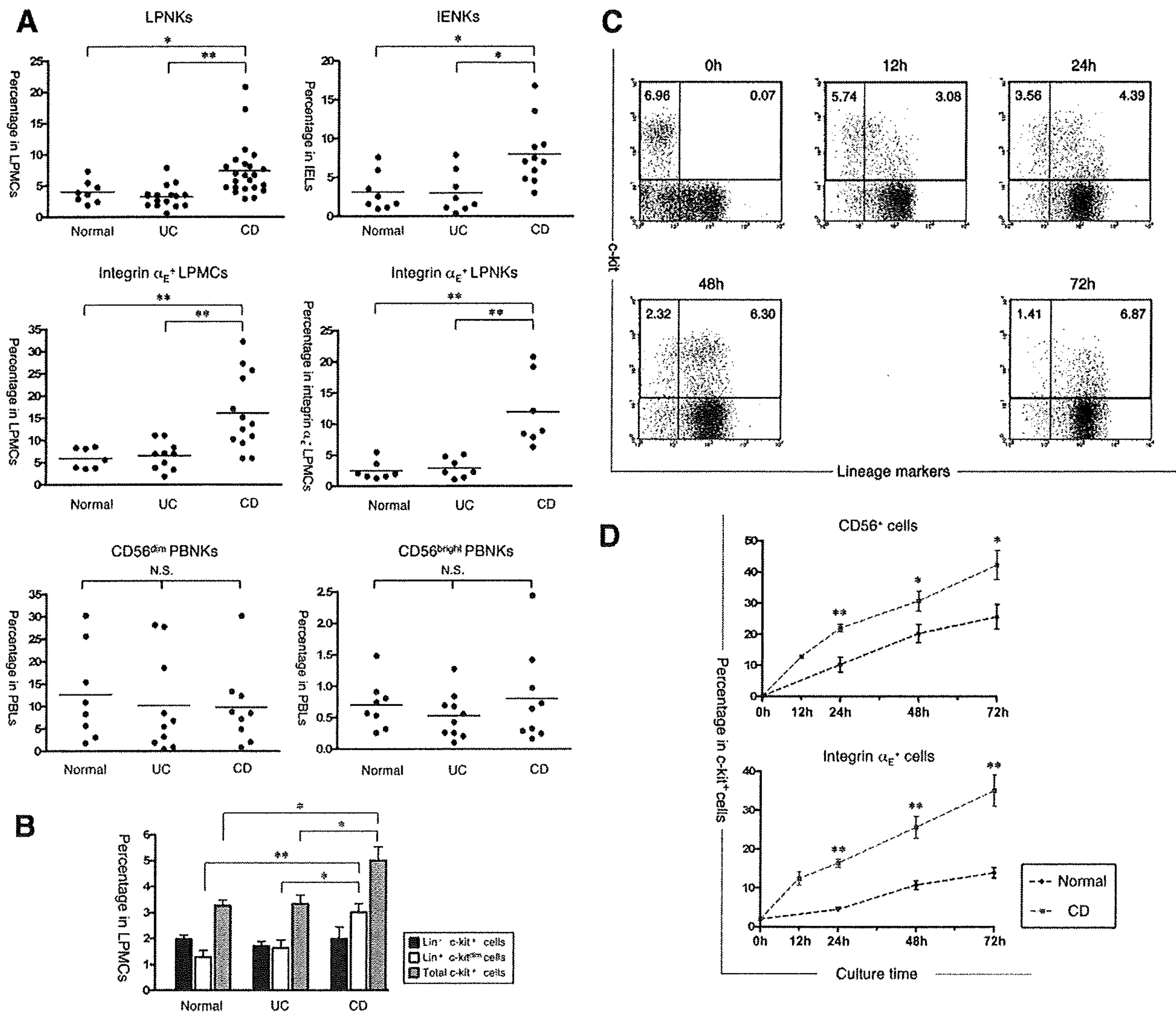


**Figure 8.** The c-kit$^{dim}$ cells, LPNKs, and IENKs are increased in CD. (*A*) Percentage of NK cells among LPMCs (normal, n = 10; UC, n = 17; CD, n = 23) or IELs (normal, n = 8; UC, n = 8; CD, n = 11) (upper panels). Percentage of integrin $\alpha_E\beta_7^+$ cells among LPMCs (normal, n = 7; UC, n = 10; CD, n = 13) or LPNKs (normal, n = 7; UC, n = 7; CD, n = 7) (middle panels). Percentage of CD56$^{dim}$ CD16$^+$ and CD56$^{bright}$ CD16– NK cells among PBLs (normal, n = 8; UC, n = 10; CD, n = 9) (bottom panels). Statistical analysis was performed with the Kruskal–Wallis 1-way analysis of variance, and the Bonferroni–Dunn test for multiple comparisons. $^*P < .05$, $^{**}P < .01$. (*B*) Percentage of total c-kit$^+$, lin$^-$ c-kit$^+$, or lin$^+$ c-kit$^{dim}$ cells among LPMCs (normal, n = 10; UC, n = 11; CD, n = 15). Results are expressed as means ± SEM. Statistical analysis was performed with the Kruskal–Wallis 1-way analysis of variance, and the Bonferroni–Dunn test for multiple comparisons. $^*P < .05$, $^{**}P < .01$. (*C*) LPMCs depleted of CD3 and CD56, obtained from CD patients, were cultured for 72 hours and analyzed for expression of c-kit and lin. The data shown are representative of 7 independent experiments. (*D*) The line graph shows the time–course changes for CD56+ or integrin $\alpha_E^+$ cells in c-kit$^+$ LPMCs from normal controls (n = 5) or CD patients (n = 7). The data are expressed as means ± SEM for the percentage of CD56$^+$ or integrin $\alpha_E^+$ cells among the c-kit$^+$ cells. Statistical analysis was performed with a 2-sided Mann–Whitney *U* test. $^*P < .05$, $^{**}P < .01$.

normal controls (Figure 8*A*). In contrast, the frequency of NK cells, both for CD56$^{dim}$ and CD56$^{bright}$, was similar in peripheral blood among the 3 groups (Figure 8*A*). Furthermore, although lin$^-$ c-kit$^+$ cells existed with similar frequency, the lin$^+$ c-kit$^{dim}$ cells were increased significantly in CD compared with UC or normal controls (Figure 8*B*). Then we repeated the in vitro culture experiments shown in Figure 5 and found that more lin$^+$ c-kit$^{dim}$ cells were detected in CD samples at each time point (Figure 8*C*), and the majority of these cells expressed CD56 and integrin $\alpha_E$ (Figure 8*D*). Taken together, the increase of LPNKs and IENKs in CD patients could have been owing to accelerated differentiation from lin$^-$ c-kit$^+$ cells. Given that the intestinal NK cells can strongly produce IFN-$\gamma$ and TNF-$\alpha$, which is a key cytokine in the pathogenesis of CD, these increased intestinal NK cells may play a pathogenic role in chronic inflammation in CD.

## Discussion

The sites of NK cell development in adults are understood poorly.[40,48] Although T/NKPs have been identified only in fetal tissues, the bone marrow is presumed to be the main site of NK cell generation in adults.[40,48] In this study, we have shown that lin$^-$ c-kit$^+$ cells in human adult intestine could differentiate into c-kit$^{dim}$ cells, which express CD56 during in vitro culture, suggesting that these cells are NK cell precursors. Moreover, further analysis showed that in vitro differentiated c-kit$^{dim}$ CD56$^+$ cells seemed to correspond to c-kit$^{dim}$ CD56$^+$ cells actually present in human adult intestine. Combined together, adult intestine may have unique NK cell differentiation system in which lin$^-$ c-kit$^+$ NK precursors undergo in situ differentiation via c-kit$^{dim}$ cells.

The newly discovered c-kit$^+$ cells in the human adult intestine also express CD34, another marker for HSCs or immune precursor cells.[17,28] In addition to c-kit and CD34, the intestinal immune precursors expressed CD38$^{dim}$, CD44, CD45RA, and Thy-1, the phenotypes of which correspond to those of common lymphoid progenitors or T/NKPs.[49] Furthermore, they had abundant mRNA transcripts for Id2, PU.1, SpiB1, and lymphotoxin, all of which are essential for HSC differentiation or NK cell development.

In the murine intestine, c-kit-expressing cells form small clusters named CP.[8] It has been reported that CP cells have the potential to differentiate mainly into extrathymic T cells in the intraepithelial space.[10] Interestingly, the intestinal c-kit$^+$ immune precursor cell expression level of RAG mRNA was very low, and the RAG expression level was similar to CP cells, which was reported previously by Oida et al.[50] Recent studies have shown that CD3$^-$ CD7$^+$ cells in the human fetal intestine express pT$\alpha$ mRNA[13] and give rise to CD3$^+$ T cells in vitro and in vivo, using SCID mice with engrafted human fetal intestine.[14] The intestinal c-kit$^+$ immune precursor cells are also CD3$^-$ CD7$^+$ and express RAG-1, RAG-2, and pT$\alpha$ mRNA. These results imply that the immune precursor cells in adult intestine include a subset similar or identical to the CD3$^-$ CD7$^+$ cells in the fetal intestine and they may differentiate into T cells in unusual environment, such as lymphopenia. However, in that they do not form aggregates and are much more committed to NK cells rather than T cells, they should be distinguished from the murine CP cells that differentiate into intraepithelial T cells. On the other hand, a recent study showed that c-kit$^+$ cells in CP represent LTi in adult mice, which organize isolated lymphoid follicles.[12] Furthermore, because LTi and T/NKPs have similar expression patterns of surface antigens and transcription factors,[51,52] they are considered to be subsets that are related closely to each other. Given that intestinal immune precursor cells also are similar to LTi in terms of surface antigen expression and transcriptional profile, it is possible that they contain an adult LTi subset.

Little information is available about intestinal NK cells. An earlier article reported on lymphokine activated killer activity in human LPMCs,[53] although they failed to identify NK cells in LPMCs, possibly because of the lack of suitable NK cell markers at that time. Some recent reports showed that human LPMCs and IELs contain NK cells capable of killing tumor cells and producing several cytokines, such as IFN-$\gamma$ and TNF-$\alpha$.[41,43] In this study, we intensively examined the intestinal NK cells to verify the hypothesis that they develop in situ from the immune precursor cells in intestinal lamina propria. In terms of NK cell markers, expression of CD56, as well as CD94, CD161, and NKG2D, was lowest in the c-kit$^+$ cells, and inversely highest in LPNKs/IENKs. In contrast, immature cell markers such as c-kit, IL-7R$\alpha$, and CD33 were highest in the intestinal immune precursor cells. Furthermore, these changes in surface marker expression also were observed during in vitro differentiation of lin$^-$ c-kit$^+$ cells into c-kit$^{dim}$ cells. Collectively, these results support the idea that intestinal immune precursor cells can give rise to intestinal NK cells via c-kit$^{dim}$ NKP-like cells.

PBNKs can be classified into 2 subsets. One subset is the conventional CD56$^{dim}$ NK cells and the other is the CD56$^{bright}$ NK cells.[43,44] Absence of CD16 expression is also a characteristic feature of the CD56$^{bright}$ NK cells. Although CD56 expression of the intestinal NK cells was not as high as the peripheral CD56$^{bright}$ NK cells, absence of CD16 expression indicates a similarity between the intestinal NK cells and the peripheral CD56$^{bright}$ NK cells. In addition, we found that both the intestinal NK cells, especially LPNKs, and peripheral CD56$^{bright}$ NK cells expressed CD33. Although CD33 is a myeloid lineage marker, it is reported that CD33$^+$ CD34$^+$ HSCs can give rise to CD16$^-$ NK cells in vitro.[54] Given that most intestinal immune precursor cells express CD33, and that intestinal NK cells are CD33$^+$ CD16$^-$, it is reasonable to assume that the intestinal NK cells may originate from the immune precursor cells. Furthermore, CD33 is reported to be expressed on CD56$^{bright}$ NK cells in umbilical cord blood[55] and on T/NKPs in the fetal thymus.[29] Although the origin of CD56$^{bright}$ NK cells still is controversial, it recently was reported that the peripheral CD56$^{bright}$ NK cells differentiate in the lymph nodes, unlike conventional CD56$^{dim}$ NK cells.[56] CD33$^+$ CD16$^-$ may be a phenotype that characterizes NK cells developing outside the bone marrow, such as lymph nodes, the thymus, and maybe the intestine.

The pathophysiologic contribution of intestinal NK cells to inflammatory bowel disease has yet to be elucidated. An interesting recent report suggested that the intestinal NK cells maintain homeostasis of intestinal mucosal immune system in mice. However, their roles have not been resolved in human beings.[57] We found that the differentiation of the intestinal immune precursor cells into NK cells was accelerated in CD, resulting in an increase in the number of intestinal NK cells in CD

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compared with UC or normal controls. According to the previous report, CD56$^{bright}$ NK cells also are enriched at inflammatory sites, such as arthritis, infectious pleuritis, and bacterial peritonitis.[58] CD is regarded as a typical T helper type 1 response (Th1) disease driven by excessive IFN-$\gamma$ production from dysregulated CD4 T cells infiltrating the inflamed tissue. However, given that NK cells constitute a considerable proportion of LPMCs or IELs (about 8%) and can highly produce IFN-$\gamma$, intestinal NK cells may contribute to the pathogenesis of CD. Overexpression of IFN-$\gamma$ in CD may modulate intestinal NK cell differentiation because it was reported that this cytokine accelerated differentiation of human HSCs.[59]

In summary, we have identified c-kit$^+$ immune precursor cells in the human adult intestine for the first time. We also have shown that these cells are committed mainly to the NK cell lineage. Because this intestinal NK cell differentiation system may contribute to the pathophysiology of CD, further clarification of the role of intestinal NK cells will help to better understand the gut immune system and may lead to new therapeutic strategies against CD.



## Appendix

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1053/j.gastro.2007.05.017.

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# Bone Marrow Retaining Colitogenic CD4+ T Cells May Be a Pathogenic Reservoir for Chronic Colitis

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BASIC-ALIMENTARY TRACT

**Background & Aims: Although bone marrow (BM) is known as a primary lymphoid organ, it also is known to harbor memory T cells, suggesting that this compartment is a preferential site for migration and/or selective retention of memory T cells. We here report the existence and the potential ability to induce colitis of the colitogenic BM CD4+ memory T cells in murine colitis models. Methods: We isolated BM CD4+ T cells obtained from colitic severe combined immunodeficient mice induced by the adoptive transfer of CD4+CD45RB$^{high}$ T cells and colitic interleukin (IL)-10$^{-/-}$ mice that develop colitis spontaneously, and analyzed the surface phenotype, cytokine production, and potential activity to induce colitis. Furthermore, we assessed the role of IL-7 to maintain the colitogenic BM CD4+ T cells. Results: A high number of CD4+ T cells reside in the BM of colitic severe combined immunodeficient mice and diseased IL-10$^{-/-}$ mice, and they retain significant potential to induce type-1 T helper-mediated colitis in an IL-7–dependent manner. These resident BM CD4+ T cells have an effector memory ($T_{EM}$; CD44$^{high}$CD62L$^{-}$IL-7R$^{high}$) phenotype and preferentially are attached to IL-7–producing BM cells. Furthermore, the accumulation of BM CD4+ $T_{EM}$ cells was decreased significantly in IL-7–deficient recipients reconstituted with the colitogenic lamina propria CD4+ $T_{EM}$ cells. Conclusions: Collectively, these findings suggest that BM-retaining colitogenic CD4+ memory T cells in colitic mice play a critical role as a reservoir for persisting lifelong colitis.**

It has long been known that T-cell precursors generated in the bone marrow (BM) migrate to the thymus, where T-cell development occurs. However, a fact often neglected is that under physiologic conditions, mature CD4+ and CD8+ T cells undergo extensive migration from the blood to the BM and vice versa. In both human beings and mice, T-cell receptor $\alpha\beta^+$ cells constitute approximately 3%–8% of nucleated BM cells.[1,2] BM CD4+ and CD8+ T-cell populations contain a high proportion of cells displaying a memory phenotype, that is, expressing low levels of CD45RA in human beings[3] and high levels of CD44 in mice.[4,5]

As early as 1974 it was documented that mouse CD4+ T cells migrate to the BM after priming, and it was proposed that BM CD4+ T cells contributed to the development of a memory antibody response in this organ.[6] Recently, T cells persisting in extralymphoid organs such as the liver, lung, and skin have attracted increasing interest because it has been recognized that these T cells contribute considerably to the long-lived memory T-cell pool.[7,8] In this context, BM has been shown to harbor a high number of antigen-specific CD8+ T cells for several months after resolution of acute infection.[9] For instance, adoptive transfer of BM cells from lymphochoriomeningitis virus–immune mice (>90 days after acute infection) to immunodeficient recipients provides antiviral protection, and thus CD8+ memory T cells from the BM are able to mount an effective secondary response.[10]

Primary T-cell responses to blood-borne antigens also can be initiated in the BM. This was shown initially in conditions of altered lymphocyte trafficking in splenectomized mice and then in individuals with normal lymphoid organs, for both CD4+ and CD8+ T-cell responses.[11] Thus, the BM resembles a secondary lymphoid organ, although it lacks the organized T- and B-cell areas found in the spleen, lymph nodes, and Peyer's patches. Although accumulating evidence suggests that BM plays an important role in the communication with mature naive/memory T cells, there is no evidence for the role of BM memory CD4+ cells in chronic immune diseases, such as inflammatory bowel diseases (ulcerative colitis and Crohn's disease) and autoimmune diseases. Crohn's disease is characterized by chronic inflammation of the small and large intestine and structures apart from the

*Abbreviations used in this paper:* Ag, antigen; APC, antigen-presenting cell; BM, bone marrow; BrdU, bromodeoxyuridine; CBA, cecal bacterial antigen; CSFE, carboxyfluoroscein succinimidyl ester; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; IFN, interferon; IL, interleukin; LP, lamina propria; mAb, monoclonal antibody; MLN, mesenteric lymph node; PE, phycoerythrin; SCID, severe combined immunodeficient; Th1, type-1 T helper.

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bowel. Surgery does not cure Crohn's disease, and recurrence after surgery is the rule rather than the exception.[12] There is also no correlation between recurrence of the disease and the dissection of regional lymph nodes and spleen.[13] The evidence suggests that other sites might play a critical role in the recurrence of diseases as reservoirs of colitogenic memory $CD4^+$ T cells.

Furthermore, it is well known that interleukin (IL)-7 is important as a critical factor for the survival and homeostatic proliferation of memory $CD4^+$ T cells, and that BM is a major site of IL-7 production.[14] We have shown previously that mucosal $CD4^+$ T cells in colitic mice express IL-7Rα highly, and they are pathogenic cells responsible for chronic colitis.[15] In vitro stimulation of these colitic lamina propria (LP) $CD4^+IL\text{-}7R^{high}$ T cells by IL-7, but not IL-15 and thymic stromal lymphopoietin, enhanced significant proliferative responses and survival of colitic $CD4^+$ T cells.[16] These backgrounds prompted us to investigate the role of the resident BM memory $CD4^+$ T cells in persisting lifelong colitis using a murine model of chronic colitis induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells.

## Materials and Methods

### *Mice*

Female BALB/c, CB-17 severe combined immunodeficient (SCID), and C57BL/6 mice were purchased from Japan Clea (Tokyo, Japan). Female C57BL/6 Rag-$2^{-/-}$ mice were provided by Central Laboratories for Experimental Animals (Kawasaki, Japan). C57BL/6 Rag-$1^{-/-}$ mice and IL-$7^{-/-}$ mice were kindly provided by Dr. Zamoyska (National Institute for Medical Research, London, UK).[17] IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice and littermate IL-$7^{+/+}$ × Rag-$1^{-/-}$ mice were generated in our laboratory. All mice were maintained under specific-pathogen-free conditions in the Animal Care Facility of the Tokyo Medical and Dental University. The Institutional Committee on Animal Research approved the experiments.

### *Antibodies and Flow Cytometry*

The following monoclonal antibodies (mAbs) other than biotin-conjugated anti-mouse IL-7Rα (A7R34; Immuno-Biological Laboratories, Takasaki, Japan) were obtained from BD PharMingen (San Diego, CA) and used for purification of cell populations and flow-cytometric analysis: Fcγ (CD16/CD32)-blocking mAb (2.4G2), phycoerythrin (PE)-, peridinin chlorophyll protein, and phycoerythrin-phycoerythrin- 5′- disulfonatoindodicarbocyanine conjugated anti-mouse CD4 (RM4-5); fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD3 (145-2C11); PE- and allophycocyanin-conjugated anti-mouse CD44 (IM7); FITC- and PE-conjugated anti-mouse CD62L (MEL-14); FITC-conjugated anti-mouse CD69 (H1.2F3); PE-conjugated anti-mouse integrin $\alpha_4\beta_7$ (DATK32); FITC-conjugated anti-mouse CD45RB (16A); FITC-conjugated hamster anti-mouse Bcl-2 (3F11); PE-conjugated streptavidin; biotin-conjugated rat IgG2; PE-conjugated mouse IgG; and PE-conjugated rat IgG. Flow cytometric 3-color analysis was performed as described.[18]

### *Induction of Colitis*

Colitis was induced in SCID/Rag-$2^{-/-}$ mice by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells as described.[18] Colitic mice were killed at 6–8 weeks after transfer, and $CD4^+$ T cells were isolated from BM, mesenteric lymph nodes (MLNs), and colonic LP.

### *Cytokine Enzyme-Linked Immunosorbent Assay*

To measure cytokine production, $3 \times 10^4$ $CD4^+$ T cells from MLN, LP, and BM were cultured in 200 μL of culture medium at 37°C in a humidified atmosphere containing 5% $CO_2$ in 96-well plates (Costar, Cambridge, MA) precoated with 5 μg/mL hamster anti-mouse CD3ε mAb (145-2C11; BD PharMingen) and 2 μg/mL hamster anti-mouse CD28 mAb (37.51; BD PharMingen) in phosphate-buffered saline (PBS) overnight at 4°C. Culture supernatants were removed after 48 hours and assayed for cytokine production. Cytokine concentrations were determined by specific enzyme-linked immunosorbent assay (ELISA) as per the manufacturer's recommendation (R&D, Minneapolis, MN).

### *Interferon-γ Production by $CD4^+$ T Cells Stimulated With APCs Pulsed With Cecal Extracts*

Colitic SCID mice were killed and their cecums were removed. The cecums were opened and placed in 1 mL of PBS, and the cecal bacteria were expelled by mixing with a vortex, and residual cecal tissue was removed. After the addition of DNase (10 μg/mL), 1 mL of this bacterial suspension was added to 1 mL of glass beads.[19] The cells were disrupted at 5000 revolutions per minute in a Mini-Bead Beater (BioSpec Products, Bartlesville, OK) for 3 minutes and then iced. The glass beads and unlysed cells were removed by centrifuging at 5000 × g for 5 minutes. The lysates were filter-processed in a similar manner. For antigen-presenting cells (APCs), spleen cells from normal BALB/c mice were prepared and treated with the appropriate concentration of cecal bacterial antigens (CBAs) as indicated at $2 \times 10^7$ cells/5 mL in a 15-mL tube overnight at 37°C. After washing twice, these APCs were treated with mitomycin-c before being added to T-cell cultures. BM, MLN, and LP $CD4^+$ T cells obtained from normal mice and colitic $CD4^+CD45RB^{high}$ T-cell–transferred SCID mice were cultured in the presence of APCs pretreated with cecal extract antigens in complete media. The culture supernatants were collected on day 3 of culture for interferon (IFN)-γ assay by ELISA.

BASIC-ALIMENTARY TRACT

*Bromodeoxyuridine Incorporation*

Colitic mice and age-matched normal BALB/c mice were given 1 mg of bromodeoxyuridine (BrdU) in PBS by intraperitoneal injection. Twenty-four hours later, mice were killed and the lymphocytes were prepared from BM, MLN, and colonic LP. Cells were first stained with PE-conjugated anti-CD4 mAbs for 2-color flow-cytometric analysis, or peridinin chlorophyll protein-conjugated anti-CD4 mAbs, APC-conjugated anti-CD44 mAbs, and PE-conjugated anti-CD62L mAbs for 4-color flow-cytometric analysis, and fixed and permeabilized with Cytofix-Cytoperm (BD PharMingen) solution according to the manufacturer's instructions. Cells were stained with FITC-conjugated anti-mouse BrdU (BD PharMingen) diluted in perm/wash buffer.

*Cell-Cycle Analysis*

A total of $1 \times 10^6$ cells from colitic mice induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells were stained for PE-conjugated anti-CD4 mAbs, and fixed and permeabilized with Cytofix-Cytoperm (BD PharMingen) solution according to the manufacturer's instructions. 7-AAD (10 $\mu$g/mL) and RNase (200 $\mu$g/mL) were added, and cells were incubated for 20 minutes at room temperature. Cells were acquired on a FACSCalibur (BD PharMingen) in their staining solution. Cell-cycle analysis of DNA histograms was performed with Cell Quest Software (BD PharMingen).

BASIC-
ALIMENTARY TRACT

*Immunohistochemistry*

Consecutive cryostat bone marrow sections (6 $\mu$m) were fixed and stained with the following rat antibodies: biotinylated CD4 (RM4-5) and polyclonal anti-IL-7 antibodies (R&D Laboratories). Alexa 594 goat anti-rat IgG, Alexa 488 goat anti-hamster IgG, and Alexa 488 rabbit anti-goat IgG (Molecular Probes, Eugene, OR) were used as second antibodies. All confocal microscopy was performed on a BioZERO BZ8000 (Keyence, Tokyo, Japan).

*Adoptive Transfer Experiments*

To assess the in vivo potential of the residual BM $CD4^+$ T cells in colitic SCID mice induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells to induce colitis, $CD4^+$ T cells ($1 \times 10^5$ cells/mouse) isolated from the BM, MLN, and LP of colitic mice or BM of age-matched normal BALB/c mice were injected into new SCID mice. In another set of experiments, BM $CD4^+$ T cells ($1 \times 10^5$ cells/mouse) isolated from colitic IL-$10^{-/-}$ mice (age, 20 wk) or age-matched normal C57BL/6 mice ($1 \times 10^5$ cells/mouse) were injected into C57BL/6 RAG$2^{-/-}$ mice. To assess the role of commensal bacteria in the development of colitis and the retention of colitogenic BM $CD4^+$ effector-memory T ($T_{EM}$) cells, we used broad-spectrum antibiotics in another adoptive transfer experiment. CB-17 SCID mice were treated with or without ampicillin (1 g/L; Sigma, St. Louis, MO), vancomycin (500 mg/L; Abbott Labs, Abbott Park, Illinois), neomycin sulfate (1 g/L; Pharmacia/Upjohn, New York, NY), and metronidazole (1 g/L; Sidmak, Gujarat, India) in drinking water 4 weeks before beginning the adoptive transfer and during the course of the development of colitis based on a variation of the commensal depletion protocol of Fagarasan et al.[20] All recipient mice were weighed initially, then 3 times/wk after the transfer. They then were observed for clinical signs of illness as previously described.[18]

*Adoptive Transfer Experiments Into IL-$7^{-/-}$ × Rag-$1^{-/-}$ Mice*

To assess the role of IL-7 in the maintenance of BM $CD4^+$ T cells, we further transferred LP $CD4^+$ T cells ($2 \times 10^6$ cells/mouse) isolated from colitic $CD4^+CD45RB^{high}$ T-cell-transferred mice into IL-$7^{-/-}$ × Rag-$1^{-/-}$ and IL-$7^{+/+}$ × Rag-$1^{-/-}$ mice. Mice were killed 5 days after transfer, and the spleen and BM cells were isolated and stained with PE-conjugated rat anti-CD3$\epsilon$ mAbs and FITC-conjugated rat anti-CD69 mAbs or isotype FITC-conjugated control antibody. Before staining for intracellular Bcl-2, cells ($2 \times 10^6$) were stained with PE-conjugated rat anti-CD3 mAbs as described earlier. After washing, cells were fixed and permeabilized with Cytofix-Cytoperm (BD PharMingen) solution according to the manufacturer's instructions. Cells were stained with either FITC-conjugated hamster anti-mouse Bcl-2 or a control antibody diluted in perm/wash buffer. To further assess the proliferative responses of $CD4^+$ T cells in IL-$7^{+/+}$ × Rag-$1^{-/-}$ and IL-$7^{-/-}$ × Rag-$1^{-/-}$ recipients, LP $CD4^+$ T cells from SCID mice with colitis induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells were labeled with carboxyfluoroscein succinimidyl ester (CFSE) (Molecular Probes) by incubating at 5 $\mu$mol/L in PBS, quenching with fetal calf serum, and washing with PBS 3 times. Cells were resuspended in PBS, and $3 \times 10^6$ total cells were transferred by intravenous injection into IL-$7^{+/+}$ × Rag-$1^{-/-}$ and IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice. In another set of experiments, we transferred with colitogenic BM $CD4^+$ T cells from colitic $CD4^+CD45RB^{high}$ T-cell-transferred Rag-$2^{-/-}$ mice into IL-$7^{+/+}$ × Rag-$1^{-/-}$ and IL-$7^{-/-}$ × Rag-$1^{-/-}$ recipients to clarify whether these mice develop colitis. Mice were killed at 10 weeks after transfer.

*Statistical Analysis*

The results were expressed as the mean ± SD. Groups of data were compared by the Mann–Whitney *U* test. Differences were considered statistically significant when the *P* value was less than .05.

## Results

*Effector Memory T Cells Reside in the BM of Colitic Mice*

To investigate the role of BM in consecutive immunopathology in immune-mediated diseases, we first compared the composition and phenotype of $CD4^+$ T

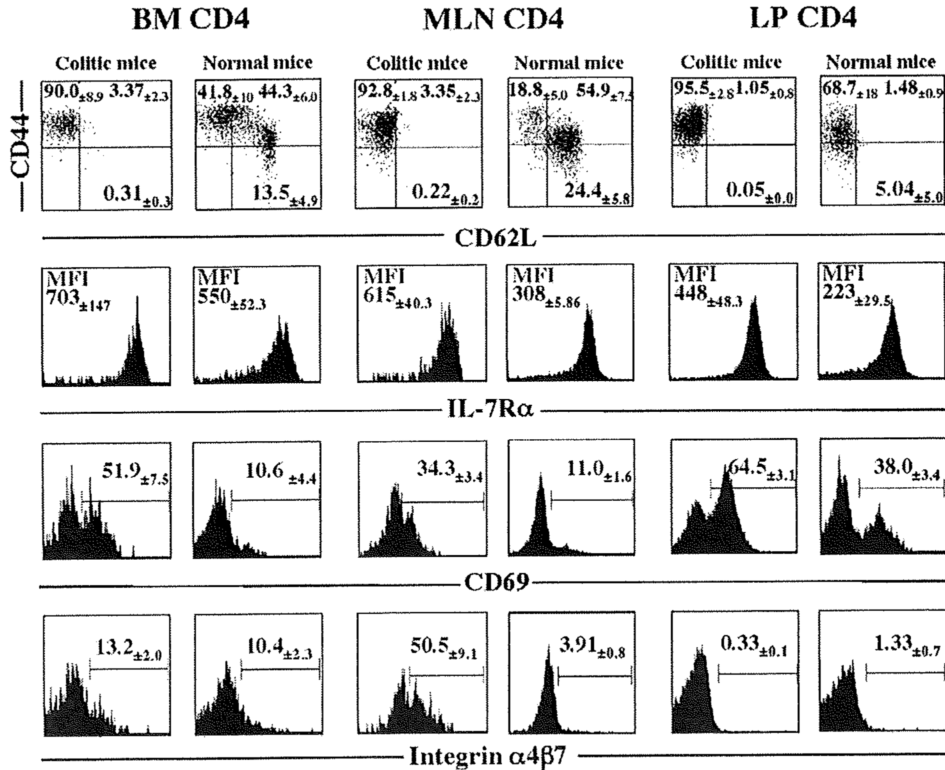


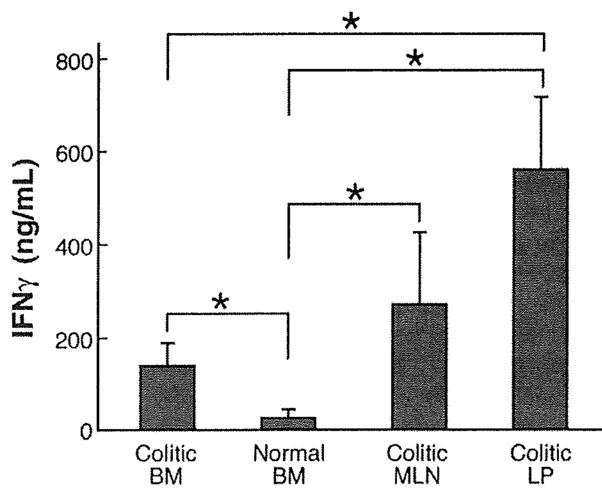

**Figure 1.** Colitic BM CD4$^+$ T cells are CD44$^{high}$CD62L$^-$IL-7Rα$^{high}$. Expression of CD44, CD62L, IL-7Rα (CD127), CD69, and integrin α4β7 on CD4$^+$ T cells obtained from spleen, MLN, LP, and BM in colitic mice induced by adoptive transfer of CD4$^+$CD45RB$^{high}$ T cells into CB-17 SCID mice (6 weeks after transfer) and normal BALB/c mice (age, 8 wk). Freshly isolated cells from colitic mice and normal BALB/c mice were stained with FITC-labeled anti-CD4, and PE-labeled anti-CD44, anti-CD62L, anti–IL-7Rα, anti-CD69, or anti-integrin α4β7 mAbs. Samples were analyzed by flow cytometry. Lymphocytes were identified by characteristic forward angle and side-scatter profiles. Data are displayed as a dotted plot (4-decade log scale) and quadrant markers were positioned to include more than 98% of control Ig-stained cells in the lower left. Percentages in each quadrant are indicated. Representative of 3 mice in each group.

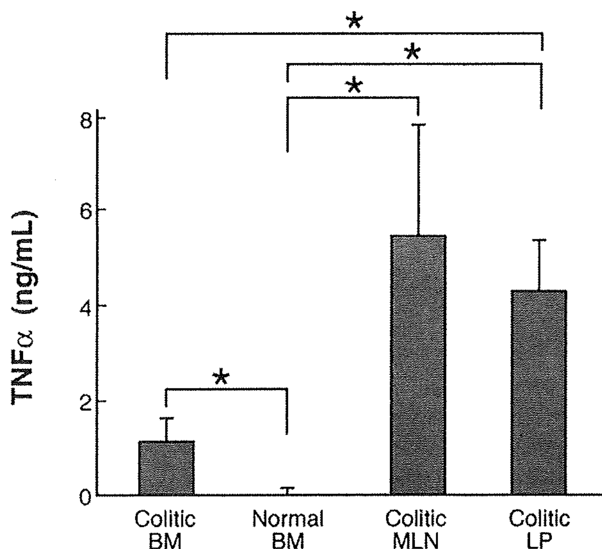
cells in BM, MLN, and colonic LP of colitic mice induced by the adoptive transfer of CD4$^+$CD45RB$^{high}$ T cells into recipient CB-17 SCID mice and with those of age-matched normal BALB/c mice. CD3$^+$CD4$^+$ mature T cells were found to reside in BM, MLN, and LP (colitic mice: BM, 12.7 ± 4.4 × $10^5$ per mouse; MLN, 7.01 ± 4.2 × $10^5$; and LP, 187 ± 99 × $10^5$; normal mice: BM, 16.6 ± 3.8 × $10^5$; MLN, 99.6 ± 18 × $10^5$; and LP, 4.17 ± 1.2 × $10^5$). As shown in Figure 1, the BM CD4$^+$ T cells, as well as MLN and LP CD4$^+$ T cells, from the colitic mice, exclusively have a phenotype of CD44$^{high}$CD62L$^-$ cells. Furthermore, these colitic BM CD4$^+$ T cells expressed IL-7Rα highly, indicating that the colitic BM CD4$^+$ T cells have a characteristic of $T_{EM}$ cells. In contrast, the BM CD4$^+$ T cells from normal mice are composed of 3 subpopulations: CD44$^{low}$CD62L$^+$ naive cells, CD44$^{high}$CD62L$^+$ central-memory T cells, and CD44$^{high}$CD62L$^-$ $T_{EM}$ cells (Figure 1). CD69, which is associated with cell activation, was expressed by a significantly higher proportion of CD4$^+$ T cells from colitic mice than from normal mice. Interestingly, BM CD4$^+$ T cells from colitic mice expressed relatively, but not significantly, high levels of integrin α4β7, a homing receptor to the gut, as compared with BM CD4$^+$ T cells from normal mice, but lower levels than did MLN CD4$^+$ T cells from colitic mice. These data indicate that the integrin α4β7-expressing CD4$^+$ memory T cells, which are instructed to express the molecule in MLN or Peyer's patches,[21,22] migrate to the BM.

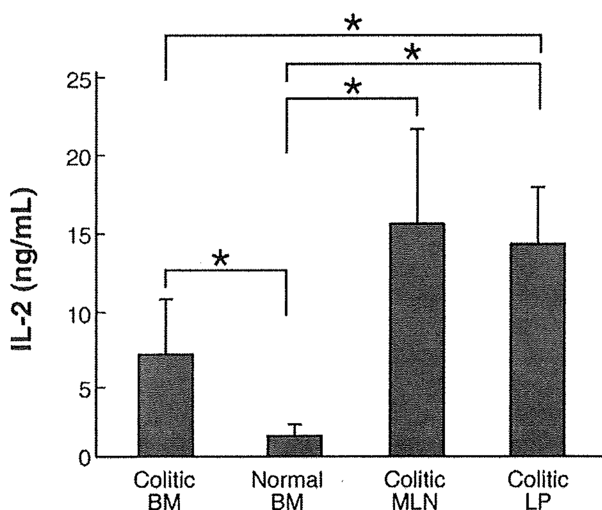
### *Colitic BM CD4$^+$ Memory T Cells Produce a Large Amount of Th1 Cytokines*

We next examined whether the colitic BM CD4$^+$ T cells retained the ability to produce type-1 T helper (Th1) cytokines as well as the colitic CD4$^+$ T cells in other sites. The production of IFN-γ, tumor necrosis factor-α, and IL-2 by anti-CD3/CD28 mAb-stimulated BM CD4$^+$ T cells from colitic mice was significantly higher than that by normal BM CD4$^+$ T cells, but lower than those by anti-CD3/CD28 mAb-stimulated LP CD4$^+$ T cells (Figure 2), indicating that the colitic BM CD4$^+$ T cells could be primed to Th1-type cells, and sustained in the BM.

To determine whether the BM CD4$^+$ T cells from colitic mice express their pathogenic potential on stim-






ulation with antigens derived from resident enteric bacteria, we examined in vitro IFN-γ secretion by normal and colitic BM, MLN, and LP $CD4^+$ T cells stimulated with various concentrations of CBA. The results show that significantly higher levels of IFN-γ were produced by colitic BM $CD4^+$ T cells in response to a high dose (1000 μg/mL) of CBA as compared with normal BM $CD4^+$ T cells, but significantly lower than those by colitic LP $CD4^+$ T cells, which responded to much lower concentrations (10, 100, 1000 μg/mL) of CBA (Figure 3). The similar result was obtained by paired samples of MLN (Figure 3) and splenic (data not shown) $CD4^+$ T cells. These results indicated that the colitic BM $CD4^+$ T cells have the potential to respond against bacterial antigens and thus have the possibility to be colitogenic similar to the colitic LP $CD4^+$ T cells as we have shown previously.[18]

### *IL-7–Expressing Cells are Scattered Throughout BM and Colocalized in Close Proximity to $CD4^+$ T Cells*

We next examined the distribution of IL-7-producing cells[23] and their interaction with $CD4^+$ T cells in the colitic BM. The IL-7-expressing cells were scattered throughout the BM as has been reported previously[24] and most $CD4^+$ T cells were in close contact with the bodies of IL-7-expressing cells (Figure 4). In contrast, IL-7 was not expressed, and $CD4^+$ T cells did not reside in the BM of IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice used as a negative control (Figure 4).

### *BM Contains the Most Actively Dividing Pool of $CD4^+$ T Cells*

To examine the homeostatic proliferation of the colitic BM $CD4^+$ T cells, 2 experimental approaches were used. First, we examined memory $CD4^+$ T cells from each tissue for evidence of active cell division by DNA staining using 7AAD (Figure 5*A*). Cells actively synthesizing DNA could be identified by their increased DNA content, allowing us to identify tissues where active cell division was occurring. A larger percentage of $CD4^+$ T cells was actively synthesizing DNA in both the colitic and normal BM than in any other tissues (Figure 5*A*). Although the difference was slight, it was reproducible over 3 independent experiments.

Second, colitic mice were injected with BrdU to provide evidence of recent DNA synthesis. To accurately examine the differences in cell proliferation in different tissues, it was necessary to give a short pulse of BrdU because

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**Figure 2.** Colitic BM $CD4^+$ T cells produce Th1 cytokines. Cytokine production by $CD4^+$ T cells. Isolated $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 mAbs for 48 hours. The indicated cytokines in these supernatants were measured by ELISA. Data are indicated as the mean ± SD of 7 mice in each group.

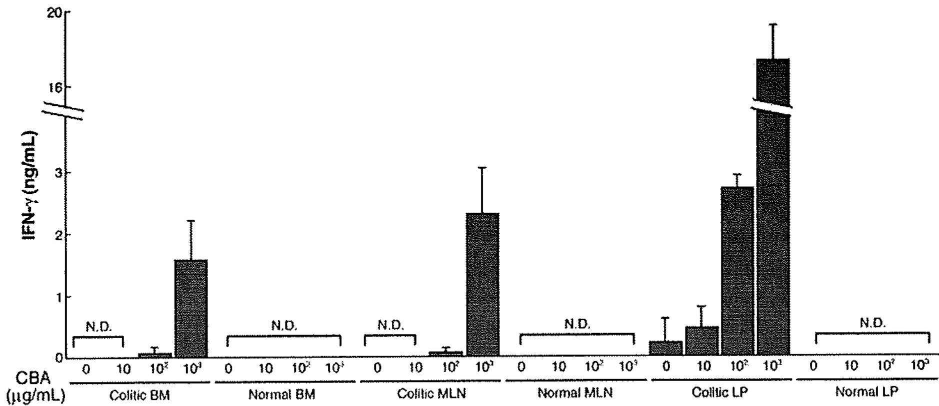


**Figure 3.** IFN-$\gamma$ production by $CD4^+$ T cells stimulated with APCs pulsed with CBA from colitic mice induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells. Supernatants collected on day 3 of culture were assayed for IFN-$\gamma$ by ELISA. Data are indicated as the mean ± SD of 5 mice in each group. $^*P < .05$. ND, not detected.

longer treatment with BrdU might obscure the differences among the various tissues, probably because of the migration of dividing cells among the tissues. Mice thus were killed 24 hours after the injection of BrdU, and BrdU incorporation was measured in the $CD4^+$ T cells obtained from BM, MLN, and LP (Figure *5B*). Significantly higher percentages of memory T cells were synthesizing DNA in the colitic BM, MLN, and LP as compared



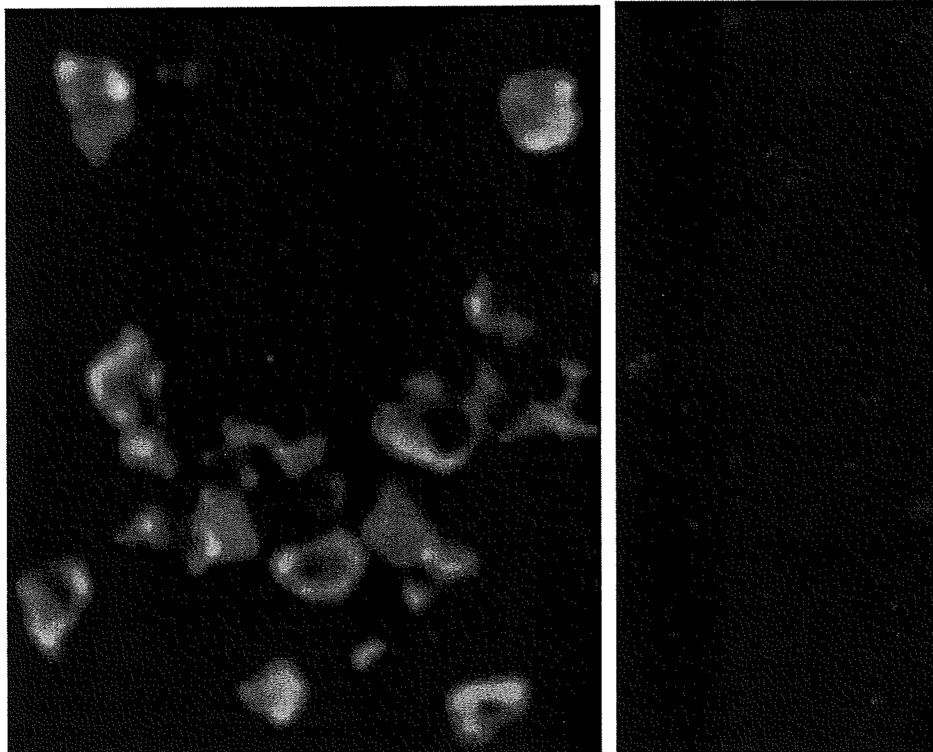


**Figure 4.** Cluster formation between $CD4^+$ T cells and IL-7–expressing stromal cells within BM. Frozen sections of BM from colitic mice induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells (*left*) and untreated IL-$7^{-/-}$ × Rag-$1^{-/-}$ control mice (*right*) were stained with corresponding monoclonal antibodies. The IL-7–expressing cells (*green*) are scattered uniformly throughout the BM $CD4^+$ T cells (*red*). $CD4^+$ T cells lie close to IL-7–expressing stromal cells.

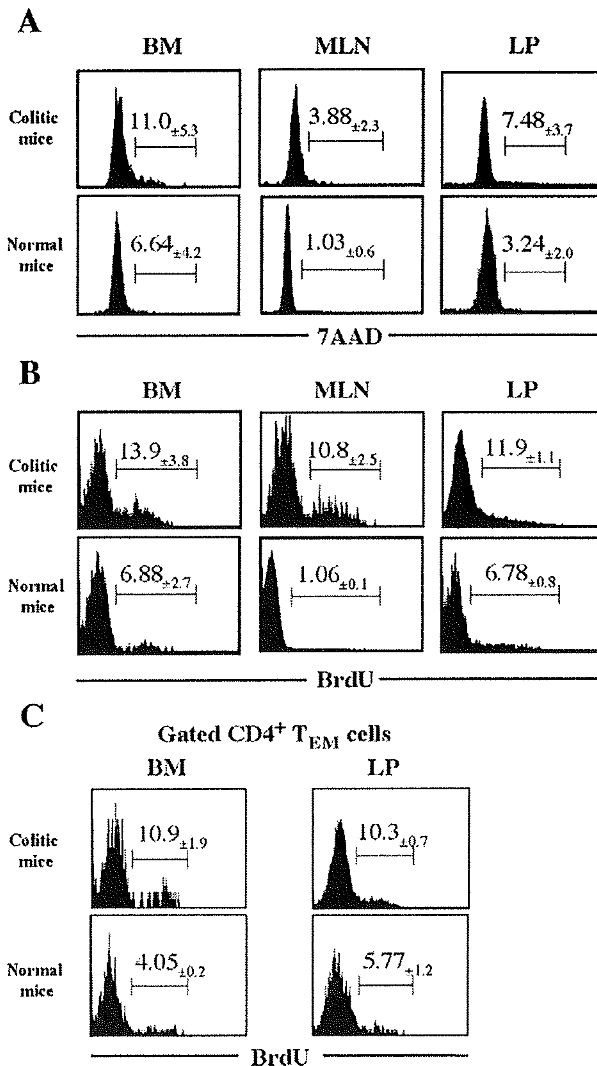


**Figure 5.** Colitic BM contains the actively dividing pool of memory $CD4^+$ T cells. (*A*) BM, MLN, and LP $CD4^+$ T cells from colitic mice or age-matched normal BALB/c mice were stained for DNA content using 7AAD. One representative mouse is shown of 5 mice analyzed. (*B*) Colitic mice and normal control mice were injected with BrdU for pulse-chase studies of BrdU incorporation. One representative mouse of 4 is shown. (*C*) Colitic mice and normal control mice were injected with BrdU as described in the Materials and Methods section. $CD4^+$ T cells were stained with CD4, CD44, and CD62L before intracellular staining for BrdU, and then the gated $CD4^+CD44^{high}CD62L^-$ $T_{EM}$ cells in the BM and LP from colitic and normal mice were assessed by the BrdU incorporation. One representative mouse of 3 is shown.

with those in the paired normal BM, MLN, and LP. Because we compared dissimilar subsets in this setting because normal BM contains all subsets, such as naive, central memory, and $T_{EM}$ $CD4^+$ T cells, yet in contrast colitic BM $CD4^+$ T cells are constituted of $T_{EM}$ cells exclusively (Figure 1), we next compared colitic BM and LP $CD4^+CD44^{high}CD62L^-$ $T_{EM}$ cells with the paired normal $T_{EM}$ cells. As shown in Figure 5*C*, DNA synthesis in colitic BM and LP $CD4^+CD44^{high}CD62L^-$ $T_{EM}$ cells was increased significantly as compared with that in the paired normal gated $T_{EM}$ cells (Figure 5*C*).

### *Transfer of the BM Memory $CD4^+$ T Cells From Colitic Mice Into SCID Mice Reproduce Th1-Mediated Colitis*

Based on the earlier-described results, we hypothesized that the colitic BM retaining $CD4^+$ $T_{EM}$ cells is a pathogenic reservoir for persisting lifelong colitis. To prove this, we performed an adoptive transfer experiment by transferring colitic BM, MLN, and LP $CD4^+$ $T_{EM}$ cells obtained from $CD4^+CD45RB^{high}$-transferred SCID mice and normal BM $CD4^+$ T cells into new SCID mice (Figure 6*A*). As shown in Figure 6*B*, mice transferred with the colitic BM, MLN, and LP $CD4^+$ T cells manifested progressive weight loss at 8 weeks after transfer. These mice had diarrhea with increased mucus in the stool, anorectal prolapse, and hunched posture by 4–6 weeks. In contrast, mice transferred with normal BM $CD4^+$ T cells appeared healthy, showing a gradual increase of body weight and no diarrhea during the period of observation (Figure 6*B* and *C*). At 8 weeks after transfer, colitic BM $CD4^+$ T-cell–transferred mice, but not mice transferred with normal BM $CD4^+$ T cells, had enlarged colons with greatly thickened walls (Figure 6*D*). The assessment of colitis by clinical scores showed a clear difference between mice transferred with colitic BM $CD4^+$ T cells and mice transferred with normal BM $CD4^+$ T cells (Figure 6*C*). In addition, the clinical scores of mice transferred with colitic BM $CD4^+$ T cells were comparable with those of mice transferred with colitic MLN or LP $CD4^+$ T cells. Histologic examination showed prominent epithelial hyperplasia with glandular elongation and massive infiltration of mononuclear cells in LP of the colon from colitic BM $CD4^+$ T-cell–transferred mice as well as colons from the colitic MLN or LP $CD4^+$ T-cell–transferred mice (Figure 6*E*). In contrast, pathologic findings were not observed in the LP of the colon from mice transferred with normal BM $CD4^+$ T cells (Figure 6*E*). This difference also was confirmed by histologic scoring of multiple colon sections (Figure 6*F*).

A further quantitative evaluation of $CD4^+$ T-cell accumulation was made by isolating $CD3^+CD4^+$ T cells. Few $CD3^+CD4^+$ T cells were recovered from the colonic LP in the normal BM $CD4^+$ T-cell–transferred mice as compared with the mice transferred with the colitic BM, MLN, or LP $CD4^+$ T cells (Figure 6*G*). Somewhat unexpectedly, the number of $CD4^+$ T cells recovered from the BM of normal BM $CD4^+$ T-cell–transferred mice was comparable with that from mice transferred with the colitic BM, MLN, or LP $CD4^+$ T cells (Figure 6*G*). Importantly, the number of $CD4^+$ cells recovered from the colitic BM $CD4^+$ T-cell–transferred mice far exceeded the number of cells originally injected ($1 \times 10^5$), indicating extensive T-cell migration and/or proliferation in each

tissue. We also examined the cytokine production by isolated LP $CD4^+$ T cells. As shown in Figure 6*H*, LP $CD4^+$ T cells from colitic BM $CD4^+$ T-cell–transferred mice produced significantly higher levels of IFN-$\gamma$ and tumor necrosis factor-$\alpha$ than those from normal BM $CD4^+$ T-cell–transferred mice on in vitro anti-CD3/anti-CD28 mAbs stimulation. In contrast, the production of IL-4 or IL-10 was not affected significantly (data not shown).

### *IL-7 Is Essential for the Survival and Homeostatic Proliferation of Colitogenic BM $CD4^+$ Memory T Cells*

To further analyze the role of IL-7 in the survival and homeostatic proliferation of the colitogenic BM $CD4^+$ T cells, we retransferred CFSE-labeled LP $CD4^+$ T cells obtained from $CD4^+CD45RB^{high}$ T-cell–transferred colitic mice into IL-$7^{+/+}$ × Rag-$1^{-/-}$ and IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice (Figure 7*A*). Rapid proliferation of donor colitic LP $CD4^+$ T cells was observed in the BM from IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice 5 days after the transfer, although the relative size of the expanded T-cell populations in IL-$7^{-/-}$ × Rag-$1^{-/-}$ BM $CD4^+$ T cells was approximately 80% of that observed in the control IL-$7^{+/+}$ × Rag-$1^{-/-}$ BM $CD4^+$ T cells (Figure 7*B*). Somewhat unexpectedly, however, the recovered cell numbers of the BM and spleen $CD4^+$ T cells from IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice were strikingly lower than those from IL-$7^{+/+}$ × Rag-$1^{-/-}$ mice (BM: IL-$7^{-/-}$ × Rag-$1^{-/-}$ 2.3 ± 1.9 × $10^5$; IL-$7^{+/+}$ × Rag-$1^{-/-}$ mice, 45 ± 19 × $10^5$; spleen: IL-$7^{-/-}$ × Rag-$1^{-/-}$ 3.8 ± .1 × $10^5$; IL-$7^{+/+}$ × Rag-$1^{-/-}$ mice, 32 ± 13 × $10^5$) (Figure 7*C*), indicating that the IL-7 was essential for the survival rather than the homeostatic proliferation of the colitogenic $CD4^+$ T cells in the BM. Consistent with this notion, we next assessed if regulation of Bcl-2 requires IL-7 at day 5 after the transfer, because induction of the anti-apoptotic protein, Bcl-2, is a hallmark of responses to IL-7.[14] As expected, the BM $CD4^+$ T cells in IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice expressed lower levels of Bcl-2 than those in IL-$7^{+/+}$ × Rag-$1^{-/-}$ mice (Figure 7*D*). Furthermore, the cell activation marker CD69 also was down-modulated significantly on the BM $CD4^+$ T cells in IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice as compared with those in IL-$7^{+/+}$ × Rag-$1^{-/-}$ mice (Figure 7*E*).

Finally, we asked whether adoptive transfer of colitogenic BM $CD4^+$ T cells into IL-$7^{-/-}$ × Rag-$1^{-/-}$ or IL-$7^{+/+}$ × Rag-$1^{-/-}$ mice induces colitis and results in the retention of BM $CD4^+$ T cells (Figure 8*A*). Expectedly, transfer of colitogenic BM $CD4^+$ T cells into the control IL-$7^{+/+}$ × Rag-$1^{-/-}$ mice led to a severe wasting disease 4–6 weeks after transfer, but IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice transferred with colitogenic BM $CD4^+$ T cells appeared healthy and continued to gain weight during 10 weeks of observation (data not shown). The clinical score of IL-$7^{-/-}$ × Rag-$1^{-/-}$ recipients was almost zero, and significantly lower than that of IL-$7^{+/+}$ × Rag-$1^{-/-}$ recipients at 10 weeks after transfer (Figure 8*B*). The colon, the spleen, and the MLN from IL-$7^{+/+}$ × Rag-$1^{-/-}$ recipients, but not those from IL-$7^{-/-}$ × Rag-$1^{-/-}$ recipients, were enlarged and had a greatly thickened wall of colon (Figure 8*C*). Consistent with the lack of clinical signs in IL-$7^{-/-}$ × Rag-$1^{-/-}$ recipients, they displayed no histologic evidence of intestinal inflammation in contrast to IL-$7^{+/+}$ × Rag-$1^{-/-}$ recipients with severe inflammation (Figure 8*D*). Histologic analysis of colonic mucosa showed development of severe colitis in IL-$7^{+/+}$ × Rag-$1^{-/-}$, but not in IL-$7^{-/-}$ × Rag-$1^{-/-}$, recipients (Figure 8*E*). The total cell numbers of isolated BM, MLN, and LP $CD3^+CD4^+$ T cells from IL-$7^{-/-}$ × Rag-$1^{-/-}$ recipients were significantly lower than those from IL-$7^{+/+}$ × Rag-$1^{-/-}$ recipients (Figure 8*F*). Collectively, these results indicated that IL-7 is essential to develop colitis for colitogenic BM $CD4^+$ T cells and to sustain these cells in the BM and in the LP and the MLN.

### *SCID Mice Transferred With $CD4^+CD45RB^{high}$ and Administered With Broad-Spectrum Antibiotics Did Not Develop Colitis, but Retained $CD4^+$ $T_{EM}$ in BM*

It generally is accepted that colitis-inducing $CD4^+CD45RB^{high}$ T cells recognize bacterial and/or self-antigens that are induced by the presence of intestinal bacteria, and germ-free conditions prevent the development of intestinal inflammation in many animal models of colitis including the $CD4^+CD45RB^{high}$ T-cell–transfer model.[25] We therefore assessed whether SCID mice transferred with $CD4^+CD45RB^{high}$ T cells and treated with or without oral administration of a mixture of antibiotics (vancomycin, neomycin, metronidazole, and ampicillin) develop colitis and the persistence of BM $CD4^+$ T cells (supplemental Figure 1*A*; supplementary material online at www.gastrojournal.org). As expected, we found that SCID mice transferred with $CD4^+CD45RB^{high}$ T cells without oral administration of antibiotics developed wasting disease (supplemental Figure 1*B*) and severe colitis (supplemental Figure 1*C*), whereas those with administration of antibiotics did not develop wasting disease and colitis 4 weeks after transfer (supplemental Figures 1*B* and *C*). The blinded histologic score of mice treated with antibiotics was almost zero in contrast to control recipient mice without administration of antibiotics (6.2 ± 1.3) (supplemental Figure 1*D*). The average number of $CD3^+CD4^+$ T cells recovered from recipient mice that transferred with $CD4^+CD45RB^{high}$ T cells and given drinking water without antibiotics was 11.0 ± 0.7 × $10^5$ per mouse in BM, 52 ± 20 × $10^5$ in MLN, and 240 ± 40 × $10^5$ in LP (supplemental Figure 1*E*). In contrast, the cell number in mice transferred with $CD4^+CD45RB^{high}$ T cells and treated with antibiotics was decreased significantly compared with mice transferred with $CD4^+CD45RB^{high}$ T cells and given the antibiotics (BM, 2.2 ± 1.8 × $10^5$ per mouse; spleen, 11 ± 11 × $10^5$;

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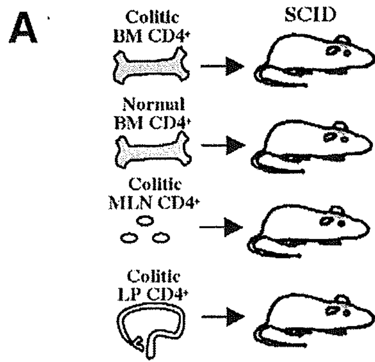


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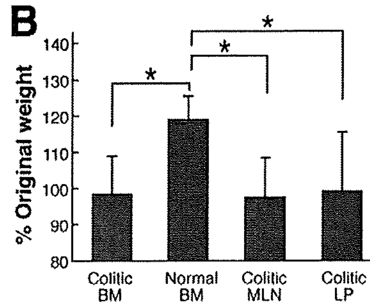


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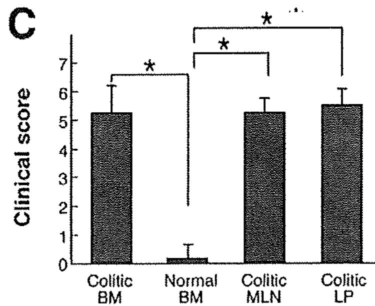


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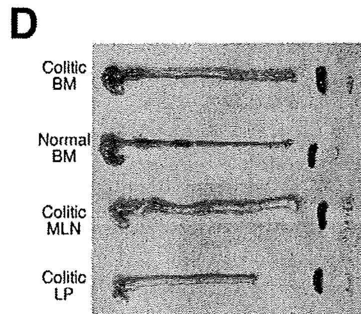


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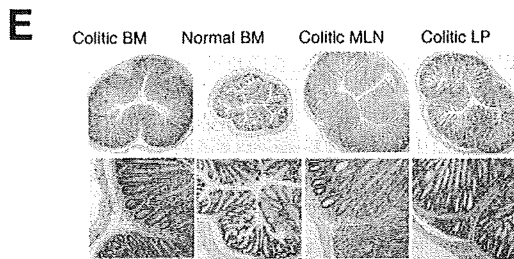


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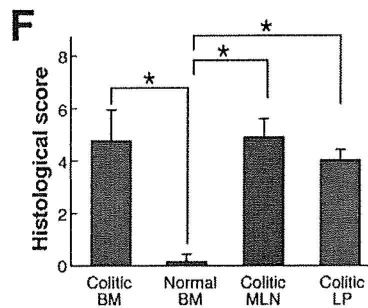


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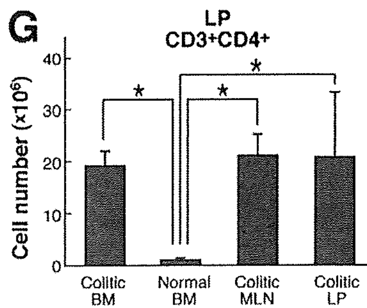


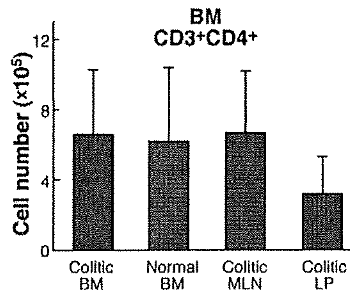


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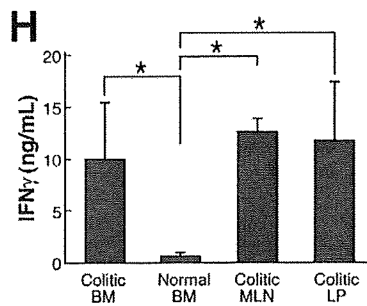


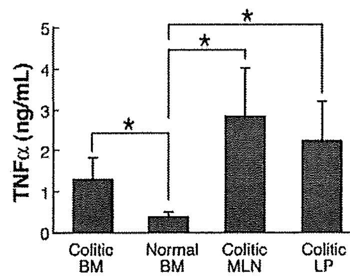


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and LP, 28 ± 24 × $10^5$) (supplemental Figure 1*E*). Therefore, the administration of antibiotics significantly suppressed colitis and resulted in the reduced expansion of BM $CD3^+CD4^+$ T cells and MLN and LP.

### *Transfer of BM $CD4^+$ T Cells From Colitic IL-10–Deficient Mice, but not Normal Mice, Into Rag-$2^{-/-}$ Mice Reproduces Th1-Mediated Colitis*

We finally addressed whether latent colitogenic $CD4^+$ T cells reside in the BM in a colitis model that develops colitis spontaneously, rather than the adoptive transfer model, in this case, IL-$10^{-/-}$ mice[26] (supplemental Figure 2*A*; supplementary material online at www.gastrojournal.org). We first isolated the BM $CD4^+$ T cells from diseased IL-$10^{-/-}$ mice and age-matched normal C57BL/6 mice, and analyzed the expression of CD44 and CD62L on $CD4^+$ T cells by flow cytometry. Similar to the BM $CD4^+$ T cells in colitic mice induced by the adoptive transfer of $CD4^+CD45RB^{high}$, $CD4^+CD44^{high}CD62L^-$ $T_{EM}$ cells preferentially resided in the BM of colitic IL-$10^{-/-}$ mice as compared with age-matched normal C57BL/6 mice (supplemental Figure 2*B*, upper). We next transferred the BM $CD4^+$ T cells from diseased IL-$10^{-/-}$ mice and age-matched normal C57BL/6 mice into recipient C57BL/6 Rag-$2^{-/-}$ mice (supplemental Figure 2*A*). Mice transferred with the colitic IL-$10^{-/-}$ BM $CD4^+$ T cells manifested progressive weight loss (wasting disease) at 10 weeks after transfer as compared with the mice transferred with normal C57BL/6 BM $CD4^+$ T cells (data not shown). These mice had significant clinical symptoms by 4–6 weeks after transfer, but mice transferred with normal BM $CD4^+$ T cells appeared healthy without diarrhea during the whole period of observation. The assessment of colitis by clinical scores showed a clear difference between the mice transferred with colitic IL-$10^{-/-}$ BM $CD4^+$ T cells and the mice transferred with normal BM $CD4^+$ T cells (supplemental Figure 2*C*). At 10 weeks after transfer, the colitic IL-$10^{-/-}$ BM $CD4^+$ T-cell–transferred mice, but not those transferred with normal BM $CD4^+$ T cells, had enlarged colons with greatly thickened walls (supplemental Figure 2*D*). Histologic examination showed severe signs of colitis, including epithelial hyperplasia and massive infiltration of mononuclear cells, in LP from the colitic IL-$10^{-/-}$ BM $CD4^+$ T-cell–transferred mice as compared with the colons from the normal BM $CD4^+$ T-cell–transferred mice (supplemental Figure 2*E*). This difference also was confirmed by histologic scoring of multiple colon sections (supplemental Figure 2*F*). Furthermore, few $CD4^+$ T cells were recovered from the colonic LP in the normal BM $CD4^+$ T-cell–transferred mice as compared with the mice transferred with the colitic IL-$10^{-/-}$ BM $CD4^+$ T cells (supplemental Figure 2*G*). As in the model of $CD4^+CD45RB^{high}$ T-cell–transferred colitis, the number of recovered BM $CD4^+$ T cells from the normal BM $CD4^+$ T-cell–transferred mice was comparable with that from mice transferred with the colitic IL-$10^{-/-}$ BM (supplemental Figure 2*G*). We finally examined the cytokine production by isolated LP $CD4^+$ T cells. LP $CD4^+$ T cells from the normal BM $CD4^+$ T-cell–transferred mice produced significantly less IFN-$\gamma$ and tumor necrosis factor-$\alpha$ than those from the colitic IL-$10^{-/-}$ $CD4^+$ T-cell–transferred mice on in vitro stimulation (supplemental Figure 2*H*). These results suggested that the colitic IL-$10^{-/-}$ BM $CD4^+$ T cells have potent colitogenic $CD4^+$ T cells to reproduce Th1-mediated colitis in normal recipient SCID mice.

## Discussion

In the present study, we showed that $CD4^+CD44^{high}CD62L^-IL\text{-}7R\alpha^{high}$ $T_{EM}$ cells, but not central-memory T cells and naive T cells, preferentially reside in the BM obtained from Th1-mediated colitic SCID/Rag-$2^{-/-}$ mice induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells. Importantly, these resident BM $CD4^+$ $T_{EM}$ cells are attached closely to IL-7–producing stromal cells in the BM, and retain significant potential to induce colitis by the adoptive retransfer into new SCID/Rag-$2^{-/-}$ mice. Of particular importance, we showed here that IL-7 is essential for the development of colitis induced by the adoptive transfer of colitogenic BM $CD4^+$ $T_{EM}$ cells using IL-$7^{-/-}$ × Rag-$1^{-/-}$ and the control IL-$7^{+/+}$ × Rag-$1^{-/-}$ mice. Furthermore, the accumulation

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**Figure 6.** SCID mice transferred with the BM $CD4^+$ T cells obtained from $CD4^+CD45RB^{high}$ T-cell–transferred colitis develop chronic colitis. (*A*) CB-17 SCID mice were injected intraperitoneally with normal splenic $CD4^+CD45RB^{high}$ T cells. Six weeks after transfer mice developed chronic colitis, and $CD4^+$ T cells were isolated from each organ. Doses of 2 × $10^5$ BM, MLN, or LP $CD4^+$ T cells were injected into new CB-17 SCID mice. As a negative control, 2 × $10^5$ BM $CD4^+$ T cells obtained from normal BALB/c mice also were injected into SCID mice. (*B*) Mice transferred with the colitic BM $CD4^+$ T cells did not gain weight. *$P < .05$. (*C*) Mice transferred with the colitic BM $CD4^+$ T cells showed severe clinical signs of colitis. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .05$. (*D*) Gross appearance of the colon, spleen, and MLN from mice transferred with the colitic BM $CD4^+$ T cells (*first row*), the normal BM $CD4^+$ T cells (*second row*), the colitic MLN $CD4^+$ T cells (*third row*), or LP $CD4^+$ T cells (*fourth row*). (*E*) Histopathologic comparison of distal colon from mice injected with the colitic BM, the normal BM, the colitic MLN, or the colitic LP $CD4^+$ T cells. Original magnification: *upper*, 40×; *lower*, 100×. (*F*) Histologic scores were determined at 8 weeks after transfer as described in the Materials and Methods section. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .05$. (*G*) LP and BM $CD4^+$ T cells were isolated from mice injected with colitic BM, normal BM, colitic MLN, or colitic LP $CD4^+$ T cells 8 weeks after transfer, and the number of $CD3^+CD4^+$ cells was determined by flow cytometry. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .05$. (*H*) Cytokine production by LP $CD4^+$ T cells. IFN-$\gamma$ and tumor necrosis factor-$\alpha$ concentrations in culture supernatants were measured by ELISA. Data are indicated as the mean ± SD of 6 mice in each group. *$P < .05$.

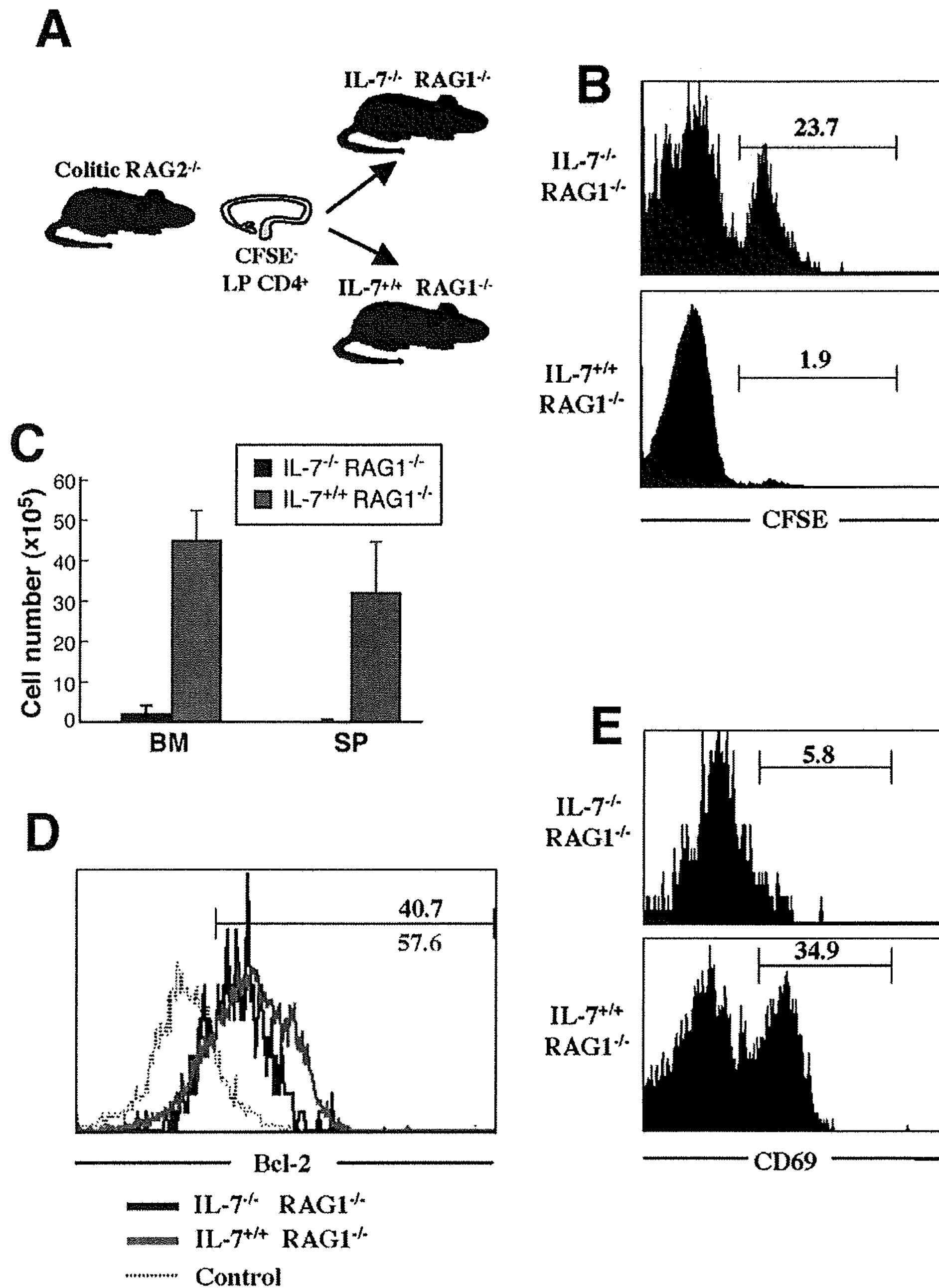


**Figure 7.** IL-7 is essential for the survival and in part for the cell turnover of colitogenic BM CD4+ T cells. (*A*) C57BL/6 Rag-2-/- mice were injected intraperitoneally with normal splenic CD4+CD45RBhigh T cells. Six weeks after transfer, the LP CD4+ T cells were isolated. Colitogenic LP CD4+ T cells were labeled with CFSE and adoptively transferred into new IL-7+/+ × Rag1-/- or IL-7-/- × Rag1-/- mice. Five days after transfer, CFSE incorporation was determined by flow cytometry. Histograms are gated on CD3+ T cells. (*C*) The BM and spleen (SP) CD4+ T cells were isolated from IL-7+/+ × Rag1-/- or IL-7-/- × Rag1-/- mice injected with the colitic LP CD4+ T cells 5 days after transfer, and the number of CD4+ cells was determined by flow cytometry. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .05$. (*D*) Representative flow-cytometric histograms showing the expression of Bcl-2 in BM CD4+ T cells from IL-7+/+ × Rag1-/- or IL-7-/- × Rag1-/- mice injected with the colitogenic LP CD4+ T cells 5 days after transfer from 3 independent similar experiments. (*E*) Representative flow-cytometric histograms showing the expression of CD69 on BM CD4+ T cells from IL-7+/+ × Rag1-/- or IL-7-/- × Rag1-/- mice injected with the colitogenic LP CD4+ T cells 5 days after transfer from 3 independent similar experiments.

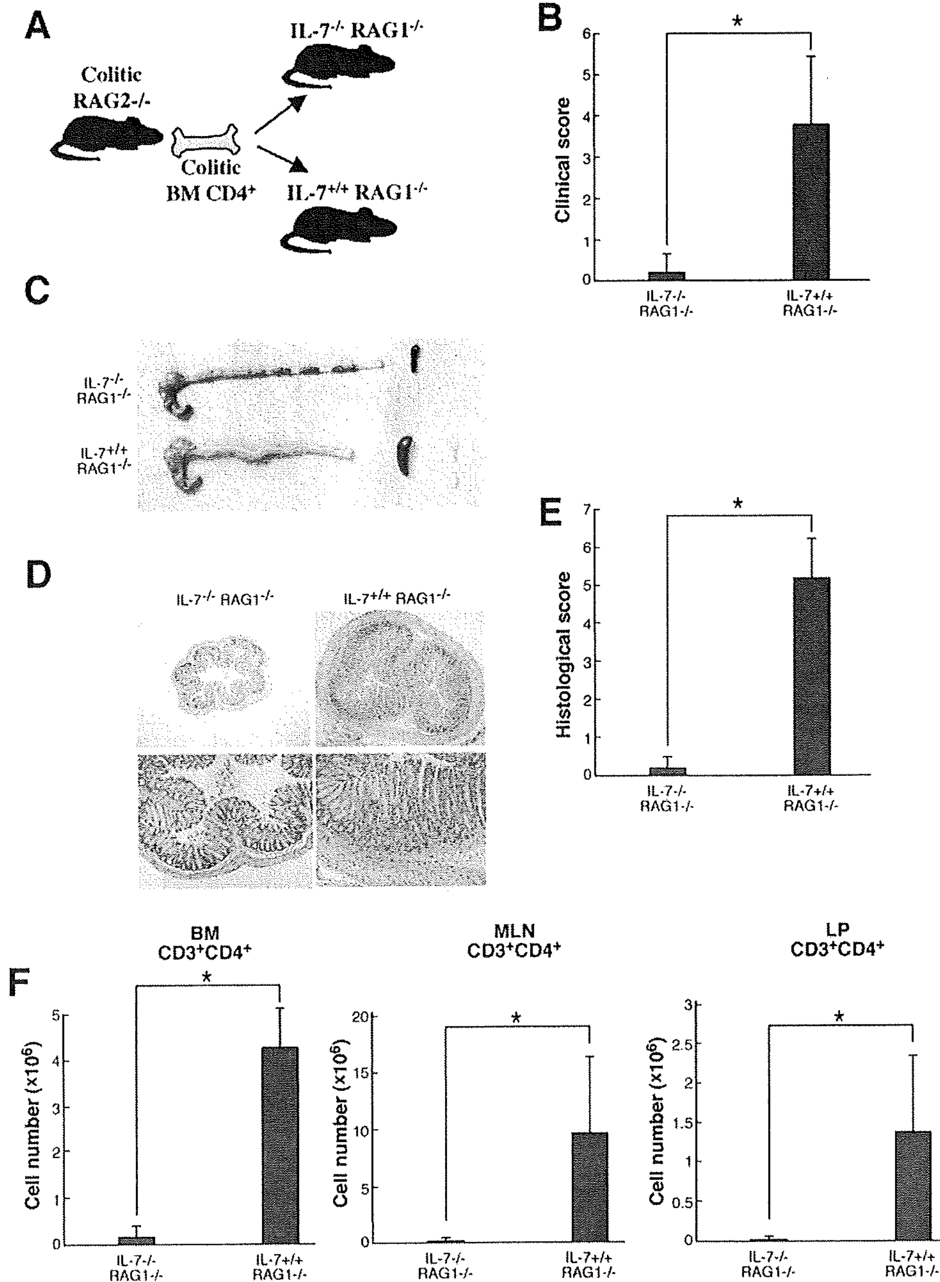


**Figure 8.** IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice transferred with colitogenic BM CD4$^+$CD44$^{high}$CD62L$^-$ $T_{EM}$ cells did not develop colitis. (*A*) IL-$7^{+/+}$ × Rag-$1^{-/-}$ (n = 5) and IL-$7^{-/-}$ × Rag-$1^{-/-}$ (n = 5) mice were transferred with colitic BM CD4$^+$ T cells. (*B*) Clinical scores were determined 10 weeks after transfer. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .005$. (*C*) Gross appearance of the colon, spleen, and MLN from IL-$7^{-/-}$ × Rag-$1^{-/-}$ (*top*) and IL-$7^{+/+}$ × Rag-$1^{-/-}$ (*bottom*) recipients 10 weeks after transfer. (*D*) Histologic examination of the colon from IL-$7^{-/-}$ × RAG-$1^{-/-}$ and IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice transferred with colitogenic BM CD4$^+$ T cells 10 weeks after transfer. Original magnification: *upper*, 40×; lower, 100×. (*E*) Histologic scoring of IL-$7^{+/+}$ × Rag-$1^{-/-}$ and IL-$7^{-/-}$ × Rag-$1^{-/-}$ recipients 10 weeks after transfer. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .005$. (*F*) BM, LP, and spleen cells were isolated from IL-$7^{+/+}$ × Rag-$1^{-/-}$ and IL-$7^{-/-}$ × Rag-$1^{-/-}$ recipients 10 weeks after transfer, and the number of CD3$^+$CD4$^+$ cells was determined by flow cytometry. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .0005$.

of BM CD4$^+$ T cells was decreased significantly in IL-7–deficient recipients reconstituted with the colitogenic LP CD4$^+$ $T_{EM}$ cells. Collectively, these findings suggest that the BM CD4$^+$ $T_{EM}$ cells residing in mice with chronic colitis play a critical role as a reservoir for persisting lifelong colitis in an IL-7–dependent manner.

The present data raise the most important question of whether the colitogenic BM CD4$^+$CD44$^{high}$CD62L$^-$ T

cells can be defined as $T_{EM}$ cells rather than effector T cells in the presence of antigens (Ags), in this case, probably intestinal bacteria. First, we found that these colitogenic BM CD4+ T cells highly expressed IL-7Rα in accordance with the evidence that IL-7Rα is one of memory, but not effector, T-cell markers. Second, it is well known that memory, but not effector, CD4+ T cells are critically controlled by the homeostatic proliferation and the survival by IL-7.[14] Consistent with this, we found that the BM CD4+ T cells were decreased markedly in $IL\text{-}7^{-/-} \times Rag\text{-}1^{-/-}$ mice transferred with the colitogenic LP or BM CD4+ T cells as compared with $IL\text{-}7^{+/+} \times Rag\text{-}1^{-/-}$ recipients. Further, we showed that $IL\text{-}7^{-/-} \times Rag\text{-}1^{-/-}$ mice transferred with the colitogenic BM CD4+ T cells did not develop colitis in contrast to $IL\text{-}7^{+/+} \times Rag\text{-}1^{-/-}$ recipients with colitis. Collectively, these data indicate that the colitogenic BM CD4+ T cells in our colitis model are $T_{EM}$ cells rather than effector T cells.

IL-7 originally was discovered in the BM stromal cells.[23] However, the role for CD4+ T cells in the BM is largely unknown, especially in pathologic conditions, although it has been recognized recently that a high number of antigen-specific CD8+ memory T cells persist in the BM for several months after resolution of acute viral infection.[7,8] Furthermore, recent accumulating evidence suggests that IL-7 is a critical factor for the survival and homeostatic proliferation of memory CD4+ T cells.[14] Thus, we hypothesized that IL-7–producing BM harbors the colitogenic memory CD4+ T cells as a reservoir, causing persistent lifelong colitis. Consistent with this hypothesis, we found that IL-7–expressing cells were scattered throughout the BM and most CD4+ T cells were in close contact with the bodies of IL-7–expressing BM cells in colitic SCID mice induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells (Figure 5). However, the possibility cannot be excluded of a recently described novel pathway for dendritic cell migration that allows dendritic cells to collect Ags in peripheral sites and traffic them to the BM to elicit recall responses by the resident BM T cells.[27] This, however, is unlikely in this case because the production of IFN-γ by anti-CD3/CD28- or CBA-stimulated colitic BM CD4+ T cells was significantly lower than that of anti-CD3/CD28- or CBA-stimulated colitic LP CD4+ T cells (Figures 2 and 3), indicating that the BM colitogenic T cells in colitic mice might be indicative of a recent encounter with Ags in the LP, and may migrate into the BM, which is abundant in IL-7, but not in Ags.

In this article we asked how CD4+ memory T cells accumulate in the BM in mice with chronic colitis. Indeed, BM stromal cells can support lymphoid precursor cell differentiation into mature T cells in vitro[28] and in athymic mice in vivo.[29] Mature T cells in the BM are probably immigrants from the blood because T cells normally are produced in the thymus. However, the mechanisms by which in vivo–generated memory cell subsets are recruited to tissues have been difficult to study in the case of polyclonal and physiologic systems rather than the monoclonal T-cell receptor transgenic system because such studies require unattainable numbers of purified cells for in vivo assay. In this study, however, we were able to circumvent this obstacle by using the $SCID/Rag\text{-}2^{-/-}$-colitis model induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells because a large number of CD4+ T cells infiltrated the colonic LP in this model, and they technically could be isolated in the order of approximately $1 \times 10^7$ cells per mouse. By using the present adoptive transfer system, we found that CD4+ T cells resided in the BM from $Rag\text{-}1^{-/-}$ mice transferred with colitogenic LP CD4+ T cells at the early time point of 5 days after transfer (Figure 7). We also found that the recovered cell number of BM CD4+ T cells was parallel to that of LP CD4+ T cells in mice given antibiotics without colitis and the control mice with colitis. These results indicate that colitogenic LP CD4+ T cells exit from the gut, and directly migrate into the BM, (Supplemental Figure 1, see supplemental material online at www.gastrojournal.org although further studies will be needed to show direct evidence for this issue.

Although the Ags driving the T-cell immune response in the experimental system of T-cell–induced IBD have not yet been identified with certainty, and thus it is impossible to chase the biological behavior of antigen-specific T cells, overwhelming evidence supports the idea that the triggering factor in this experimental system is of bacterial origin. Furthermore, the present study significantly complements recent reports that BM harbors Ag-specific memory CD8+ T cells.[2,30–31] A recent report has shown very efficient interactions between T cells and dendritic cells in the BM microenvironment.[11] It may be that the similar environment that promotes T-cell priming also triggers homeostatic proliferation and survival of the colitogenic BM $T_{EM}$ cells by IL-7. Perhaps, as has been suggested for plasma cells and Ag-specific CD8+ memory T cells, a unique combination of the cytokine milieu including IL-7 and contact-dependent interactions in the BM supports the colitogenic BM $T_{EM}$ cells. Furthermore, the possibility that other sites, such as MLN and spleen, also might play a role as other reservoirs for colitogenic CD4+ $T_{EM}$ cells, as well as the BM in colitic mice, cannot be excluded. Further studies will be needed to address this issue.

In conclusion, our findings show that a proportion of colitogenic CD4+ T cells in colitic mice may leave peripheral tissues, such as LP and MLN, and gain access to the IL-7–abundant BM via the bloodstream. By using adoptive transfer protocols, we have shown that these BM CD4+ $T_{EM}$ cells possess the ability to induce colitis, suggesting that the colitogenic BM CD4+ T cells residing in colitic mice play a critical role as a reservoir for persisting lifelong colitis and participate in relapses after remissions in IBDs.[17]

## Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi;10.1053/j.gastro.2006.10.035.

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# Reciprocal Targeting of Hath1 and β-Catenin by Wnt Glycogen Synthase Kinase 3β in Human Colon Cancer

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***Background & Aims:*** **The transcription factor Hath1 plays a crucial role in the differentiation program of the human gut epithelium. The present study was conducted to investigate the molecular mechanism of Hath1 expression and its close association with β-catenin/glycogen synthase kinase 3β (GSK3β) under the Wnt pathway in human colonocytes.** ***Methods:*** **Tissue distribution of Hath1 messenger RNA in human tissues was examined by Northern blot. Stability of Hath1 protein was analyzed by expression of FLAG-tagged Hath1 in human cell lines. Targeting of Hath1 protein by GSK3β was determined by specific inhibition of GSK-3β function. Expression of Hath1 protein in colorectal cancers was examined by immunohistochemistry.** ***Results:*** **Hath1 messenger RNA expression was confined to the lower gastrointestinal tract in human adult tissues. In colon cancer cells, although Hath1 messenger RNA was also detected, Hath1 protein was positively degradated by proteasome-mediated proteolysis. Surprisingly, the GSK3β-dependent protein degradation was switched between Hath1 and β-catenin by Wnt signaling, leading to the dramatic alteration of cell status between proliferation and differentiation, respectively. Hath1 protein was detected exclusively in normal colon tissues but not in cancer tissues, where nuclear-localized β-catenin was present.** ***Conclusions:*** **The present study suggests a novel function of the canonical Wnt signaling in human colon cancer cells, regulating cell proliferation and differentiation by GSK3β-mediated, reciprocal degradation of β-catenin or Hath1, respectively, which further emphasizes the importance of aberrant Wnt signaling in colonocyte transformation.**

The gut epithelium undergoes continual renewal throughout adult life, maintaining the proper architecture and function of the intestinal crypts. This process involves highly coordinated regulation of the induction of cellular differentiation and the cessation of proliferation, and vice versa.[1–3] The intestinal epithelium consists of cells of 4 lineages: goblet cells, enteroendocrine cells, Paneth cells, and enterocytes.[4] Cellular differentiation into the former 3 lineages is believed to be regulated by a basic helix-loop-helix transcription factor called "Math1" in mice and "Hath1" in humans (officially termed as "ATOH1"). Math1 and Hath1 are known to play crucial roles in differentiation of various cells in other tissues, such as dorsal interneurons in the spinal cord,[5] granule cells in the cerebellum,[6] Merkel cells in the skin,[7] and inner hair cells in the auditory systems.[8]

In mice intestine, the *Math1* gene promotes the differentiation of epithelial cells to secretory lineage cells without affecting absorptive cell differentiation and is expressed in Ki-67–positive proliferating cells of the crypt, indicating a role of Math1 at an early stage of lineage commitment.[9] Expression of Math1 seems to be regulated at its transcriptional level, because forced expression of Notch intracellular domain in murine intestinal epithelial cells causes a decrease of Math1 messenger RNA (mRNA) expression and subsequent depletion of goblet cells in vivo.[10] Conversely, depletion of Hes1, another basic helix-loop-helix transcription factor known as a downstream target of Notch intracellular domain, up-regulates Math1 mRNA expression in murine intestine.[11] Thus, it is likely that *Math1* gene expression is regulated at the mRNA level by Notch signaling, leading to subsequent control of intestinal epithelial cell lineage decision of the crypt cells. It was recently reported that Hath1, a human homologue of Math1, up-regulates gastric mucin gene expression in gastric cells[12]; however, the regulation of Hath1 expression is less understood in human intestine.

The canonical Wnt signaling is another signaling pathway known to regulate cell differentiation and proliferation of the intestinal crypt cells.[13] It is believed that Wnt proteins induce inactivation of glycogen synthase kinase 3β (GSK3β), a component of the so-called destruction complex that also contains adenomatous polyposis coli (APC) and Axin, and the resultant dephosphorylation and stabilization of its substrate β-catenin leads to the transcription of genes targeted by the nuclear β-catenin/

*Abbreviations used in this paper:* APC, adenomatous polyposis coli; EGFP, enhanced green fluorescent protein; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GSK3β, glycogen synthase kinase 3b; RIPA, radioimmunoprecipitation assay; RT-PCR, reverse-transcription polymerase chain reaction; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA; TCF, T-cell factor.

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T-cell factor (TCF) complex.[14,15] However, in intestinal cells, it has not been shown whether activation of Wnt signaling simply inactivates general kinase activity of GSK3β or could possibly change the substrate specificity instead of kinase activity, thereby stabilizing the β-catenin protein. Constitutive activation of Wnt signaling is assumed to be essential for both continuous proliferation and maintenance of the undifferentiated state in intestinal stem cells.[16,17] Of note, the biological impact of the Wnt pathway lies in its close association with the carcinogenesis of colorectal cancer. Mutations that perturb the assembly or function of the destruction complex, such as truncation of APC, are present in approximately more than 90% of colorectal tumors. These mutations lead to constitutive activation of Wnt signaling, and the downstream genes that are transcriptionally up-regulated by the β-catenin/TCF complex are implicated in the growth-promoting properties of the tumor cells.[15,18] However, it has not been well understood how constitutive Wnt signaling could maintain colorectal cancer cells at an undifferentiated state.

A previous study reported that inhibition of Wnt signaling in a human colon cancer–derived cell line, HT-29, up-regulated both *Hath1* and *MUC2* gene mRNA expression.[19] This suggested that Hath1 expression may be suppressed at the mRNA level by the aberrant Wnt signaling, thereby maintaining the undifferentiated state of colorectal cancer cells. However, in the same study, it was also suggested that some colorectal cancers did express Hath1 mRNA at an amount comparable to the neighboring normal colon tissue but maintained an undifferentiated state at the same time.

These data prompted us to prove that *Hath1* gene function is regulated by the aberrant Wnt signaling, not only by the mRNA level but also by an unknown posttranscriptional or posttranslational mechanism in human colon cancer cells. Here, we present the evidence that Hath1 protein expression is regulated by Wnt signaling via GSK3β-mediated protein degradation. Our results suggest that the reciprocal regulation of Hath1 and β-catenin protein stability is mediated by GSK3β, which functions as a molecular switch regulating the proliferation and differentiation of colon cancer cells in vitro and in vivo. These results present a novel function of the Wnt-GSK3β pathway and further emphasize the importance of aberrant Wnt signaling in colonocyte transformation.

## Materials and Methods

### *Cell Culture*

Human colon cancer–derived SW480, DLD-1, and HT-29 cells and human embryonic kidney-derived 293T cells were grown in Dulbecco's modified Eagle medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. In all experiments, $1 \times 10^6$ cells were seeded onto 6-cm culture dishes 36 hours before the experiment. All transfection experiments of DNA constructs and small interfering RNA (siRNA) oligonucleotides were performed by using TransIT transfection reagent (Mirus, Madison, WI) according to the manufacturer's instructions.

### *DNA Constructs*

pcDNA3-Myc-ubiquitin[20] was a kind gift from Dr K. Tanaka (Tokyo Metropolitan Institute, Tokyo, Japan). pMX-IRES-GFP[21] was a kind gift from Dr T. Kitamura (University of Tokyo, Tokyo, Japan). Series of expression vectors encoding mutants for *APC* genes (pCS2-APC2, -APC3, and -APC25)[22] and a pRL5-Wnt1[23] were kind gifts from Dr H Shibuya (Tokyo Medical and Dental University, Tokyo, Japan). Expression plasmids encoding N-terminally Flag-tagged WT-Hath1 (pCMV-Flag-WT-Hath1) or enhanced green fluorescent protein (EGFP) (pCMV-Flag-EGFP) were generated by inserting the polymerase chain reaction (PCR)-amplified *Hath1* gene or *EGFP* gene, respectively, into the *Eco*RI/*Bam*HI site of a pCMV-Flag vector (Stratagene, La Jolla, CA) in frame. Plasmids for various mutants that lack either the N- or C-terminal region of Hath1 (N1-5, C1, and C2 mutants; Figure 3*A*) and the mutant 54/58SA-Hath1, in which both 54S and 58S are substituted to alanines, were constructed by PCR-mediated mutagenesis by using pCMV-Flag-WT-Hath1 as a starting material. pMX-Flag-WT-Hath1-IRES-GFP was generated by inserting a fragment encoding the N-terminally Flag-tagged *Hath1* gene, which was amplified by PCR using pCMV-Flag-WT-Hath1 as a template, into the pMX-IRES-GFP vector. A reporter plasmid E-box Luc was generated by inserting a 77–base pair oligonucleotide containing 7 repeats of the E-box (kE sites) (AGGCAGGTGGC) into an *Sma*I site of the pTA-Luc vector (Clontech, Mountain View, CA). Reporter plasmids TOPflash and FOPflash were obtained from Upstate Biotechnology (Charlottesville, VA). All plasmids constructed were verified by sequencing.

### *Immunoblottings and Immunoprecipitations*

Cells were transfected with 1 μg of pCMV-Flag vector (control), pCMV-Flag-EGFP, pCMV-Flag-Hath1, or various mutants of pCMV-Flag-Hath1. In cotransfection experiments, 1 μg of either pcDNA3-Myc-ubiquitin or one of the expression plasmids for mutant APC (pCS2-APC2, -APC25, or -APC3) or pRL5-Wnt1 was transfected along with 1 μg of pCMV-Flag vector (control) or pCMV-Flag-Hath1. In each cotransfection experiment, the total amount of DNA was equalized by adding the appropriate amount of empty expression vector. After 12 hours of transfection, cells were cultured for 12 hours under the usual conditions or in the presence of 10 μmol/L lactacystin (Calbiochem, San Diego, CA), 10 μmol/L MG132 (Calbiochem), 5 μmol/L calpain inhibitor (Calbiochem),

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