit, and OptEIA human IL-12p40 ELISA kit (BD Biosciences, San Jose, CA) were used according to the manufacturer's instructions. Samples were analyzed using FACSCalibur (BD Pharmingen).

Cell Viability Assay

Cells were incubated with 200 μL (1×10^5 cells) $well^{-1}$ with 20 μL of supplemented medium containing MTT [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2-H-tetrazolium] (Nacalai Tesque, Kyoto, Japan), for 2 hours at 37°C and 5% CO_2. Absorbency was read at 450 nm. Cell viability was calculated as a relative index of LPS-stimulated cells.

Quantitative (RT-PCR) Analysis

After 4 and 6 hours of stimulation by LPS (100 ng/mL), total RNA was isolated from monocytes using an RNeasy Mini Kit (Qiagen, Chatsworth, CA). cDNA was synthesized

with OmniScript reverse transcriptase (Qiagen). For quantitative reverse-transcriptase polymerase chain reaction (RT-PCR), TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays for human TNF-α, IL-12p40, and β-actin (Applied Biosystems, Foster City, CA) were used. PCR amplifications were conducted in a thermocycler DNA Engine (OPTICON2; MJ Research, Cambridge, MA). Cycling conditions for PCR amplification were 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Inhibition of Cytokine Synthesis

PKA inhibitor, H-89 (5 μM) was added to monocyte cultures in titration assays and incubated for 30 minutes at 37°C and 5% CO_2. The cultures were incubated with LPS (100 ng/mL) in the presence or absence of tetomilast. The cultures were maintained at 37°C and 5% CO_2, and 24-hour supernatants were collected for TNF-α and IL-12 measurements. After 60 minutes of LPS stimulation plus H-89, the phosflow method was used to confirm that H-89 (5 μM) avoid CREB phosphorylation.

Phosphorylation of Transcription Factors

After 30 or 60 minutes of LPS stimulation, cells were harvested for phosphorylation of p65, ERK, p38, and STAT3 quantification using the phosflow method (BD Biosciences) according to the manufacturer's instructions. Samples were analyzed using FACSCalibur (BD Pharmingen).

Preparation of BM-derived Mϕ

BM cells were isolated from femora of 7–12-week-old IL-$10^{-/-}$ mice. After separation of BM mononuclear cells by gradient centrifugation, $CD11b^+$ cells were purified using a magnetic cell separation system (MACS; Miltenyi Biotec) with antimouse CD11b microbeads. To generate BM-derived macrophages, $CD11b^+$ cells (5×10^5 cells/mL) were cultured for 7 days with M-CSF (20 ng/mL).

Activation of Mϕ by Whole Bacteria Ags

BM-derived Mϕs were plated on 96-well tissue culture plates (1×10^5 cell/well) in RPMI-1640 medium supplemented with 10% FBS and antibiotics and stimulated by heat-killed bacterial Ags (MOI = 100) for 24 hours at 37°C. Culture supernatants were collected for determination of cytokine levels (TNF-α, Il-12p70) by a mouse inflammatory cytometric beads array (CBA) kit (BD Biosciences) according to the manufacturer's instructions. Samples were analyzed using FACSCalibur (BD Pharmingen).

Animals

Specific pathogen-free IL-10 gene-deficient mice, generated on C57BL/6 background, were obtained from Dr. Hiroshi Kiyono (Osaka University, Osaka, Japan) and were housed at the animal center of Kitasato Institute Hospital (Tokyo, Japan) before initiating the study.

Tetomilast Treatment in a Murine Chronic Colitis Model

For in vivo experiments a tetomilast suspension was prepared with 0.5% tragacanth solution (Torabanto, Nippon Funmatsu Yakuhin, Osaka, Japan), and was kept at 4°C. Under conventional conditions, 8-week-old IL-$10^{-/-}$ mice were orally administered tetomilast (10 mg/kg) or vehicle for 12 weeks. After 12 weeks of treatment mice were killed to assess the colonic lesions, e.g., stool consistency, histological score, colon cytokine synthesis, and serum amyloid A.

Histological Score

IL-10 gene-deficient mice were killed at 20 weeks of age. (vehicle group $n = 16$, tetomilast group $n = 14$) Colons were harvested and fixed in 10% phosphate-buffered formalin. These samples were paraffin-embedded sectioned and stained with hematoxylin and eosin. Tissues were reviewed in a blinded fashion and were assigned a histological score for intestinal inflammation using a scheme modified from Saverymuttu et al.[26] Histological grades (ranging from 0–10) represent the numerical sum of 4 scoring criteria: mucosal ulceration, epithelial hyperplasia, lamina propria mononuclear infiltration, and lamina propria neutrophilic infiltration.

ELISA for Serum Amyloid A

Blood samples were collected and the serum fraction was separated. Serum was analyzed for serum amyloid A (SAA) protein by enzyme-linked immunosorbent assay (ELISA) kit (BioSource).

Analysis of Proinflammatory Cytokines Expression by Quantitative RT-PCR

Total RNA was isolated from colon tissues using RNeasy Mini kit (Qiagen). cDNA was synthesized from 1 μg total RNA with Omniscript reverse transcriptase (RT; Qiagen). For quantitative RT-PCR, equivalent amounts of cDNA (2 μL), 0.5 μM of the forward and reverse primers, and DyNAmoSYBR Green qPCR Kit (MJ Research) were used. Cycling conditions for PCR amplification were 95°C for 10 seconds and 58°C for 50 seconds. Murine-specific primers for tumor necrosis factor (TNF)-α were forward, 5′-CATCTTCTCAAAATTCGAGTGACAA-3′, and reverse, 5′-TGGGAGTAGACAAGGTACAACCC-3′. β-Actin primers were forward 5′-AGAGGGAAATCGTGCGTGAC-3′, and reverse, 5′-CAATAGTGATGACC-TGGCCGT-3′.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software v. 4.0 (San Diego, CA). The statistical significance of differences between 2 groups was tested using a

Student's *t*-test. For comparison of more than 2 groups, analysis of variance (ANOVA) was used. If the ANOVA was significant, Dunnett's multiple comparison test was used as a post-hoc test. Differences at $P < 0.05$ were considered significant. All data are expressed as means ± SEM, $*P < 0.05$, $**P < 0.01$

Ethical Considerations

All experiments were approved by the local ethics committees. Informed consent was obtained from all healthy volunteers before obtaining samples.

RESULTS

Tetomilast Suppressed the Production of Inflammatory Cytokines from Human Monocytes

Recent evidence suggests that the increase in intracellular levels of cyclic AMP is accompanied by a significant decrease of cytokine production by LPS-activated macrophages.[27,28] In this context we first examined whether tetomilast could modulate TNF-α and IL-12 synthesis by human monocytes derived from peripheral blood. Tetomilast effectively inhibited LPS induced TNF-α and IL-12p40 production in a concentration-dependent manner (Fig. 1A,B). The effect was comparable to that of 8-Br-cAMP. On the other hand, tetomilast did not affect the synthesis of IL-10 when added to monocytes 30 minutes before stimulation with LPS (Fig. 1C). The indicated concentrations of tetomilast or 8-Br-cAMP had no influence on cell viability (Fig. 1D). We next examined IL-12 p70 production when monocytes were stimulated by LPS and IFN-γ. Tetomilast also significantly suppressed IL-12p70 production (Fig. 1E).

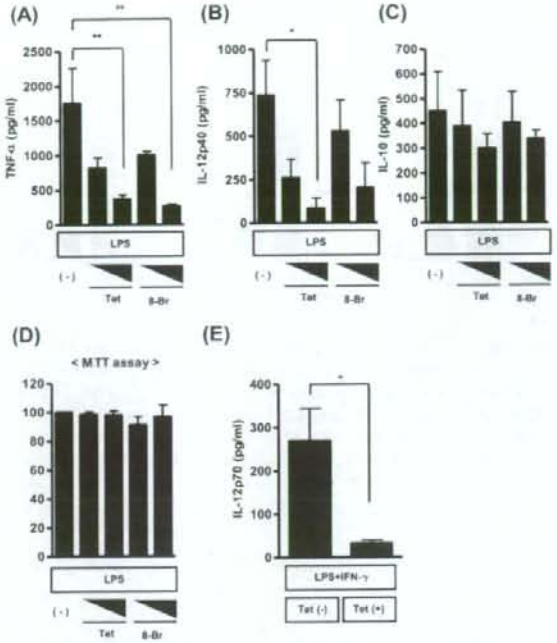

FIGURE 1. Tetomilast suppressed the production of inflammatory cytokines from human peripheral monocytes. Monocytes were isolated from peripheral blood and incubated with graded doses of tetomilast (1, 5 μM) or 8-Br-cAMP (50, 250 μM) for 30 minutes before stimulation with LPS. After 24-hour stimulation the supernatants were recovered and the concentration of TNF-α (A), IL-12 p40 (B), and IL-10 (C) were measured by CBA or ELISA. Results are expressed as mean ± SE. D: The cell viability was assessed using an MTT assay. E: For IL-12 p70 measurement, cells were pretreated with tetomilast (10 μM) and stimulated with LPS plus IFN-γ. Tet; tetomilast, 8-Br; 8-Br-cAMP.

Tetomilast Suppressed Cytokine Production from Human Peripheral CD4 Cells

It has been reported that cAMP-elevating agents are also potent suppressors of cytokine production from T cells.[29] We next examined the effect of tetomilast against T cells. When CD4 cells were activated with plate-coated anti-CD3/28 antibody, tetomilast at 5 μM suppressed all the cytokines tested, i.e., TNF-α, IFN-γ, and IL-10 (Fig. 2). Tetomilast did not affect the CD4 cell viability at this concentration (data not shown).

Tetomilast Suppressed the Production of Inflammatory Cytokines at the mRNA Level

Previous studies reported that cAMP and cAMP-elevating agents such as PDEs inhibit LPS-stimulated TNF-α production through a posttranscriptional mechanism.[28,30] To determine whether tetomilast affects TNF-α transcription, human monocytes were treated with LPS in the presence or absence of tetomilast and the expression of TNF-α mRNA was quantified by RT-PCR analysis at several timepoints. Tetomilast significantly reduced TNF-α mRNA expression at 4 hours (Fig. 3A) and IL-12p40 expression at 6 hours (Fig. 3B).

Tetomilast Did Not Affect Phosphorylation of NF-κB, ERK, p38, and STAT3

We next performed some experiments to clarify how tetomilast regulates the transcription of these inflammatory cytokines. LPS activates numerous signaling cascades including the NF-κB pathway.[31,32] To examine the effect of tetomilast on p65, ERK, p38, and STAT3 activation, the phosphorylation of the transcription factors was assessed with the phosflow technique (see Materials and Methods). As shown in Figure 4, LPS-induced p65, ERK, p38, and STAT3 phosphorylation was not altered in the presence of tetomilast (5 μM).

Inhibitory Effect of Tetomilast on Both TNF-α and IL-12p40 Synthesis Did Not Involve PKA Activation

As it is believed that cAMP exerts its multiple functions through activation of protein kinase A (PKA), we examined

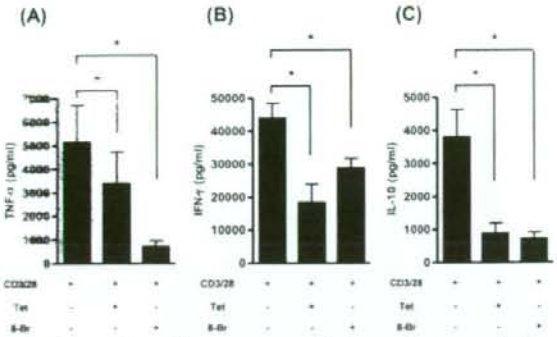

FIGURE 2. Tetomilast suppressed the cytokine production from human peripheral CD4 cells. CD4 cells were isolated from peripheral blood and treated with tetomilast (5 μM) or 8-Br-cAMP (250 μM) for 30 minutes before stimulation with plate-bound CD3/CD28 antibody. After 48 hours, supernatants were recovered and TNF-α (A), IFN-γ (B), and IL-10 (C) concentrations were measured by CBA.

whether PKA activation was essential to the activity of tetomilast. Human monocytes were incubated in the presence or absence of the PKA inhibitor H-89 (at 5 μM) for 30 minutes before addition of indicated concentration of tetomilast. In these experiments, tetomilast significantly decreased TNF-α and IL-12p40 production even in the presence of H-89 (Fig. 5A,B). Figure 5C shows that H-89 completely blocked CREB phosphorylation, which reflected that PKA activation was efficiently inhibited at this concentration of H-89.

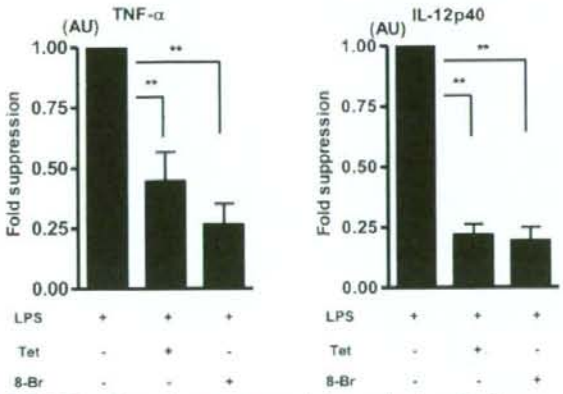

FIGURE 3. Tetomilast suppressed the production of inflammatory cytokines at the transcriptional level. Monocytes were stimulated as indicated in Figure 1. Total RNA was isolated and the expression of mRNA for TNF-α at 4 hours (A) and IL-12 p40 at 6 hours (B) was examined with real-time PCR. The effect of tetomilast (5 μM) or 8-Br-cAMP (250 μM) on the expression of transcripts are shown as fold suppression (mean ± SE) against the control LPS stimulation (left column).

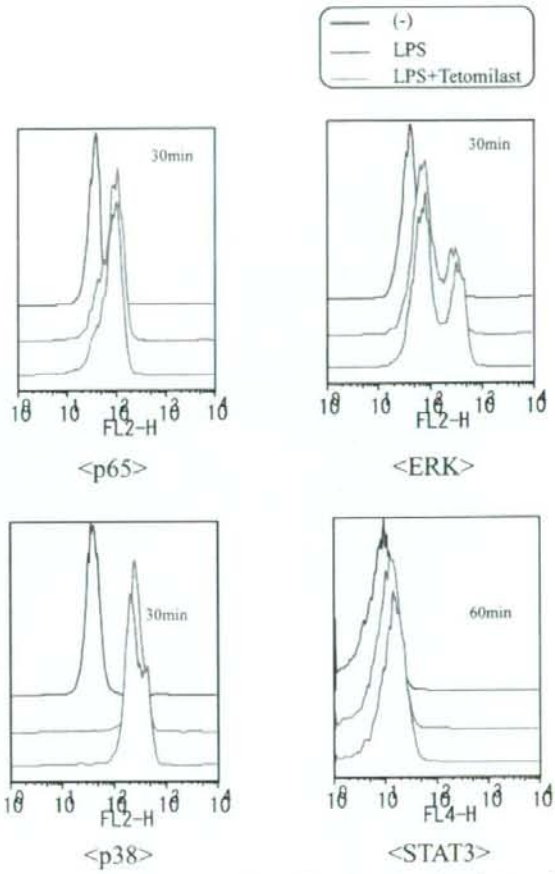

FIGURE 4. Tetomilast did not affect the phosphorylation of transcription factors. Nuclear extracts were prepared from human monocytes stimulated as indicated in Figure 1 for 30 or 60 minutes. Phosflow was performed to assess the status of phosphorylation of ERK, p38, NF-κB p65 (30 minutes), and STAT3 (60 minutes) as described in Materials and Methods.

Endogenous IL-10 Was Not Necessary for the Inhibitory Effect of Tetomilast on TNF-α and IL-12p70 Synthesis

It is suggested that IL-10 produced from macrophages may endogenously inhibit TNF-α and IL-12 production. Although controversial, there have been some reports suggesting that this mechanism may account for the inhibitory effect of cyclic AMP on these inflammatory cytokines.[27,28,30] We examined whether endogenous IL-10 was essential for the antiinflammatory effect of tetomilast by taking advantage of IL-$10^{-/-}$ macrophages. We previously reported that M-CSF-induced macrophages from IL-$10^{-/-}$ bone marrow cells produced extremely high levels of TNF-α and IL-12p70 responding to heat-killed *E. coli*.[8] As shown in Figure 6,

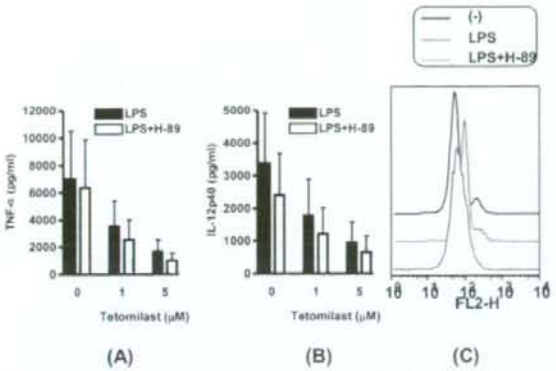

FIGURE 5. Tetomilast suppressed the production of proinflammatory cytokines in a protein kinase A (PKA)-independent manner. The same experiments as shown in Figure 1 were repeated in the presence of 5 μM H-89, a PKA inhibitor. TNF-α (A) and IL-12 p40 (B) at 24 hours were quantified by CBA or ELISA. (C) Phosflow demonstrated that 5 μM H-89 effectively inhibited CREB phosphorylation.

tetomilast dramatically decreased the production of these cytokines. These data clearly demonstrated that the antiinflammatory activity of tetomilast was independent of IL-10. In addition, these data allowed us to use IL-$10^{-/-}$ mice as a chronic colitis model for in vivo experiments.

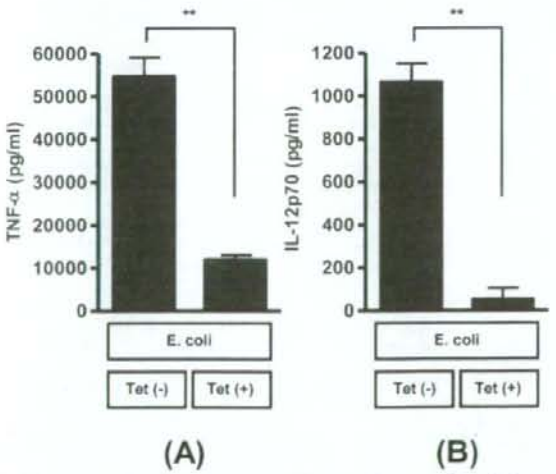

FIGURE 6. Tetomilast suppressed the production of proinflammatory cytokines in an IL-10-independent manner. Macrophages were differentiated from bone marrow cells of IL-10-deficient mice as described in Materials and Methods. Cells were stimulated with heat-killed *E. coli* (MOI = 100) for 24 hours and TNF-α (A) and IL-12p70 (B) were analyzed by CBA. Date are expressed as the mean ± SEM from 4 independent experiments.

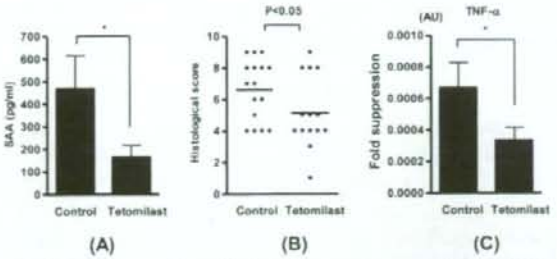

FIGURE 7. Tetomilast alleviated spontaneous colitis in IL-10 deficient mice. The mice were orally administered with vehicle or tetomilast between 12–20 weeks of age and sacrificed for the evaluation of the antiinflammatory effect of the agent. A: The serum amyloid A concentration was determined by ELISA. Values are expressed as mean ± SEM. B: Resected colonic tissues were examined by immunohistochemistry and the grade of inflammation was determined by 2 pathologists in a blind manner (0 for no inflammation, 10 for maximal tissue damage and cell infiltration). Scores are expressed as mean ± SEM. C: TNF-α mRNA in colonic mucosa was quantified with real-time PCR. Relative TNF-α mRNA expression against β-actin are expressed as mean ± SEM.

Tetomilast Ameliorated Spontaneous Chronic Colitis in IL-$10^{-/-}$ Mice

Next we examined whether tetomilast could exert its antiinflammatory effect in an animal model of IBD. To this end we used IL-$10^{-/-}$ mice as a model of chronic colonic inflammation. IL-$10^{-/-}$ mice developed spontaneous colitis under specific pathogen-free (SPF) conditions, which is characterized by passage of mucus, rectal prolapse, and diarrhea.[33] In this experimental period none of the mice treated with tetomilast developed apparent diarrhea and rectal prolapse, while some among the control mice showed these symptoms (4/16, 2/16, respectively). (Table 1). We did not observe significant alteration in body weight in each group (date not shown). The SAA protein concentrations, which could be a marker of inflammation, were significantly lower in tetomilast-treated mice (Fig. 7A). Histological scores for intestinal inflammation were also significantly reduced in tetomilast-treated mice (Fig. 7B). In this model, TNF-α has been thought to be a key cytokine to drive spontaneous colitis. Then we examined the expression of this cytokine and

TABLE 1. Clinical Findings of IL-10-Deficient Mice Treated with Tetomilast

	Control	Tetomilast
Normal stool	4/16	5/14
Loose stool	8/16	9/14
Diarrhea	4/16	0/14
Rectal prolapse	2/16	0/14

found that tetomilast significantly suppressed TNF-α mRNA production in resected colonic specimens (Fig. 7C).

DISCUSSION

Phosphodiesterases (PDEs) are enzymes that degrade cyclic nucleotide monophosphates, the intracellular second messenger, and at least 11 members constitute PDE superfamily. Among them, PDE4 is predominantly expressed in immune cells.[29] Increased intracellular cAMP level by PDE4 inhibition or cAMP analog exerts antiinflammatory effects in neutrophils,[34] lymphocytes,[35] and monocytes.[36] It is widely recognized that increased cAMP displays multiple functions through activation of its receptor, PKA.[37] For instance, activated PKA phosphorylates cAMP responding element (CRE)-binding protein, which is involved in IL-10 transcription. However, the exact mechanism of cAMP-mediated inhibition of TNF-α and IL-12 production in monocytes or macrophages remains unclear. It has been proposed that this antiinflammatory effect is IL-10-dependent,[27,30,38,39] but others reported the opposite observations.[27,28,40] As shown in Figure 6, we clearly demonstrated that the downregulation of TNF-α and IL-12 production by tetomilast is an IL-10-independent effect by using IL-10 deficient macrophages. Moreover, tetomilast showed therapeutic efficacy in IL-10$^{-/-}$ chronic colitis (Fig. 7). We also examined whether classical cAMP/PKA signaling is essential for the downregulation of proinflammatory cytokine production by tetomilast in monocytes. There are some reports showing that the same effect of cAMP analog is not necessarily PKA-dependent in neutrophils[41] and lymphocytes,[42,43] as well as nonimmune cells.[44] As shown in Figure 5, tetomilast could downregulate TNF-α and IL-12 production even in the presence of H-89, which clearly demonstrated that the effect is PKA-independent. As we found that downregulation of this cytokine production occurred at the transcriptional level (Fig. 3), we examined the phosphorylation status of relevant transcriptional factors but could not observe any significant change by tetomilast. Further studies are necessary to clarify how tetomilast or cAMP analog could downregulate the transcripts of TNF-α and IL-12.

Besides its inhibitory effect on proinflammatory cytokines, other functions of PDE4 inhibitors have been reported. Videla et al[45] reported that rolipram ameliorated intestinal fibrosis by downregulating TGF-β1. Sanz et al[46] demonstrated that roflumilast inhibited leukocyte–endothelial cells interaction. Banner and Trevethick[29] implied that PDE4 inhibitors may decrease contractility of gut smooth muscle and help to maintain mucosal blood flow.

Recently, Schreiber et al[47] reported the result of a randomized placebo-controlled phase II study of tetomilast in active UC which was conducted in 35 outpatient clinics in the United States. Unfortunately, tetomilast did not achieve statistical significance for the primary endpoint, which was defined as clinical improvement at 8 weeks. However, post-hoc analysis suggested its potential efficacy especially in patients with higher disease severity. A large and adequately powered phase III program for the treatment of active UC is currently under way.

This study revealed the antiinflammatory effects of tetomilast against monocytes and CD4 cells in addition to neutrophils. Its potent inhibitory effect on TNF-α, IL-12, and IFN-γ, which are believed to drive Th1 disease, may also imply its clinical application to CD. Once-daily oral dosing as used for tetomilast will provide great advantages for outpatient clinic of IBD. Further studies will clarify its antiinflammatory mechanisms and may increase our understanding of the therapeutic implication of this drug in IBD as well as many other inflammatory disorders.

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Colitogenic CD4$^+$ Effector-memory T Cells Actively Recirculate in Chronic Colitic Mice

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Background: Although the clinical usefulness of leukocytapheresis for patients with inflammatory bowel disease (IBD) has been reported as a selective removal therapy targeting pathogenic immune cells in blood circulation, it remains unclear whether colitogenic CD4$^+$ T cells continuously recirculate in peripheral blood during the chronic phase of colitis.

Methods: To resolve this question we conducted a series of in vivo experiments using a murine chronic colitis model induced by adoptive transfer of CD4$^+$CD45RB^{high} cells into SCID mice in combination with a parabiosis system.

Results: In colitic SCID recipients, first, almost all CD4$^+$ CD45RB^{high} donor cells were converted to CD4$^+$CD44^{high}CD62L$^-$ IL-7Rα^{high} effector-memory T (T_{EM}) cells at 8 weeks after transfer and were distributed throughout the whole body, including colonic lamina propria, mesenteric lymph nodes, thoracic duct, peripheral blood, spleen, and bone marrow. Second, SCID mice retransferred with the colitic peripheral blood CD4$^+$ T cells developed colitis that is identical to the original colitis. Third, CD4$^+$ cells in parabionts between established colitic RAG-2$^{-/-}$ mice induced by adoptive transfer of Ly5.1$^+$ or Ly5.2$^+$ CD4$^+$CD45RB^{high} T cells were well mixed in almost equal proportions at various sites 2 weeks after parabiosis surgery, and the redistribution of Ly5.1$^+$ and Ly5.2$^+$ CD4$^+$ T cells was significantly suppressed in FTY720-treated parabionts.

Conclusions: Together, these findings indicate that colitogenic CD4$^+$ T_{EM} cells continuously recirculate in established colitic mice, suggesting that therapeutic approaches targeting systemic CD4$^+$ T_{EM} cells, such as bone marrow transplantation, rather than those targeting only intestinal CD4$^+$ T cells, may be feasible for the treatment of IBD.

(Inflamm Bowel Dis 2008;14:1630–1640)

Key Words: colitogenic memory T cells, FTY720, parabiosis, colitis, recirculation

Inflammatory bowel diseases (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), are characterized by a wasting disease with chronic intestinal inflammation.[1–6] Importantly, it is well recognized that surgery never cures CD, as relapses are the rule after remissions,[7] and extraintestinal disorders often occur in UC patients even after total colectomy,[8] suggesting that IBD may not be a circumscribed disease, but rather a systemic disease that colitogenic memory lymphocytes, which might memorize the disease prototype, distribute throughout the body via the bloodstream as if they were 'benign leukemia cells,' and might hide in a reservoir other than the inflamed intestine. Consistent with this hypothesis, recent findings of the usefulness of leukocytapheresis for IBD patients[9–11] suggest that the recirculation of colitogenic memory lymphocytes between the gut and some reservoir may play a role in the perpetuation of IBD. Furthermore, we have recently demonstrated that FTY720, which is able to inhibit the circulation of lymphocytes,[12–14] prevents the development of SCID colitis induced by adoptive transfer of lamina propria (LP) colitogenic CD4$^+$ effector-memory T (T_{EM}) cells and suppresses IFN-γ, IL-2, and TNF-α production by LP CD4$^+$ T cells.[15] The findings reported in the literature just cited suggest that the hemodynamics of colitogenic CD4$^+$ T_{EM} cells in IBD is sufficiently active to cause colitogenic CD4$^+$ T_{EM} cells to circulate continuously in the peripheral blood.

Egress of immune cells from nonlymphoid tissues is an important step in lymphocyte migration as well as lympho-

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cyte homing to nonlymphoid tissues.[12] Draining lymphatics of tissues contains substantial numbers of lymphocytes, some of which are cells with memory phenotype.[16] Early studies demonstrated that sheep thoracic duct lymph (TDL), which drains from the intestine and empties into the blood, contains many lymphocytes,[16] although the exact source and fate of this population are of considerable controversy. Furthermore, in a landmark experiment Gowans and Knight[17] demonstrated that labeled lymphocytes in TDL that were intravenously transferred into syngenic recipients were detected in their TDL again. This indicates that lymphocytes recirculate continuously between blood and lymph. In the intestine, however, it is also thought that the altered phenotype of memory cells in intestinal LP and the lack of such cells elsewhere suggest that the memory T cells do not exit the tissue.[18,19]

Based on such complex backgrounds we conducted a series of adoptive transfer experiments in combination with parabiosis using mice to assess the hemodynamics of colitogenic $CD4^+$ T_{EM} cells in chronic colitis.

MATERIALS AND METHODS

Animals

BALB/c, C.B-17 SCID, and C57BL/6-Ly5.2 mice were purchased from Japan Clea (Tokyo, Japan). C57BL/6-Ly5.1 and C57BL/6-Ly5.2-RAG-2 deficient (RAG-$2^{-/-}$) mice were obtained from Taconic Farms (Hudson, NY) and Central Laboratories for Experimental Animals (Kawasaki, Japan). Mice were maintained under specific pathogen-free (SPF) conditions in the Animal Care Facility of Tokyo Medical and Dental University. Donors and littermate recipients were used at 6–12 weeks of age. All experiments were approved by the regional animal study committees and were done according to institutional guidelines and Home Office regulations.

Antibodies

The following mAbs other than biotin-conjugated antimouse IL-7Rα (A7R34; eBioscience, San Diego, CA) were obtained from BD PharMingen (San Diego, CA) for purification of cell populations and flow cytometry analysis: 145-2C11, FITC-conjugated antimouse CD3; RM4-5, PE-conjugated antimouse CD4; C363.16A, FITC-conjugated antimouse CD45RB; 104, FITC-conjugated antimouse Ly5.1 (CD45.1); A20, FITC-conjugated antimouse Ly5.2 (CD45.2); IM7, Allophycocyanine-conjugated antimouse CD44; MEL-14, PE-conjugated antimouse CD62L; H1.2F3, PE-conjugated antimouse CD69; DATK32, PE-conjugated antimouse $\alpha4\beta7$. Biotinylated antibodies were detected with PE-streptavidin (BD PharMingen).

In Vivo Adoptive Transfer Experiments

We performed a series of in vivo experiments to assess the role of circulating colitogenic $CD4^+$ effector-memory T (T_{EM}) cells in the perpetuation of chronic colitis induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells. For adoptive transfer, $CD4^+$ T cells were first isolated from SP cells of C57BL/6-Ly5.2 mice using the anti-CD4 (L3T4)-MACS system (Miltenyi Biotec) according to the manufacturer's instruction. Enriched $CD4^+$ T cells (96-97% pure, as estimated by FACSCalibur (BD Biosciences)) were then labeled with PE-conjugated antimouse CD4 (RM4-5; BD Pharmingen) and FITC-conjugated antimouse (16A; BD Pharmingen). $CD4^+$ $CD45RB^{high}$ cells were purified using a FACSAria (BD Biosciences). This population was 98.0% pure on reanalysis. Experiment 1: The distribution of $CD4^+$ T cells in LP, mesenteric lymph nodes (MLN), thoracic duct (TD), peripheral blood (PB), spleen (SP), and bone marrow (BM) after the establishment of colitis at 8 weeks after transfer was first assessed using flow cytometry. Experiment 2: To assess whether circulating PB $CD4^+$ T cells in established colitic SCID mice at the late stage of colitis are colitogenic, new SCID mice were transferred with colitic PB or LP $CD4^+$ T cells (3×10^5) obtained from the established colitic SCID mice. Mice were monitored during the course and sacrificed at 6 weeks after transfer. Experiment 3: To assess the hemodynamics of colitogenic $CD4^+$ T_{EM} cells in established colitic mice, an adoptive transfer experiment was performed in combination with a parabiosis system[20] between established RAG-$2^{-/-}$ mice transferred with C57BL/6-Ly5.1- or C57BL/6-Ly5.2-$CD4^+CD45RB^{high}$ T cells at 8 weeks after transfer. Briefly, sex-matched colitic RAG-$2^{-/-}$ mice were anesthetized prior to surgery and incisions were made in the skin on the opposing flanks of the donor and recipient animals. Surgical sutures were used to bring the body walls of the 2 mice into direct physical contact. The outer skin was then attached with surgical staples. In another set of experiments, colitic parabionts were treated with FTY720 (1.0 mg/kg) or phosphate-buffered saline (PBS) daily over a period of 4 weeks starting 1 day before parabiosis surgery and were monitored for clinical signs such as hunched posture, piloerection, diarrhea, and blood in the stool. Mice were sacrificed 4 weeks after surgery and assessed for a clinical score as the sum of 4 parameters: 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; and an additional point was added if gross blood was noted).[21]

T-cell Preparation

For the preparation of TD cells, mice were fed 500 μL of olive oil by gavage. Then, 45 minutes later, mice were anesthetized by intraperitoneal injection of ketamine (50 mg/kg, Alexis Biochemicals, San Diego, CA) and xylazine (10

mg/kg, Sigma-Aldrich, St. Louis, MO) and subjected to laparotomy. A heparinized PE-10 polyethylene catheter (Natsume Seisakusho, Japan) was inserted into the cysterna chili and lymph was collected for 30 minutes. For isolation of PB, 600 μL of PB was collected from each mouse and diluted 1:1 with PBS. The diluted blood was layered over Lymphosepar II (IBL, Gunma, Japan) and centrifuged at 400*g* for 30 minutes at room temperature. The lymphocytes were then isolated from the plasma-Ficoll interface. SP and MLN were mechanically disrupted into single-cell suspensions. BM was collected from the femur by flushing with sterile PBS. For the preparation of colonic LP cells, the colon was first flushed extensively to eliminate the lumen content, then longitudinally opened and cut into small pieces. The dissected mucosa was incubated with Ca^{++} Mg^{++}-free Hanks' BSS containing 1 mM DTT (Sigma-Aldrich) for 30 minutes to remove mucus, then treated with 3 mg/mL collagenase (Roche, Nutley, NJ) and 0.01% DNase (Worthington Biochemical, Freehold, NJ) for 2 hours. After filtering through gauze, cells were pelleted twice through a 40% isotonic Percoll solution, and then subjected to Ficoll-Hypaque density gradient centrifugation (40%/75%). Enriched $CD4^+$ T cells were obtained by positive selection using anti-CD4 (L3T4) MACS magnetic beads. The resultant cells contained >94% $CD4^+$ cells when analyzed by FACS Calibur ((BD Biosciences).

Histological Examination and Immunohistology

Tissue samples were fixed in PBS containing 10% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with hematoxylin and eosin (H&E). The sections were analyzed without prior knowledge of the type of T-cell reconstitution and recipients. The area most affected was graded by the number and severity of lesions. The mean degree of inflammation in the colon was calculated using a modification of a previously described scoring system.[22]

Flow Cytometry

To detect the surface expression of a variety of molecules, isolated splenocytes, MLN, PB, TD, PC, BM, or LP mononuclear cells (LPMCs) were preincubated with an FcγR-blocking mAb (CD16/32; 2.4G2, BD PharMingen) for 15 minutes, then incubated with specific FITC-, PE-, PerCP-, Allophycocyanine-, or biotin-labeled antibodies for 20 minutes on ice. Biotinylated antibodies were detected with PE-streptavidin. Standard 3- or 4-color flow cytometric analyses were obtained using the FACS Calibur with CellQuest software. Background fluorescence was assessed by staining with control-irrelevant isotype-matched mAbs.

Statistical Analysis

The results were expressed as the mean ± standard error of mean (SEM). Groups of data were compared by Mann–Whitney *U*-test. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

$CD4^+CD44^{high}CD62L^-IL\text{-}7R\alpha^{high}$ T_{EM} Cells Were Distributed Throughout Whole Body in Established Colitic Mice

Although the SCID transfer model of colitis induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells is characterized by a marked infiltration of colitogenic $CD4^+$ T cells in the colonic tissues, we previously showed that SP and BM $CD4^+$ T cells in the colitic mice were also colitogenic, because their adoptive transfer into new SCID mice induces a similar colitis to the original one.[23] This suggests a link between the inflamed colon and other reservoir sites such as BM through the continuous circulation of colitogenic $CD4^+$ T cells. To investigate this notion, we first checked the distribution of $CD4^+$ T cells in various sites of established colitic $CD4^+CD45RB^{high}$ T-cell-transferred SCID mice at the late stage of this model (8 weeks after transfer). As depicted in Figure 1, $CD4^+$ T cells resided in all sites of colitic mice examined including PB, LP, SP, TD (efferent lymphatics side), MLN, and BM, and had solely the character of $CD44^{high}CD62L^-IL\text{-}7R\alpha^{high}$ T_{EM} cells. Importantly, the fact that the detection of such cells in TD and PB indicates continuous recirculation of $CD4^+$ T cells even after the establishment of colitis. In contrast, CD4-gated T cells obtained from normal mice consisted of $CD44^{low}CD62L^+$ naïve, $CD44^{high}CD62L^+$ central-memory (T_{CM}), and $CD44^{high}CD62L^-$ T_{EM} cells in all the examined sites except LP, which contained solely $CD44^{high}CD62L^-$ T_{EM} cells (Fig. 1).

Adoptive Transfer of Colitic PB $CD4^+$ T Cells Induces Colitis

We next attempted to show whether continuously circulating PB $CD4^+$ T cells in the established colitic mice are also colitogenic, in the same way that colitic LP $CD4^+$ T cells were previously shown to be.[21] To this end, we transferred colitic PB or LP $CD4^+$ T cells obtained from colitic $CD4^+CD45RB^{high}$ T-cell-transferred SCID mice into new SCID mice as illustrated in Figure 2A. As expected, SCID mice transferred with colitic LP $CD4^+$ T cells (positive control) manifested progressive weight loss from 3 weeks after transfer (data not shown). These mice had diarrhea with increased mucus in the stool, anorectal prolapse, and hunched posture by 5–6 weeks after transfer, in sharp contrast to age-matched control BALB/c (Fig. 2B). Similarly, SCID mice transferred with colitic PB $CD4^+$ T cells also developed a wasting disease with symptoms of colitis to a similar extent to those with colitic LP $CD4^+$ T cells (Fig. 2B). At 6 weeks after transfer the colon from colitic PB or LP $CD4^+$ T cells was enlarged and had a greatly thickened wall, in sharp contrast to the control BALB/c mice (data not shown). Histo-

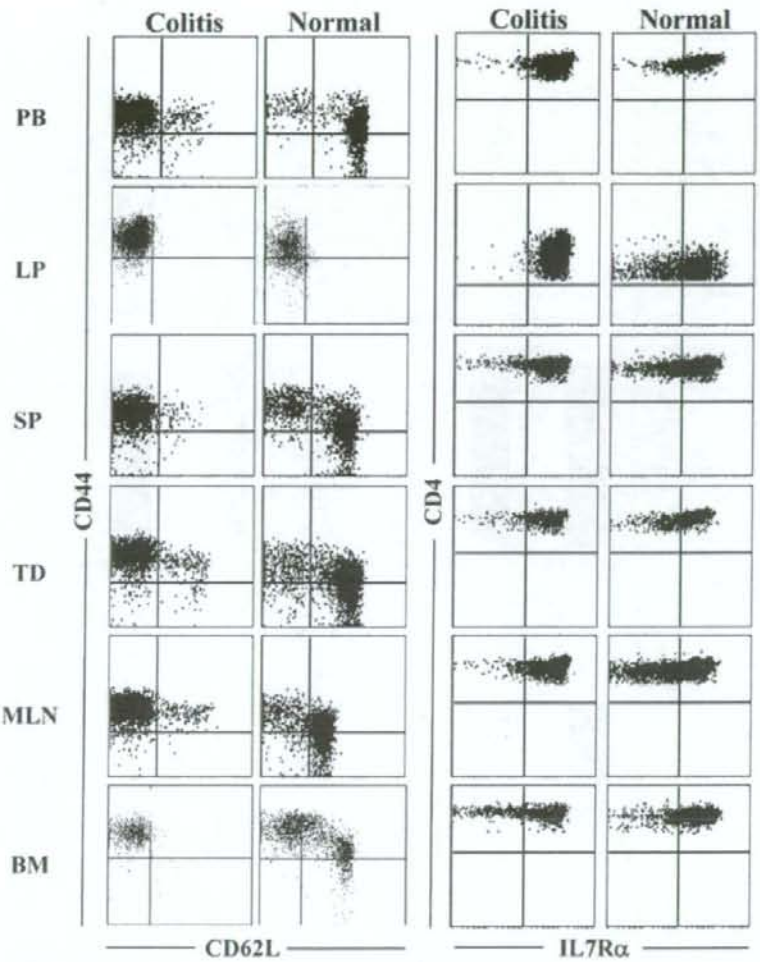

FIGURE 1. $CD4^+CD44^{high}CD62L^-$ T_{EM}-like cells reside in various sites including LP, MLN, TD, PB, SP, and BM, of established colitic SCID mice at 8 weeks after adoptive transfer of $CD4^+CD45RB^{high}$ T cells. Samples were analyzed by flow cytometry. Lymphocytes were identified by characteristic forward angle and sidescatter profiles. Data are displayed as dotted plots (4-decade log scale), and quadrant markers were positioned to include >98% of control IgG-stained cells in the lower left. Percentages in each quadrant are indicated. Representatives of 3 mice in each group.

logical examination showed prominent epithelial hyperplasia with massive infiltration of mononuclear cells in the colonic LP from mice transferred with colitic PB or LP $CD4^+$ T-cell-transferred SCID mice, but not from the control BALB/c mice (Fig. 2C). This difference was also confirmed by histological scoring of multiple colon sections, the scores being 14.5 ± 1.0 in mice transferred with colitic LP $CD4^+$ T cells, 15.3 ± 1.2 in mice transferred with colitic PB $CD4^+$ T cells, and 0.7 ± 0.7 in control BALB/c mice ($P < 0.005$) (Fig. 2D).

A further quantitative evaluation of $CD4^+$ T-cell infiltration was made by isolating LP $CD4^+$ T cells. Significantly more LP $CD4^+$ T cells were recovered from SCID mice transferred with colitic LP or PB $CD4^+$ T cells than from the control BALB/c mice (Fig. 2E). The number of $CD4^+$ cells recovered from the colonic LP of mice transferred with colitic PB $CD4^+$ T cells ($202 \pm 42 \times 10^5$) far exceeded the number of originally injected cells (3×10^5), indicating an extensive T cell migration and/or proliferation in the inflamed colon.

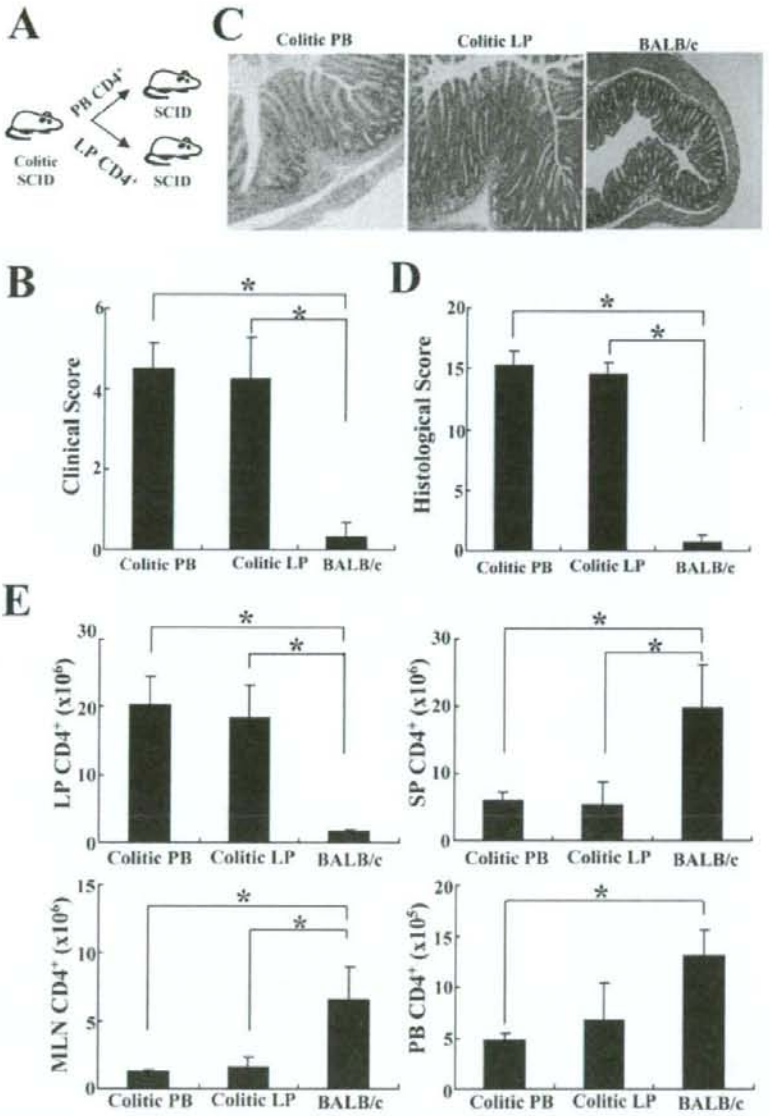

FIGURE 2. New SCID mice transferred with circulating PB $CD4^+$ T cells obtained from established colitic $CD4^+CD45RB^{high}$ T cell-transferred SCID mice develop colitis. A: New SCID mice were transferred with colitic PB $CD4^+$ T cells ($n = 6$) or colitic LP $CD4^+$ T cells ($n = 6$). B: Clinical scores were determined at 6 weeks after transfer. Data are indicated as mean ± SEM of 6 mice in each group. *Versus age-matched control BALB/c mice, $P < 0.01$. C: Histological examination of the colon from mice were transferred with colitic PB $CD4^+$ T cells (left) or colitic LP $CD4^+$ T cells at 6 weeks after transfer (middle). Original magnification, ×100. D: Histological scoring of mice transferred with colitic PB $CD4^+$ T cells or colitic LP $CD4^+$ T cells at 6 weeks after transfer. Data are indicated as mean ± SEM of 6 mice in each group. *$P < 0.01$. E: LP, SP, MLN, and PB cells were isolated from the colon at 6 weeks after transfer, and the number of $CD4^+$ cells was determined by flow cytometry. Data are indicated as mean ± SEM of 7 mice in each group. *$P < 0.05$.

Collectively, these results clearly showed that colitogenic $CD4^+$ T cells continuously circulate in PB of established colitic mice.

Colitogenic $CD4^+$ T Cells Dynamically Recirculate Even After the Establishment of Colitis

To further assess the hemodynamics of colitogenic $CD4^+$ T cells in colitic mice, we next conducted an in vivo experiment combining adoptive transfer and a parabiosis system. To this end, RAG-$2^{-/-}$ recipient mice and donor cells from C57BL/6-Ly5.1- or C57BL/6-Ly5.2-mice were used. First, RAG-$2^{-/-}$ mice were transferred with C57BL/6-Ly5.1- or C57BL/6-Ly5.2-$CD4^+CD45RB^{high}$ T cells (Fig. 3A). After confirming the establishment of colitis in these mice at 6 weeks after transfer, we performed parabiosis surgery between these colitic RAG-$2^{-/-}$ mice (Fig. 3A,B). At 2 weeks after surgery, both parabionts in each pair were consistently diseased, with greatly thickened colon wall and enlarged spleen and MLN, in sharp contrast to the control C57BL/6 mice (Fig. 3C). Both parabionts also showed severe colitis with crypt elongation, surface erosion, and a marked infiltration of mononuclear cells (Fig. 3D). Importantly, $Ly5.1^+$ and $Ly5.2^+$ cells in both the donor and recipient sides were well mixed in almost equal proportions in LP, as well as in SP, MLN, BM, PB, PC, and TD (Fig. 3E), suggesting that colitogenic $CD4^+$ T_{EM} cells continuously recirculate even after the establishment of chronic colitis.

FTY720 Treatment Inhibited the Recirculation of Colitogenic $CD4^+$ T_{EM} Cells, But Did Not Ameliorate the Established Colitis

To finally assess whether FTY720, which has the ability to turn off the circulation of lymphocytes by promoting their sequestration and inhibiting their egress,[12,13] suppresses the continuous recirculation of colitogenic $CD4^+$ T cells and thereby ameliorates the established colitis in this parabiosis system, both parabionts in each pair of established colitic RAG-$2^{-/-}$ mice transferred with $Ly5.1^+$ or $Ly5.2^+$ $CD4^+CD45RB^{high}$ T cells were next treated intraperitoneally with FTY720 (1.0 mg/kg) or control PBS daily for 4 weeks starting 1 day before parabiosis surgery, and were monitored for clinical signs such as hunched posture, piloerection, diarrhea, and blood in the stool (Fig. 4A). Unexpectedly, both parabionts treated with FTY720 were consistently diseased, with clinical symptoms of colitis to a similar extent to PBS-treated parabionts (data not shown). The colon, spleen, and MLN from all these mice, whether treated with FTY720 or PBS, were enlarged and had a greatly thickened wall due to severe colonic inflammation (Fig. 4B). Histological examination also showed a severe colitis with massive infiltration of mononuclear cells in LP of the colon from all the parabionts (Fig. 4C), and the scores of histology confirmed that FTY720 did not ameliorate the established colitis in parabionts (Fig. 4D). Interestingly, however, the ratio of donor cells in all the sites examined, namely, SP, LP, MLN, and BM, was significantly suppressed by FTY720 treatment (Fig. 4E).

To further assess the change of surface markers of the donor and recipient cells in various sites (SP, LP, MLN, and BM) by FTY720 treatment, we performed flow cytometry analysis (Fig. 5). First, $Ly5.1^+$ and $Ly5.2^+$ cells, whether donor or recipient cells, and irrespective of FTY720 treatment, showed the character of $CD44^{high}CD62L^-$ T_{EM} cells. Second, the expression pattern of cell activation marker CD69 was dependent on the site, but not on donor or recipient cells, as CD69 upregulated in LP and BM $CD4^+$ T cells of FTY720- or PBS-treated parabionts as compared to that in other sites. Furthermore, the expression of gut-homing receptor integrin $\alpha4\beta7$ was not affected by FTY720 treatment. These results indicate that the effect of FTY720 treatment is to suppress the recirculation of lymphocytes, but not to modulate the cell phenotype.

DISCUSSION

In the present study we demonstrated that colitogenic $CD4^+$ T_{EM} cells continuously circulate in whole body through the blood vessels even after the establishment of colitis by showing that colitis can be induced by adoptive retransfer of colitic PB $CD4^+$ T_{EM} cells, and that $CD4^+$ T cells in parabionts between established colitic RAG-$2^{-/-}$ mice induced by adoptive transfer of $Ly5.1^+$ or $Ly5.2^+$ $CD4^+CD45RB^{high}$ T cells were well mixed in various sites including PB, TD, and LP.

Since colitogenic effector or memory T cells are thought to be generated in regional LN and from there migrate into the inflamed mucosa, it is logical to conclude that they probably circulate in the peripheral blood. Curiously, however, the question of whether they actively recirculate in PB has not hitherto been experimentally solved, although we and others previously showed that SP, MLN, and BM $CD4^+$ T cells obtained from colitic mice are colitogenic, as adoptive transfer of those cells induces colitis that is similar to the original colitis of donor mice.[15,23–25] The present model of colitis induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells is useful for this purpose, because 1) primarily transferred $CD4^+CD45RB^{high}$ T cells in recipient mice can be tracked over time under conditions that allow us to exclude the recruitment of naïve $CD4^+$ T cells that are continuously supplied from thymus; and 2) this model induces the extremely rapid proliferation of colitogenic LP $CD4^+$ T cells, which presumably respond to commensal bacterial antigens or self-antigens by lymphopenia-driven proliferation, and thereby accounts for almost all $CD4^+$ T cells in the body.[26,27] Using this adoptive transfer model, we here found that 1) $CD4^+CD44^{high}CD62L^-$ T_{EM} cells do indeed reside in PB of colitic mice; and 2) adoptive transfer of those cells success-

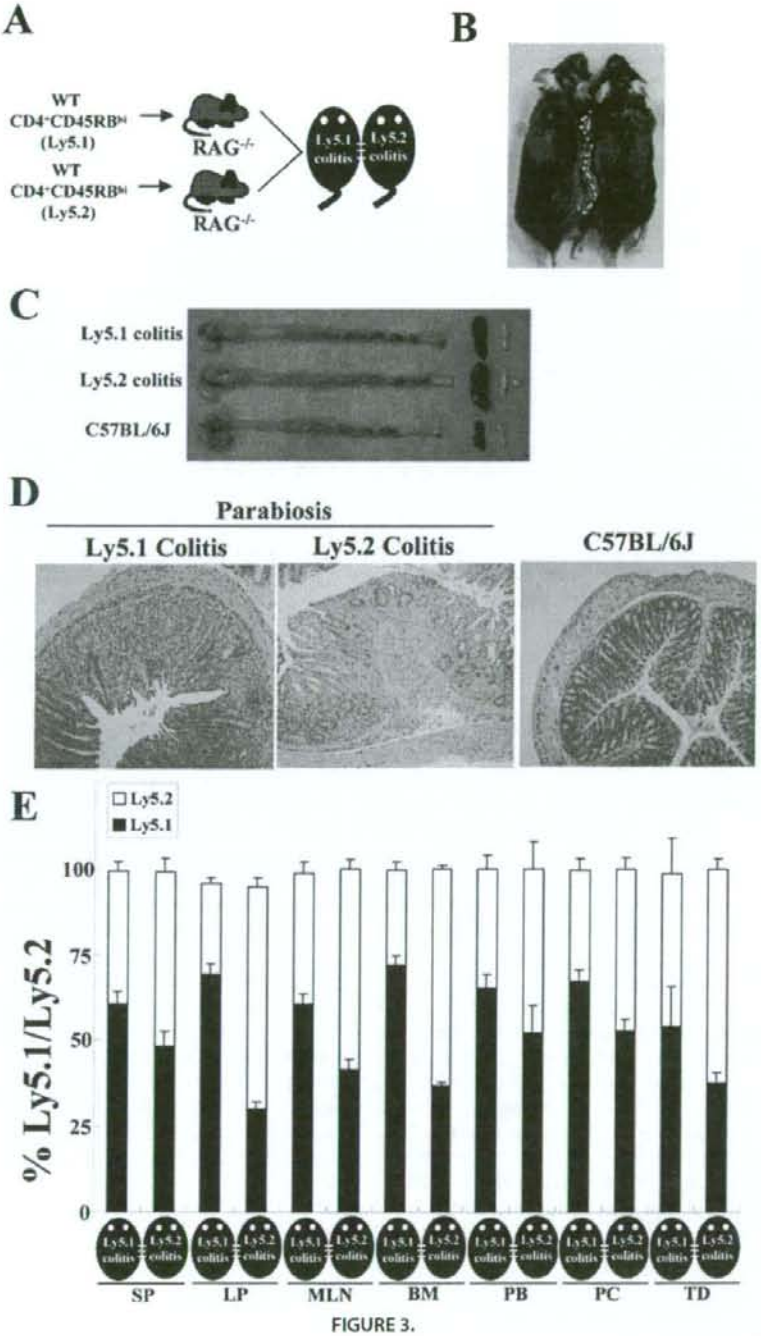
A
WT
CD4+CD45RBhi
(Ly5.1)
WT
CD4+CD45RBhi
(Ly5.2)
RAG-/-
RAG-/-
Ly5.1 colitis
Ly5.2 colitis
B
C
Ly5.1 colitis
Ly5.2 colitis
C57BL/6J
D
Parabiosis
Ly5.1 Colitis
Ly5.2 Colitis
C57BL/6J
E
Ly5.2
Ly5.1
% Ly5.1/Ly5.2
100
75
50
25
0
SP
LP
MLN
BM
PB
PC
TD

FIGURE 3.