Stimulation of interferon-γ production by CD11b$^+$ macrophages and CD4$^+$ T cells

To assess the effect of IL-12 and IL-18 on interferon-γ (IFN-γ) production by splenic CD11b$^+$ macrophages and CD4$^+$ T cells,[15] 1×10^5 cells per well were cultured in the presence or absence of either IL-12 (10 ng/mL) or IL-18 (100 ng/mL) in a total volume of 0.2 mL in 96-well round plates for 72 h. At the end of each culture period, supernatant was collected. The IFN-γ production was determined by specific ELISA according to the manufacturer's instructions (R&D, Minneapolis, MN, USA).

Proliferation assays

Proliferation assays[15] were performed by culturing splenocytes (1×10^5 per well) in 96-well round plates for 72 h in the presence of saporin-conjugated anti-CD11b mAb (1 μg/mL), a mixture of free anti-CD11b mAb and saporin, or PBS alone. After incubation, the cultures were pulsed for 7 h with [^{3}H]-thymidine (1.85×10^4 Bq/well; New England Nuclear, Boston, MA, USA), harvested on glass fiber filters, and radioactivity was counted (in counts per minute) in a liquid scintillation system.

Reverse transcription–polymerase chain reaction for cytokine expression

The expressions of IFN-γ and TNF-α were determined by reverse transcription (RT) of total RNA, followed by polymerase chain reaction (PCR).[16] Total RNA was isolated from the colonic tissue by the acid guanidium thiocyanate–phenol chloroform extraction method. The cDNA were synthesized by incubating 5 μg total RNA with 600 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD, USA) in the presence of 0.05 U oligo (dT) primers (Pharmacia, Piscataway, NJ, USA), 3 mg acetylated BSA (Life Technologies), and 40 U RNase inhibitor (Promega, Madison, WI, USA) in a volume of 30 mL for 1 h at 37°C and then for 5 min at 96°C to stop the reaction. The PCR of the cDNA was performed in a final volume of 50 μL containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.001% (w/v) gelatin, 0.2 mmol/L dNTP (Pharmacia), 1.25 U ampliTaq DNA polymerase (Perkin-Elmer, Branchburg, NJ, USA), and each primer at 0.5 μmol/L, using the GeneAmp 2400 PCR system (Perkin-Elmer). The amplification cycles were 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min. The PCR products were separated by electrophoresis on 1.5% agarose gel after 30 cycles for IFN-γ, TNF-α and glyceraldehyde-3-phosphate dehydrogenase (G3PDH), and were visualized by ethidium bromide staining. The following primers were used: IFN-γ sense, 5′-GAA-AGC-CTA-GAA-AGT-CTG-AAT-AAC-T-3′; IFN-γ antisense, 5′-ATC-AGC-AGC-GAC-TCC-TTT-TCC-GCT-T-3′; TNF-α sense, 5′-ATG-AGC-ACA-GAA-AGC-ATG-ATC-CGC-3′; TNF-α antisense, 5′-CCA-AAG-TAG-ACC-TGC-CCG-GAC-TC-3′; and G3PDH sense, 5′-CGG-TGC-TGA-GTA-TGT-CGT-GGA-GTC-T-3′; G3PDH antisense, 5′-GTT-ATT-ATG-GGG-GTC-TGG-GAT-GGA-A-3′.

Statistical analysis

Significant differences between the two groups were determined using the Mann–Whitney *U*-test. $P < 0.05$ was considered to be statistically significant.

Results

Infiltrating CD11b-expressing macrophages in colitic mice

To assess the involvement of macrophages in the development of chronic colitis, we used the Th1-type model induced by the adoptive transfer of CD4$^+$CD45RB^{high} T cells to SCID mice, which is considered to be a representative T-cell-mediated colitis model. We first examined the expression of CD11b and CD11c in the colitic mice by immunohistochemical analysis. As shown in Fig. 1(a), CD11b$^+$ macrophages and CD11c$^+$ DC from the colitic mice were markedly increased in the LP as compared with normal mice. Similarly, flow cytometric analysis in macrophage gate

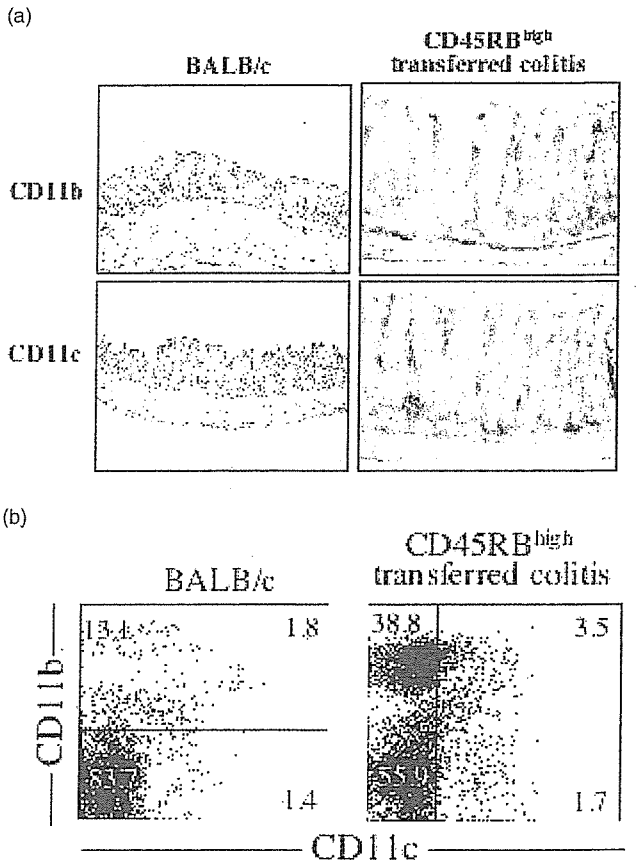

Figure 1 Expression of CD11b and CD11c on lamina propria mononuclear cells (LPMC) and splenocytes. (a) Immunohistochemical analysis of CD11b$^+$ and CD11c$^+$ cells in the colons. Immunohistochemical analysis showed the markedly increased CD11b$^+$ and CD11c$^+$ cell infiltration to colonic tissue in colitic mice at 4 weeks after the T-cell transfer. (b) Freshly isolated splenocytes from colitic mice and normal BALB/c mice were stained with fluorescein isothiocyanate (FITC)-labeled anti-CD11b and phycoerythrin (PE)-labeled anti-CD11c. Samples were analyzed by FACSCalibur. The subpopulation for macrophages/dendritic cells was identified by characteristic forward angle and side scatter profiles. Data are displayed as dotted plot (4-decade log scale) and quadrant markers were positioned to include >98% of control Ig-stained cells in the lower left. Representatives of three mice in each group.

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revealed that $CD11b^+CD11c^-$ macrophages, and $CD11b^+CD11c^+$ and $CD11b^-CD11c^+$ DC were significantly increased in spleens from colitic mice as compared with those from normal BALB/c mice (Fig. 1b).

$CD11b^+$ macrophages produce a large amount of IFN-γ on stimulation by IL-12 and IL-18

Because pro-inflammatory cytokines, such as IL-12 and IL-18, are critically involved in the initial immune response of Th1-mediated diseases, we next analyzed the induction of IFN-γ production *in vitro* by exogenously added IL-12 and/or IL-18 in normal splenic BALB/c $CD4^+$ T cells and $CD11b^+$ macrophages. Although both cells produced little IFN-γ in the presence of IL-12 or IL-18 alone, they produced comparably large amounts of IFN-γ when stimulated by the cytokines in combination (Fig. 2), indicating that macrophages also play a critical role in the Th1-mediated immune response in the early stage of the development of colitis.

Targeting of $CD11b^+$ macrophages by saporin-conjugated anti-CD11b mAb *in vitro*

Given the increased number of macrophages and the associated potential for IFN-γ production, we were interested to address the functional role of macrophages in this model of colitis. First, we examined the effect of saporin-conjugated anti-CD11b mAb on the function of LPMC *in vitro*. To this end, proliferation assays were performed by culturing splenocytes from normal mice and colitic mice induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells in the presence of saporin-conjugated anti-CD11b mAb, a mixture of free anti-CD11b mAb and saporin, or PBS alone. Proliferative responses of splenocytes from normal mice (Fig. 3a) and colitic mice (Fig. 3b) with concanavalin A (Con A) in the presence of saporin-conjugated anti-CD11b mAb were significantly decreased as compared with those in the presence of a mixture of free anti-CD11b mAb and saporin, or PBS alone.

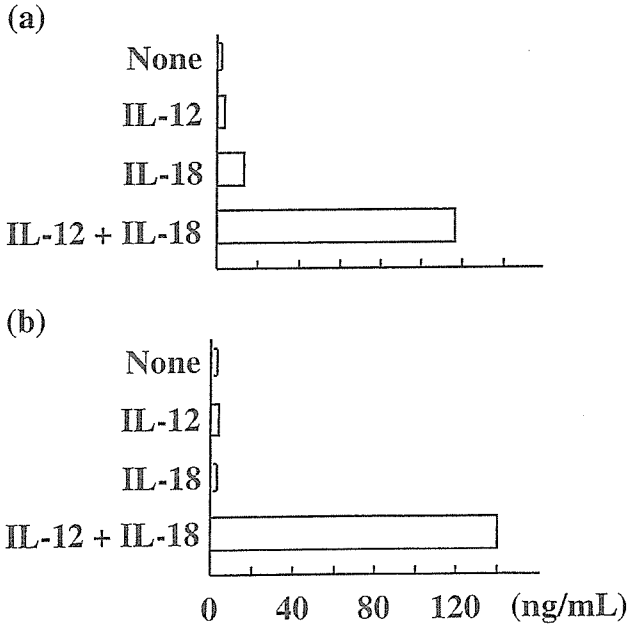

Figure 2 Normal macrophages produce a large amount of interferon (IFN)-γ by the combined stimulation with interleukin (IL)-12 and IL-18. Recombinant murine IL-18 (100 ng/mL) and/or murine IL-12 (10 ng/mL) was added to cultures of (a) isolated splenic $CD4^+$ T cells or (b) $CD11b^+$ macrophages for 48 h, then IFN-γ production was determined by ELISA. Data (mean ± SEM) are the pooled results of seven samples each.

Targeting of $CD11b^+$ macrophages by saporin-conjugated anti-CD11b mAb *in vivo*

To explore the contribution of macrophages to the development of chronic colitis, a saporin-conjugated anti-CD11b antibody was administered to the recipient mice reconstituted with $CD4^+CD45RB^{high}$ T cell on the day of T-cell transfer and 7 days later. As shown in Fig. 4(a) mice treated with a dose-matched mixture of free anti-CD11b mAb and saporin or with PBS manifested progressive weight loss (wasting disease) from 2 weeks after T-cell transfer. These mice had diarrhea with increased mucus in the stool, anorectal prolapse, and hunched posture by 3 weeks. In contrast, the mice treated with saporin-conjugated anti-Mac-1 antibody appeared healthy, showing no decrease of bodyweight and no occurrence of diarrhea during the whole period of observation (Fig. 4a). At 4 weeks after the transfer, the colons from the mixture-treated mice and PBS-treated mice, but not those from the saporin-conjugated anti-Mac-1 antibody-treated mice, were enlarged and had a greatly thickened wall (Fig. 4b). Totally, the assessment of colitis by clinical scores showed a clear difference between the control (mixture- or PBS-treated) mice and saporin-conjugated anti-Mac-1 antibody-treated mice (data not shown). Histological examination showed a marked elongation of the villi with a massive infiltration of mononuclear cells in LP of the colon from the control mice (Fig. 4b). In contrast, the elongation of the villi was mostly abrogated and only few mononuclear cells were observed in the LP of the colon from the saporin-conjugated anti-Mac-1 antibody-treated mice (Fig. 4b). This difference was also confirmed by histological scoring of multiple colon sections, the scores being 1.5 ± 0.15 in the saporin-conjugated anti-Mac-1 antibody-treated mice versus 3.5 ± 0.70 in the mixture of free anti-CD11b mAb-treated mice and 3.0 ± 0.70 in the PBS-treated mice ($P < 0.05$). The $CD11b^+$ macrophage infiltration was also evaluated by immunohistochemical analysis. Few $CD11b^+$ macrophages were found in the colonic tissue of the saporin-conjugated anti-Mac-1 antibody-treated mice as compared with the mixture of free anti-CD11b mAb-treated mice and the PBS-treated mice (Fig. 4c).

Colonic expression of IFN-γ and TNF-α was also reduced by treatment with saporin-conjugated anti-Mac-1 antibody

Wasting disease is a Th1-mediated colitis, and Th1 cytokines, especially IFN-γ and TNF-α, have been demonstrated to play an important role in its pathogenesis.[17] Therefore, using RT-PCR, we compared the cytokine expression in the colons of the colitic mice that developed wasting disease after treatment with a mixture of free anti-CD11b mAb and saporin or with PBS, with that in the mice successfully treated with the saporin-conjugated anti-Mac-1 antibody. As shown in Fig. 5, the mixture-treated mice and

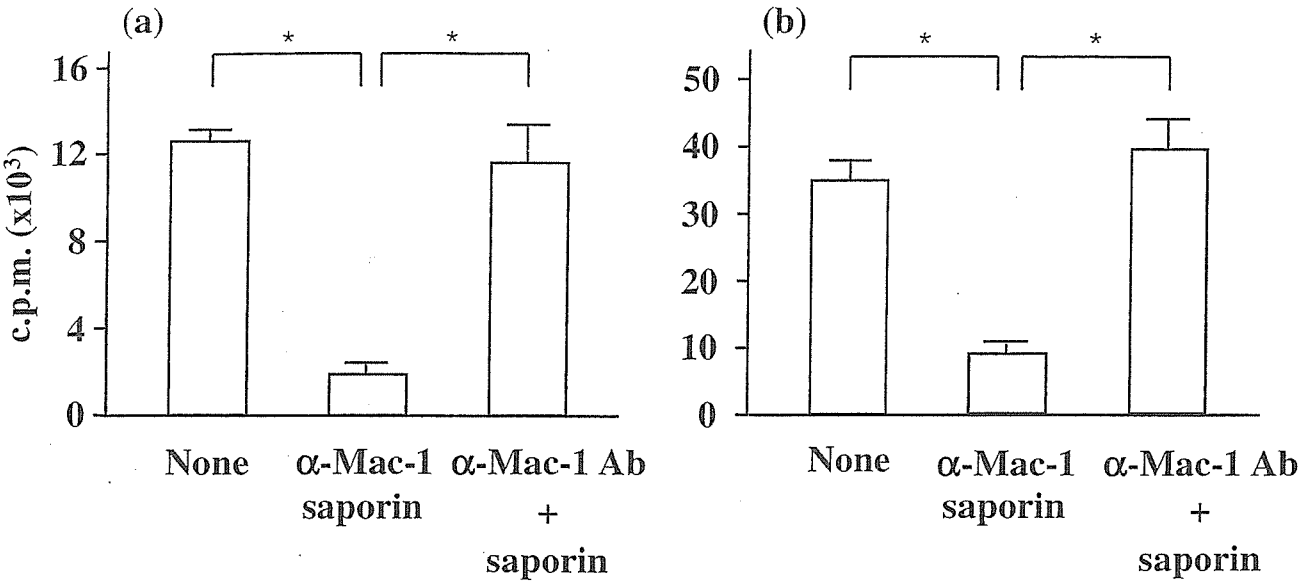

Figure 3 *In vitro* addition of saporin-conjugated anti-Mac-1 antibody inhibits the proliferative responses of concanavalin A (Con A)-stimulated splenocytes from both (a) normal mice and (b) colitic mice. Proliferation assays were performed by culturing splenocytes (1×10^5 per well) in 96-well round plates for 72 h in the presence of saporin-conjugated anti-CD11b mAb (1 μg/mL), a mixture of free anti-CD11b mAb and saporin, or phosphate-buffered saline (PBS) alone. After incubation, the cultures were pulsed for 7 h with [^{3}H]-thymidine (1.85×10^4 Bq/well), harvested on glass fiber filters, and radioactivity was counted (c.p.m.) in a liquid scintillation system. Data are indicated as the mean ± SEM of seven samples in each group. $^*P < 0.01$.

PBS-treated mice showed strong expression of both IFN-γ and TNF-α mRNA, whereas mice successfully treated with the saporin-conjugated anti-Mac-1 antibody exhibited only slight expression of these cytokines.

Saporin-conjugated anti-Mac-1 antibody reduced CD11b$^+$ cells *in vivo*

Finally, to determine whether saporin-conjugated anti-Mac-1 antibody reduces CD11b$^+$ cells *in vivo*, we administered 10 μg saporin-conjugated anti-Mac-1 antibody or an equal volume of PBS to normal BALB/c mice and normal C.B17 SCID mice. Mice were killed 72 h after the injection, and the expression of CD11b was analyzed by flow cytometry. As shown in Fig. 6, the expression of CD11b$^+$ cells was significantly decreased in the spleen of the antibody-treated mice as compared with PBS-treated mice, indicating that the target for saporin conjugated anti-Mac-1 antibody is CD11b-expressing cells.

Discussion

The present study demonstrates the possible contribution of macrophages to the pathogenesis of the murine CD model, by showing that administration of saporin-conjugated anti-Mac-1 antibody effectively prevented the onset of CD4$^+$CD45RB^{high}-transferred colitis and significantly abrogated infiltration of both macrophages and CD4$^+$ T cells and local Th1 cytokine production in the inflamed colon. Although this model of colitis is induced by the transfer of CD4$^+$ naïve T cells, the evidence that the primary targeting of macrophages was effectively prevented in the development of colitis indicates the substantial role of macrophages in CD4$^+$ T cell-mediated inflammatory responses and tissue damage in intestinal mucosa.

Knowledge of the human and the rodent immune systems has evolved to include an understanding of a variety of immunologic pathways and mechanisms including T-cell activation resulting from antigen presentation and costimulation; the differentiation of CD4$^+$ T cells into Th1, Th2, Th3, and T-regulatory-1 (Tr1) subsets; lymphocyte trafficking; TNF-α-mediated inflammation; and nuclear factor κB (NF-κB)-mediated inflammation.[1,2,17] A fundamental question addressed in the current study involves the potential role of macrophages in the induction and/or maintenance of IBD. In humans it has been shown that colonic macrophages are concentrated in a band immediately beneath the luminal epithelium.[6] In this anatomical context, one can envisage that macrophages might normally serve as a first line of defense by non-specifically eliminating particles or organisms from the intestinal lumen. In the various experimental models of mucosal inflammation, as in human IBD, recruitment and enhanced activation of macrophages is a constant feature of the immunopathology.[17,18] This raises the possibility that initial disturbances of macrophages may be important in the disease pathogenesis. In support of this possibility, we demonstrated that macrophages did produce a large amount of IFN-γ in response to combined stimulation by pro-inflammatory IL-12 and IL-18, to a similar extent to CD4$^+$ T cells (Fig. 2). Thus, it is possible that, when stimulated by bacterial components, such as lipopolysaccharide and CpG-DNA, macrophages can themselves produce pro-inflammatory cytokines, such as IL-12 and IL-18, following the induction of IFN-γ production. In the next step, IFN-γ might stimulate macrophages in an autocrine IFN-γ-productive manner. Importantly, these processes might not require the involvement of CD4$^+$ T cells. In

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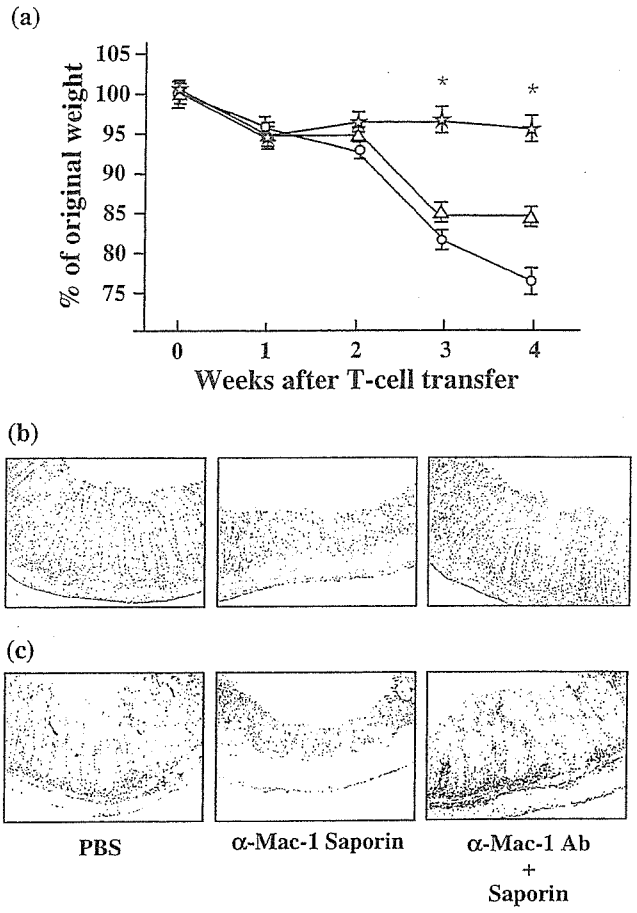

Figure 4 Preventive effect of the saporin-conjugated anti-CD11b monoclonal antibodies (mAb) *in vivo*. The recipient mice were given saporin-conjugated anti-CD11b mAb, a mixture of free anti-CD11b mAb and saporin or phosphate-buffered saline (PBS) on the day of and 7 days after T-cell transfer. (a) Change in bodyweight over time is expressed as percent of the original weight. Data are given as mean ± SEM of six mice in each group. $*P < 0.05$ compared to the control mice. (○) PBS; (☆) anti-Mac-1 saporin; (△) anti-Mac-1 mAb + saporin; (b) Histologic analysis of the colonic specimens. The colons of the mice reconstituted with the saporin-conjugated anti-CD11b mAb, mixture of free anti-CD11b mAb and saporin, or PBS alone were stained with HE. Original magnification, ×100. (c) Immunohistochemical analysis of $CD11b^+$ cells in the colons. Tissue samples for immunohistochemistry were stained with biotinylated antimouse CD11b mAb, followed by detection using a Vectastain ABC kit. The sections were finally counterstained with hematoxylin. Original magnification, ×100.

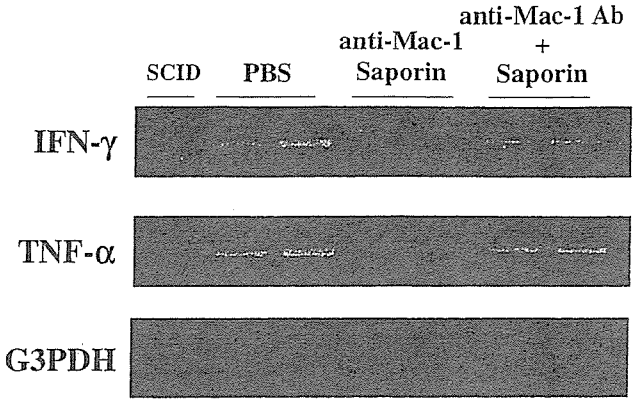

Figure 5 Saporin-conjugated anti-Mac-1 antibody inhibits colonic expression of interferon (IFN)-γ and tumor necrosis factor (TNF)-α mRNA. The expression of IFN-γ and TNF-α mRNA in the colons was analyzed by reverse transcription–polymerase chain reaction (RT-PCR). Amplification of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) serves as a control for sample loading and integrity. The data are representative of three independent experiments. SCID, severe combined immunodeficiency; PBS, phosphate-buffered saline.

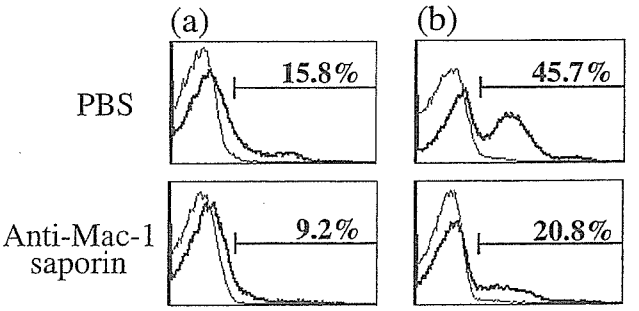

Figure 6 Saporin-conjugated anti-Mac-1 antibody reduced the number of $CD11b^+$ cells *in vivo*. To determine whether saporin-conjugated anti-Mac-1 antibody eliminates $CD11b^+$ cells *in vivo*, we administered 10 μg of saporin conjugated anti-Mac-1 antibody into (a) normal BALB/c mice and (b) normal C.B17 severe combined immunodeficiency (SCID) mice. Control mice were given volume-matched phosphate-buffered saline (PBS). Mice were killed 72 h after the injection, and freshly isolated splenocytes were stained with phycoerythrin (PE)-labeled anti-CD11b and analyzed by flowcytometry. Representatives of three mice in each group are shown.

support of this hypothesis, it has been reported that Rag-1-deficient mice that are lacking in T and B cells could develop dextran sodium sulfate (DSS)-induced colitis.[19]

Furthermore, mice with myeloid-specific deficiency of signal transducer and activator of transcription 3 (STAT3) resulting from gene targeting exhibited an inability to produce several STAT3-dependent cytokines, most notably IL-10.[20] Macrophages from such mice exhibited the phenotype of IFN-γ-primed macrophages that cannot be inhibited by regulatory cytokines such as IL-10. As a result, the mice developed chronic colitis, which was characterized by the depletion of goblet cells and marked infiltration of inflammatory cells in the LP. In line with this, Corazza *et al.* recently showed the importance of TNF-α production by macrophages, but not by T cells, of colonic mucosa in the pathogenesis of colitis using the $CD4^+CD45RB^{high}$-transferred colitis model.[21]

The availability of the prototypical anti-TNF-α agent Infliximab has offered an important advance in therapy for patients with CD.[22,23] Although Infliximab's mechanism of action is not completely understood, its effectiveness suggests that TNF-α, a product of activated macrophages, may have a pivotal role among the many regulatory cytokines with altered expression in association

with IBD. This chimeric monoclonal antibody, composed of a complement-fixing human IgG_1 constant region and a murine-derived antigen-binding variable region, binds soluble TNF-α, but its action is thought to be dependent in part on macrophage apoptosis.[2] First, the treatment with Infliximab has been reported to induce monocytopenia rapidly after infusion, and also to profoundly downregulate monocytes in CD mucosa.[2] Second, Lugering *et al.* have recently shown that Infliximab bound specifically to membrane-bound TNFα, as well as to TNF-α receptor-bound TNF-α, and also that Infliximab induced apoptosis in peripheral monocytes of patients with chronic active CD in a dose-dependent manner.[24] Collectively, these studies show quite clearly that a primary dysregulation of macrophages can result in mucosal inflammation, indicating that macrophages (and macrophage-derived products) are important therapeutic targets in the treatment of IBD.

In summary, the present findings suggest that the regulation of macrophages may be of key importance in successful treatment of CD, and that macrophage targeting may be useful for the treatment of refractory CD.

Acknowledgments

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FEATURE: REGENERATIVE MEDICINE

Regeneration of the intestinal epithelia: Regulation of bone marrow-derived epithelial cell differentiation towards secretory lineage cells

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Abstract

The intestinal epithelia consists of four lineages of differentiated cells, all of which arise from stem cells residing in the intestinal crypt. For proper regeneration from epithelial damage, both expansion of the epithelial cell number and appropriate regulation of lineage differentiation from the remaining stem cells are thought to be required. In a series of studies, we have shown that bone-marrow derived cells could promote the regeneration of damaged epithelia in the human intestinal tract. Donor-derived epithelial cells substantially repopulated the gastrointestinal tract of bone-marrow transplant recipients during epithelial regeneration after graft-versus-host disease. Furthermore, precise analysis of epithelial cell lineages revealed that during epithelial regeneration, secretory lineage epithelial cells that originated from bone-marrow significantly increased in number. These findings may lead to a novel therapy to repair damaged intestinal epithelia using bone marrow cells, and provide an alternative therapy for refractory inflammatory bowel diseases.

Key words: bone marrow cell, epithelial cell, gastrointestinal tract, inflammatory bowel disease, regeneration.

MAINTENANCE AND REGENERATION OF THE GASTROINTESTINAL EPITHELIA

The epithelium of the gastrointestinal (GI) tract covers the entire inner surface of the GI tract, which is continuously and completely renewed every 3–4 days.[1–5] This rapid regeneration is maintained by intestinal epithelial stem cells, which reside in the lowest part of the intestinal crypt (Fig. 1). The intestinal stem cells could both self-renew and also give rise to the four main constituent cell lineages of the intestinal epithelia (enterocytes, goblet cells, enteroendocrine cells and Paneth cells).[2] A single stem cell provides progenitor cells residing in the mid-crypt region. Progenitor cells proliferate rapidly to increase epithelial cell number, and at the same time gradually mature into functional, differentiated cells as they migrate along the crypt–villus axis. Differentiated epithelial cells reside and constitute the epithelial layer of the villi, but drop off into the gut lumen as they migrate and reach the tip of the villi. Disruption of this continuous maintenance of the intestinal epithelia leads to pathologic changes such as ulcer formation in the GI tract and also loss of various critical epithelial functions.

In clinical practice, we face several diseases that cause multiple refractory ulcers in the GI tract. For example, inflammatory bowel disease is a common disease both in Western countries and in Japan, and in certain populations this disease is associated with severe failure to regenerate the damaged intestinal epithelia.[6] In these diseases, not only is the suppression of mucosal inflammation

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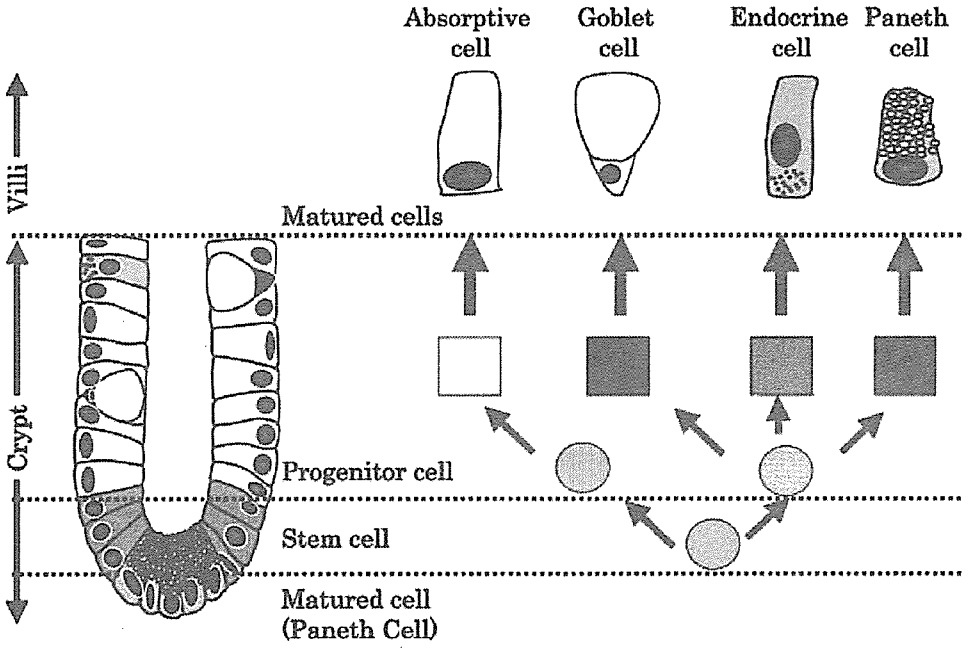

Figure 1 Maintenance of intestinal epithelial cells: The gastrointestinal epithelium is maintained by intestinal stem cells residing in the lower part of the intestinal crypt. The intestinal stem cells give rise to the four lineages of differentiated epithelial cells via production of proliferating progenitor cells.

required to restore the complete function of the GI tract but also proper regeneration of the intestinal epithelium is necessary. Thus, it is important to understand how the intestinal epithelia is maintained and regenerated.[7]

Recent studies have revealed various cells, factors or molecules that are important during the process of regeneration of the intestinal epithelium.[8] In the following sections, we would like to propose bone marrow cells as an extra-intestinal source for intestinal epithelial repair, and discuss the possibility of a novel therapy using bone marrow cells for intestinal epithelial repair.[9]

REPOPULATION OF THE GASTROINTESTINAL EPITHELIA OF BONE-MARROW TRANSPLANT RECIPIENTS BY BONE-MARROW DERIVED CELLS

In our recent series of studies, we sought to determine whether human bone marrow cells have the potential to repopulate the gastrointestinal epithelia. For this purpose, we examined biopsy specimens of the GI tract taken during endoscopic examinations of women (XX) who had received bone-marrow transplants (BMT) from male (XY) donors.[10] Fluorescent *in situ* hybridization (FISH) specific for human Y-chromosome was employed to identify GI-epithelial cells that contain a Y-chromosome and thus must have descended from transplanted bone marrow cells. Surprisingly, donor-derived epithelial cells were detected in every part of the gastrointestinal tract.[10] The frequency of bone-marrow derived epithelial cells was less than 1% of epithelial cells in regions where no inflammation had occurred. The donor-derived epithelial cells we observed were not clustered. However, with sharp contrast, bone-marrow derived epithelial cells significantly increased in number, and repopulated the gastrointestinal tract of bone-marrow transplant recipients during epithelial regeneration. We examined specimens from the small intestine taken at four different times after BMT from a single female recipient who developed acute GVHD. No bone-marrow derived cell was detected in the tissue obtained 25 days before BMT. The number of bone-marrow derived epithelial cells was low (0.4 cells per 100 nucleated cells) 26 days after BMT at the beginning of an episode of acute GVHD, but the number had increased up to ninefold (3.6 cells per 100 nucleated cells) by day 77 as GVHD ran its course. By 777 days after BMT, the patient recovered from GVHD, but bone-marrow derived epithelial cells were still present, although less abundant

(1.0 cell per 100 nucleated cells). We examined another case that developed a gastric ulcer by day 586 after BMT. At that time, there were 40–50 times as many bone-marrow derived epithelial cells in the regenerating epithelia of the stomach compared to the surrounding normal epithelia. These findings suggest that when tissue repair is required to reconstitute human GI epithelia, bone-marrow derived cells can support the healing process by transiently increasing epithelial cell number, thereby promoting the repair of damaged mucosa related to GVHD or gastric ulcer.

BONE-MARROW CELLS ARE DIRECTED TO DIFFERENTIATE INTO SECRETORY LINEAGE EPITHELIAL CELLS DURING EPITHELIAL REGENERATION OF THE GI TRACT

In the next study, we asked whether bone-marrow derived GI epithelial cells support the epithelial regeneration merely by increasing the epithelial cell number or whether there are specific functions of epithelial cells that support functions of the epithelia at the same time.

To examine whether bone-marrow derived epithelial cells have specific functions of differentiated GI epithelial cells, we evaluated the expressions of functional lineage markers within bone-marrow derived cells. For goblet cells, alcian blue staining was used to detect acid mucin production.[11] For Paneth cells, hematoxylin-eosin staining was used to detect eosinophilic granules. For enteroendocrine cells and absorptive cells, immunostaining for Chromogranin A and CD10, respectively, were used. Results showed that bone-marrow derived epithelial cells could express all four lineage markers, suggesting that bone-marrow derived epithelial cells could obtain the function of any epithelial lineage. Another surprising finding was that the expression of different lineage markers within bone-marrow derived epithelial cells showed remarkable change during epithelial regeneration. Bone-marrow derived absorptive cells dominated the bone-marrow derived epithelial cells in a region where no inflammation or epithelial damage had occurred. In sharp contrast, in the region where epithelial damage due to GVHD had occurred, bone-marrow derived epithelial cells increased in total number and at the same time increases in the numbers of bone-marrow derived cells expressing lineage markers of goblet cells, enteroendocrine cells and Paneth cells were observed.[11]

These observations suggest that bone-marrow derived cells not only support epithelial regeneration by increasing the epithelial cell number, but also support various epithelial functions during regeneration. Also, dramatic changes of lineage determination in bone-marrow derived epithelial cells during epithelial regeneration suggests that there may be an unknown mechanism regulating cell lineage determination during inflammation or epithelial regeneration. The shift in the development of bone-marrow derived epithelial cells towards goblet cells, enteroendocrine cells and Paneth cells (collectively called secretory lineage cells) apart from absorptive cells during regeneration is reasonable, as these lineages have specific functions that are indispensable for prompt epithelial regeneration. For example, goblet cells secrete trefoil factors, which are indispensable for epithelial restitution.[12] Enteroendocrine cells secrete GLP-2, an essential growth factor for the expansion of absorptive cell progenitor.[13]

HOW BONE MARROW CELLS DEVELOP INTO INTESTINAL EPITHELIAL CELLS

The mechanism that generates bone-marrow derived epithelial cells still remains to be explained, but some of our observations suggest several possibilities. The first question is whether bone-marrow cells reside as epithelial stem cells and subsequently generate various lineages of differentiated epithelial cells. The answer is possibly "no". Our observation showed that there were no linear clusters of bone-marrow derived epithelial cells in the longitudinal sections of the intestinal crypt. Instead, they distributed mostly as individual cells in diffuse patches around the epithelia. Examination of the expression of a candidate intestinal stem cell marker, Musashi-1, showed that bone-marrow derived epithelial cells virtually never express this marker.[11] Bone-marrow derived epithelial cells were detected in tissue samples obtained more than 8 years after BMT, suggesting that these cells are continuously generated from bone marrow cells and also continuously renewed at the same time.[10] However, bone-marrow derived epithelial cells were not detected in tissue samples obtained from a female patient whose bone marrow had been replaced by a female donor bone marrow following the first BMT from a male donor.[11] These observations collectively suggest that bone-marrow derived cells virtually never reside as intestinal stem cells (Fig. 2).

However, certain populations of bone-marrow derived epithelial cells coexpress proliferative cell marker, Ki-67. Also, up to two bone-marrow derived epithelial cells expressing Ki-67 have been observed as a cluster within

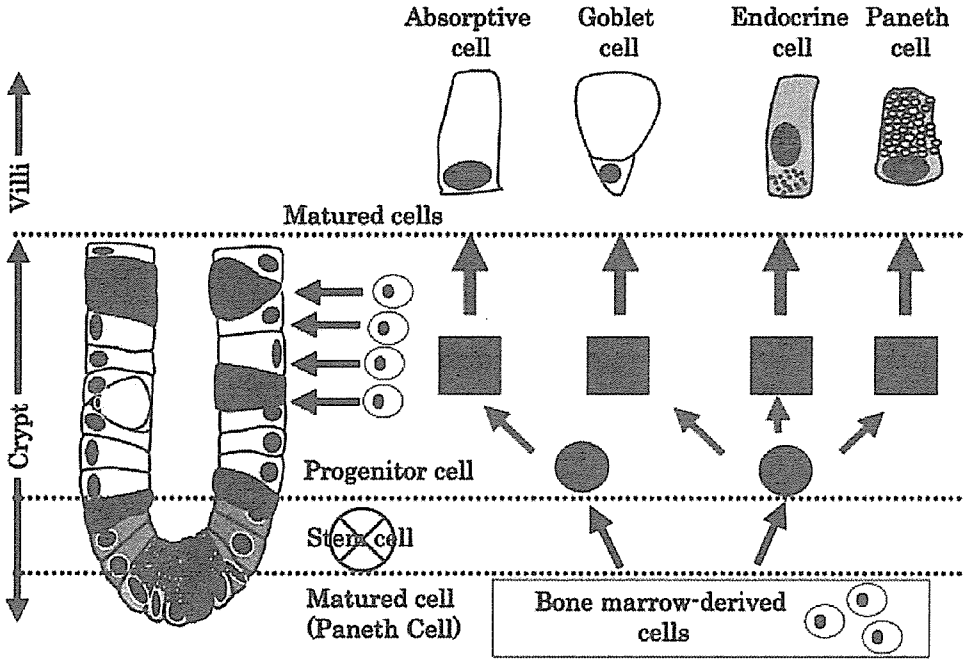

Figure 2 Bone-marrow derived epithelial cells are generated from proliferative progenitor cells, but not from stem cells. Bone-marrow derived cells are not integrated into the epithelia as stem cells, but are integrated as progenitor cells residing in the proliferating zone of the crypt. These cells subsequently divide and mature into terminally differentiated epithelial cells.

the epithelia. This suggests that bone-marrow derived cells could reside as cells of the proliferative region of the intestinal crypt, and possibly proliferate within the epithelia.[11] Thus, bone marrow cells may first reside as immature, proliferating cells, but not as stem cells, within the epithelia and subsequently divide and differentiate into mature epithelial cells. Thus, the change in lineage differentiation during epithelial regeneration may occur at the level of progenitor cells, but not at the stem cell level.

The next question is whether bone-marrow derived epithelial cells are generated from a fusion between two cells of different origin or from *trans*-differentiation of bone marrow cells.[14–19] The answer to this question is still under discussion. As far as we have observed, cell fusion may not be the major mechanism. Using multicolor FISH technique, we have examined the ploidity of bone-marrow derived intestinal epithelial cells. Results have shown that all of the cells examined were euploid, and not a single cell could be proved to be aneuploid.[11] However, this observation does not exclude the possibility of cell fusion followed by reductive division, and also does not provide positive evidence that bone-marrow derived epithelial cells are generated from *trans*-differentiation.

Another question arises about the origin of bone-marrow derived epithelial cells. All cases that we have examined received bone-marrow transplantation using almost the whole population of bone marrow cells, but others have shown that in patients who had received only hematopoietic stem cells (CD34 positive cells) for transplantation, the same results were observed.[20] This suggests that CD34 positive hematopoietic stem cells may be the origin of bone-marrow derived intestinal epithelial cells, at least in humans. However, this issue remains controversial, as it is technically difficult to track the origin of generated epithelial cells within the human body. So far, it is also controversial in animal models.[21]

PROSPECTS FOR THE THERAPEUTIC USE OF BONE-MARROW DERIVED CELLS IN HUMAN GASTROINTESTINAL DISEASES

Several case reports have suggested that both allogeneic and autologous bone-marrow transplantation have therapeutic effects in autoimmune diseases such as inflammatory bowel diseases.[22–26] One report described

two cases of refractory Crohn's disease treated by high-dose immunosuppressive therapy followed by autologous hematopietic stem cell transplantation that resulted in long-term remission of the disease.[27] The therapeutic effect of bone-marrow transplantation in these cases is proposed to occur through the elimination of pathogenic immune cells. Considering our observation,[25] bone-marrow transplantation may also have a therapeutic effect in regenerating the damaged intestinal epithelia. However, whether bone-marrow transplantation itself is the best way to use bone-marrow derived cells to regenerate the intestinal epithelia is not clearly determined. If there is a humoral factor which could be proved to facilitate introduction of bone marrow cells into the intestinal epithelia, these factors may also be used. A possible candidate is GM-CSF, which was reported to have therapeutic effect for Crohn's disease patients.[28] Further study of the molecular mechanism by which bone-marrow derived epithelial cells are generated from bone marrow cells may lead to discovery of the unknown factors involved in the regeneration process.

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Journal of Gastroenterology

Editorial

Do fatty acids influence functions of intestinal dendritic cells?

Article on page 209
Differential modulation in the functions of intestinal dendritic cells by long- and medium-chain fatty acids
Tsuzuki Y, Miyazaki J, Matsuzaki K, et al.

It is well known that dendritic cells (DCs) are present in the gut-associated lymphoid tissue (GALT), and in the intestinal lamina propria, lying in close proximity to the large and dynamic antigenic load in the gut lumen.[1] DCs are the professional antigen-presenting cells (APCs) that take up antigens in peripheral tissues and migrate to draining lymph nodes. After encountering antigens, DCs undergo a maturation process involving the loss of phagocytotic ability, the acquisition of APC activity and the capacity to migrate into draining lymph nodes for efficient activation of T cells. In the gut, DCs in Peyer's patches sample commensal bacteria and external antigens, but this is not the only site at which antigen uptake occurs. Lamina propria DCs sometimes pass their dendrites between epithelial tight junctions and interact directly with luminal antigens.[2] Thus, it is reasonable that DCs are affected and modulated by dietary stimuli. Indeed, dietary deficiencies or excesses are often associated with perturbations of functional responses of GALT cells engaged in maintaining immunity and mediating inflammation. Among dietary components, research on fatty acids (FAs) and their effect on the immune system has been progressing for more than two decades. FAs serve as essential fuel for energy production, as an integral structural part of cell membrane structure, and as a substrate for the generation of biologically active mediator molecules. Total fats, different types of fats and their ratios, specific FAs, and even modified (oxidized, conjugated, hydrogenated) FAs, all have an impact on immune cell function. Nevertheless, little is known regarding the direct effects of FAs on DC functions in the intestinal mucosa.

Tsuzuki and coworkers in this issue of the *Journal of Gastroenterology* focus on intestinal DCs to investigate the role of FAs on modulating intestinal immune responses.[3] They demonstrate that long- and medium-chain FAs in the intestine differentially modulate the immune functions of intestinal DCs by showing that exposure to long- and medium-chain FAs maintains the phagocytic function of intestinal DCs, whereas exclusively long-chain FAs abrogate (1) the antigen presentation ability, accompanied by decreased expression of major histocompatibility complex (MHC) class II molecules, and (2) the chemotactic ability of mature DCs toward CCL21. These data suggest that intraluminal exposure to FAs, especially long-chain FAs, may modulate downward the intestinal immune responses through the dysregulated DCs, suggesting a possible therapeutic strategy for the treatment of patients with inflammatory bowel disease (IBD) with long-chain FAs. However, we sometimes observe that the intake of long-chain FAs aggravates inflammation in IBD with increased immune cell infiltration. Consistent with this observation, but contrary to the results of Tsuzuki and colleagues,[3] it has been reported that some saturated long-chain FAs upregulate the expression of costimulatory molecules (CD40, CD80, and CD86), MHC class II molecules, and cytokines [interleukin (IL)-12p70 and IL-6] in bone marrow-derived DCs.[4] Furthermore, in clinical fields, an elemental diet with a lower FA content has been established as the primary treatment for patients with Crohn's disease. To resolve this issue, it will be necessary to assess other types of FAs by the same assays, especially using n-3 polyunsaturated FAs such as eicosapentaenoic acid and docosahexaenoic acid, which have recently received much attention as anti-inflammatory factors.

Another important point should be discussed. Is the origin of the DCs isolated by the method of Tsuzuki and coworkers[3] the intestine? Although they isolated intestinal DCs from the thoracic duct lymph of mesenteric lymphadenectomized rats by thoracic duct cannulation, it is still possible that these DCs were derived from circulating DCs that had migrated directly into the me-

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senteric lymph nodes (MLNs) from the blood, but not from the intestine, because it seems to be technically impossible to remove all MLNs. The prevalent model of DC migration is a unidirectional pathway whereby precursor DCs arise from progenitors in the bone marrow, enter the blood, and traffic into secondary lymphoid organs, including MLNs, as well as into peripheral tissues such as skin and gut, where they contribute to the frontline defense against pathogens. When DCs encounter inflammatory stimuli in the local tissues, they undergo a switch in chemokine receptor expression, enabling their egress into lymphatic vessels and transport to draining lymph nodes. Since there is ample experimental evidence that draining lymph nodes are the terminal targets for most DCs that leave peripheral tissues, it is unclear whether DCs egress further into efferent lymphatics and into the thoracic duct. Furthermore, it is likely that the isolated DCs, if all of them are derived from the intestine, are more activated and more differentiated, because these cells egress from the intestine after encountering inflammatory stimuli such as pathogen-associated molecular patterns, followed by the switching on of chemokine receptor expression. In the light of this knowledge regarding the migration and activation pattern of DCs, the authors may need to sample DCs from other sites, such as Peyer's patches and the lamina propria, because those DCs should be the more immature types of DCs, such as would physiologically make contact with FAs in the gut.

In proportion to the rapid increase in fat intake in the diets of people in Japan and other Asian countries, the numbers of new IBD patients will rapidly increase. Thus, this timely important study of this issue by Tsuzuki and colleagues may shed light on the development of new strategies for the treatment of IBD patients in the near future.

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T_H1/T_H2–Mediated Colitis Induced by Adoptive Transfer of $CD4^+CD45RB^{high}$ T Lymphocytes Into Nude Mice

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Background: Transfer of $CD4^+CD45RB^{high}$ T cells from normal donors to SCID/Rag-1, 2–deficient mice, which lack T and B cells, leads to the development of a T_H1-mediated inflammatory bowel disease (IBD)–like syndrome characterized by extensive mononuclear cell infiltrates and epithelial cell hyperplasia. Because it is well known that B cells are also involved in a multitude of mechanistic pathways in human IBD, this study attempts to establish a new model of colitis in nude mice.

Methods: We transferred $CD4^+CD45RB^{high}$ T cells into athymic nude mice, which lack thymus-dependent T cells but retain normal B cells, to establish and investigate a B cell–involving chronic colitis model. As a control, $CD4^+CD25^-$ T cells were also used.

Results: Mice reconstituted with $CD4^+CD45RB^{high}$ but not $CD4^+CD25^-$ T cells developed a wasting disease, with severe infiltrates of B cell aggregates as well as T cells, macrophages, and dendritic cells into the colon and elevated levels of interferon-γ, tumor necrosis factor-α, interleukin (IL)-4, IL-5, and IL-10, by 7 weeks after T cell transfer. Furthermore, the infiltrated lamina propria B cells in colitic nude mice consisted predominantly of massive aggregated immunoglobulin (Ig) M- and scattered IgG-positive cells, but not IgA-positive cells. In contrast, mice reconstituted with $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ did not develop wasting disease or colitis.

Conclusions: Collectively, the power of the colitis model induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells into nude mice is that one can investigate the roles of T_H2-type cells and B cells in a regulatory T cell–depleted condition.

Key Words: nude mice, colitis model, Crohn's disease, T_H1/T_H2

(*Inflamm Bowel Dis* 2006;12:89–99)

Evidence from a growing number of rodent models of inflammatory bowel disease (IBD) supports a central role for dysregulated $CD4^+$ T cell responses.[1–3] Despite the potential immune stimulus, however, it is also well known that immune responses in the intestine remain in a state of controlled inflammation.[1] Regulation of the immune response is a balance between the need to mount protective immunity toward pathogens and not activating damaging inflammatory responses to the plethora of harmless antigens present, including those derived from resident bacteria.[1] To maintain the intestinal homeostasis including immunological tolerance, functionally distinct subsets have been clearly defined in T cells.[4,5] Among these subsets, the regulatory T (T_R) cell subset down-regulates immune responses for both foreign- and self-antigens and effectively participates in the suppression of autoimmune disorders.[6–8] Indeed, targeted deletion of essential factors to maintain regulatory function, such as interleukin (IL)-10, IL-2, and transforming growth factor (TGF)-β, their receptors, and other T_R markers (cytotoxic T-lymphocyte antigen [CTLA-4] and α_E integrin), revealed the development of autoimmune diseases including intestinal inflammation.[9]

A variety of cells that display regulatory function in vitro or in vivo have been described. These cells can be subdivided into different subsets based on the expression of cell surface markers, production of cytokines, and mechanisms of action. Recent studies focused on CD25 as the best marker for T_R cells in mice and humans.[10–12] $CD4^+CD25^+$ T cells, which

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constitute ≈10% of peripheral murine $CD4^+$ T cells, express $CD45RB^{low}$.[13–16] Increasing evidence suggests, however, that the peripheral $CD4^+CD25^-CD45RB^{low}$ T cell population also possesses some regulatory activity.[17–22] Furthermore, the $CD4^+CD25^+$ cell population is heterogeneous and although a relatively high proportion of these cells may be T_R cells, however, one should not presume that this is true for the entire population or that all T_R cells express CD25.

Consistent with this notion, it is well established that transfer of $CD4^+CD45RB^{high}$ T cells, which lacks $CD4^+CD45RB^{low}$ T_R cells regardless of CD25 expression, to SCID/Rag-1,2–deficient mice leads to the development of wasting disease and colitis 6 to 8 weeks after T cell transfer.[23] The features of immune pathology in the intestine included epithelial cell hyperplasia, goblet cell depletion, and transmural inflammation. Characteristically, the colitis involved the differential activation of T_H1 cells responding to intestinal bacterial antigens, but not T_H2 cells, as there is a 20- to 30-fold accumulation of T_H1 cells in the intestine when compared with normal mice. These cells were involved in the immune pathology because treatment with anti-interferon (IFN)-γ, anti-tumor necrosis factor (TNF)-α or anti-IL-12 monoclonal antibodies (mAbs) inhibited disease.[24–26] In marked contrast, transfer of the reciprocal $CD4^+CD45RB^{low}$ population not only fails to induce colitis but also actually prevents it because colitis fails to develop in SCID/Rag-1,2–deficient mice reconstituted with a mixture of potentially pathogenic $CD4^+CD45RB^{high}$ plus $CD4^+CD45RB^{low}$ T cells.[23] Herein lies the power of the model. One can immediately identify 2 populations: one ($CD4^+CD45RB^{high}$) is a source of effector cells and the other ($CD4^+CD45RB^{low}$) is a source of T_R cells, and analyses of each population can identify the cells necessary for each type of function. Similarly, BALB/c/C.B. 17 SCID mice reconstituted with $CD4^+CD25^-$ T cells also develop wasting disease and also suffer from colitis, albeit to a less extent than SCID mice reconstituted with $CD4^+CD45RB^{high}$ T cells.[22] It is true, however, that these SCID-transfer models are too simple because these models neglect the role of B cells, which are believed to critically involve IBD pathophysiology at some phase. In contrast, athymic BALB/c nude mice reconstituted with $CD4^+CD25^-$ T cells spontaneously develop histologically and serologically evident autoimmune diseases (e.g., thyroiditis, gastritis, insulitis, sialoadenitis, adrenalitis, oophoritis, glomerulonephritis, polyarthritis), but not colitis.[27] These complicated findings suggest that colitis would be established by the balance between a type of recipient and transferred cells.

In this study, we attempt to transfer normal $CD4^+CD45RB^{high}$ T cells into athymic nude mice, which lack thymus-dependent T cells but retain normal B cells, to establish a new colitis model that involves B cell dysfunction and should be closer to human IBD pathology.

METHODS

Animals

Female BALB/c normal and athymic nude mice were purchased from Japan CLEA (Tokyo, Japan). Mice were maintained under specific pathogen-free conditions in the animal care facility of Tokyo Medical and Dental University. Mice were used at 7 to 12 weeks of age. All experiments were approved by the regional animal study committees.

Antibodies

The following mAbs and reagents were purchased from BD PharMingen (San Diego, Calif) and used for purification of cell populations and cell surface analysis: RM4-5, phycoerythrin equivalent (PE)–conjugated anti-mouse CD4 mAb; 7D4, fluorescein isothiocyanate conjugated (FITC)–anti-mouse CD25 mAb; 16A, FITC-conjugated anti-mouse CD45RB mAb; RA3-6 B2, FITC or PE-conjugated anti-mouse B220 mAb; 16-10A1, PE-conjugated anti-mouse CD5 mAb; PE-conjugated streptavidin; Cy-Chrome-conjugated streptavidin. FITC-conjugated anti-mouse immunoglobulin M (IgM) mAb was purchased from Southern Biotechnology Associates (SBA) Inc. (Birmingham, Ala).

Purification of T Cell Subsets

$CD4^+$ T cells were isolated from spleen cells from BALB/c mice using the anti-CD4 (L3T4)-magnetic cell sorting (MACS) system (Miltenyi Biotec, Auburn, Calif) according to the manufacturer's instruction. Enriched $CD4^+$ T cells (96%–97% pure, as estimated by FACS Calibur [Becton Dickinson, Sunnyvale, Calif]) were then labeled with PE-conjugated anti-mouse CD4 (RM4-5), FITC-conjugated anti-CD45RB (16A), and FITC-conjugated anti-CD25 (7D4). Subpopulations of $CD4^+$ cells were generated by 2-color sorting on a FACS Vantage (Becton Dickinson). All populations were >98.0% pure on reanalysis.

$CD4^+CD45RB^{high}$ T Cell Transferred Colitis in Nude Mice and Disease Monitoring

To establish a new murine colitis model by cell transfer, athymic BALB/c nude mice were injected intraperitoneally with 1 or 2 subpopulations of sorted $CD4^+$ T cell in 250 μL phosphate-buffered saline (PBS), $CD4^+CD45RB^{high}$ cells (brightest staining 20%, 5×10^5/body), $CD4^+CD25^-$ cells (5×10^5/body), and $CD4^+CD45RB^{high}$ + $CD4^+CD45RB^{low}$ (dullest staining 20%) cells (each 5×10^5/body). The recipient nude mice after T cell transfer were weighed initially and then 3 times/week thereafter. They were observed for clinical signs of illness: hunched-over appearance, piloerection of the coat, diarrhea, and blood in the stool. Mice were killed and assessed for a clinical score that is the sum of 4 parameters: hunching and wasting, 0 or 1; colon thickening, 0 to 3 (0, no colon thickening; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); and stool consistency, 0 to 3 (0, normal

beaded stool; 1, soft stool; 2, diarrhea; and an additional point was added if gross blood was noted).[28]

Histological Examination and Immunohistochemical Staining

Tissue samples were fixed in PBS containing 10% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with hematoxylin and eosin. Three tissue samples from the proximal, middle, and distal parts of the colon were prepared. The sections were analyzed without prior knowledge of the type of T cell reconstitution or treatment. 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.[29] Colonic samples for immunohistochemistry were embedded in OCT compound (SAKURA, Tokyo, Japan), snap-frozen in liquid nitrogen, and stored at −80°C. The staining of the sections was performed by the avidin-biotin complex method.[30] Six-micron sections were incubated with purified anti-mouse CD4 mAb (RM4-5, rat IgG1, BD PharMingen), anti-mouse B220 mAb (RA3-6 B2, rat IgG2a, BD PharMingen), anti-mouse CD11b (M1/70, rat IgG2b, BD PharMingen), anti-mouse CD11c (HL-3, hamster IgG, BD PharMingen), and anti-mouse F4/80 (A3-1, rat IgG, Serotec Inc, Oxford, UK). Biotinylated isotype-matched control antibodies (BD PharMingen) were also used. Biotinylated antibodies were then detected by immersing the sections for 30 min in a solution of streptavidin-enzyme conjugates (Vectastain ABC kit, Vector, Burlingame, Calif). The localization of antigens was visualized by incubation with diaminobenzidine solution and counterstained with hematoxylin. In some experiments, frozen sections of colon were prepared and fixed with cold acetone and then incubated with Block Ace (Dainippon-Pharmaceuticals, Tokyo, Japan). Sections were then incubated with FITC-labeled goat anti-mouse IgM (×500 dilution, SBA), tetramethyl rhodamine (TRITC)–labeled goat anti-mouse IgG (×500, SBA) and biotin-labeled goat anti-mouse IgA (×400, BD-PharMingen), followed by incubation with aminocumarin-labeled streptavidin (×500, SBA).[31] Anti-colon autoantibodies in sera from mice reconstituted with $CD4^+CD45RB^{high}$ T cells 7 weeks after T cell transfer were examined by indirect immunofluorescence using colonic tissues from 6-week-old noncolitic nude mice. Sections were blocked with 10% goat serum in PBS for 30 min at room temperature, incubated with mouse serum diluted in 10% goat serum in PBS for 1 hour, and then bound antibody was detected with TRITC-labeled anti-mouse IgG or FITC-labeled anti-mouse IgM antibody (SBA). Sections were examined with a fluorescence microscope (Olympus BX50/BXFLA, Tokyo, Japan) equipped with a charge coupled device camera (Olympus) and an image-capture system (Olympus). Combination images for multicolor staining were created using Photoshop 4.0 (Adobe Systems, San Jose, Calif).

Preparation of Splenocytes, Mesenteric Lymph Node Cells, and Mucosal Lamina Propria Mononuclear Cells

Spleens and mesenteric lymph nodes (MLNs) were pressed through a nylon cell strainer to isolate single cell suspensions. Cells were washed twice and the cell number was counted before analysis for flow cytometry for the isolation of lamina propria mononuclear cells (LPMCs) from colon,[32] after which the entire length of intestine was opened longitudinally, washed with PBS and cut into small (~5mm) pieces. The dissected mucosa was incubated with Ca^{2+} Mg^{2+}-free Hanks' balanced salt solution containing 1 mmol/L dithiothreitol (Sigma-Aldrich, St. Louis, Mo) for 30 min to remove mucus and then serially incubated 2 times in medium containing 0.75 mmol/L EDTA (Sigma-Aldrich) for 60 min at each incubation. The supernatants from these incubations containing epithelium and intraepithelial lymphocyte population were deserted, and the residual fragments were pooled and treated with 2 mg/mL collagenase A (Worthington Biomedical, Freehold, NJ) and 0.01% DNase (Worthington) in humidified air at 37°C for 2 hours. The cells were then pelleted 2 times through a 40% isotonic Percoll solution, after which they were purified further by Ficoll-Hypaque density gradient centrifugation (40%/75%). Enriched $CD4^+$ lamina propria T cells were obtained by positive selection using an anti-CD4 (L3T4) MACS (Miltenyi Biotec). The resultant cells, when analyzed by FACS Calibur, contained >95% $CD4^+$ cells.

Flow Cytometry

To detect the surface expression of a variety of molecules, isolated splenocytes, MLN cells, or LPMCs were preincubated with an FcγR-blocking mAb (CD16/32; 2.4G2, BD PharMingen) for 20 min followed by incubation with specific FITC-, PE-, or biotin-labeled antibodies for 30 min on ice. Biotinylated antibodies were detected with PE-streptavidin. Standard 2-color flow cytometric analyses were obtained using the FACS Calibur using CellQuest software (Becton Dickinson). Background fluorescence was assessed by staining with irrelevant control isotype-matched mAbs.

Cytokine Assays

To measure cytokine production, 1×10^5 lamina propria $CD4^+$ T cells were cultured in 200-μL culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in 96-well plates (Costar, Cambridge, Mass) pre-coated with 5 μg/mL hamster anti-mouse CD3ε mAb (145-2C11, BD PharMingen) and hamster 2 μg/mL anti-mouse CD28 mAb (37.51, BD PharMingen) in PBS overnight at 4°C. Culture supernatants were removed after 48 hours and assayed for cytokine production. IFN-γ, TNF-α, IL-4, and IL-5 concentrations in culture supernatants were determined by a cytometric bead array (CBA) cytokine detection assay (BD PharMingen), and

IL-10 concentration by a specific enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, Minn) per the manufacturer's recommendation.

Statistical Analysis

The results were expressed as the mean ± SEM. Groups of data were compared by Mann-Whitney U test. Differences were considered to be statistically significant when $P < .05$.

RESULTS

Athymic Nude Mice Reconstituted With CD4$^+$CD45RB^{high} T Cells Developed Colitis With Wasting Disease

To establish a new murine chronic colitis model by the adoptive cell transfer into athymic nude mice, we first strictly sorted the 20% brightest CD45RB^{high} CD4$^+$ T cells using a FACS Vantage to avoid contamination of CD45RB$^{low\text{-}intermediate}$ cells, which retain some immunosuppressive property[23] (Fig. 1A). BALB/c nude mice were reconstituted with the CD4$^+$CD45RB^{high} (5×10^5/body) alone or CD4$^+$CD45RB^{high} + CD4$^+$CD45RB^{low} T cells (each 5×10^5/body; Fig. 1A). As a control, they were also reconstituted with the CD4$^+$CD25$^-$ (5×10^5/body), which include some CD45RB$^{low\text{-}intermediate}$ cells but not classic CD4$^+$CD25$^+$ T_R cells (Fig. 1A). Mice reconstituted with the CD4$^+$CD45RB^{high} T cells developed wasting disease (Fig. 1B), and these mice had diarrhea with increased mucus in the stool, anorectal prolapse, and hunched-over posture by 4 to 7 weeks. In contrast to the CD4$^+$CD45RB^{high} T cell–reconstituted mice, mice reconstituted with both CD4$^+$CD45RB^{high} and CD4$^+$CD45RB^{low} T cell subset or with no transfer appeared healthy, with a gradual increase of body weight (Fig. 1B) and an absence of diarrhea and other clinical symptoms during the period of observation. Of importance, mice reconstituted with the CD4$^+$CD25$^-$ cell subset also appeared healthy, with a gradual increase of body weight (Fig. 2A). The assessment of colitis by clinical scores showed a clear difference between mice transferred with CD4$^+$CD45RB^{high} T cells and other groups (Fig. 1C). Consistent with the clinical findings, the colons from mice reconstituted with the CD4$^+$CD45RB^{high} T cells were enlarged and had a greatly thickened wall (data not shown), and the colonic weight of mice transferred with CD4$^+$CD45RB^{high} T cells was significantly increased as compared with those of other groups (Fig. 1D). In contrast, the weight of the spleen was not significantly different in each group (Fig. 1E).

Microscopically, the transmural inflammation was common in the ascending and transverse colons of mice transferred with CD4$^+$CD45RB^{high} T cells (Fig. 2A[b]), characterized by prominent epithelial hyperplasia with glandular elongation and loss of goblet cells and extensive leukocytic infiltrates (Fig. 2A[b]). The infiltrates were also seen in the lamina propria and submucosa and, to a lesser degree, in the muscularis, serosa, and mesentery (Fig. 2B[a]). Inflammatory infiltrates consisted mainly of mononuclear cells with smaller numbers of neutrophils (Fig. 2B[b]). In addition, epithelial lesions included mucin depletion, loss of goblet cells, crypt abscesses (Fig. 2B[a]), and ulceration, occasionally with a mountain-peak appearance (Fig. 2B[c]). Furthermore, we found a higher number of hypertrophic lymphoid patches in the colon (Fig. 2B[d]). In contrast, the colons from mice reconstituted with both CD4$^+$CD45RB^{high} and CD4$^+$CD45RB^{low} T cells (Fig. 2A[c]), with CD4$^+$CD25$^-$ cells (Fig. 2A[d]), or with no transfer (Fig. 2A[a]), exhibited no detectable pathological changes. Consistent with these findings, histological scores of mice transferred with CD4$^+$CD45RB^{high} T cells were significantly increased as compared with those in mice reconstituted with both CD4$^+$CD45RB^{high} and CD4$^+$CD45RB^{low} T cells, with CD4$^+$CD25$^-$ cells, or with no transfer (Fig. 2C).

To examine the phenotypical cell surface markers of infiltrated mononuclear cells, the sections of colon were immunohistochemically stained with antibodies against CD4, B220, F4/80, CD11b, and CD11c. Although major populations of cell infiltrates in the inflamed mucosa from mice transferred with CD4$^+$CD45RB^{high} T cells were CD4$^+$ T cells, B220$^+$ cell aggregates, which were structurally different from colonic patches, were also markedly infiltrated in the lamina propria (Fig. 3). In addition, F4/80$^+$ (macrophages), CD11b$^+$ (macrophages and granulocytes), and CD11c$^+$ (dendritic cells) cells in the inflamed mucosa from mice transferred with CD4$^+$CD45RB^{high} T cells were also increased as compared with those from the other groups (Fig. 3).

A further quantitative evaluation of infiltrates in the LP was made by isolating LPMCs from the resected bowels. The number of CD4$^+$ cells recovered from the colons of mice transferred with CD4$^+$CD45RB^{high} T cells ($119.3 \pm 21.9 \times 10^5$) far exceeded the number of originally injected cells (5×10^5), indicating an extensive T cell proliferation in the inflamed colon (Fig. 4). In contrast, only a few CD4$^+$ T cells were recovered from the colonic tissue of mice reconstituted with both CD4$^+$CD45RB^{high} and CD4$^+$CD45RB^{low} T cells ($14.7 \pm 8.1 \times 10^5$), CD4$^+$CD25$^-$ cells ($28.5 \pm 6.5 \times 10^5$), or with no transfer ($0.3 \pm 0.1 \times 10^5$). Like the cell number recovered from the colon, the number of CD4$^+$ cells recovered from the spleen and MLNs of mice transferred with CD4$^+$CD45RB^{high} T cells ($56.4 \pm 8.1 \times 10^5$, $21.5 \pm 4.3 \times 10^5$, respectively) was significantly increased as compared with that in mice reconstituted with both CD4$^+$CD45RB^{high} and CD4$^+$CD45RB^{low} T cells ($21.3 \pm 2.9 \times 10^5$, $3.4 \pm 0.9 \times 10^5$, respectively) or with no transfer ($14.9 \pm 4.0 \times 10^5$, $1.0 \pm 0.1 \times 10^5$, respectively). Interestingly, the number of CD4$^+$ cells recovered from the spleen and MLNs of mice transferred with CD4$^+$CD25$^-$ T cells ($47.2 \pm 9.4 \times 10^5$, $11.9 \pm 0.7 \times 10^5$, respectively) was also significantly increased as compared with that in mice reconstituted with both CD4$^+$CD45RB^{high}

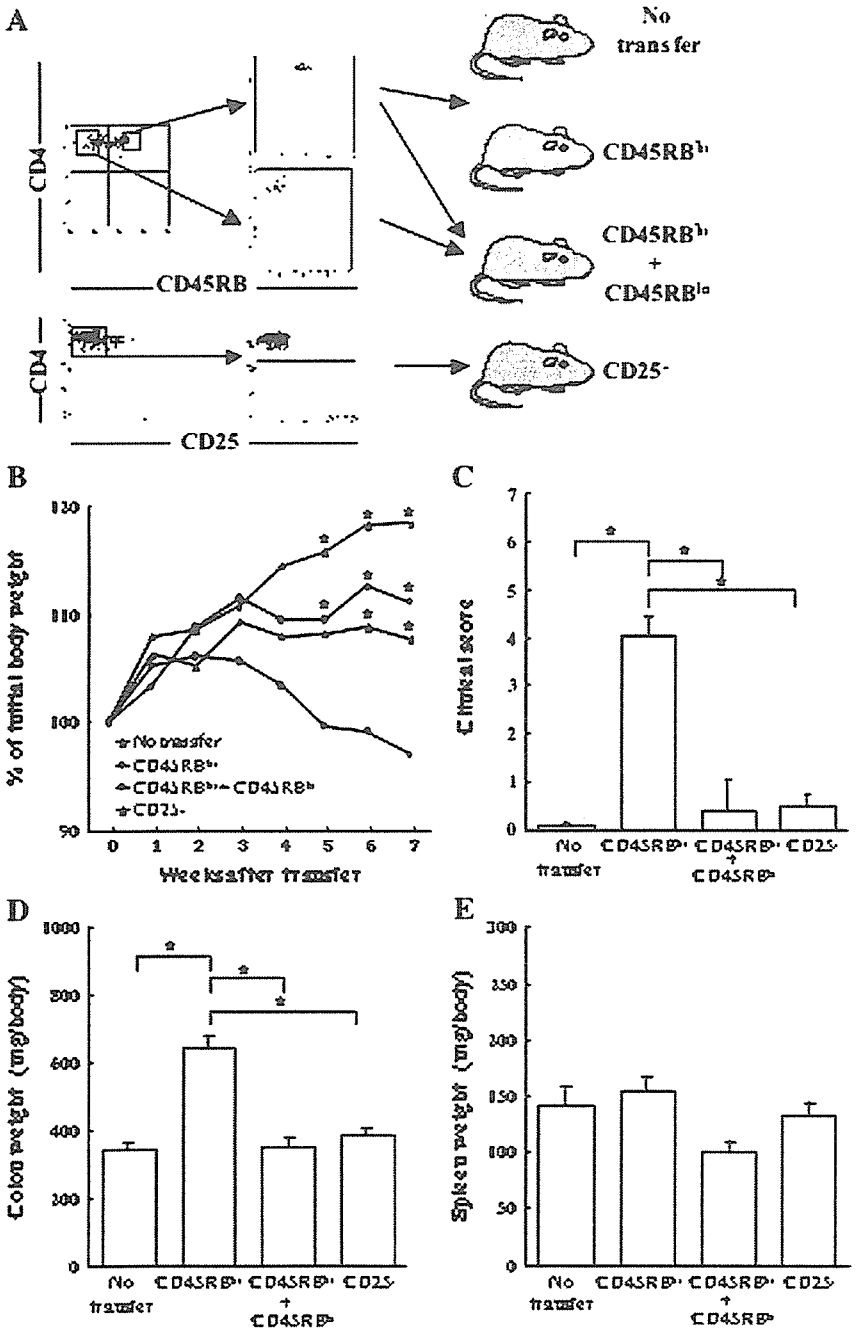

FIGURE 1. Severe wasting disease in BALB/c nude mice restored with $CD4^+CD45RB^{high}$ T cells. A, In vivo experimental design for the induction of colitis in athymic nude mice. To establish a new murine colitis model by T cell transfer, athymic BALB/c nude mice were injected intraperitoneally with 1 or 2 subpopulations of sorted $CD4^+$ T cells: no transfer, $CD4^+CD45RB^{high}$ T cells (brightest staining 20%, 5×10^5/body), $CD4^+CD45RB^{high}$ + $CD4^+CD45RB^{low}$ T cells (each 5×10^5/body), or $CD4^+CD25^-$ cells (5×10^5/body). B, Change in body weight over time is expressed as percent of the original weight (mice reconstituted with $CD4^+CD45RB^{high}$ cells, n = 28; with $CD4^+CD45RB^{high}$ cells + $CD4^+CD45RB^{low}$ cells, n = 11; with $CD4^+CD25^-$ cells, n = 12; with no transfer, n = 13). $^*P < .05$ as compared with $CD4^+CD45RB^{high}$ group. C, Clinical scores were determined at 7 wk after transfer as described in Methods. Data are indicated as the mean ± SEM of mice in each group. $^*P < .05$. D, Colon weight was determined at 7 wk after transfer. Data are indicated as the mean ± SEM of mice in each group. $^*P < .05$. E, Spleen weight was determined at 7 wk after transfer. Data are indicated as the mean ± SEM of mice in each group.

and $CD4^+CD45RB^{low}$ T cells or with no transfer (Fig. 4). Similarly, with the recovered cell number of $CD4^+$ cells, the number of $B220^+$ cells recovered from the colon and the MLNs of mice transferred with $CD4^+CD45RB^{high}$ T cells ($118.2 \pm 20.1 \times 10^5$, 95.4 ± 21.7, respectively) was significantly increased as compared with that in mice reconstituted with both $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ T cells ($31.1 \pm 8.3 \times 10^5$), with $CD4^+CD25^-$ cells ($27.9 \pm 4.1 \times 10^5$), or with no transfer ($25.6 \pm 4.2 \times 10^5$; Fig. 4). There were no differences, however, in the number of $B220^+$ cells recovered from the spleens among each group (Fig. 4).

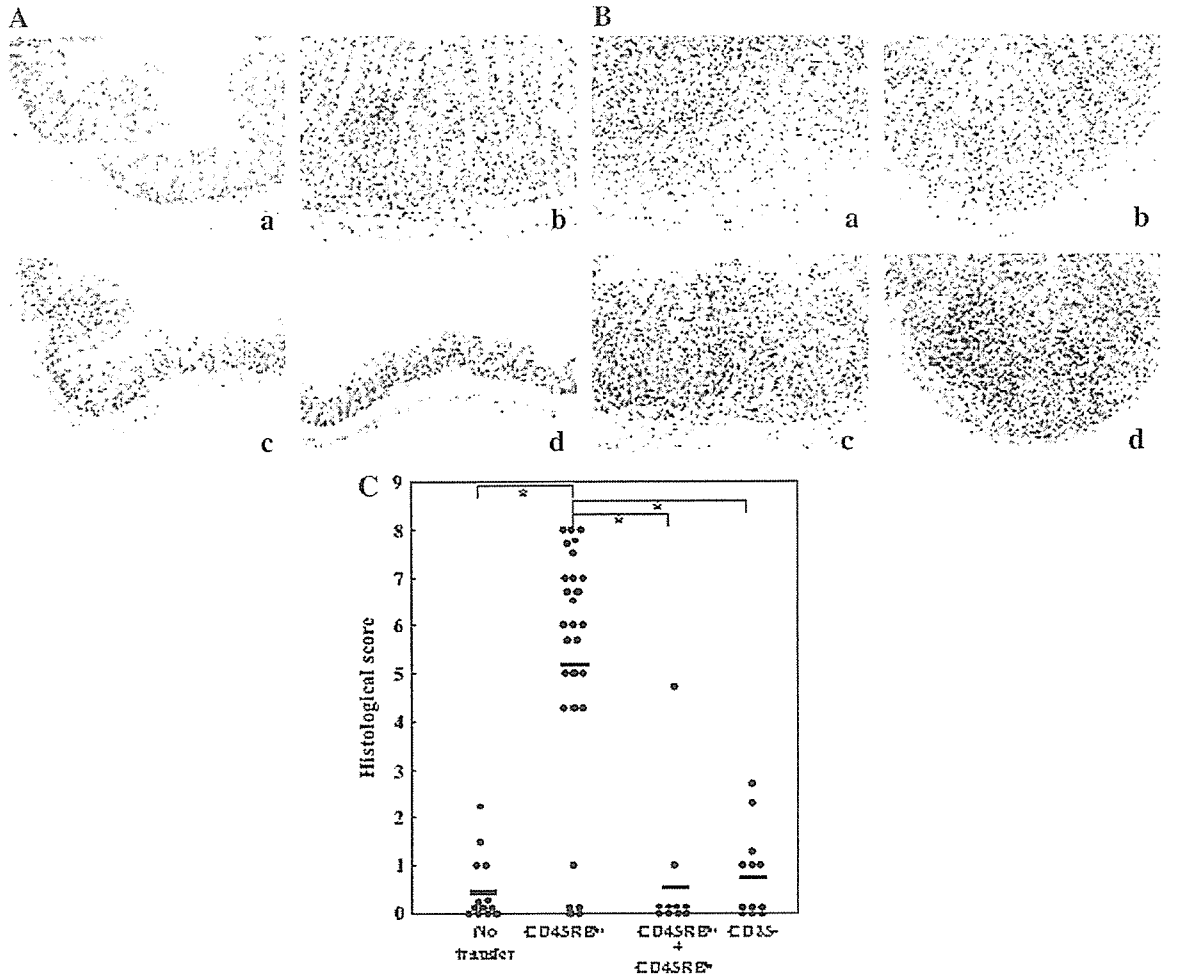

FIGURE 2. Severe chronic colitis in BALB/c nude mice restored with $CD4^+CD45RB^{high}$ T cells. A, Histological examination of the colon from each mice. Original magnification ×100. a, Mice with no transfer; b, mice reconstituted with $CD4^+CD45RB^{high}$ cells; c, mice reconstituted with $CD4^+CD45RB^{high}$ cells +$CD4^+CD45RB^{low}$ cells; d, mice reconstituted with $CD4^+CD25^-$ cells. B, Histopathological features of mice reconstituted with $CD4^+CD45RB^{high}$ T cells 7 wk after transfer. The infiltrates were also seen in the submucosa and, to a lesser degree, in the muscularis, serosa, and mesentery (a). b, Inflammatory infiltrates consisted of mainly mononuclear cells with smaller numbers of neutrophils. c, Epithelial lesions included ulceration, occasionally with a mountain-peak appearance. d, A higher number of hypertrophic lymphoid patches in the colon were also seen. Original magnification ×100. C, Histological scoring of colitis in each mice 7 wk after transfer. $*P < .05$.

Increased Expression of IgM- or IgG-Expressing B cells in Inflamed Mucosa From Colitic Athymic Mice

To further investigate the role of infiltrating lamina propria B cells in the development of colitis in nude mice reconstituted with $CD4^+CD45RB^{high}$ T cells, we assessed the immunoglobulin-producing cells using fluorescent immunohistochemistry. There were a markedly increased number of the surface IgM-positive cells observed in the inflamed mucosa of mice reconstituted with $CD4^+CD45RB^{high}$ T cells (Fig. 5). In addition, the surface IgG-producing cells, which were rarely present in normal lamina propria, were often detected in these mice compared with mice in other groups (Fig. 5). In contrast, somewhat odd, surface IgA-producing cells disappeared in these mice as compared with those in other groups (Fig. 5). To assess the involvement of anti-colon autoantibodies in the pathogenesis of colitis, we tested whether sera obtained from colitic nude mice reacted with epithelial cells in the colons of young nude mice with no transfer. Neither anti-colon IgG nor IgM autoantibodies were detected in sera from the colitic nude mice transferred with $CD4^+CD45RB^{high}$ T cells (data not shown), suggesting that

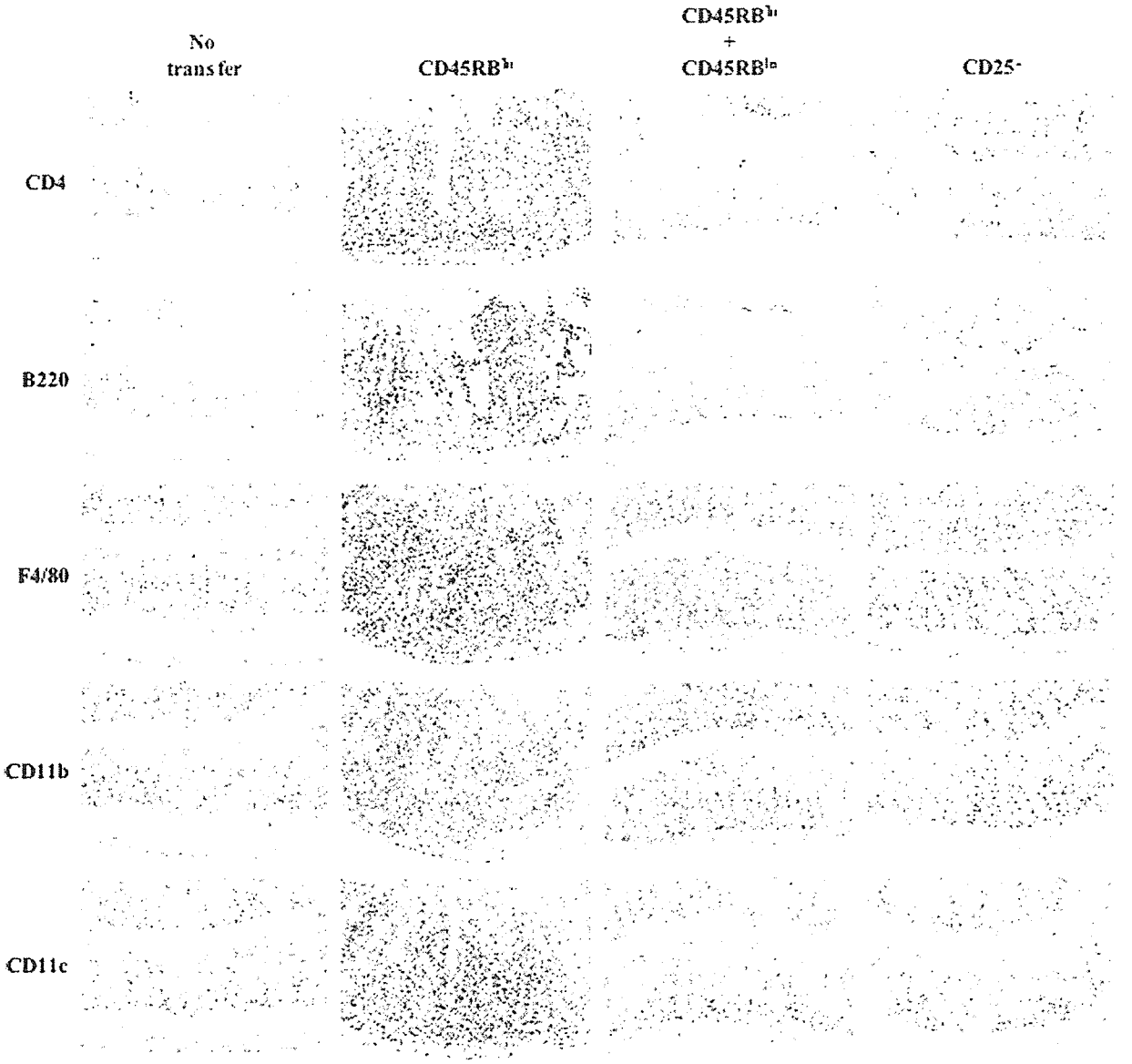

FIGURE 3. Immunohistochemical analysis showed the massive B220$^+$ cell infiltration of colonic tissue in mice reconstituted with CD4$^+$CD45RB^{high} T cells 7 wk after transfer. A large number of aggregated B220$^+$ cells, which were structurally different from B cells in colonic patches, were infiltrated in the lamina propria in the mice reconstituted with CD4$^+$CD45RB^{high} T cells 7 wk after transfer. In addition, CD4$^+$, CD8$^+$, F4/80$^+$, and CD11c$^+$ cells were increased in the inflamed colonic mucosa of these mice as compared with those of other groups. Original magnification ×100.

other helper T cells are required, and these may be not derived from CD4$^+$CD45RB^{high} T cells. We further assessed the B1 B cells in inflamed mucosa because B1 cells appears to be involved in B cell–mediated autoimmunity.[33] However, flow cytometric analysis of LPMC from the colitic nude mice showed no significant absolute increase in CD5$^+$IgM$^+$ B1 B cells on LPMC from colitic nude mice (data not shown).

Both T_H1 and T_H2 Mediated Immune Responses in Colitic Athymic Mice

Because, as shown in Figures 3 and 4, CD4$^+$ and B220$^+$ cells were increased in inflamed mucosa from nude mice reconstituted with CD4$^+$CD45RB^{high} T cells, we assessed the involvement of cytokines in the development of colitis. To this end, isolated lamina propria CD4$^+$ T cells were cultured, and

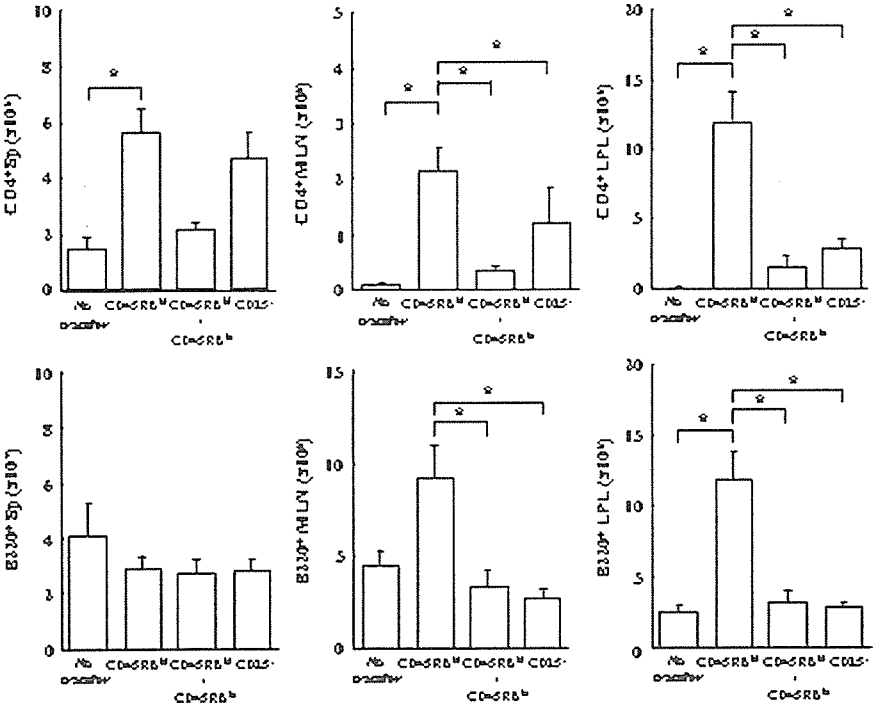

FIGURE 4. A marked increase in CD4+ T cells and B220+ B cells in inflamed mucosa in mice reconstituted with CD4+CD45RB^{high} T cells 7 wk after transfer. The number of CD4+ T and B220+ B cells in the spleen (Sp), MLN, and colonic lamina propria were determined by flow cytometry. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .05$.

the supernatants were analyzed for concentration of various cytokines by a specific ELISA for IL-10 and by CBA for other cytokines. IFN-γ and TNF-α production by anti-CD3/anti-CD28 mAbs-stimulated lamina propria CD4+ T cells was significantly increased in mice reconstituted with CD4+CD45RB^{high} T cells as compared with other groups, indicating that the colitis induced by the adoptive transfer of CD4+CD45RB^{high} T cells into nude mice could be mediated in T_H1-immune responses (Fig. 6). T_H2 cytokine (IL-4, IL-5, and IL-10) production was also significantly increased in mice reconstituted with CD4+CD45RB^{high} T cells as well as in mice reconstituted with CD4+CD25− T cells as compared with that in mice reconstituted with CD4+CD45RB^{high} and CD4+CD45RB^{low} T cells or with no transfer (Fig. 6). This indicated that this new model could also be involved in T_H2–immune responses.

DISCUSSION

In the present study, we established a new chronic colitis model associated with the deleted T_R function by transferring CD4+CD45RB^{high} T cell subset into athymic nude mice. These mice developed wasting disease with severe leukocyte infiltration in the colon, accompanied by marked epithelial hyperplasia, loss of goblet cells, and crypt abscesses. More important, unlike a classic SCID-transfer model, the colitis induced by the adoptive transfer of CD4+CD45RB^{high} T cell subset into nude mice has the hallmarks of both T_H1 and T_H2 responses as indicated by the overexpression of IFN-γ, TNF-α, IL-4, and IL-5. In clear contrast, mice reconstituted with CD4+CD45RB^{high} and CD4+CD45RB^{low} T cells or CD4+CD25− T cells showed no significant intestinal inflammation when followed up to 30 weeks after T cell reconstitution (data not shown). The co-transfer of CD4+CD45RB^{high} and CD4+CD45RB^{low} T cell populations did not cause wasting and also inhibited the induction of colitis, suggesting that this model should be controlled by T_R cells in the CD4+CD45RB^{low} T cell population. This population contains classic CD4+CD25+ T_R cells and the recently identified CD4+CD25−CD45RB^{low} T_R cells, which constitutively express glucocorticoid-induced TNF receptor–related gene (GITR), CD103, or membranous TGF-β.[20,32,34] Furthermore, this nude colitis model is unique because the model is accompanied by the feature that a massive IgM-producing B cell is infiltrated in the inflamed mucosa similar to a human IBD but unlike the classic colitis model induced by the adoptive transfer of CD4+CD45RB^{high} T cells into SCID/Rag-1,2–deficient mice.[23]

Laroux and colleagues have provided data contrary to those presented here in that they reported that the transfer of CD4+CD45RB^{high} T cells into syngeneic athymic nude mice does not induce the development of wasting disease and colitis,[35] whereas the transfer into syngeneic SCID mice does. Therefore, the question arises: why is the present study in disagreement with the Laroux et al study? We believe that the