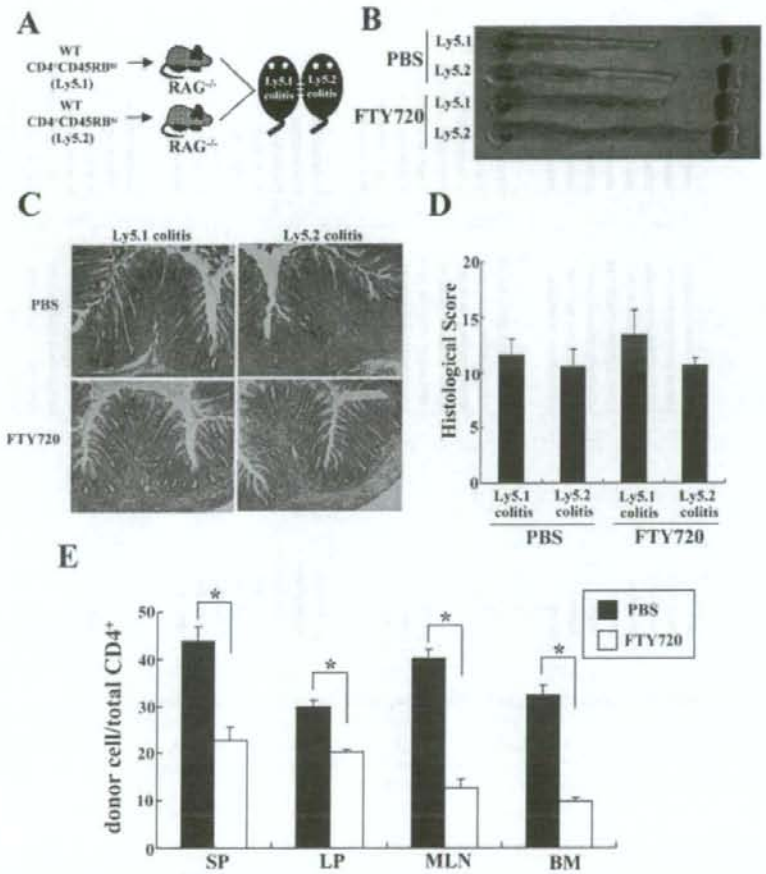

FIGURE 4. FTY720 treatment suppresses recirculation of colitogenic $CD4^+$ T cells, but does not ameliorate the established colitis in parabionts. A: Parabionts between established colitic RAG-$2^{-/-}$ mice transferred with $Ly5.1^+$ or $Ly5.2^+$ $CD4^+CD45RB^{high}$ T cells were treated with FTY720 (1.0 mg/kg) or control PBS daily for up to 6 weeks starting 1 day before parabiosis surgery, and were monitored for clinical signs such as hunched posture, piloerection, diarrhea, and blood in the stool. B: Gross appearance of the colon, spleen, and MLNs obtained from parabionts treated with PBS (top) or FTY720 (bottom) after 6 weeks of treatment. C: Histological examination of the colon from PBS-treated (upper) or FTY720-treated (lower) mice. Original magnification; ×100. D: Histological scoring of colitis in PBS-treated and FTY720-treated mice. Data are indicated as mean ± SEM of 6 mice in each group. NS, not significant. E: Ratio of donor-derived $CD4^+$ T cells per total $CD4^+$ T cells of each parabiont. *$P < 0.05$. Data are indicated as mean ± SEM of 6 parabionts in each group.

FIGURE 3. Colitogenic $CD4^+$ T cells dynamically recirculate even after the establishment of colitis. A: Parabiosis experimental design. For adoptive transfer, splenic $CD4^+CD45RB^{high}$ T cells were isolated from C57BL/6-Ly5.1 or -Ly5.2 mice and transferred into RAG-$2^{-/-}$ mice ($n = 6$ per group). Six weeks after transfer, when both groups of recipient mice had developed wasting disease and colitis, intergroup pairs were joined by parabiosis surgery. B: Photo of parabionts. C: Gross appearance of colon, spleen, and MLN obtained from parabionts between RAG-$2^{-/-}$ mice transferred with C57BL/6-Ly5.1- or C57BL/6-Ly5.2-$CD4^+CD45RB^{high}$ T cells at 4 weeks after surgery or age-matched control C57BL/6 mice. D: Histological examination of the colon from parabiont pairs at 6 weeks after transfer or control C57BL/6 mice. Original magnification, ×100. E: LP, MLN, TD, PB, SP, and BM cells were isolated from each mouse at 4 weeks after surgery, and the number of $Ly5.1^+$ and $Ly5.2^+$ $CD4^+$ cells was determined by flow cytometry. Data are indicated as mean ± SEM of 6 mice in each group.

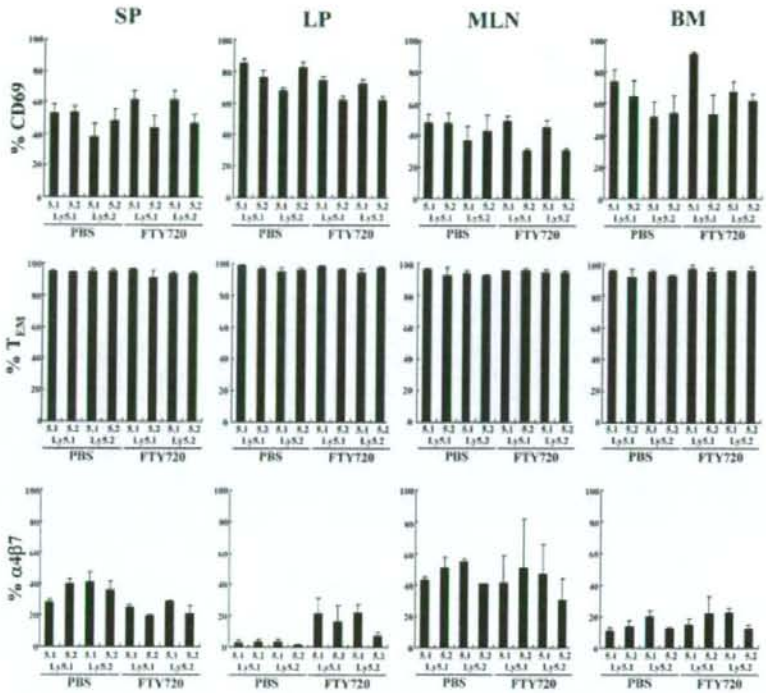

FIGURE 5. FTY720 treatment does not modulate the phenotype of $CD4^+$ T cells in parabionts. Expression of CD44, CD62L, CD69, and integrin $\alpha4\beta7$ on $CD4^+$ T cells obtained from SP, MLN, LP, and BM in colitic parabionts treated with FTY720 or PBS. Freshly isolated cells were stained with FITC-labeled anti-CD45.1 or CD45.2, and PerCP-labeled anti-CD4, and PE-labeled anti-CD62L, anti-CD69, or anti-integrin $\alpha4\beta7$ mAb and allophycocyanin-labeled anti-CD44. Samples were analyzed by flow cytometry. Lymphocytes were identified by characteristic forward angle and sidescatter profiles.

fully induces chronic colitis, even after the full establishment of colitis, indicating that colitogenic $CD4^+$ T_{EM} cells actively and continuously recirculate in chronic colitic mice.

Although the parabiosis system seems to be somewhat artificial and problematic on some level, as 2 mice are under the stress of surgery and behavioral limitation, the surgical joining of mice harboring colitogenic $CD4^+$ T cells results in prompt development of anastomoses of blood vessels within a few days. This model is also useful to assess the hemodynamics of circulating or resident $CD4^+$ T cells in colitic parabionts, these cells being distinguishable by using the Ly5.1/Ly5.2 system.[28] We showed that $Ly5.1^+$ and $Ly5.2^+$ cells in both parabionts had the character of $CD44^{high}CD62L^-$ T_{EM} cells and were well mixed and in almost equal proportions in LP, as well as in SP, MLN, BM, PB, PC, and TD 4 weeks after parabiosis surgery. This result also indicated that colitogenic $CD4^+$ T cells continuously egress from the inflamed mucosa, migrate into afferent lymphatics→MLN→efferent lymphatics→PB in sequence, and return to the intestine in the established colitic mice.

Hence, the present results support the notion that circulating colitogenic $CD4^+$ T cells might be appropriate targets for IBD therapy. In this regard, leukocytapheresis has been applied to the treatment of various autoimmune diseases including, in recent years, UC.[29] It is thought that leukocytapheresis removes pathogenic cells that contribute to the pathogenesis of IBD from the body. Interestingly, we recently demonstrated that leukocytapheresis treatment for patients with UC selectively removed $CD4^+CD45RO^+CD62L^-$ T_{EM} cells rather than $CD4^+CD45RO^+CD62L^+$ T_{CM} cells or $CD4^+CD25^{bright}$ regulatory T (T_{REG}) cells,[30] suggesting that the removal of circulating $CD4^+$ T_{EM} cells in PB of patients with IBD has logical advantages for the treatment of IBD.

As an essential factor in the persistence of T-cell-mediated colitis, we have focused on IL-7 in a series of studies.[23,27,32,35–37] IL-7 is secreted by stromal cells in BM and thymus, and epithelial cells including intestinal epithelia.[31–33] Also, accumulating evidence shows that IL-7 is an important cytokine involved in supporting the survival of memory $CD4^+$ T cells.[34] We have previously demonstrated

that: 1) IL-7 is constitutively produced by intestinal goblet epithelial cells in human[32]; 2) IL-7 transgenic (Tg) mice, where strong promoters drive systemic overexpression, develop chronic colitis that mimics the histopathological characteristics of human IBD[35]; 3) LP $CD4^+$IL-$7R\alpha^{high}$ T cells in $CD4^+CD45RB^{high}$ T cell-transferred colitic mice are colitogenic[36]; 4) IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice transferred with colitogenic $CD4^+$ T cells isolated from the inflamed LP of $CD4^+CD45RB^{high}$ T cell-transferred colitic mice do not develop colitis[37]; 5) nevertheless, intestinal IL-7 is not essential for the perpetuation of colitis, since IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice parabiosed with colitic $CD4^+CD45RB^{high}$ T-cell-transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ donor mice develop severe colitis despite lacking intestinal IL-7[27]; 6) colitogenic $CD4^+CD44^{high}CD62L^-$IL-$7R\alpha^{high}$ T_{EM} cells preferentially reside in the BM of colitic $CD4^+CD45RB^{high}$ T cell-transferred SCID/RAG-1/$2^{-/-}$ mice[23]; and 7) paradoxically, IL-7-producing goblet cells are easily decreased or depleted (so-called 'goblet depletion') in colitic IL-7 Tg mice[35] and in the present model of colitis,[37] resulting in the decreased IL-7 production in the intestine. These findings might have a close bearing on the present data, because it appears that colitogenic LP $CD4^+$ T_{EM} cells may intermittently have to egress from the inflamed mucosa, where IL-7 is absent or decreasing, and actively circulate to IL-7-producing reservoirs such as BM in order to survive and continue to cause the chronic inflammation.

Finally, the reason should be discussed why FTY720 could not ameliorate the colitis in parabionts between 2 established RAG-$2^{-/-}$ mice transferred with $Ly5.1^+$ or $Ly5.2^+$ $CD4^+CD45RB^{high}$ T cells. One possibility is that FTY720 treatment is not appropriate for therapeutic use, as almost all previous reports show the preventive, but not therapeutic, effect of FTY720 on the development of various diseases including autoimmune diseases and allo-transplantation.[38–42] It is also possible that more time is needed for the therapeutic effect of FTY720 to become evident in our parabiosis model, as we observed FTY720-treated parabionts for only 6 weeks because of the limitation of the parabiosis system. Consistent with this possibility, we previously demonstrated that colitogenic LP $CD4^+$ T cells are quite long-lived, because sequential adoptive transfers of colitogenic LP $CD4^+$ T cells into new SCID mice could stably induce colitis at least 6 times totally in experiments extending over 3 years. Thus, although FTY720 might inhibit the recruitment of colitogenic LP $CD4^+$ T cells into the inflamed mucosa in this parabiosis system (Fig. 4), the resident colitogenic LP $CD4^+$ T cells might to be able to maintain intestinal inflammation for an extended period, as FTY720 is unable to induce apoptosis of those cells (unpubl. data).[15]

In summary, we demonstrated that colitogenic $CD4^+$ T_{EM} cells actively recirculate in fully established colitic mice. Although IBD have hitherto been classified solely as intestinal disorders, unlike other autoimmune diseases such as connective tissue diseases, the present study suggests not only that IBD are not curable by local resection, but also that they should be reclassified as systemic diseases that pathogenic lymphocytes disseminate outside the intestine.

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Negative Feedback Regulation of Colitogenic CD4$^+$ T Cells by Increased Granulopoiesis

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Background: Chronic inflammatory diseases are characterized by massive infiltration of innate and acquired immune cells in inflammatory sites. However, it remains unclear how these cells cooperate in the development of disease. Although bone marrow (BM) is a primary site for hematopoiesis of immune cells except T cells, BM recruits memory T cells from the periphery. We have recently demonstrated that colitogenic CD4$^+$ memory T cells reside in BM of colitic CD4$^+$CD45RB^{high} T-cell-transferred SCID mice. Based on this background we here investigate whether granulocytes promote or suppress the expansion of colitogenic CD4$^+$ T cells.

Methods: First, we show that Gr-1^{high}CD11b$^+$ granulocytes were significantly increased in colitic BM along with a significant increase of peripheral granulocytes. Consistently, the colony-forming unit (CFU) assay revealed that granulocyte colony formation was dominantly induced by supernatants from anti-CD3-stimulated colitic BM CD4$^+$ T cells.

Results: Administration of granulocyte-depleting anti-Gr-1 mAb to colitic mice did not ameliorate the colitis, but exacerbated the wasting disease with an increased expansion of systemic, but not lamina propria, CD4$^+$ T cells with activated phenotype.

Conclusions: These results suggest that the increased granulopoiesis by colitogenic BM CD4$^+$ T cells represent a negative feedback mechanism to control systemic inflammation.

(Inflamm Bowel Dis 2008;14:1491–1503)

Key Words: CD4$^+$ T cells, bone marrow, granulocytes, immune regulation, immunopathology

Inflammatory bowel diseases (IBDs) are caused by excessive tissue damage by chronic inflammatory responses in the gut wall and commonly take persistent courses.[1,2] According to our current understanding the diseases are caused by activated macrophages and effector CD4$^+$ T cells infiltrated in inflamed mucosa, which are presumably primed by commensal bacterial antigen-presenting dendritic cells in lymphoid tissues, such as mesenteric lymph nodes and Payer's patches.[3–8] Thus, recent studies mainly focus on lymphocytes, macrophages, and dendritic cells in addition to epithelial cells for the pathogenesis of IBD. Granulocytes are thought to be the first emerging cells in inflamed mucosa of human IBD in response to danger signals, such as epithelial damage, before the establishment of antigen-specific T-cell-mediated immune responses.[9–12]

Clinicopathologically, however, it is well known that abundant granulocytes are observed in inflamed mucosa of active IBD patients, and the degree of infiltrated granulocytes/neutrophils is a criteria for disease severity in the pathological scoring systems of both Crohn's disease and ulcerative colitis.[13] In addition, it has also been reported that the circulating activated granulocytes are elevated with increased survival time in patients with severe IBD.[14,15] Thus, we investigated the role of granulocytes in the pathogenesis of the chronic phase of colitis using a murine T-cell-mediated model of colitis induced by adoptive transfer of CD4$^+$CD45RB^{high} T cells into immunodeficient SCID mice.[16,17]

MATERIALS AND METHODS

Mice

BALB/c and C.B-17 SCID mice were purchased from Japan Clear (Tokyo, Japan). Mice were maintained under

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specific pathogen-free (SPF) conditions in the Animal Care Facility of Tokyo Medical and Dental University. Donors and littermate recipients were used at 6–12 weeks of age. All experiments were approved by the regional animal study committees and were done according to institutional guidelines and Home Office regulations.

Antibodies

The following mAbs other than purified antimouse Gr-1 mAb (RB6-8C5), biotin-conjugated antimouse IL-7Rα (A7R34; eBioscience, San Diego, CA), PE-conjugated antimouse CD11b (M1/70; eBioscience), and PE-conjugated anti-F4/80 (CI:A3-1; Serotec, Bicester, UK) were obtained from BD Pharmingen (San Diego, CA) and used for purification of cell populations and flow cytometric analysis: FcγR (CD16/CD32)-blocking mAb (2.4G2), FITC-, PE-, PECy5-, PerCP-, or APC-conjugated antimouse CD4 (RM4-5); FITC- or PerCP-conjugated antimouse CD3 (145-2C11); FITC-conjugated antimouse CD45RB (16A); FITC-conjugated antimouse CD62L (MEL-14); FITC-conjugated antimouse CD69 (H1.2F3); FITC-conjugated antimouse Gr-1 (RB6-8C5); FITC-conjugated antimouse CD11c (7D4); PE-conjugated anti-Ly6G (1A8); biotin-conjugated anti-Ly6C (AL-21); PE-conjugated anti-CD31 (MEC13.3); biotin-conjugated rat IgG2; PE-conjugated mouse IgG; and PE-conjugated rat IgG.

Induction of Colitis

Colitis was induced in SCID mice by adoptive transfer of $CD4^+CD45RB^{high}$ T cells (17). Briefly, $CD4^+$ T cells were isolated from splenocytes using the anti-CD4 (L3T4) MACS magnetic separation system (Miltenyi Biotec, Auburn, CA). Enriched $CD4^+$ T cells were labeled with PE-conjugated antimouse CD4 mAb and FITC-conjugated anti-CD45RB mAb, then sorted to yield the $CD45RB^{high}$ (highest staining 30%) fraction on a FACS Vantage SE (Becton Dickinson, Sunnyvale, CA). Each SCID mouse was injected intraperitoneally with 3×10^5 $CD4^+CD45RB^{high}$ T cells. The colitic mice induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells were sacrificed at 6–8 weeks after transfer.

T-cell Preparation

For isolation of peripheral lymphocytes, 600 μL of peripheral blood was collected from each mouse and diluted 1:1 with phosphate-buffered saline (PBS). The diluted blood was layered over Lymphosepar II (IBL, Gunma, Japan) and centrifuged at 400*g* for 20 minutes at room temperature. Spleen (SP) was mechanically disrupted into single cell suspensions. BM was collected from the femur by flushing with sterile PBS. To assess cells for the preparation of colonic LP cells,[18] colon was first flushed extensively to eliminate the lumen content, then longitudinally opened and cut into small pieces. The dissected mucosa was incubated with Ca++ Mg++-free Hank's BSS containing 1 mM DTT (Sigma-Aldrich, St. Louis, MO) for 30 minutes to remove mucus, then treated with 1 mg/mL collagenase (Worthington Biomedical, Freehold, NJ) and 0.01% DNase (Worthington) for 2 hours. After filtering through gauze, cells were pelleted twice through a 40% isotonic Percoll solution and then subjected to Ficoll-Hypaque density gradient centrifugation (40%/75%). Enriched $CD4^+$ T cells were obtained by positive selection using anti-CD4 (L3T4) MACS magnetic beads. The resultant cells contained >94% $CD4^+$ cells when analyzed by FACS Calibur.

Flow Cytometry

To detect the surface expression of a variety of molecules, isolated cells were preincubated with the FcγR-blocking mAb for 20 minutes followed by incubation with specific FITC-, PE-, PerCP-, APC-, or biotin-labeled antibodies for 30 minutes on ice. Biotinylated antibodies were detected with PE-streptavidin. Standard 2-, 3-, or 4-color flow cytometric analyses performed on FACS Calibur and CellQuest software. Background fluorescence was assessed by staining with control isotype-matched mAbs.

Cytokine ELISA

To measure cytokine production, 1×10^5 $CD4^+$ T cells from 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 antimouse CD3e mAb (145-2C11, BD Pharmingen) and 2 μg/mL hamster antimouse CD28 mAb (37.51, BD Pharmingen) in PBS overnight at 4°C. Culture supernatants were removed after 48 hours and assayed for cytokine production. Cytokine concentrations were determined by specific ELISA as per the manufacturer's recommendation (R&D, Minneapolis, MN).

Immunohistochemistry

Consecutive cryostat bone marrow sections (6 μm) were fixed and stained with the following rat antibodies by a 4-step method. First, the sections were stained with purified anti-Gr-1 antibodies (RB6-8C5), followed by staining with Alexa 594 goat antirat IgG (Molecular Probes, Eugene, OR). The sections were then stained with biotinylated anti-CD4 (RM4-5; BD Pharmingen), following by staining with Alexa 488 streptavidin (Molecular Probes). All confocal microscopy was carried out on a BioZERO BZ8000 (Keyence, Tokyo).

Colony-forming Unit (CFU) Assay

Unseparated femoral BM from wildtype BALB/c mice were plated at 5×10^5 cells/mL in MethoCult without cytokines (Stem Cell Technologies, Vancouver, BC, Canada) in the presence of 2000-fold diluted supernatants obtained from

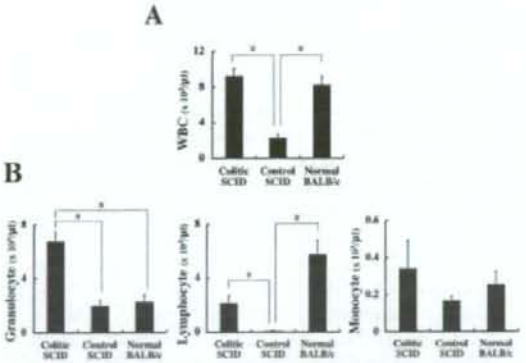

FIGURE 1. Neutrophils are markedly increased in peripheral blood of mice with established chronic colitis. A: WBC counts in colitic SCID mice 6 weeks after transfer, age-matched control SCID mice, and normal BALB/c mice. B: Cell counts of different compartments in colitic SCID mice 6 weeks after transfer, age-matched control SCID mice and normal BALB/c mice were determined by flow cytometry. Error bar indicates SEM for 10 mice of each group. $^*P < 0.05$.

anti-CD3/CD28 mAbs-stimulated colitic or normal BM or LP $CD4^+$ T cells for 48 hours. Colonies were scored 7 days after incubation of cells at 5% CO_2 in a humidified atmosphere.

In Vivo Experimental Design

We first checked whether in vivo anti-Gr-1 treatment preferentially depletes granulocytes, but not dendritic cells and macrophages, and myeloid suppressor cells by assessing the expression of Ly-6G molecule that is a more specific marker for granulocytes, CD11c, F4/80, and CD31 in addition to CD11b and Gr-1 on cells obtained from various sites of colitic mice administered anti-Gr-1 mAb (250 μg) or control IgG (250 μg) every 8 hours 3 times for 1 day before sacrifice. Then the expression of F4/80 (macrophage marker), CD11c (dendritic cell), and CD31 (myeloid suppressor cells)[19] on $CD11b^+Gr\text{-}1^{high}$ or $CD11b^+Gr\text{-}1^{low}$ cells was examined using flow cytometry. As a long-term administration of anti-Gr-1 mAb, each SCID mouse was injected i.p. with 200 μL of PBS containing 3×10^5 $CD4^+CD45RB^{high}$ T cells. These mice were then i.p. administered 250 μg of anti-Gr-1 mAb in 250 μL PBS 3 times per week, starting at the day of transfer or at 3 weeks after T-cell transfer. An equivalent amount of control rat IgG (Sigma) was administered in positive control mice. Negative control SCID mice were transferred with a mixture of 3×10^5 $CD4^+CD45RB^{high}$ and 3×10^5 $CD4^+CD45RB^{low}$ T cells. In another set of in vivo experiments, SPX SCID mice were used as recipient mice to exclude a possible contribution of the spleen to granulopoiesis in this model.

The recipient mice after transfer were weighed initially, then 3 times per 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 sacrificed at the indicated timepoint and assessed for a clinical score that is the sum (0–8 points) of 4 parameters as follows: hunching and wasting, 0 or 1; colon thickening, 0–3 (0, no colon thickening; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); stool consistency, 0–3 (0, normal beaded stool; 1, soft stool; 2, diarrhea); and an additional point was added if gross blood was noted.[20] To monitor the clinical signs during the observed period over time, the disease activity index is defined as the sum (0–5 points) of the above-mentioned parameters except colon thickening. To assess the histological scores, tissue samples were fixed in PBS containing 6% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with hematoxylin and eosin (H&E). The sections were analyzed without prior knowledge of the type of T-cell reconstitution and recipients. The area most affected was graded by the number and severity of lesions. The mean degree of inflammation in

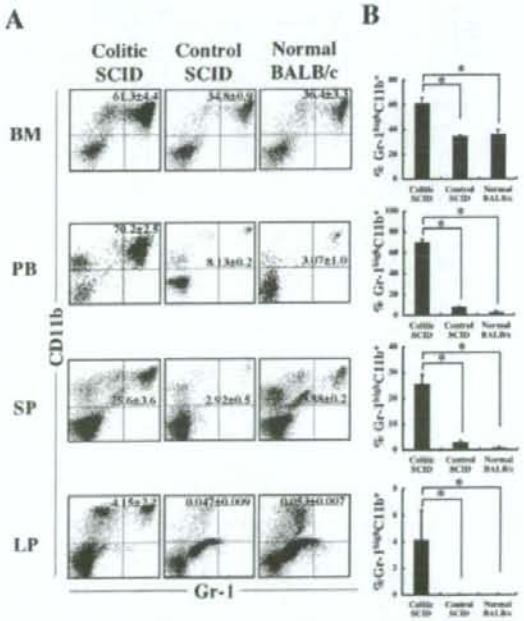

FIGURE 2. Granulocytes in colitic BM are markedly increased. A: BM, PB, SP, and LP cells of colitic SCID mice 6 weeks after transfer, age-matched control SCID mice, and normal BALB/c mice were analyzed by FACS for the expression of Gr-1 and CD11b cell-surface markers. B: Percentages of $Gr\text{-}1^{high}CD11b^+$ mature granulocytes. Error bar indicates SEM for 10 mice of each group. $^*P < 0.05$.

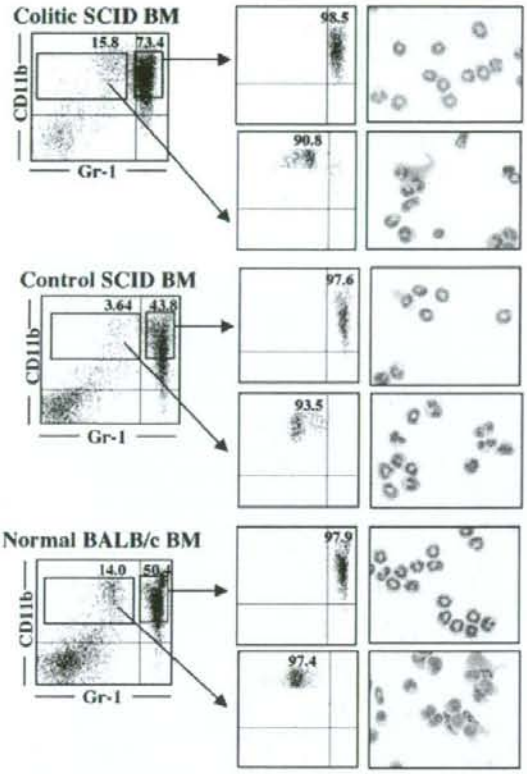

FIGURE 3. BM Gr-1^{high}CD11b^{+} cells are morphologically granulocytes. Cytospins staining with Wright was performed using isolated BM Gr-1^{high}CD11b^{+} and Gr-1^{low}CD11b cells. BM cells were isolated from colitic SCID mice, age-matched normal SCID mice, and normal BALB/c mice. Then Gr-1^{high}CD11b^{+} and Gr-1^{low}CD11b cells isolated using FACS Vantage were stained with Wright after cytospin procedures. Original, ×40. Data represent 3 independent experiments.

the colon was calculated using a modification of a previously described scoring system.[19]

Statistical Analysis

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

RESULTS

Peripheral Granulocytes Are Markedly Increased in Mice with Chronic Colitis

Although SCID-transfer model of colitis induced by adoptive transfer of CD4^{+}CD45RB^{high} T cells is one of the representative CD4^{+} T-cell-mediated colitis models accompanied by the activation of antigen-presenting cells and CD4^{+} T cells,[3,16] the role of granulocytes in this model is largely unknown. Since it is generally believed that granulocytes are the early emerging innate immune cells that are involved in the acute phase of colitis in an antigen-nonspecific manner before the establishment of T-cell-mediated immune responses, little is known about the role of granulocytes in this model especially after developing colitis. Thus, to investigate the role of granulocytes in this chronic model of colitis we first examined immune cell compartments in the peripheral blood (PB) by a conventional blood examination of cell compartment. Expectedly, the total number of white blood cells (WBCs) in colitic SCID mice at 6 weeks after transfer was significantly increased as compared with that in nontransferred age-matched SCID (control SCID) and was comparable to that in normal BALB/c mice (Fig. 1A). Surprisingly, however, the major compartment of the increased peripheral WBC in colitic SCID mice was predominantly the granulocyte population rather than the lymphocyte population (Fig. 1B, left). In contrast, the number of lymphocytes in colitic mice was indeed significantly increased as compared with that in control SCID mice, but was significantly lower than that in normal BALB/c mice (Fig. 1B, middle). In addition, no significant differences in the numbers of monocytes among the 3 groups were observed (Fig. 1B, right).

BM Granulocytes Are Markedly Increased in Mice With Chronic Colitis

Given that peripheral granulocytes were significantly increased even after the chronic phase (6 weeks after transfer) of the T-cell-mediated colitis, we next assessed the granulocytes in various sites 6 weeks after transfer using flow cytometry. Interestingly, we found that the proportion of CD11b^{+}Gr-1^{high} granulocytes was significantly increased not only in PB, SP, and LP, but also in BM in colitic mice as compared with age-matched control SCID mice or normal BALB/c mice (Fig. 2A,B). We further confirmed that the population of CD11b^{+}Gr-1^{high} cells was morphologically categorized as granulocyte by showing that almost all the isolating BM CD11b^{+}Gr-1^{high} cells from colitic SCID, control SCID, and normal BALB/c mice were preferentially polymorphonuclear-shaped (Fig. 3), while isolated BM CD11b^{+}Gr-1^{low} cells from 3 groups of mice were a mixture of mononuclear and polynuclear cells (Fig. 3), suggesting that the increase of CD11b^{+}Gr-1^{high} granulopoiesis stimulated by the infiltrating colitogenic CD4^{+} T cells in BM is a cause of the systemic increase of granulocytes in colitic mice.

To further assess the association between increased numbers of granulocytes and CD4^{+} T cells in various sites of colitic mice, we assessed the numbers of CD11b^{+}Gr-1^{high} granulocytes and CD3^{+}CD4^{+} T cells in various sites at 4 and 10 weeks after transfer. During this course the clinical score

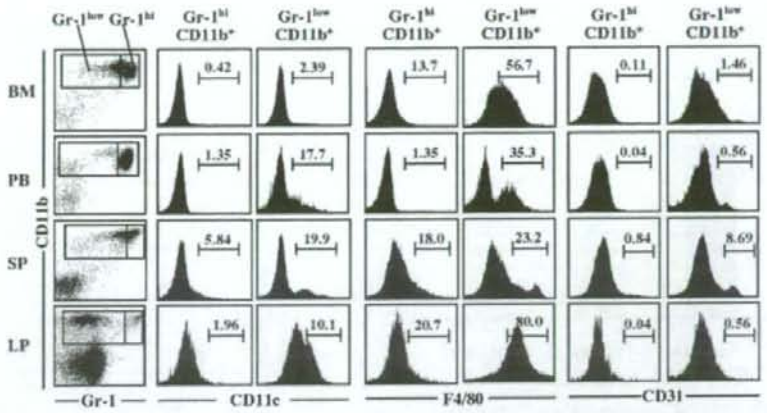

FIGURE 4. BM Gr-1^{high}CD11b^{+} cells are phenotypically granulocytes. BM cells were isolated from colitic SCID mice previously transferred with CD4^{+}CD45RB^{high} T cells. Then cells were stained with mAbs against CD11c, F4/80, or CD31 in addition to mAgs against CD11b and Gr-1. Data represent FACS profiles from 5 independent experiments.

of colitis was gradually increased (data not shown). Concomitant with the increase of CD3^{+}CD4^{+} T cells, the absolute number of Gr-1^{high}CD11b^{+} granulocytes was also gradually increased in various sites including BM (data not shown), suggesting that increased granulocytes in colitic mice may be involved in the pathogenesis in chronic colitis at the late stage of inflammation.

Since it is well known that immature dendritic cells and macrophages and myeloid suppressor cells[19] also express CD11b and/or Gr-1 molecules, we next assessed whether our isolated CD11b^{+}Gr-1^{high} cells obtained from various sites of colitic mice are solely granulocytes by flow cytometry using other specific mAbs. As shown in Figure 4, the majority of colitic CD11b^{+}Gr-1^{high} cells from BM, PB, SP, or LP did not express F4/80 (macrophage marker), CD11c (dendritic cell), and CD31 (myeloid suppressor cell)[19] in sharp contrast to the paired CD11b^{+}Gr-1^{low} cells that contained such nongranulocyte cells, suggesting that our CD11b^{+}Gr-1^{high} cells in colitic mice are mainly granulocytes, but not immature dendritic cells and macrophages, and myeloid suppressor cells.

Colitic BM CD4^{+} T Cells Produce Granulopoietic Cytokines

Given the evidence that CD11b^{+}Gr-1^{high} granulocytes are increased in various sites of colitic mice even at the late stage of colitis development, we next investigated how these cells are maintained in the colitic mice. To this end we focused on colitogenic CD4^{+} T cells residing in colitic BM and hypothesized that they produce granulopoietic cytokines such as IL-3, G-CSF, GM-CSF, IL-6, and IL-17 in addition to Th1 cytokines.[21] Among these cytokines the production of IL-3, IL-17, or GM-CSF, but not IL-6 and G-CSF, by anti-CD3/CD28 mAbs-stimulated colitic BM CD4^{+} T cells was significantly increased as compared with that by normal BM and LP CD4^{+} T cells, and this was to a similar extent to those by colitic LP CD4^{+} T cells (data not shown). Interestingly, the production of Th1 cytokines (IFN-γ and TNF-α) by colitic BM CD4^{+} T cells was significantly increased as compared with that by normal BM CD4^{+} T cells, but this was significantly lower than that by colitic LP CD4^{+} T cells (data not shown).

Colitic BM CD4^{+} Cells Secrete Colony-forming Factors

To further assess the ability of colitic BM CD4^{+} T cells to promote granulopoiesis, we next performed in vitro CFU assay using supernatants from anti-CD3/CD28 mAb-stimulated colitic BM CD4^{+} T cells. Expectedly, we found that the supernatant from colitic BM CD4^{+} T cells significantly increased the frequencies of CFU-G, -M, and -GM, as compared with that from normal BM CD4^{+} T cells (Fig. 5).

In Vivo Depletion of Granulocytes Exacerbates Wasting Disease in Chronic Colitic Mice

Given the findings of increased granulopoiesis in the BM of chronic colitic mice, we finally assessed the role of granulocytes in the pathogenesis of chronic colitis by in vivo administration of a granulocyte-depleting anti-Gr-1 mAb (RB6-8C5). Before starting the long-term administration of anti-Gr-1 mAb to colitic mice, we first checked whether the short-term anti-Gr-1 treatment preferentially depletes granulocytes, but not dendritic cells and macrophages, and myeloid suppressor cells, by assessing the expression of Ly-6G mol-

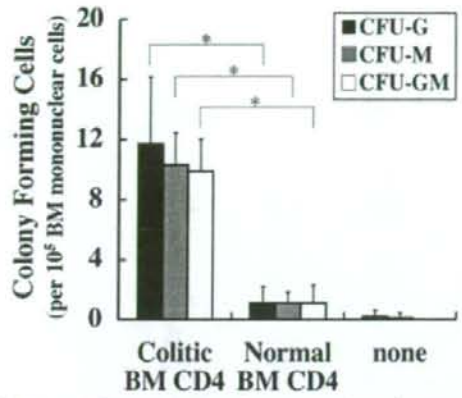

FIGURE 5. Colitic BM $CD4^+$ cells secrete colony-forming factors. BM mononuclear cells from normal BALB/c mice were plated in methylcellulose-containing media with 2000-fold diluted supernatants obtained from anti-CD3/CD28-stimulated colitic or normal BM $CD4^+$ cells. Hematopoietic colonies containing greater than 30 cells were scored after 7 days. Data are indicated as mean ± SD of 6 mice in each group.

ecule that are more specific markers for granulocytes, F4/80 (macrophage marker), CD11c (dendritic cell), and CD31 (myeloid suppressor cell) in addition to CD11b and Gr-1 on cells obtained from various sites of colitic mice administered anti-Gr-1 mAb or control IgG every 8 hours 3 times for 1 day before sacrifice. As expected, we found that anti-Gr-1 mAb treatment preferentially depleted Ly-$6G^+CD11b^+$ cells, but not Ly-$6C^+CD11b^+$ cells (Fig. 6A), showing that anti-Gr-1 treatment depletes granulocytes, but not other cells. Consistently we also found that anti-Gr-1 treatment does not deplete $CD11b^+F4/80^+$ macrophages, $CD11b^+CD11c^+$ dendritic cells, and $CD11b^+CD31^+$ myeloid suppressor cells (Fig. 6B).

Given the evidence that in vivo anti-Gr-1 mAb treatment preferentially depletes granulocytes, we then administered anti-Gr-1 mAb or control rat IgG to the recipient mice from the day of transfer of $CD4^+CD45RB^{high}$ T cells and then 3 times per week for 4 weeks (Fig. 7A). As a negative control, SCID mice were transferred with the same number of $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ T cells. As shown in Figure 7B, the control IgG-treated mice manifested weight loss (wasting disease) from 3 weeks after transfer. These mice had diarrhea with increased mucus in the stool, anorectal prolapse, and hunched posture by 4 weeks after transfer. Surprisingly, the mice treated with anti-Gr-1 mAb developed markedly severer wasting disease with diarrhea, and the average weight loss at 4 weeks after transfer (percent original weight 87.9 ± 9.29%) was significantly higher than that of the control IgG-treated mice (104.8 ± 6.53%) (Fig. 7B). On the contrary, the mice transferred with $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ cells appeared healthy, with a gradual increase of body weight and without diarrhea during the whole period of observation (Fig. 7B).

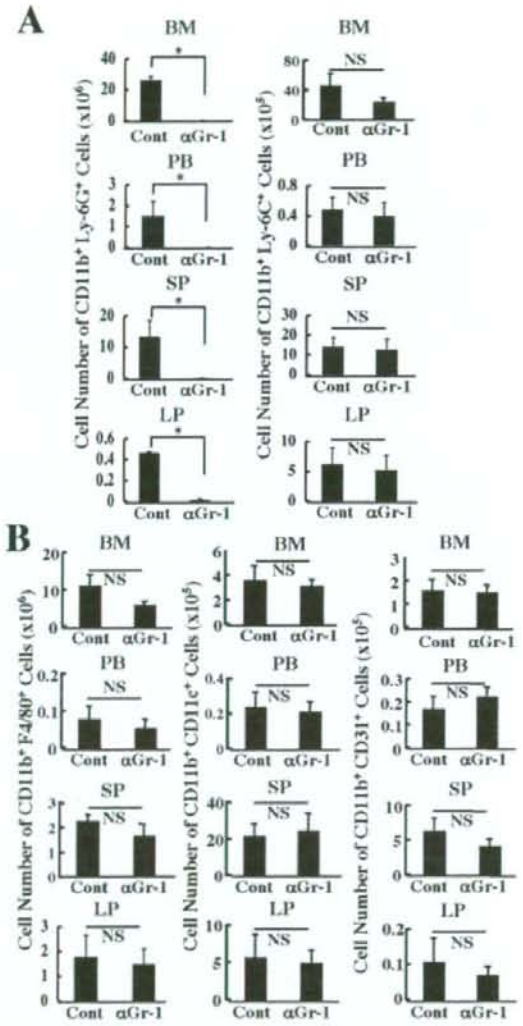

FIGURE 6. Anti-Gr-1 mAb treatment depletes Gr-$1^{high}CD11b^+$Ly-$6C^+$ cells, but not $CD11b^+F4/80^+$ macrophages, $CD11b^+CD11c^+$ dendritic cells, and $CD11b^+CD31^+$ myeloid suppressor cells. Expression of Ly-6G, Ly-6C, F4/80, CD11c, or CD31 in addition to CD11b and Gr-1 on cells obtained from various sites of colitic mice administered anti-Gr-1 mAb or control IgG every 8 hours 3 times for 1 day before sacrifice was examined using flow cytometry. Data are indicated as mean ± SD of 6 mice in each group. *$P < 0.05$. NS, not significantly different.

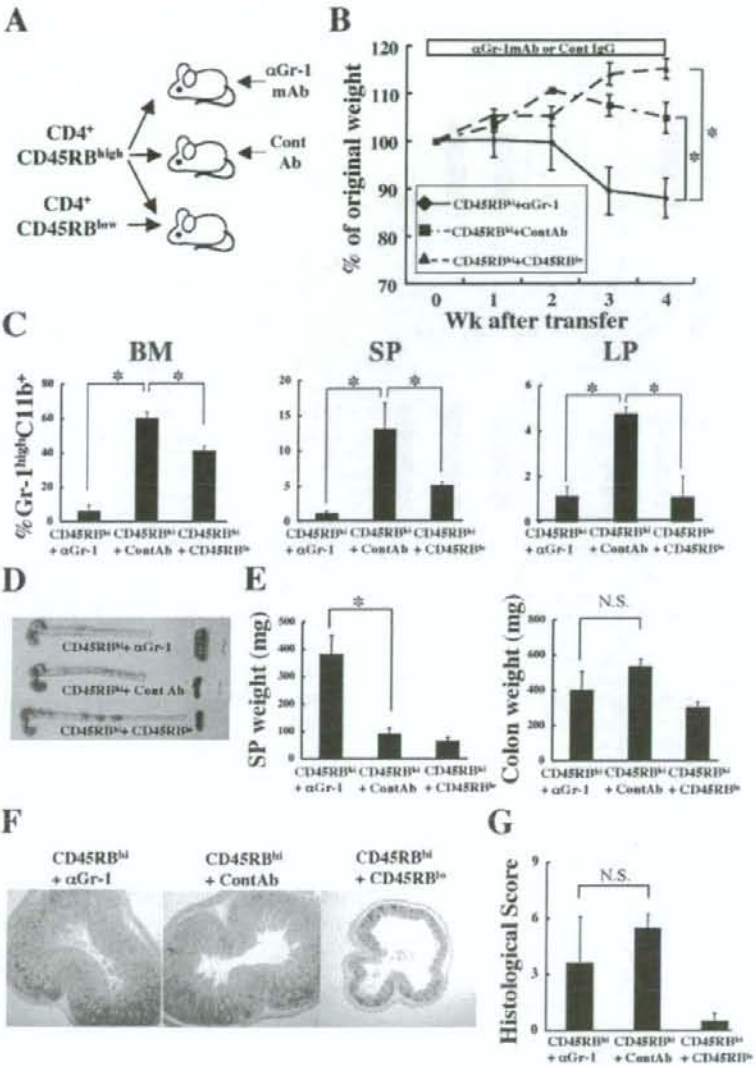

FIGURE 7. Anti-Gr-1 mAb treatment does not ameliorate colitis, but exacerbated wasting disease. A: Recipient SCID mice were administered anti-Gr-1 mAb or control rat IgG for 4 weeks starting from the time of $CD4^+CD45RB^{high}$ T-cell transfer. Other SCID control mice were transferred with $CD4^+CD45RB^{high}$ T cells and $CD4^+CD45RB^{low}$ T cells. All mice were sacrificed at 4 weeks after transfer. B: Change in body weight over time is expressed as percent of the original weight. Data are represented as mean ± SEM of 7 mice in each group. *$P < 0.05$. C: Mice were analyzed by FACS for the expression of Gr-1 and CD11b cell-surface markers. D: Proportion of $Gr\text{-}1^{high}CD11b^+$ cells in BM, SP, and LP. Data are represented as mean ± SEM of 7 mice in each group. E: Gross appearance of the colon, spleen, and mesenteric lymph nodes. F: Weight of the colon and spleen. Data are represented as mean ± SEM of 7 mice in each group. *$P < 0.05$. NS, not significantly different. G: Histological examination of the colon. Original magnification, ×100. H: Histological scoring. Data are indicated as the mean ± SEM of 7 mice in each group. NS, not significantly different.

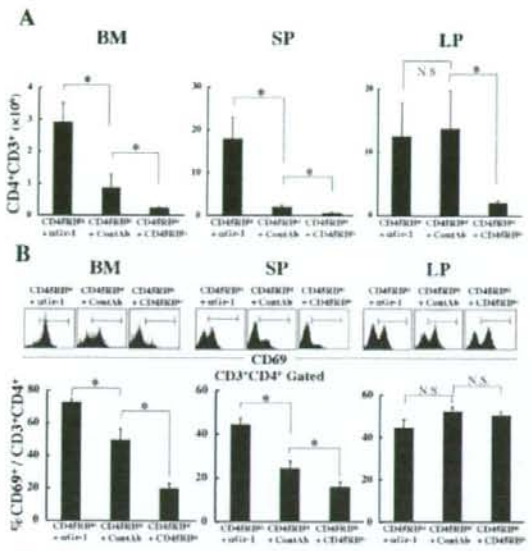

FIGURE 8. Anti-Gr-1 mAb treatment induces marked expansion of $CD4^+$ T cells in bone marrow and spleen of colitic mice. A: BM, SP, and LP $CD4^+$ T cells were isolated from SCID mice 4 weeks after transfer of $CD4^+CD45RB^{high}$ cells and administration with anti-Gr-1 mAb or control rat IgG, or transfer of $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ cells. The absolute number of $CD3^+CD4^+$ cells was determined by flow cytometry. Data are indicated as mean ± SEM of 7 mice in each group. $^*P < 0.05$. NS, not significantly different. B: The ratio of $CD69^+$ activated cells per total $CD4^+$ cells in BM, SP, and LP. Data are indicated as mean ± SEM of 7 mice in each group. $^*P < 0.05$. NS, not significantly different.

We again confirmed that administration of anti-Gr-1 mAb preferentially depleted $Gr\text{-}1^{high}CD11b^+$ granulocytes, but not $Gr\text{-}1^{low}CD11b^+$ monocytes in SP (Fig. 7C). Consistent with the above-mentioned results (Figs. 1, 2), the proportion of $Gr\text{-}1^{high}CD11b^+$ granulocytes at 4 weeks after transfer was significantly increased in the BM, SP, and LP in the control IgG-treated mice as compared with the mice transferred with $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ cells (Fig. 7D). Furthermore, the depletion of granulocytes in the anti-Gr-1 mAb-treated mice was confirmed by the marked decrease of $Gr\text{-}1^{high}CD11b^+$ cells in these mice (Fig. 7C,D). At 4 weeks after transfer the colon from the control IgG- or anti-Gr-1-treated mice transferred with $CD4^+CD45RB^{high}$ cells, but not that from mice transferred with $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ cells, was shortened and enlarged with a greatly thickened wall (Fig. 7E). Of note, the spleen of anti-Gr-1-treated mice was markedly enlarged as compared with that of the control IgG-treated mice and the mice transferred with $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ cells. This difference statistically confirmed that the weight of the spleen (Fig. 7F, left), but not the colon (Fig. 7F, right), of the anti-Gr-1-treated mice was significantly increased as compared with that of the control IgG-treated mice.

Histological examination showed prominent epithelial hyperplasia and glandular elongation with a massive infiltration of mononuclear cells in LP of the colon from the control IgG- or anti-Gr-1-treated mice (Fig. 7G). In contrast, the inflammation was mostly abrogated and only a few mononuclear cells were observed in LP of the colon from the mice transferred with $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ cells (Fig. 7G). This difference was also confirmed by histological scoring of multiple colon sections, which was 5.5 ± 0.71 in the control IgG-treated mice, 3.6 ± 2.46 in the anti-Gr-1-treated mice, and 0.5 ± 0.41 in the mice transferred with $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ T cells (Fig. 7H). Of note, the difference of histological score between the control IgG-treated mice and the anti-Gr-1-treated mice was not significant (Fig. 7H).

In Vivo Depletion of Granulocytes Induces Marked Expansion of $CD4^+$ T Cells with Activated Phenotype

A further quantitative evaluation of $CD4^+$ T-cell expansion was made by isolating mononuclear cells from various sites. The recovered cell number of LP $CD4^+$ T cells was significantly increased in the control IgG- or anti-Gr-1-treated mice as compared with that in the mice transferred with $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ cells, but the differences between the control IgG- and anti-Gr-1-treated mice was not significant (Fig. 8A, right). In contrast to the colonic infiltration of $CD4^+$ cells, the numbers of SP and BM $CD4^+$ T cells from the anti-Gr-1-treated mice were markedly increased as compared with those from the control IgG-treated mice (Fig. 8A, left and middle). Furthermore, the ratio of $CD69^+$ cells in total $CD4^+$ cells in the SP and BM, but not in the LP, from the anti-Gr-1-treated mice was significantly increased as compared with that from the control-IgG-treated mice (Fig. 8B), indicating that the granulocyte depletion induced a marked expansion of $CD4^+$ cells with activated phenotype.

Increased Granulopoiesis Is Induced in Splenectomized $CD4^+CD45RB^{high}$ T-cell-transferred SCID Mice

Although we so far focused on the BM for the increased granulopoiesis in colitic mice, it remained possible that the marked increase of granulocytes in various sites of $CD4^+CD45RB^{high}$ T-cell-transferred SCID mice was mainly due to extramedullary granulopoiesis in the spleen that is a representative site for it, but not in the BM. To evaluate this possibility we finally prepared age-matched SCID mice with or without splenectomy (SPX). Two weeks after recovery

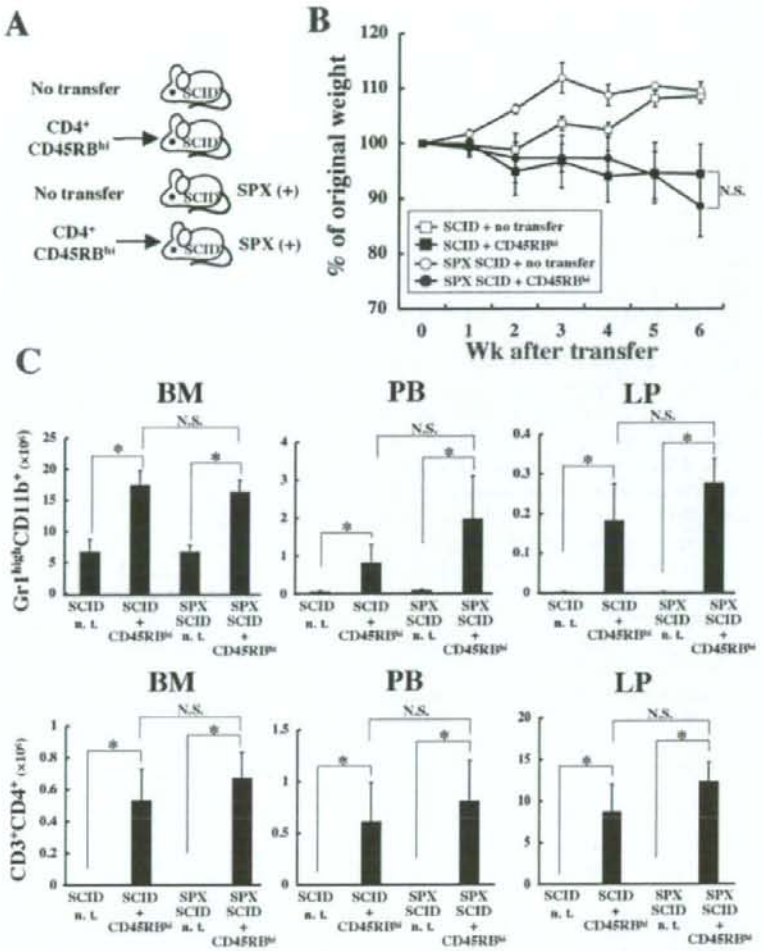

FIGURE 9. Splenectomized SCID mice transferred with $CD4^+CD45RB^{high}$ T cells develop marked granulopoiesis. A: SCID mice were splenectomized 2 weeks before $CD4^+CD45RB^{high}$ T-cell transfer. Mice were divided into 4 groups (each, $n = 5$) as follows; Group 1, SCID mice without splenectomy and without the transfer of $CD4^+CD45RB^{high}$ T cells; Group 2, SCID mice without splenectomy and with the transfer of $CD4^+CD45RB^{high}$ T cells; Group 3, SCID mice with splenectomy and without the transfer of $CD4^+CD45RB^{high}$ T cells; and Group 4, SCID mice with splenectomy and with the transfer of $CD4^+CD45RB^{high}$ T cells. All mice were sacrificed at 6 weeks posttransfer. SPX, splenectomy. B: Change in body weight over time is expressed as percent of the original weight. Data are represented as the mean ± SEM of 7 mice in each group. *$P < 0.05$. C: Absolute number of $Gr\text{-}1^{high}CD11b^+$ granulocytes or $CD3^+CD4^+$ T cells in the BM, PB, and LP. Data are represented as mean ± SEM of 7 mice in each group. nt, no transfer.

from surgery the SCID mice with or without SPX were transferred with $CD4^+CD45RB^{high}$ T cells and were monitored for 6 weeks posttransfer (Fig. 9A). We found that SCID mice transferred with $CD4^+CD45RB^{high}$ T cells irrespective of SPX developed a wasting disease (Fig. 9B) and clinical signs of colitis (data not shown). Also of note, the number of $CD11b^+Gr\text{-}1^{high}$ granulocytes (Fig. 9C, upper) or $CD3^+CD4^+$ T cells (Fig. 9C, lower) was significantly increased in BM, PB, and LP to a similar extent irrespective of SPX, as compared with the paired SCID mice with no transfer, suggesting little contribution of spleen for the increased number of granulopoiesis in this colitis model.

Delayed Administration of Anti-Gr-1 mAb Induces Marked Expansion of Systemic CD4$^+$ T Cells

We next evaluated the effect of delayed administration of anti-Gr-1 mAb on the expansion of systemic CD4$^+$ T cells to assess the role of granulocytes in the process of ongoing disease (Fig. 10A). Since a wasting disease started 3 weeks after transfer (Fig. 10B), and the infiltration of lymphocytes and colitis was already detectable at 2 weeks (data not shown), we started the anti-Gr-1 mAb treatment from 3 weeks after transfer. As shown in Figure 10B, the anti-Gr-1 mAb-treated mice exhibited a significant exacerbation of weight loss as compared with the control IgG-treated mice. In contrast, control mice transferred with both CD4$^+$CD45RB^{high} and CD4$^+$CD45RB^{low} T cells did not exhibit a wasting disease (Fig. 10B). Furthermore, the proportion of Gr-1^{high}CD11b$^+$ granulocytes at 6 weeks after transfer was significantly increased in the SP and LP in the control IgG-treated mice as compared with the mice transferred with CD4$^+$CD45RB^{high} and CD4$^+$CD45RB^{low} cells (Fig. 10C), and the depletion of granulocytes by the anti-Gr-1 mAb treatment induced the marked decrease of Gr-1^{high}CD11b$^+$ cells in those mice (Fig. 10C). Nevertheless, the spleen of anti-Gr-1-treated mice was markedly enlarged as compared with that of the control IgG-treated mice and the mice transferred with CD4$^+$CD45RB^{high} and CD4$^+$CD45RB^{low} T cells (data not shown). Furthermore, histological assessment revealed that both mice treated with control IgG or anti-Gr-1 mAb developed severe colitis to a similar extent (Fig. 10D), with no statistical difference (data not shown). As seen in the protocol treated antibodies from 0 weeks after transfer (Figs. 8, 9), the number of CD3$^+$CD4$^+$ T cells in SP, but not in LP, from anti-Gr-1-treated mice was significantly increased as compared with control IgG-treated mice in spite of the delayed administration protocol (Fig. 10E).

DISCUSSION

In this study we demonstrated that the increased granulopoiesis in colitic BM plays a negative regulatory role to suppress the expansion of colitogenic T cells and wasting disease in a murine model of chronic colitis induced by adoptive transfer of CD4$^+$CD45RB^{high} T cells into SCID mice. We found that Gr-1^{high}CD11b$^+$ granulocytes in colitic PB were significantly increased even after the establishment of chronic colitis, and the depletion of granulocytes allowed the expansion of colitogenic CD4$^+$ T cells with activated phenotype, resulting in severe wasting disease.

Most studies have so far focused on the capacity of innate immune cells to shape adaptive immunity, and thus relatively little attention has been paid to the potential influence of acquired immunity on the innate immune system. For example, in the pathogenesis of chronic colitis it is thought that the early emerging immune cells in inflamed mucosa at the initial attack and at the recurrence of the diseases are granulocytes and macrophages before the activation of antigen-specific CD4$^+$ T cells.[1,2] These innate immune cells are thought to function as a first defense in injured mucosal tissues, and produce chemokines and proinflammatory cytokines to recruit acquired-immune CD4$^+$ T cells.[9] At the chronic phase of colitis after the establishment of coliotgenic effector CD4$^+$ T cells, however, the role of granulocytes is almost ignored. Clinicopathologically, however, it has been established as a clinical score system that the degree of granulocyte infiltration in inflamed mucosa is a criteria for disease severity in both Crohn's disease[22] and ulcerative colitis.[23] According to these score systems, the degree of granulocyte infiltration correlates with pathological severity. Furthermore, it has also been reported that the circulating activated granulocytes are elevated with increased survival time in patients with severe IBD.[14,15] However, it still remains unclear whether the local and systemic activation of the granulocytes in severe IBD is pathogenic or protective.

In this regard, we adopted a direct approach using granulocyte-depleting anti-Gr-1 mAb in an in vivo study, and found that the administration of granulocyte-depleting anti-Gr-1 mAb did not affect the development of chronic colitis by assessing the histological scores and the expansion of LP CD4$^+$ T cells, but surprisingly induced a marked expansion of CD4$^+$ T cells with activated phenotype (CD69$^+$) in the SP and BM, resulting in a severer wasting disease as compared with the control IgG-treated mice. Interestingly, we observed that the anti-Gr-1-treated mice revealed splenomegaly in spite of almost complete depletion of Gr-1$^+$ granulocytes in SP, and surprisingly a marked increased compartment in SP was CD4$^+$ T cell. In addition, we observed that the delayed administration of anti-Gr-1 mAb starting at 3 weeks after CD4$^+$CD45RB^{high} T cell transfer also induced the systemic expansion of CD4$^+$ T cells as compared with the recipient with no treatment. These results suggest that the expansion of pathogenic T cells is negatively regulated by the presence of granulocytes in the BM and SP of chronic colitic mice. In a very recent publication, however, Kühl et al[24] demonstrated that RAG-1$^{-/-}$ mice transferred with CD4$^+$CD45RB^{high} T cells and treated with anti-Gr-1 mAb had a more increased mortality than the control IgG-treated RAG-1$^{-/-}$ mice transferred with CD4$^+$CD45RB^{high} T cells, but the difference of histological score between the 2 groups was not significant. Although they did not assess the systemic immune system in their study, they concluded that the discrepancy was due to the higher mortality and that the colitis in anti-Gr-1-treated mice was so severe that all mice eventually died from colitis. However, it remains possible that the marked increase of the systemic expansion of coliotgenic CD4$^+$ T cells that produce a large amount of cachexia-inducing cytokines including TNF-α[17] as demonstrated in our present study induced a severe wasting disease rather than severe local inflammation.

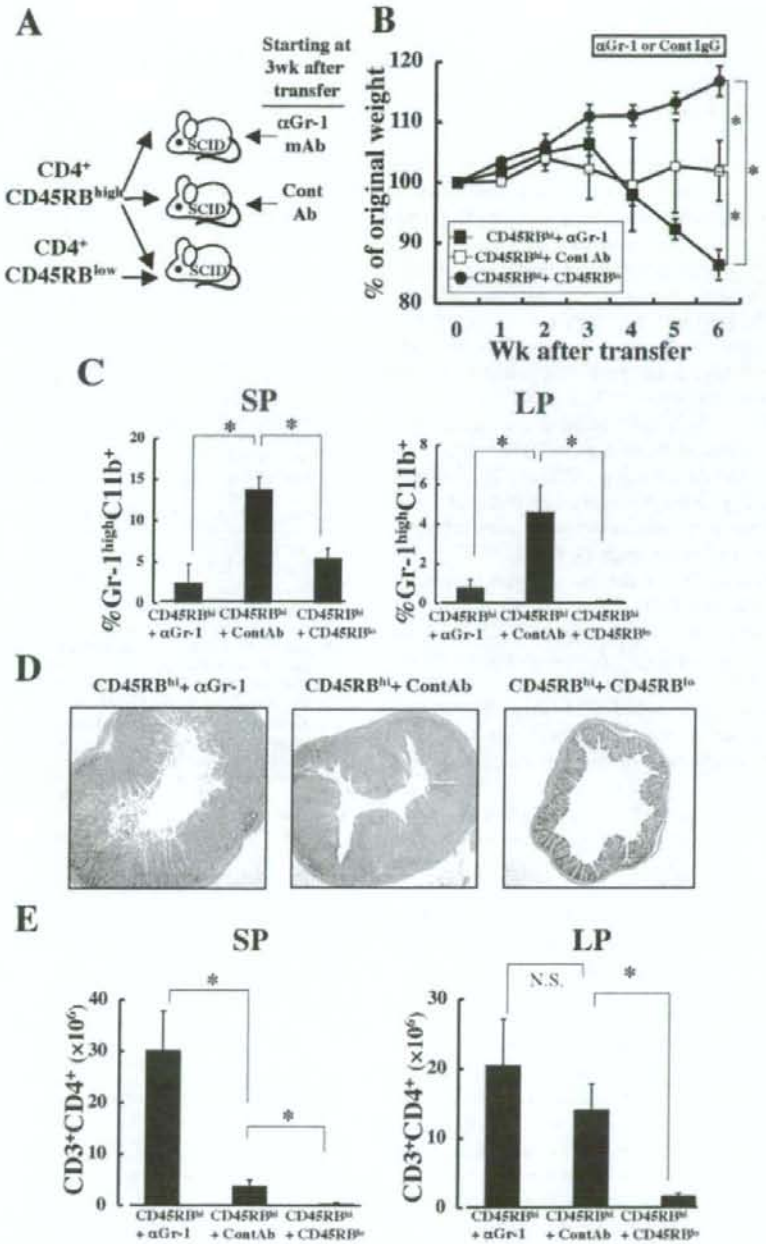

FIGURE 10. Delayed anti-Gr-1 mAb treatment does not ameliorate colitis, but increased systemic $CD4^+$ T cells. A: Recipient SCID mice were administered anti-Gr-1 mAb or control rat IgG for 3 weeks starting from 3 weeks after transfer. Other SCID control mice were transferred with $CD4^+CD45RB^{high}$ T cells and $CD4^+CD45RB^{low}$ T cells. All mice were sacrificed at 6 weeks after transfer. B: Change in body weight over time is expressed as percent of the original weight. Data are represented as mean ± SEM of 7 mice in each group. *$P < 0.05$. C: Proportion of $Gr\text{-}1^{high}CD11b^+$ cells in SP and LP. Data are represented as mean ± SEM of 7 mice in each group. D: Histological examination of the colon. Original magnification, ×100. E: SP and LP $CD4^+$ T cells were isolated from SCID mice 6 weeks after transfer. The absolute number of $CD3^+CD4^+$ cells was determined by flow cytometry. Data are indicated as mean ± SEM of 7 mice in each group. *$P < 0.05$. NS, not significantly different.

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Consistent with this, we here showed that the main increased sites of granulocytes were PB, SP, and BM rather than LP in colitic mice (Fig. 2). Further study will be needed to conclude this issue.

Although granulocytes make an important contribution to the recruitment of antigen-presenting cells, resulting in the T-cell activation and expansion at least in the initial phase of inflammation,[1,2] it has been proposed that Gr-1$^+$CD11b$^+$ cells induced in various pathological conditions including tumor, traumatic stress, bacterial, and parasitic infections, designated myeloid-derived suppressor cells or myeloid suppressor cells, have an ability to suppress T-cell activation.[25–27] For example, it has been demonstrated that activated granulocytes can impair TCR ζ-chain expression and cytokine production by T cells in advanced cancer.[28] Although we demonstrated that Gr-1^{high}CD11b$^+$ cells in the present colitis model do not express CD31, it is conceivable that the increased granulocytes at the late phase of chronic colitis also function as a novel suppressor against colitogenic CD4$^+$ T cells by a negative feedback loop.

Furthermore, the notion that granulocytes suppress the expansion of colitogenic CD4$^+$ T cells in chronic colitis may be relevant to the recent clinical usage of G-CSF and GM-CSF for patients with IBD.[29,30] A recent randomized controlled trial of 124 patients with severe-moderate Crohn's disease revealed a significant benefit of GM-CSF administration in response and in remission.[31] Although the authors speculated that normalization of innate immune function by the administration is one of the reasons for its effectiveness, our current study may suggest a possibility that the secondarily increased granulocytes directly suppress the activation and expansion of colitogenic CD4$^+$ T cells as "a regulatory granulocytes" as found in this study.

We have recently demonstrated that colitogenic CD4$^+$ memory T cells reside in the BM in chronic colitic mice.[21] Although it was initially thought that this model of colitis is mediated by Th1-type immune responses,[32] it has recently been recognized that Th17-mediated immune responses are also involved in this model.[33] Furthermore, it is well known that IL-17 induces G-CSF production by BM stromal cells, and is recognized as a promoting factor for granulopoiesis.[34] We thus focused on granulopoiesis in the colitic BM in this model. Although it was possible that extramedullary granulopoiesis in SP is also involved in the systemic increase of granulocytes in colitic mice, we also clearly demonstrated that splenectomized SCID mice transferred with CD4$^+$CD45RB^{high} T cells also had a marked increase of granulocytes in BM to a similar extent of the non-splenectomized SCID recipients (Fig. 6), indicating a more specific contribution of BM for the granulopoiesis in colitic mice. Furthermore, our current results in colitic mice may coincide with a recent report by Monteiro et al[35] showing that resident CD4$^+$ T cells in BM support the granulopoiesis in the normal BM environment.

In conclusion, we have demonstrated that granulocytes may play a pivotal role in the suppression of expansion of systemic colitogenic CD4$^+$ T cells in the late phase of colitis by a feedback loop induced by colitogenic BM CD4$^+$ T cells themselves. This finding also may provide the therapeutic rationale of the G-CSF and GM-CSF therapies for IBD.

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Endoscopic and Chromoendoscopic Atlas Featuring Dysplastic Lesions in Surveillance Colonoscopy for Patients with Long-Standing Ulcerative Colitis

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Abstract: Clinical and epidemiological studies have revealed that the incidence of colorectal cancer associated with ulcerative colitis increases with long-term chronic inflammation. Careful endoscopic observation and histological studies to check for dysplasia in the colon are important in detecting neoplasia. Current surveillance protocols mainly involve frequent step biopsies to yield a reasonable rate of dysplasia detection. However, recent studies using chromoendoscopy or magnifying endoscopy have proposed that neoplastic changes may be detected efficiently. Therefore, it is very important to understand the typical endoscopic findings found in neoplastic changes in patients proven to have long-standing ulcerative colitis. In this review, we demonstrate the typical endoscopic findings by conventional endoscopy and chromoendoscopy.

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Key Words: ulcerative colitis, dysplasia, cancer surveillance, chromoendoscopy, endoscopy

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It is well accepted that there is a high prevalence of colonic cancer in patients with long-standing ulcerative colitis. Eaden et al[1] reported that the cumulative incidence of colonic cancer in patients with ulcerative colitis reaches approximately 18% after 30 years. Patients with early-onset disease present with extensive involvement of colitis and complications of primary sclerosing cholangitis. Further, the cancer risk associated with ulcerative colitis is believed to be higher.[2] Because colorectal cancer in patients with ulcerative colitis arises because of continuous or repeated chronic mucosal inflammation, multicentric generation of colorectal cancer is frequently observed throughout the colon. Therefore, surveillance colonoscopy that detects the early stages of colitis-associated neoplastic changes is believed to be indispensable for the diagnosis of early-stage colonic cancer in patients eligible for curative surgery.[3–5]

The guidelines of the American Gastroenterological Association[6] for surveillance colonoscopy recommend an annual total colonoscopy for high-risk patients with ulcerative colitis, with a minimum of 4 biopsies from every 10 cm of the colon, and the British Society for Gastroenterology[7] guidelines recommend 2–4 biopsies from every 10 cm of the whole colon.

Neoplastic changes are believed to be associated with chronic inflammatory changes that develop in patients with ulcerative colitis[8]; however, genetic abnormalities commonly occurring in such neoplastic changes remain controversial.

In contrast, recent studies[10,11] using chromoendoscopy suggest that dysplastic lesions may be detected by close observations based on the pit pattern diagnosis proposed by Kudo et al.[12] For patients with a long history of inflammation, dysplastic histology is rarely detectable by classical endoscopic imaging even if more than 30 biopsies from the entire colon are taken because the lesions often appear flat or normal. Since the introduction of high-resolution videoendoscopy as a surveillance technique for patients with long-standing ulcerative colitis, it has often been possible to identify lesions potentially related to dysplasia.[13] To facilitate the identification of lesions related to neoplastic histology derived from long-term inflammation with ulcerative colitis, we

have designed an endoscopic atlas that characterizes the candidate endoscopic features of regions from which surveillance biopsies should be sampled.

Clinical and Pathological Features of Ulcerative Colitis-Associated Colorectal Cancer

The clinical and pathological features of ulcerative colitis–associated colorectal cancer have been well documented. Briefly, ulcerative colitis–associated colorectal cancer is often multicentric in origin and frequently associated with dysplastic lesions. Typical endoscopic findings of dysplastic lesions are termed dysplasia-associated lesions or mass (DALM); these are mainly observed as elevated or mass lesions under conventional endoscopy. Patients presenting with high-grade dysplasia are usually referred for surgery because invasive colorectal cancer often coexists elsewhere in the body. It is still controversial whether patients presenting with low-grade dysplasia should undergo immediate surgery; however, it is accepted that there should be frequent observation of these patients.

To efficiently distinguish such neoplastic lesions in surveillance endoscopy in daily clinics, it is important to understand the typical endoscopic findings that could be associated with dysplasia. In this article, we review the potential application of recently developed high-resolution endoscopy as a surveillance technique for colorectal neoplasia associated with ulcerative colitis. By examining conventional and chromoendoscopic findings in patients with confirmed dysplasia, we detail the typical endoscopic findings optimal for biopsies. Histological diagnosis of dysplasia is confirmed by routine hematoxylin and eosin (H&E) staining and p53 immunohistochemistry.[11]

Typical Endoscopic Findings of Neoplastic Lesions Associated with Long-Standing Ulcerative Colitis

Protruded Lesions

Protruded lesions are one of the most common features of ulcerative colitis–associated neoplastic lesions. It has been well documented that dysplasia-associated lesions or mass (DALM), surface irregularity, and uneven redness in protruded lesions are suggestive of dysplastic pathology. Other important findings associated with dysplasia can be obtained from the surrounding mucosa. As shown in Figure 1, slightly elevated lesions are sometimes observed around the protruded lesion, which may be easier to identify using dye-spraying endoscopy. However, careful observation by conventional endoscopy enables recognition of such lesions, which have to be further confirmed by dye spraying. Details about these endoscopic features are explained in the section on mixed-type lesions.

Slightly Elevated Lesions

Dysplastic pathology is often detected in biopsy specimens obtained from flat elevated lesions. Slightly elevated lesions with irregular granular surfaces and uneven redness can be markers for obtaining biopsy specimens (Figs. 2–6). When compared with the villous adenoma observed in patients without ulcerative colitis, dysplastic lesions with slightly elevated morphology are usually surrounded by inflammatory mucosa. As noted in Figures 2–6, it is sometimes still difficult, although important, to distinguish between dysplastic lesions and chronic inflammation. Neoplastic lesions in the mucosa may be a factor indicating deformity of the intestinal lumen. In slightly elevated lesions and in flat lesions (mentioned next), invasive cancer is sometimes observed in resected specimens with less neoplastic pathology of the surface mucosal epithelial cells.

FIGURE 1. Protruded lesions in a 65-year-old male patient with an 18-year history of pancolitis. Colonoscopy revealed a protruding lesion in the sigmoid colon. This type of lesion was well documented as a dysplasia-associated lesion or mass (DALM). Histological diagnosis of the resected specimen was well-differentiated adenocarcinoma extending into the submucosa.

Flat Lesions

Flat lesions with dysplasia are usually identified as areas of reddish mucosa with a defined boundary (Figs. 7 and 8). However, it is difficult to differentiate flat dysplastic lesions from inflammatory mucosa. Therefore, it is strongly recommended that such lesions be sampled for biopsies. The

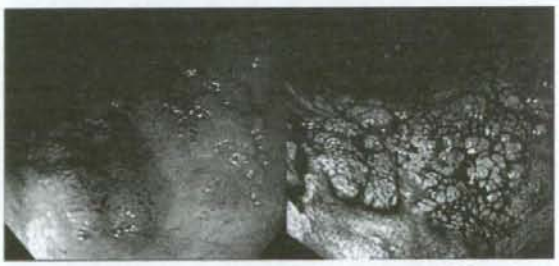

FIGURE 2. Slightly elevated lesions in a 50-year-old female patient with a 21-year history of extensive colitis. Colonoscopy revealed a slightly elevated lesion in the sigmoid colon with gathered granular mucosa. Mucosa around the lesion were slightly atrophic without elevation. Histological diagnosis of the biopsy specimen was high-grade dysplasia: left, conventional endoscopy; right, chromoendoscopy.

FIGURE 3. Slightly elevated lesions. A 45-year-old female patient with a 14-year history of extensive colitis. Colonoscopy revealed a slightly elevated lesion in the rectum. A flat elevated lesion showed a larger granular pattern compared to that around the lesion. Histological diagnosis of the biopsy specimen was high-grade dysplasia: left, conventional endoscopy; right, chromoendoscopy.

pit pattern diagnosis based on Kudo's criteria is not always applicable to these flat lesions, even if dye-spraying magnifying endoscopy is used. Therefore, it is necessary to investigate the usefulness of magnifying endoscopy for the detection of these lesions.

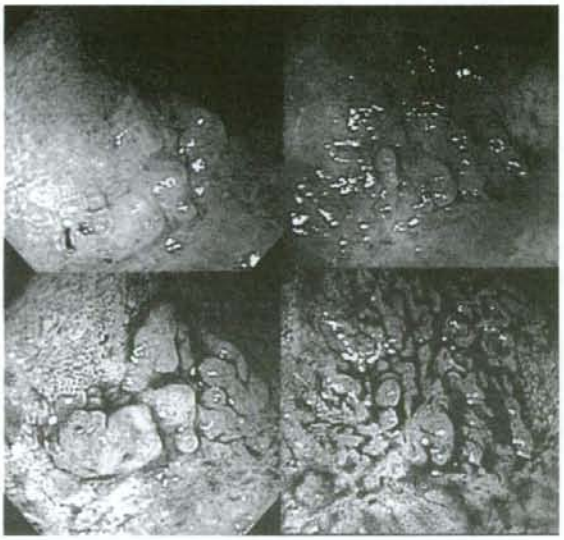

FIGURE 4. Slightly elevated lesion in a 40-year-old male patient with a 16-year history of left-sided colitis. Colonoscopy revealed a slightly elevated lesion in the sigmoid colon. Slightly elevated lesions with irregular size of granularity were focused; however, colonic mucosa around the lesion showed atrophy with reconstituted vascular pattern. Note that the lesion showed enlargement at the second endoscopy. Histological diagnosis of the biopsies and resected specimen was low-grade dysplasia: left upper and lower, the first endoscopy; right upper and lower, the second endoscopy 11 months later.

FIGURE 5. Slightly elevated lesion in a 50-year-old female patient with a 19-year history of extensive colitis. Colonoscopy revealed a villous, slightly elevated lesion in the sigmoid colon. Slightly elevated lesions with redness were observed in the reddish mucosa of the background. Note that a pit pattern resembling type IV was observed in the lesions; however, background mucosa showed a small, round pit pattern with redness. Histological diagnosis of the biopsy specimen was high-grade dysplasia.

Depressed Lesions

Depressed neoplastic lesions are uncommon and are frequently accompanied by slightly elevated lesions (Fig. 9) that are not easily identified by conventional colonoscopy (without dye spray). Therefore, dye-spraying colonoscopy can reveal important information about neoplastic changes.

Mixed-Type Lesions

Thus far, most mixed lesions have been identified as protruded lesions accompanying slightly elevated lesions (Figs. 10 and 11). This strongly suggests that protruded lesions arise from flat elevated lesions, which often include neoplastic changes developed through long-standing chronic inflammation with ulcerative colitis.

Role of Chromoendoscopy in Surveillance

Typical neoplastic lesions including the dysplasia associated with chronic ulcerative colitis can be identified by

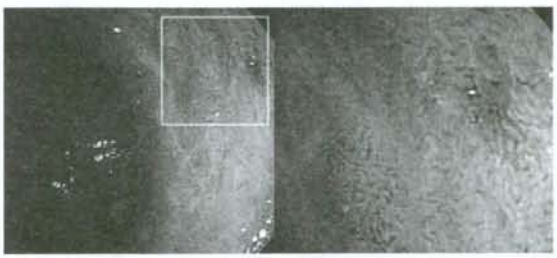

FIGURE 6. Slightly elevated lesion in a 70-year-old female patient with a 15-year history of left-sided colitis. Colonoscopy revealed a slightly elevated lesion in the rectum. A flat elevated lesion with redness was observed in the atrophic background mucosa. Note that pit pattern showed long but unclassified type somewhat different from typical type IV or type III. Histological diagnosis of the biopsy specimen was low-grade dysplasia.