

## Materials and methods

### Animals

Female BALB/c and C.B-17 SCID mice were purchased from Japan Clea (Tokyo, Japan). Mice were maintained under specific pathogen-free (SPF) conditions in the Animal Care Facility of Tokyo Medical and Dental University. Donors and littermate recipients were used at 6–12 weeks of age. All experiments were approved by the regional animal study committees and were done according to institutional guidelines and Home Office regulations.

### Antibodies

The following mAb except biotin-conjugated anti-mouse IL-7R $\alpha$  (A7R34; eBioscience, San Diego) were obtained from BD Pharmingen (San Diego, CA) for purification of cell populations and flow cytometry analysis: 145-2C11, FITC-conjugated anti-mouse CD3; RM4-5, FITC- or PE-conjugated anti-mouse CD4; 16A, FITC-conjugated anti-mouse CD45RB; 7D4, FITC-conjugated anti-mouse CD25; IM7, PE-conjugated anti-mouse CD44; MEL-14, FITC- or PE-conjugated anti-mouse CD62L; H1.2F3, FITC-conjugated anti-mouse CD69; 37.51, PE-conjugated anti-mouse CD28; J43, PE-conjugated anti-mouse PD-1 and the following FITC-conjugated antibodies; V $\beta$ 2 (B20.6), V $\beta$ 3 (KJ25), V $\beta$ 4 (KT4), V $\beta$ 5 (MR9-4), V $\beta$ 6 (RR4-7), V $\beta$ 7 (TR310), V $\beta$ 8.1/2 (MR5-2), V $\beta$ 8.3 (B21.14), V $\beta$ 9 (MR10-2), V $\beta$ 10b (B21.5), V $\beta$ 11 (RR3-15), V $\beta$ 12 (MR11-1), V $\beta$ 13 (IN12.3), V $\beta$ 14 (14.2), and V $\beta$ 17 (KJ23). Biotinylated antibodies were detected with PE- or Cy-Chrome<sup>TM</sup>-streptavidin (BD Pharmingen).

### T cell preparation

SP and LP CD4<sup>+</sup> T cells were isolated from mice as previously described [15]. The resultant cells contained > 94% CD4<sup>+</sup> cells when analyzed by FACSCalibur. SP CD4<sup>+</sup> T cells were then labeled with PE-conjugated anti-mouse CD4 mAb and FITC-conjugated anti-CD45RB mAb, and then sorted to yield the CD45RB<sup>high</sup> (highest staining 30%) fraction by the FACS Vantage SE (Becton Dickinson, Sunnyvale, CA).

### Sequential adoptive transfer experiments

Colitis was induced in C.B-17 SCID mice by adoptive transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells [15]. Each SCID mouse was injected intraperitoneally with  $3 \times 10^5$  CD4<sup>+</sup>CD45RB<sup>high</sup> T cells. All the recipient mice were weighed initially, and three times per week after transfer. Mice were killed when their ongoing clinical score after transfer reached four points or more as mentioned below, and LP CD4<sup>+</sup> T cells were isolated for the next transfer. The isolated colitic LP CD4<sup>+</sup> T cells ( $3 \times 10^5$ /mouse) were then

transferred into new SCID mice [16]. After seven sequential transfers, we found that some mice failed to develop colitis within 40 weeks from transfer. To characterize these non-colitic LP CD4<sup>+</sup> T cells, we transferred CD4<sup>+</sup>CD45RB<sup>high</sup> T cells ( $3 \times 10^5$ ) alone (group 1), CD4<sup>+</sup>CD45RB<sup>high</sup> T cells ( $3 \times 10^5$ ) and LP CD4<sup>+</sup> T cells obtained from colitic mice originally transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells ( $1 \times 10^5$ ) (group 2), or CD4<sup>+</sup>CD45RB<sup>high</sup> T cells ( $3 \times 10^5$ ) and LP CD4<sup>+</sup> T cells obtained from the non-colitic mice after seven transfers ( $1 \times 10^5$ ) (group 3) into new SCID mice. Mice were killed 6 weeks after transfer.

### Clinical and histological scorings

The recipient mice were weighed initially, then three times per week after transfer. They were observed for clinical signs of illness [40]: hunched over appearance, piloerection of the coat, diarrhea, and blood in the stool. When mice were killed at a predetermined time point, their clinical score was assessed as the sum (0–8 points) of four parameters: 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 gross blood, 0 or 1 [17]. For the sequential adoptive transfers, we monitored the clinical signs during the observation period, and the ongoing clinical score was defined as the sum (0–5 points) of the above-parameters other than colon thickening [40]. Mice were killed when their ongoing clinical score reached four points or more, and isolated LP CD4<sup>+</sup> T cells were transferred into new SCID mice. Transfers were repeated as long as the mice continued to develop colitis within 40 weeks post transfer. We judged recipient mice to be 'non-colitic' when they did not develop colitis within 40 weeks post transfer, and killed them at this time for further analysis. Tissue samples were fixed in PBS containing 10% neutral-buffered formalin. Paraffin-embedded sections (5  $\mu$ m) were stained with H&E. The mean degree of inflammation in the colon was calculated as previously described [15].

### Flow cytometry

To detect surface expression of various molecules, isolated splenocytes or LP mononuclear cells (LPMC) were preincubated with an Fc $\gamma$ R-blocking mAb (CD16/32; 2.4G2, BD Pharmingen) for 15 min, then incubated with specific FITC-, PE-, PECy5- or biotin-labeled antibodies for 20 min on ice. Biotinylated antibodies were detected with PE- or Cy-Chrome<sup>TM</sup>-streptavidin. Intracellular Foxp3 staining was performed with the PE-anti-mouse Foxp3 staining set (eBioscience) according to the manufacturer's instructions. Standard two- or three-color flow cytometric analyses were obtained using the FACSCalibur equipped with CellQuest software. Background fluorescence was assessed by staining of the control irrelevant isotype-matched mAb.

### Cytokine ELISA

To measure cytokine production,  $1 \times 10^5$  LP CD4<sup>+</sup> T cells were cultured in 200  $\mu$ L culture medium at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in 96-well plates (Costar, Cambridge, MA) pre-coated with 5  $\mu$ g/mL hamster anti-mouse CD3<sub>c</sub> mAb (145–2C11, BD PharMingen) and 2  $\mu$ g/mL hamster anti-mouse CD28 mAb (37.51, BD PharMingen) in PBS overnight at 4°C. Culture supernatants were removed after 48 h and assayed for cytokine production. Cytokine concentrations were determined by specific ELISA per the manufacturer's recommendation (R&D, Minneapolis, MN).

### In vitro functional analysis for Treg cells

CD4<sup>+</sup> cells were prepared from BALB/c splenocytes as APC by depleting CD4<sup>+</sup> cells with anti-CD4 MACS and treatment with 50  $\mu$ g/mL MMC for 45 min at 37°C. In co-culture experiments, CD4<sup>+</sup>CD25<sup>+</sup> T cells ( $0-1 \times 10^4$  as Treg) or isolated LP CD4<sup>+</sup> T cells ( $0-1 \times 10^4$ ) were cultured with CD4<sup>+</sup>CD25<sup>-</sup> responder cells ( $1 \times 10^5$ ) and MMC-treated CD4<sup>+</sup> cells ( $5 \times 10^5$ ) in the presence of anti-CD3 mAb (1  $\mu$ g/mL). To determine proliferation, each well was pulsed with 1  $\mu$ Ci [<sup>3</sup>H]thymidine (NEN, Boston, MA) for the last 9 h of 72-h culture.

### Statistical analysis

The results were expressed as the mean  $\pm$  SEM. Groups of data were compared by the Mann-Whitney U test. Differences were considered to be statistically significant when  $p < 0.05$ .

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Abbreviations: BM-DC: bone marrow-derived DC - LSEC: liver sinusoidal endothelial cells - MSC: mesenchymal stem cell

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## Continuous generation of colitogenic CD4<sup>+</sup> T cells in persistent colitis

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Inflammatory bowel diseases take chronic courses due to the expansion of colitogenic CD4<sup>+</sup> cells. However, it is unclear whether the persistent disease is driven by continuous reactivation of colitogenic memory CD4<sup>+</sup> cells to generate effector CD4<sup>+</sup> cells or by continuous generation of effector CD4<sup>+</sup> cells from naïve cells. To clarify this issue, we performed a series of sequential adoptive transfers of Ly5.2<sup>+</sup> and Ly5.1<sup>+</sup> CD4<sup>+</sup>CD45RB<sup>high</sup> cells into RAG-2<sup>-/-</sup> mice at different time points. We show here that the secondarily transferred CD4<sup>+</sup>CD45RB<sup>high</sup> cells can be converted to CD4<sup>+</sup>CD44<sup>high</sup>CD62L<sup>low</sup>IL-7R $\alpha$ <sup>high</sup> effector-memory T cells even in the presence of pre-existing effector-memory CD4<sup>+</sup> cells. Although the total cell numbers of CD4<sup>+</sup> cells in established colitic mice were consistently equivalent irrespective of the number of primarily transferred cells, the ratio of primarily and secondarily transferred cells was dependent on the ratio of the transferred cell numbers, but not on the order of the transfer. Of note, we found that primarily transferred CD4<sup>+</sup> cells produced significantly lower amounts of IFN- $\gamma$  and IL-17 than CD4<sup>+</sup> cells arising from secondary transfer. In conclusion, the continuous generation of colitogenic CD4<sup>+</sup> cells that compensate for exhausted CD4<sup>+</sup> cells may be one of the mechanisms involved in the persistence of colitis.

**Key words:** Colitis · Colitogenic memory T cells · Mucosal immunity

### Introduction

Intestinal mucosal surfaces are continuously exposed to antigens of the intestinal flora [1]. However, the gut-associated immune system defends against systemic circulation of harmful intestinal antigens and induces systemic tolerance toward intestinal commensal antigens by various mechanisms including suppression by regulatory CD4<sup>+</sup> T cells [2–7]. In contrast, inflammatory bowel disease (IBD) is associated with activation of the local and systemic immune responses due to a lack of tolerance to intestinal bacterial antigens [6, 7]. Although the etiology of IBD is uncertain, there is much evidence suggesting that the pathogenesis of IBD involves dysregulated recognition of the intestinal bacterial

antigens, resulting in the generation of colitogenic CD4<sup>+</sup> effector and memory T cells. Nevertheless, the nature of the colitogenic CD4<sup>+</sup> T cells over time is not fully understood especially in terms of the perpetuation of chronic colitis.

In general, IBD progresses steadily or relapses after remission throughout life [8, 9]. Although it is likely that the persistent disease is caused by the activation and expansion of colitogenic CD4<sup>+</sup> effector T cells, several possible mechanisms may be involved. One possibility is that the persistency is driven by the initial colitogenic CD4<sup>+</sup> memory T clones acting like memory stem cells [10] throughout the entire course of disease. In this case, colitogenic CD4<sup>+</sup> effector T cells would be generated from colitogenic CD4<sup>+</sup> memory T cells that are established at the initial onset, but are presumably suppressed by regulatory CD4<sup>+</sup> T cells in remission [5, 7]. However, this scenario has one obstacle, namely that memory T cells are believed to be generated for the first time after antigen clearance, but not in the persistent presence of

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antigens. Certainly, such is the case in models of chronic viral infections, such as lymphocytic choriomeningitis virus (LCMV) and influenza A virus infections [11]. Since the possible antigens for colitogenic CD4<sup>+</sup> T cells are derived from intestinal bacteria and/or self antigens in the intestine that are never eliminated from the body, it is doubtful whether colitogenic CD4<sup>+</sup> memory T cells could actually be established in such a situation. A second possibility is that the same or different epitope-specific colitogenic CD4<sup>+</sup> effector T cells are generated and expand after priming from newly recruited naive CD4<sup>+</sup> T cells from the thymus. In the case of multiple sclerosis, for example, it is thought that recruitment and activation of new autoimmune T cells evoke repeated disease episodes [12]. Newly recruited T cells could recognize distinct autoantigenic epitopes on the same antigen, or even be specific for different autoantigens through the epitope-spreading cascade. In the case of persistent colitis, it is also unclear whether pre-existing colitogenic CD4<sup>+</sup> effector or memory T cells prevent or ignore the priming, expansion, and phenotypic and functional conversion of newly recruited naive CD4<sup>+</sup> T cells to effector or memory CD4<sup>+</sup> T cells. To evaluate these unsolved, but critical, issues, we performed sequential adoptive transfers of Ly5.1<sup>+</sup> and Ly5.2<sup>+</sup> CD4<sup>+</sup>CD45RB<sup>high</sup> T cells into immunodeficient RAG-2<sup>-/-</sup> mice at different time points to induce chronic colitis.

## Results

### Newly transferred CD4<sup>+</sup>CD45RB<sup>high</sup> cells are converted into effector-memory T cells in colitic mice

To assess the possibility that continuous generation of new colitogenic CD4<sup>+</sup> T cells from naive CD4<sup>+</sup> T cells occurs routinely in colitic mice, we performed sequential adoptive transfers of Ly5.2<sup>+</sup> and Ly5.1<sup>+</sup> CD4<sup>+</sup>CD45RB<sup>high</sup> T cells into RAG-2<sup>-/-</sup> mice at different time points. To do this, we divided RAG-2<sup>-/-</sup> recipient mice into three groups (Fig. 1A): Group 1, RAG-2<sup>-/-</sup> mice transferred with Ly5.2<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells at 0 wk; Group 2, RAG-2<sup>-/-</sup> mice transferred with Ly5.2<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells at 0 wk and with Ly5.1<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells at 5 wk; and Group 3, RAG-2<sup>-/-</sup> mice transferred with Ly5.1<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells at 5 wk. Mice were observed for 10 wk after the first transfer of Ly5.2<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells. In Group 1 and Group 2, mice developed colitis over time after the first transfer of Ly5.2<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells, and ongoing clinical scores estimated by diarrhea with increased mucus in the stool, anorectal prolapse, hunched posture, and weight loss gradually increased after transfer, reaching to the maximum score 10 wk after the first transfer regardless of whether the second transfer was made (Fig. 1B). Similarly, Group 3 mice gradually developed colitis with time after the transfer of Ly5.1<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells, and the ongoing clinical score reached a similar level to that of Groups 1 and 2 mice at 10 wk (Fig. 1B). Histological examination showed prominent epithelial hyperplasia with glandular elongation with a massive infiltration of mononuclear cells in the lamina propria (LP) of the colon from all groups of mice at 10 wk (Fig. 1C).

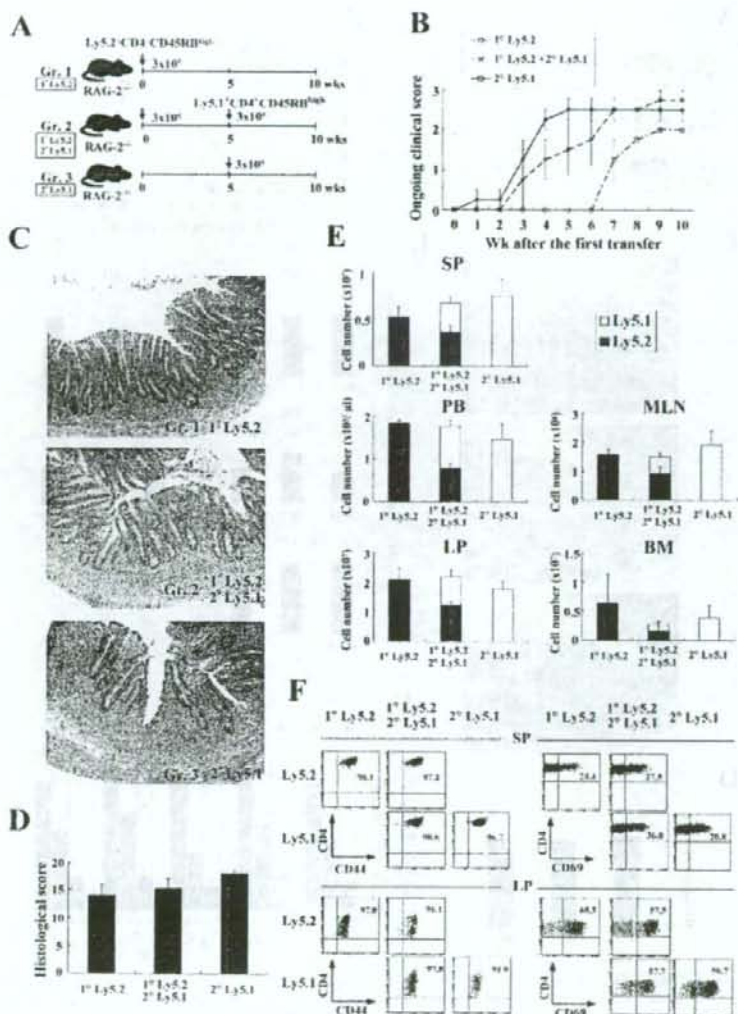
Histological scorings revealed no significant differences between the three groups (Fig. 1D).

A further quantitative evaluation of CD4<sup>+</sup> T cell accumulation was made by isolating CD4<sup>+</sup> T cells from various sites, such as spleen (SP), peripheral blood (PB), LP, mesenteric lymph nodes (MLN), and bone marrow (BM). Consistent with the similar clinical and histological severity of colitis between the three groups, the total recovered cell numbers in all sites were equivalent among the groups at 10 wk after the first transfer (Fig. 1E). Interestingly, in Group 2 mice, the ratio of Ly5.2<sup>+</sup> and Ly5.1<sup>+</sup> CD4<sup>+</sup> T cells in all sites was almost 1:1, in accordance with the 1:1 ratio of the transferred cell numbers (Fig. 1E). Furthermore, we found that all the transferred CD4<sup>+</sup>CD45RB<sup>high</sup> T cells, whether Ly5.1<sup>+</sup> or Ly5.2<sup>+</sup>, were converted to CD4<sup>+</sup>CD44<sup>high</sup> effector or memory cells both in SP and LP (Fig. 1F). In addition, substantial numbers of CD4<sup>+</sup> T cells both in SP and LP, especially in LP, expressed the activation marker CD69 (Fig. 1F). These data indicated two findings. First, secondarily transferred Ly5.1<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> naive T cells could be converted to CD4<sup>+</sup>CD44<sup>high</sup> effector or memory cells even in the presence of pre-existing Ly5.2<sup>+</sup> effector or memory cells that had previously expanded in colitic mice. Second, newly (secondarily transferred) and previously (primarily transferred) established CD4<sup>+</sup>CD44<sup>high</sup> effector or memory cells compete with each other and the already present CD4<sup>+</sup> T cells to occupy the space available to a constant number of CD4<sup>+</sup> T cells in established chronic colitis at 10 wk.

### Competition between colitic CD4<sup>+</sup> T cells is dependent on the transferred cell numbers

To further assess the mechanism of the competition between the first (old) and the second (new) transferred CD4<sup>+</sup> T cells in colitic mice, we next divided RAG-2<sup>-/-</sup> mice into three groups according to the number of Ly5.2<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells transferred at the first transfer (Fig. 2A): Group 1,  $3 \times 10^5$  cells (named "10<sup>4</sup>"), Group 2,  $3 \times 10^5$  cells ("10<sup>5</sup>"), and Group 3,  $3 \times 10^6$  cells ("10<sup>6</sup>"). All groups of mice were secondarily transferred with the same number ( $3 \times 10^5$ ) of Ly5.1<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells at 5 wk after the first transfer and killed at 10 wk after the first transfer. At 5 wk, the assessment of ongoing clinical scores revealed that Group 2 ("10<sup>5</sup>") and Group 3 ("10<sup>6</sup>") mice started to develop wasting disease and colitis, but the clinical severities of all groups were not significantly different at 5 wk after the first transfer (Fig. 2B), although the severity assessed by ongoing clinical scores of Group 1 ("10<sup>4</sup>") tended to be low. After the second transfer, all groups of mice progressively developed wasting disease and colitis to a similar extent until 10 wk after the first transfer (Fig. 2B). Like ongoing clinical scores, histological findings at 10 wk revealed that all groups of mice developed severe colitis with a massive infiltration of mononuclear cells in the LP (Fig. 2C), and the histological scores confirmed this finding in multiple colon sections (Fig. 2D).

Interestingly, the total cell numbers recovered from various sites at 10 wk were not significantly different between the groups

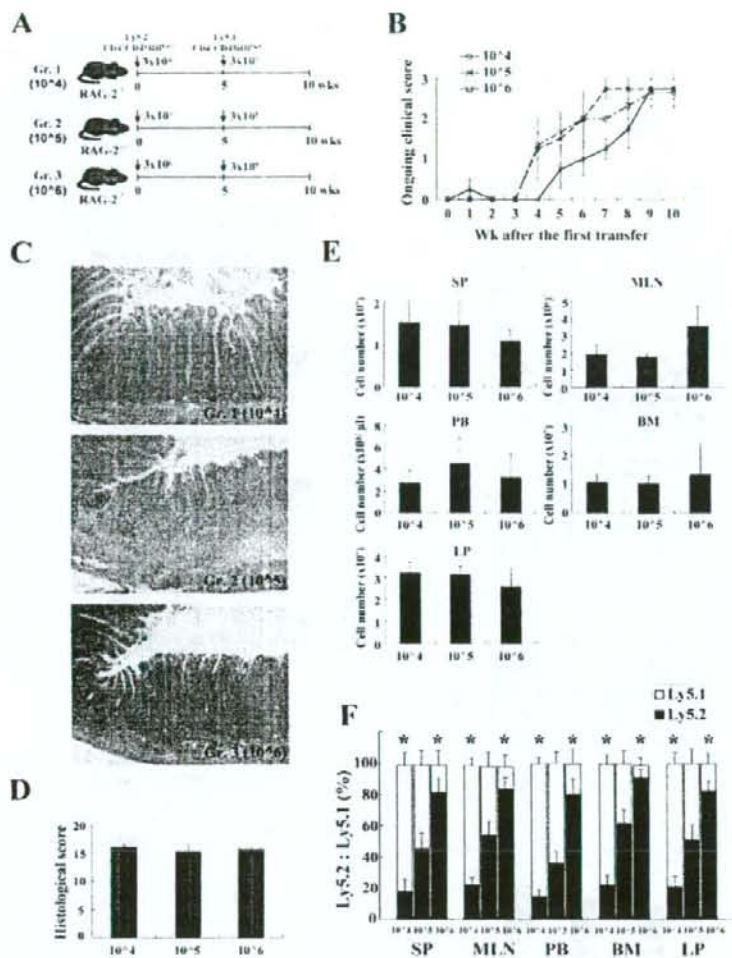


**Figure 1.** Newly recruited CD4<sup>+</sup>CD45RB<sup>high</sup> T cells are primed and converted to CD4<sup>+</sup>CD44<sup>high</sup> T cells. (A) Experimental design. C57BL/6-Ly5.2-RAG-2<sup>-/-</sup> mice were divided into three groups as described in the *Materials and Methods*. (B) Ongoing clinical scores for the three groups were determined at the indicated times. (C) Histopathological findings of colon. Original magnification, ×100. (D) Histological scores were determined at 10 wk after the first transfer. (E) Recovered cell numbers of CD3<sup>+</sup>CD4<sup>+</sup> T cells from SP, PB, LP, MLN, and BM. (F) Phenotypic characterization of SP and LP CD4<sup>+</sup> T cells after the transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells. Representative results shown are from six mice per group.

in spite of the different numbers of the transferred cells in the first transfer (Fig. 2E), indicating that the space occupied by colitic CD4<sup>+</sup> T cells in each tissue of established colitic mice is equivalent. Furthermore, the ratio of Ly5.2<sup>+</sup> cells to Ly5.1<sup>+</sup> cells in colitic mice at 10 wk was dependent on the ratio of the transferred cell numbers: it was less than unity in Group 1 (10<sup>6</sup>) mice and greater than unity in Group 3 (10<sup>6</sup>), whereas in Group 2

(10<sup>6</sup> / 5<sup>6</sup>) mice transferred with the same numbers of Ly5.2<sup>+</sup> cells and Ly5.1<sup>+</sup> cells at different times, the ratio of these cell types was almost 1:1 at various sites (Fig. 2F).

We next examined whether the transferred CD4<sup>+</sup>CD45RB<sup>high</sup> T cells could be differentiated into effector or memory CD4<sup>+</sup> T cells in this sequential transfer experiment. As shown in Fig. 3, almost all CD4<sup>+</sup> T cells in any tissues, whether Ly5.1<sup>+</sup> or Ly5.2<sup>+</sup> cells, had

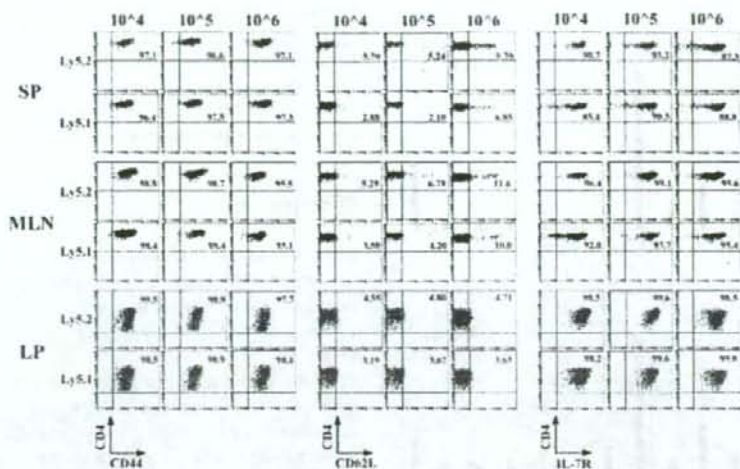


**Figure 2.** Newly generated CD4<sup>+</sup>CD44<sup>high</sup> T cells compete with pre-existing CD4<sup>+</sup>CD44<sup>high</sup> T cells depending on the transferred cell numbers. (A) Experimental design. C57BL/6-Ly5.2-RAG-2<sup>-/-</sup> mice were divided into three groups as described in the *Materials and methods*. (B) Ongoing clinical scores for the three groups were determined at the indicated times. (C) Histopathological findings of colon. Original magnification,  $\times 100$ . (D) Histological scores were determined at 10 wk after the first transfer. (E) Recovered cell numbers of CD3<sup>+</sup>CD4<sup>+</sup> T cells from SP, PB, LP, MLN, and BM. Cell numbers of Ly5.1<sup>+</sup> or Ly5.2<sup>+</sup> CD3<sup>+</sup>CD4<sup>+</sup> T cells were determined by four-color flow cytometry. (F) Phenotypic characterization of SP, MLN, PB, BM and LP CD4<sup>+</sup> T cells after the transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells. Results shown are from six mice per group. \* $p < 0.05$  vs. Ly5.2 ratio in same group.

a phenotype of CD4<sup>+</sup>CD44<sup>high</sup>CD62L<sup>+</sup>IL-7R $\alpha$ <sup>+</sup> effector-memory T ( $T_{EM}$ )-like cells, in contrast to the originally transferred CD4<sup>+</sup>CD45RB<sup>high</sup>CD44<sup>low</sup> T cells. Interestingly, in Group 3 ( $3 \times 10^6$ ), which received a high number ( $3 \times 10^6$  cells/mouse) of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells at the first transfer, higher numbers of central-memory CD4<sup>+</sup> T ( $T_{CM}$ )-like cells were generated in the SP and the MLN both in Ly5.2<sup>+</sup> cells and Ly5.1<sup>+</sup> populations. This agrees with a recent report that precursor numbers can impact the differentiation and the homeostasis of the resultant memory cells [13].

#### TCR V $\beta$ repertoire is constant between first and second transferred cells

Although we found that the secondarily transferred CD4<sup>+</sup>CD45RB<sup>high</sup> T cells could differentiate into CD4<sup>+</sup>  $T_{EM}$ -like cells *in vivo*, even in the persistent presence of previously developed CD4<sup>+</sup>  $T_{EM}$ -like cells from the first transfer (Fig. 1–3), it was unclear whether newly recruited CD4<sup>+</sup> T cells recognize the same antigenic epitopes as the previously recruited CD4<sup>+</sup> T cells at



**Figure 3.** Phenotypic characterization of SP, MLN and LP CD4<sup>+</sup> T cells after a sequential adoptive transfer as described in Fig. 2. Flow cytometric analysis shows that most of the transferred CD4<sup>+</sup>CD45RB<sup>high</sup> T cells in Group 1 (10<sup>4</sup>), Group 2 (10<sup>5</sup>), and Group 3 (10<sup>6</sup>) mice have the characteristics of CD4<sup>+</sup>CD44<sup>high</sup>CD62L<sup>+</sup>IL-7R<sup>+</sup> T<sub>EM</sub> cells. Results shown are from six mice per group.

the first transfer. To clarify this issue, splenic Ly5.1<sup>+</sup> and Ly5.2<sup>+</sup> CD4<sup>+</sup> T cells from three groups were analyzed for their TCR V $\beta$  repertoire by three-color flow cytometry. It is reasonable to use SP CD4<sup>+</sup> T cells in place of LP CD4<sup>+</sup> T cells for this assay, since we previously demonstrated that colitic SP CD4<sup>+</sup> T cells had similar characteristics in that they were CD4<sup>+</sup>CD44<sup>high</sup>CD62L<sup>+</sup>IL-7R<sup>+</sup> T<sub>EM</sub>-like cells [14], and were also colitogenic cells by which colitis can be transferred to new recipient mice [15]. As shown in Fig. 4, a polyclonal dominant TCR V $\beta$  repertoire with dominant V $\beta$ 2, V $\beta$ 4, V $\beta$ 8.1/8.2, and V $\beta$ 14 was almost constant between previously and newly transferred cells, regardless of the different numbers of the first transfer, indicating that colitogenic CD4<sup>+</sup> T cells recognizing the same or similar antigenic epitopes developed in accordance with the frequency or number of colitogenic antigen-specific naive CD4<sup>+</sup> T cells. In addition, the pattern of TCR V $\beta$  repertoire in colitic mice, whether with older Ly5.2<sup>+</sup> and newer Ly5.1<sup>+</sup> CD4<sup>+</sup> T cells, was similar to that of originally transferred CD4<sup>+</sup>CD45RB<sup>high</sup> T cells (Fig. 4).

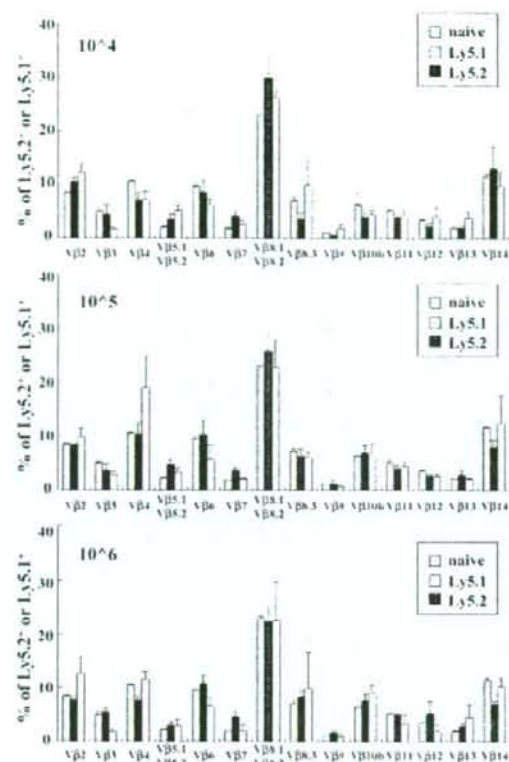
#### Previously generated, older CD4<sup>+</sup> T cells produce less IFN- $\gamma$ and IL-17

It has recently been demonstrated that, during chronic viral infection, the functions of virus-specific CD8<sup>+</sup> T cells often become impaired and exhausted in the persistent presence of viral antigens, in contrast to the highly functional effector and memory CD8<sup>+</sup> T cells generated after virus clearance in acute infection [11, 16]. Since colitogenic CD4<sup>+</sup> T cells in colitic RAG-2<sup>-/-</sup> mice induced by an adoptive transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells are likely to expand by responding to resident enteric bacterial

antigens that are persistently resident in the colonic lumen, it was possible that colitogenic CD4<sup>+</sup> T<sub>EM</sub>-like cells gradually become exhausted over time after the transfer. To assess whether newly recruited T<sub>EM</sub>-like cells that are differentiated from the secondarily transferred CD4<sup>+</sup>CD45RB<sup>high</sup> T cells can compete and prevail against the previously established and possibly exhausted T<sub>EM</sub>-like cells in colitic mice, we next divided RAG-2<sup>-/-</sup> mice into two groups (Fig. 5A): Group 1, RAG-2<sup>-/-</sup> mice transferred with Ly5.2<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells at 0 wk, and Group 2, RAG-2<sup>-/-</sup> mice transferred with Ly5.2<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells at 0 wk, and secondarily transferred with Ly5.1<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells at 13 wk after fully establishing colitis by the first transfer. Mice were observed for 17 wk after the first transfer.

The assessment of clinical scores revealed that Group 1 and 2 mice developed wasting disease and severe colitis at the plateau level from approximately 6 wk after the first transfer and remained at this level until 13 wk after the first transfer without dropout by death (data not shown). At autopsy at 17 wk, the total clinical scores of both groups were not significantly different (Fig. 5B). Histological findings revealed that both groups of mice developed severe colitis with a massive infiltration of mononuclear cells in the LP (Fig. 5C), and histological scores also confirmed no differences between two groups in multiple colon sections (Fig. 5D). Interestingly, the cell numbers recovered from the SP and LP were not significantly different between the groups regardless of the second transfer (Fig. 5E), indicating again that the space occupied by colitogenic CD4<sup>+</sup> T cells in each colitic mouse is equivalent. As shown in Fig. 5F, almost all LP CD4<sup>+</sup> T cells in any tissues, whether old Ly5.2<sup>+</sup> cells or new Ly5.1<sup>+</sup> cells, had a phenotype of CD4<sup>+</sup>CD44<sup>high</sup>CD62L<sup>+</sup>IL-7R<sup>+</sup> T<sub>EM</sub>-like cells.





**Figure 4.** Flow cytometric analysis of V $\beta$  families on the surface of the splenic CD4<sup>+</sup> T cells in the Group 1 ( $10^4$ ), Group 2 ( $10^5$ ), and Group 3 ( $10^6$ ) mice described in Fig. 2. To analyze the TCR V $\beta$  family repertoire, splenic cells were four-color-stained with PerCP-conjugated anti-CD3 mAb, allophycocyanin-conjugated anti-CD4 mAb, PE-conjugated anti-Ly5.1 or Ly5.2 mAb, and the indicated mAb of a panel of 14 FITC-conjugated V $\beta$  mAb. Each percentage value indicates the frequency of each V $\beta$  ( $n=6$ ). Naive, CD4<sup>+</sup>CD45RB<sup>high</sup> T cells serves as a control.

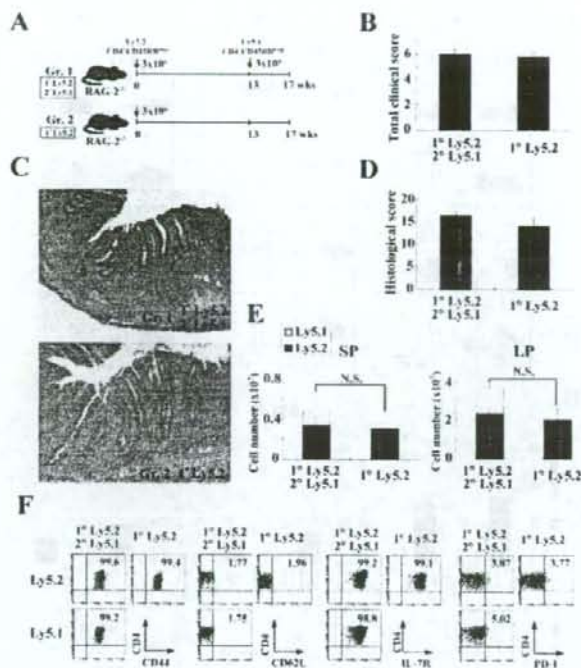
Of note, we found that the old LP Ly5.2<sup>+</sup> cells produced significantly less IFN- $\gamma$  (Th1) and IL-17 (Th17) cytokines than the new LP Ly5.1<sup>+</sup> cells (Fig. 6A and B), indicating that the old LP Ly5.2<sup>+</sup> cells long after the first transfer had fallen into exhaustion. In addition, the ratios of IL-17<sup>+</sup>, but not IFN- $\gamma$ <sup>+</sup>, cells in the old MLN and SP Ly5.2<sup>+</sup>CD4<sup>+</sup> cells were significantly decreased as compared with those of new Ly5.1<sup>+</sup> cells (Fig. 6A and B), suggesting that colitogenic CD4<sup>+</sup> T cells in various sites became exhausted over time. In particular, the LP shows the greatest level of cell exhaustion, at least in the expression of IFN- $\gamma$ . Since it has been reported that PD-1 is one of markers for exhausted CD8<sup>+</sup> T cells in chronic LMCV infection in mice and HIV infection in human [17–19], we also checked this molecule in old Ly5.2<sup>+</sup> cells and new Ly5.1<sup>+</sup> cells. Contrary to the above data of cytokine

production and previous reports of exhausted PD-1-expressing CD8<sup>+</sup> T cells [17–19], no difference in PD-1 expression was found between old Ly5.2<sup>+</sup> cells and new Ly5.1<sup>+</sup> cells at 17 wk (Fig. 5F), indicating that PD-1 is not an appropriate marker for the exhaustion of murine CD4<sup>+</sup> T cells, at least in the present time course of the sequential adoptive transfer protocol.

## Discussion

In the present study, we demonstrated that newly recruited naive CD4<sup>+</sup>CD45RB<sup>high</sup> T cells can be primed, expand and differentiate into CD4<sup>+</sup>CD44<sup>high</sup>CD62L<sup>+</sup>IL-7R $\alpha$ <sup>high</sup>T<sub>EM</sub>-like cells in colitic RAG-2<sup>-/-</sup> mice induced by adoptive transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells in competition with previously established colitogenic T<sub>EM</sub>-like CD4<sup>+</sup> T cells. Of note, the patterns of TCR V $\beta$  repertoire were constantly similar between the first and the second transfers, indicating that a set of colitogenic polyclonal CD4<sup>+</sup> T cells compete for each clone-specific survival signal. This is most easily explained by TCR recognition of a specific and limiting antigen epitope-MHC class II ligand and homeostatic cytokines, such as IL-7 [20, 21]. Furthermore, we found that old LP CD4<sup>+</sup> T<sub>EM</sub>-like cells produced less IFN- $\gamma$  and IL-17 than newly developed LP CD4<sup>+</sup> T<sub>EM</sub>-like cells, suggesting that old colitic LP CD4<sup>+</sup> T cells showed immunological exhaustion over time. These findings suggest that continuous generation of colitogenic CD4<sup>+</sup> T cells from newly recruited naive CD4<sup>+</sup> T cells to compensate for the older exhausted CD4<sup>+</sup> T cells is one of the mechanisms sustaining chronic colitis.

Although IBD is thought to be caused by colitogenic effector CD4<sup>+</sup> T cells, which probably respond to intestinal bacterial antigens and damage the target intestine, the nature of the colitogenic CD4<sup>+</sup> T cells over time remains largely unclear. How are these colitogenic CD4<sup>+</sup> T cells generated and maintained in the body of patients with IBD? Are these colitogenic CD4<sup>+</sup> T cells just effector CD4<sup>+</sup> T cells, which are generated from naive CD4<sup>+</sup> T cells or from colitogenic memory CD4<sup>+</sup> T cells acting like memory stem cells [10], and which are established at the initial attack and reside in the body throughout life? This question arises basically from the concept that 'true' memory T cells are established for the first time after antigen clearance from the body as often assessed in memory CD8<sup>+</sup> T cells in animal models of acute viral infection and vaccination [22–25]. According to this scenario, it seems that colitogenic 'true' memory CD4<sup>+</sup> T cells cannot be built up in our colitis model, since the possible target intestinal bacteria are never eliminated, but persist throughout life in chronic colitis both in animal models and human IBD. However, recent evidence suggests that maintenance of the CD8<sup>+</sup> and CD4<sup>+</sup> T cells in chronic infection is dependent on antigens [26, 27], although it seems inappropriate to call such cells memory cells. We here showed that colitic CD4<sup>+</sup> T cells of this transfer model strongly expressed CD44 and IL-7R $\alpha$ , which is a reliable marker for memory, but not effector, CD4<sup>+</sup> T cells; and also we recently demonstrated that IL-7, which is an important factor for survival of memory CD4<sup>+</sup> T cells [21], is essential for the persistence of colitis by showing that IL-7<sup>-/-</sup>  $\times$  RAG-1<sup>-/-</sup> mice transferred with colitogenic LP



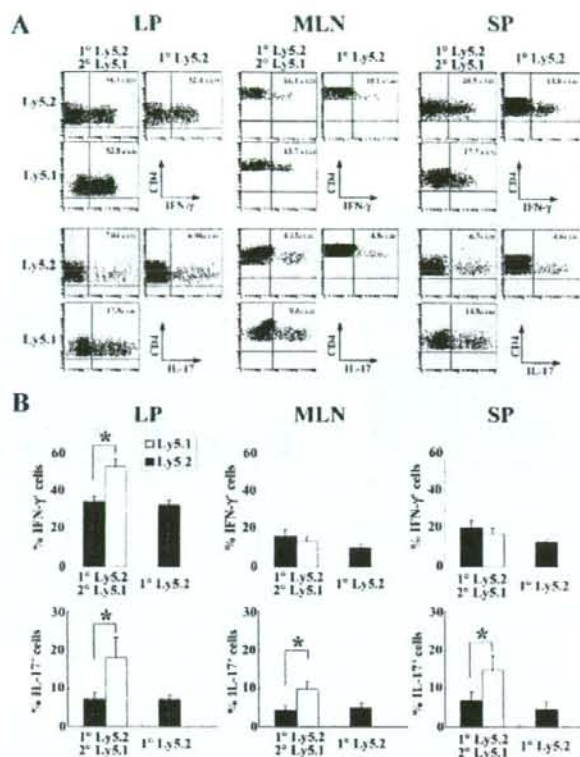
**Figure 5.** Pre-existing CD4<sup>+</sup>CD44<sup>high</sup> T cells are gradually exhausted. (A) Experimental design. CS7BL/6-Ly5.2-RAG-2<sup>-/-</sup> mice were divided into two groups: Group 1, RAG-2<sup>-/-</sup> mice transferred with Ly5.2-derived CD4<sup>+</sup>CD45RB<sup>high</sup> T cells at 0 wk (n=6), and Group 2, RAG-2<sup>-/-</sup> mice transferred with Ly5.1-derived CD4<sup>+</sup>CD45RB<sup>high</sup> T cells at 13 wk after the first transfer (n=6). Mice were observed for 17 wk after the first transfer. (B) Total clinical scores were determined at 17 wk after the first transfer as described in Materials and Methods. Data are indicated as the mean ± SEM of six mice per group. (C) Histopathological findings of colon. Original magnification, ×100. (D) Histological scores were determined at 17 wk after the first transfer. Data are indicated as the mean ± SEM of six mice in each group. (E) Recovered cell numbers of CD3<sup>+</sup>CD4<sup>+</sup> T cells from SP and LP. The cell number of Ly5.1<sup>+</sup> or Ly5.2<sup>+</sup> CD3<sup>+</sup>CD4<sup>+</sup> T cells was determined by four-color flow cytometry. Data are indicated as the mean ± SEM of six mice per group. N.S., not significant. (F) Phenotypic characterization of LP CD4<sup>+</sup> T cells after the transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells. Representative results shown are from one mice per group (six mice per group were analysed).

CD4<sup>+</sup>CD44<sup>high</sup>IL-7R $\alpha$ <sup>high</sup> cells did not develop colitis [28]. Since the survival of memory, but not effector, CD4<sup>+</sup> T cells is believed to be dependent on IL-7 [21], our results suggest that colitogenic CD4<sup>+</sup>CD44<sup>high</sup>CD62L<sup>-</sup>IL-7R $\alpha$ <sup>high</sup> T<sub>EM</sub> cells are sustained at least in part in colitic mice even in the persistent presence of intestinal bacteria. Consistent with the present findings, we recently demonstrated that substantial numbers of colitogenic CD4<sup>+</sup>CD44<sup>high</sup>CD62L<sup>-</sup>IL-7R $\alpha$ <sup>high</sup> T<sub>EM</sub>-like cells reside in colitic BM, which is believed to lack intestinal bacterial antigens but produce IL-7 [28].

Naive CD4<sup>+</sup> T cells are known to proliferate extensively, probably in response to foreign or self antigens, and are converted to memory-like CD4<sup>+</sup>CD44<sup>high</sup> T cells in lymphopenic immunodeficient mice, such as SCID and RAG-2<sup>-/-</sup> mice, in the process of 'lymphopenia-driven proliferation' [29]. Thus, it is likely that CD4<sup>+</sup>CD44<sup>high</sup>IL-7R $\alpha$ <sup>high</sup> cells in CD4<sup>+</sup>CD45RB<sup>high</sup> T cell-transferred colitic SCID mice represent memory-like cells, but not 'true' memory cells. Otherwise, it is also possible that the IL-7-dependent

colitogenic CD4<sup>+</sup> T cells in the presence of intestinal bacterial antigens in colitic mice have unique characteristics that differentiate them from conventional effector or 'true' memory CD8<sup>+</sup> and CD4<sup>+</sup> T cells defined mainly by studies of models of acute viral infection. Further studies will be needed to address this issue.

Colitogenic CD4<sup>+</sup> T cells may also be continuously generated from naive CD4<sup>+</sup> T cells that reside in the periphery or are newly generated from the thymus, because it is still unknown whether memory CD4<sup>+</sup> T cells, either 'true' memory or memory-like (T<sub>EM</sub>-like) cells, are long-lived. Thus, it was of interest to determine whether the generation of new colitogenic CD4<sup>+</sup> T cells from naive CD4<sup>+</sup> T cells is inhibited by the pre-existence of colitogenic CD4<sup>+</sup> T cells, and also whether it is involved in the persistence of disease, although it is known that adoptively transferred naive CD4<sup>+</sup> T cells extensively proliferate and are converted to T<sub>EM</sub>-like CD4<sup>+</sup> T cells presumably in response to intestinal bacteria under lymphopenic conditions like those in immunodeficient SCID and RAG-2<sup>-/-</sup> mice [25]. In a setting in the absence of antigens, it has recently been



**Figure 6.** Preexisting CD4<sup>+</sup>CD44<sup>high</sup> T cells are gradually exhausted. (A) Expression of IFN- $\gamma$  and IL-17 on freshly isolated cells from LP, MLN, and SP in the Group 1 and Group 2 mice described in Fig. 5. Cells were labeled for Ly5.1, Ly5.2, CD4, and intracellular IFN- $\gamma$  or IL-17. Ly5.2<sup>+</sup> and Ly5.1<sup>+</sup> CD4<sup>+</sup> cells were gated and analyzed for the presence of CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells or CD4<sup>+</sup>IL-17<sup>+</sup>. Number in upper quadrant represents the percentage of IFN- $\gamma$ <sup>+</sup> or IL-17<sup>+</sup> cells among CD4<sup>+</sup> cells. (B) The ratios of IFN- $\gamma$ <sup>+</sup> or IL-17<sup>+</sup> cells among Ly5.1<sup>+</sup> or Ly5.2<sup>+</sup> cells were analyzed by gating Ly5.1 or Ly5.2 on CD4<sup>+</sup> cells. Results shown are from six mice per group. \* $p < 0.05$ .

demonstrated that pre-existing 'true' memory CD4<sup>+</sup> T cells in RAG-2<sup>-/-</sup> mice after adoptive transfer prevent the proliferation and conversion of newly transferred naive CD4<sup>+</sup> T cells to the memory-like phenotype [30]. Furthermore, the same group previously demonstrated that RAG<sup>-/-</sup> mice transferred with a large number of naive CD4<sup>+</sup>CD45RB<sup>high</sup> T cells ( $12 \times 10^6$  cell/mouse) do not develop colitis [31]. This finding indicates that two mechanisms, cytokine (IL-7) competition and clonal competition, may restrain the activation and overgrowth of a small number of transferred naive T cells in the lymphopenic condition. Nevertheless, the fact that newly recruited naive CD4<sup>+</sup>CD45RB<sup>high</sup> T cells can be activated and expand in established colitic mice, in which space available to them should already be occupied by a large number of colitogenic CD4<sup>+</sup> T cells, may suggest the older colitogenic effector or memory CD4<sup>+</sup> T cells are exhausted and permit the expansion of newly recruited colitogenic CD4<sup>+</sup> T cells in our model. By contrast, surprisingly, we found that newly recruited CD4<sup>+</sup>CD45RB<sup>high</sup> T cells can be primed and expand extensively

even in established colitic mice in which pre-existing colitogenic CD4<sup>+</sup> T cells were fully expanded and occupied the space of CD4<sup>+</sup> T cells. Thus, our present results may link the frequent recurrence in natural history of IBD to the continuous generation of colitogenic CD4<sup>+</sup> effector and memory T cells, because the frequency of recurrence gradually decreases in accordance with immunosenescence in patients with a decreased supply of naive T cells by thymic involution. Interestingly, in addition, the total cell number of CD4<sup>+</sup> T cells was constant, and the ratio of pre-existing and newly recruited CD4<sup>+</sup>CD44<sup>high</sup> T cells in the body was dependent on the ratio of the transferred cell numbers, indicating that both old and new CD4<sup>+</sup> T cells compete for space and a possibly constant amount of IL-7 depending on the frequency of transferred colitogenic naive CD4<sup>+</sup> T cells.

Of note, we also demonstrated that production of IFN- $\gamma$  and IL-17 by older LP CD4<sup>+</sup>CD44<sup>high</sup> T cells was significantly less as compared with newly developed LP CD4<sup>+</sup>CD44<sup>high</sup> T cells (Fig. 5), indicating that these LP CD4<sup>+</sup>CD44<sup>high</sup>IL-7R<sup>high</sup> cells become

exhausted over time, as seen in CD8<sup>+</sup> T cells in persistent virus infection [17, 18]. Thus, it appears that colitogenic LP CD4<sup>+</sup> T cells in colitic mice are a mixture of IL-7-dependent CD4<sup>+</sup> T<sub>EM</sub>-like cells and exhaustion-facing effector cells derived from these CD4<sup>+</sup> T<sub>EM</sub>-like cells after encountering intestinal bacterial antigens. Consistent with this, the most affected site of cell exhaustion as indicated by cytokine production seemed to be the LP (Fig. 6), which is thought to be the effector site. Nevertheless, it remains unknown why mice transferred secondarily with CD4<sup>+</sup> CD45RB<sup>high</sup> T cells did not show exacerbation of the disease as compared with mice without the second transfer in clinical and histological evaluations (Fig. 5B and D). As a clue, we recently performed over seven sequential transfers of colitic LP CD4<sup>+</sup> cells obtained from colitic CD4<sup>+</sup> CD45RB<sup>high</sup> cell-transferred SCID mice into new SCID mice. Although SCID mice transferred with colitic LP CD4<sup>+</sup> cells stably developed colitis over several transfers, the severity of colitis declined with the increasing number of transfers [32]. Thus, it is likely that the exhaustion of CD4<sup>+</sup> T cells requires longer to become clinically and histologically evident than does the decline in cytokine production. Further study will be needed to address this issue.

In summary, we propose that continuous generation of colitogenic CD4<sup>+</sup> T cells from naive CD4<sup>+</sup> T cells is critically involved in the persistence of chronic colitis, suggesting that it is important not only to target the pre-existing colitogenic CD4<sup>+</sup> T cells but also to suppress and control the new generation of colitogenic CD4<sup>+</sup> T cells in developing a strategy for the treatment of IBD.

## Materials and methods

### Animals

C57BL/6N-Ly5.2 mice were purchased from Japan Clea (Tokyo, Japan). C57BL/6N-Ly5.1 and C57BL/6N-Ly5.2-RAG-2 deficient (RAG-2<sup>-/-</sup>) mice were obtained from Taconic Laboratory (Hudson, NY) and Central Laboratories for Experimental Animals (Kawasaki, Japan). Mice were maintained under specific pathogen-free (SPF) conditions in the Animal Care Facility of Tokyo Medical and Dental University. All donors and recipients were used for adoptive transfer experiments at 6–10 wk of age. All experiments were approved by the regional animal study committees (permission number: 2006-049) and were done according to institutional guidelines and Home Office regulations.

### Antibodies

The mAb other than biotin-conjugated anti-mouse IL-7R $\alpha$  (A7R34; eBioscience, San Diego) were obtained from BD Pharmingen (San Diego, CA) and used for purification of cell populations and flow cytometry analysis; 145-2C11, FITC-, PE- and PerCP<sup>565</sup>-conjugated anti-mouse CD3; RM4-5, PE- and allophycocyanin-

conjugated anti-mouse CD4; 16A, FITC-conjugated anti-mouse CD45RB; IM7, allophycocyanin-conjugated anti-mouse CD44; MEL-14, PE-conjugated anti-mouse CD62L; H1.2F3, FITC- and PE-conjugated anti-mouse CD69; A20, FITC- and PE-conjugated anti-mouse Ly5.1 (CD45.1); 104, FITC-conjugated anti-mouse Ly5.2 (CD45.2); J43, PE-conjugated anti-mouse PD-1; XMG1.2, PE-conjugated anti-mouse IFN- $\gamma$ ; TC11-18H10, PE-conjugated anti-mouse IL-17; B20.6, FITC-conjugated anti-mouse V $\beta$ 2; KJ25, FITC-conjugated anti-mouse V $\beta$ 3; KT4, FITC-conjugated anti-mouse V $\beta$ 4; MR9-4, FITC-conjugated anti-mouse V $\beta$ 5.1/2; RR4-7, FITC-conjugated anti-mouse V $\beta$ 6; TR310, FITC-conjugated anti-mouse V $\beta$ 7; MR5-2, FITC-conjugated anti-mouse V $\beta$ 8.1/2; B21.14, FITC-conjugated anti-mouse V $\beta$ 8.3; MR10-2, FITC-conjugated anti-mouse V $\beta$ 9; B21.5, FITC-conjugated anti-mouse V $\beta$ 10<sup>9</sup>; RR3-15, FITC-conjugated anti-mouse V $\beta$ 11; MR11-1, FITC-conjugated anti-mouse V $\beta$ 12; IN12.3, FITC-conjugated anti-mouse V $\beta$ 13; 14.2, FITC-conjugated anti-mouse V $\beta$ 14. Biotinylated antibodies were detected with PE-streptavidin (BD Pharmingen).

### T cell preparation

For isolation of peripheral lymphocytes, 600  $\mu$ L PB was collected from each mouse and diluted 1:1 with PBS. The diluted blood was layered over Lymphosepar II (IBL, Gunma, Japan) and centrifuged at 400  $\times$  g for 30 min at room temperature. The lymphocytes were then isolated from the plasma-Ficoll interface. SP and MLN were mechanically disrupted into single cell suspensions. BM was collected from the femur by flushing with sterile PBS. For the preparation of colonic LP cells [33], 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<sup>2+</sup>/Mg<sup>2+</sup>-free Hanks' BSS containing 1 mM DTT (Sigma-Aldrich, St. Louis, MO) for 30 min to remove mucus, then treated with 3 mg/mL collagenase (Roche Diagnostics GmbH, Germany) and 0.01% DNase (Worthington Biomedical Co., Freehold, NJ) for 2 h. After filtering through gauze, cells were pelleted two times through a 40% isotonic Percoll solution, and then subjected to Ficoll-Hypaque density gradient centrifugation (40%/75%). Enriched CD4<sup>+</sup> T cells were obtained by positive selection using anti-CD4 (L3T4) MACS magnetic beads. The resultant cells contained >94% CD4<sup>+</sup> cells when analyzed by FACSCalibur.

### Adoptive transfer protocols

In the first set of adoptive transfer experiments, we divided C57BL/6-Ly5.2-RAG-2<sup>-/-</sup> mice into three groups: Group 1, RAG-2<sup>-/-</sup> mice transferred with  $3 \times 10^5$  Ly5.2-derived CD4<sup>+</sup> CD45RB<sup>high</sup> T cells at 0 wk of the starting point ( $n=6$ ); Group 2, RAG-2<sup>-/-</sup> mice transferred with  $3 \times 10^5$  Ly5.2-derived CD4<sup>+</sup> CD45RB<sup>high</sup> T cells at 0 wk and with  $3 \times 10^5$  Ly5.1-derived CD4<sup>+</sup> CD45RB<sup>high</sup> T cells at 5 wk after the first transfer ( $n=6$ );

Group 3, RAG-2<sup>-/-</sup> mice transferred with  $3 \times 10^5$  Ly5.1-derived CD4<sup>+</sup>CD45RB<sup>high</sup> T cells at 5 wk after the starting point ( $n=6$ ). Briefly, CD4<sup>+</sup> T cells were isolated from splenocytes using the anti-CD4 (L3T4) MACS magnetic separation system (Miltenyi Biotec, Auburn, CA). Enriched CD4<sup>+</sup> T cells were labeled with PE-conjugated anti-mouse CD4 mAb and FITC-conjugated anti-CD45RB mAb, then sorted to yield the CD45RB<sup>high</sup> (highest staining 30%) fraction on a FACS Aria (Becton Dickinson, Sunnyvale, CA). Each mouse was injected intraperitoneally with  $3 \times 10^5$  CD4<sup>+</sup>CD45RB<sup>high</sup> T cells once. Mice were observed and killed at 10 wk after the starting point.

In the second set of adoptive transfer experiments, we divided C57BL/6-Ly5.2-RAG-2<sup>-/-</sup> mice into three groups: Group 1, RAG-2<sup>-/-</sup> mice transferred with  $3 \times 10^5$  Ly5.2<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells at 0 wk ( $10^4$ ,  $n=6$ ); Group 2, RAG-2<sup>-/-</sup> mice transferred with  $3 \times 10^5$  Ly5.2<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells at 0 wk ( $10^5$ ,  $n=6$ ); and Group 3, RAG-2<sup>-/-</sup> mice transferred with  $3 \times 10^6$  Ly5.2<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells at 0 wk ( $10^6$ ,  $n=6$ ). All groups of mice were transferred with  $3 \times 10^5$  Ly5.1<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells at 5 wk after the first transfer. Mice were killed at 10 wk after the starting point.

In the third set of adoptive transfer experiments, we divided C57BL/6-Ly5.2-RAG-2<sup>-/-</sup> mice into two groups: Group 1, RAG-2<sup>-/-</sup> mice transferred with  $3 \times 10^5$  Ly5.2<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells at 0 wk of the starting point ( $n=6$ ); and Group 2, RAG-2<sup>-/-</sup> mice transferred with  $3 \times 10^5$  Ly5.2<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells at 0 wk and with  $3 \times 10^5$  Ly5.1<sup>+</sup>CD4<sup>+</sup>CD45RB<sup>high</sup> T cells at 13 wk after the first transfer ( $n=6$ ). Mice were killed at 17 wk after the starting point.

### Disease monitoring and clinical scoring

The recipient mice after T cell transfer were weighed initially, then three times per week thereafter. They were observed for clinical signs of illness: hunched appearance, piloerection of the coat, diarrhea, and blood in the stool. Mice were killed at the indicated times and assessed for a total clinical score as the sum (0–6 points) of three parameters: hunching and wasting, 0 or 1; colon thickening, 0–3 (0, no colon thickening; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); and stool consistency, 0–2 (0, normal beaded stool; 1, soft stool; 2, diarrhea). To monitor clinical signs during the observation period, the ongoing clinical score is defined as the sum (0–3 points) of the two parameters other than colon thickening [34].

### Histological examination

Tissue samples were fixed in PBS containing 10% neutral-buffered formalin. Paraffin-embedded sections (5  $\mu$ m) were stained with H&E. The sections were analyzed without prior knowledge of the type of T cell reconstitution and recipients. The area most affected was graded by the number and severity of lesions. The mean degree of inflammation in the colon was calculated using a modification of a previously described scoring system [33].

### Flow cytometry

To detect the surface expression of a variety of molecules, isolated cells were preincubated with an Fc $\gamma$ R-blocking mAb (CD16/32; 2.4G2, BD Pharmingen) for 20 min followed by incubation with specific FITC-, PE-, PerCP-, allophycocyanin-, or biotin-labeled antibodies for 30 min on ice. Biotinylated antibodies were detected with PE-streptavidin. For intracellular staining for IFN- $\gamma$  and IL-17 [35], cells were stimulated with 50 ng/mL phorbol-12-myristate-13 acetate (PMA; Calbiochem, CA) and 500 ng/mL ionomycin (Sigma-Aldrich) for 10 h, then 5  $\mu$ g/mL brefeldin A (GolgiPlug; BD Pharmingen) was added. Cells were first preincubated with Fc $\gamma$ R-blocking mAb for 20 min, and then stained with PerCP-anti-CD3 mAb, allophycocyanin-anti-CD4 mAb, and FITC-anti-CD45.1 or anti-CD45.2 mAb. The stimulated cells were fixed and permeabilized with Cytotfix/Cytoperm<sup>TM</sup> (BD Pharmingen) at 4°C for 30 min. Staining and washing were performed in Perm/Wash Buffer<sup>TM</sup> (BD Pharmingen), and cells were stained with PE-conjugated anti-IFN- $\gamma$  or anti-IL-17 mAb.

### Statistical analysis

The results were expressed as the mean  $\pm$  SEM. Groups of data were compared by Mann-Whitney U test. Differences were considered to be statistically significant when  $p < 0.05$ .

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Abbreviations: IBD: inflammatory bowel disease; LP: lamina propria; MLN: mesenteric LN; PB: peripheral blood; SP: spleen; T<sub>CM</sub>: central-memory T; T<sub>EM</sub>: effector-memory T

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# Negative Feedback Regulation of Colitogenic CD4<sup>+</sup> 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<sup>+</sup> memory T cells reside in BM of colitic CD4<sup>+</sup>CD45RB<sup>high</sup> T-cell-transferred SCID mice. Based on this background we here investigate whether granulocytes promote or suppress the expansion of colitogenic CD4<sup>+</sup> T cells.

**Methods:** First, we show that Gr-1<sup>high</sup>CD11b<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cells with activated phenotype.

**Conclusions:** These results suggest that the increased granulopoiesis by colitogenic BM CD4<sup>+</sup> T cells represent a negative feedback mechanism to control systemic inflammation.

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**Key Words:** CD4<sup>+</sup> 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.<sup>1,2</sup> According to our current understanding the diseases are caused by activated macrophages and effector CD4<sup>+</sup> 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 Peyer's patches.<sup>3–8</sup> 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.<sup>9–12</sup>

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.<sup>13</sup> In addition, it has also been reported that the circulating activated granulocytes are elevated with increased survival time in patients with severe IBD.<sup>14,15</sup> 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<sup>+</sup>CD45RB<sup>high</sup> T cells into immunodeficient SCID mice.<sup>16,17</sup>

## 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-7R $\alpha$  (A7R34; eBioscience, San Diego, CA), PE-conjugated antimouse CD11b (M170; eBioscience), and PE-conjugated anti-F4/80 (Cl:A3-1; Serotec, Bicester, UK) were obtained from BD Pharmingen (San Diego, CA) and used for purification of cell populations and flow cytometric analysis: Fc $\gamma$ R (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<sup>+</sup>CD45RB<sup>high</sup> T cells (17). Briefly, CD4<sup>+</sup> T cells were isolated from splenocytes using the anti-CD4 (L3T4) MACS magnetic separation system (Miltenyi Biotec, Auburn, CA). Enriched CD4<sup>+</sup> T cells were labeled with PE-conjugated antimouse CD4 mAb and FITC-conjugated anti-CD45RB mAb, then sorted to yield the CD45RB<sup>high</sup> (highest staining 30%) fraction on a FACS Vantage SE (Becton Dickinson, Sunnyvale, CA). Each SCID mouse was injected intraperitoneally with  $3 \times 10^5$  CD4<sup>+</sup>CD45RB<sup>high</sup> T cells. The colitic mice induced by the adoptive transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells were sacrificed at 6–8 weeks after transfer.

### T-cell Preparation

For isolation of peripheral lymphocytes, 600  $\mu$ L 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 400g 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,<sup>18</sup> 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<sup>+</sup> T cells were obtained by positive selection using anti-CD4 (L3T4) MACS magnetic beads. The resultant cells contained >94% CD4<sup>+</sup> cells when analyzed by FACS Calibur.

### Flow Cytometry

To detect the surface expression of a variety of molecules, isolated cells were preincubated with the Fc $\gamma$ R-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,  $1 \times 10^5$  CD4<sup>+</sup> T cells from LP and BM were cultured in 200  $\mu$ L of culture medium at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in 96-well plates (Costar, Cambridge, MA) precoated with 5  $\mu$ g/mL hamster antimouse CD3e mAb (145-2C11, BD Pharmingen) and 2  $\mu$ g/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).

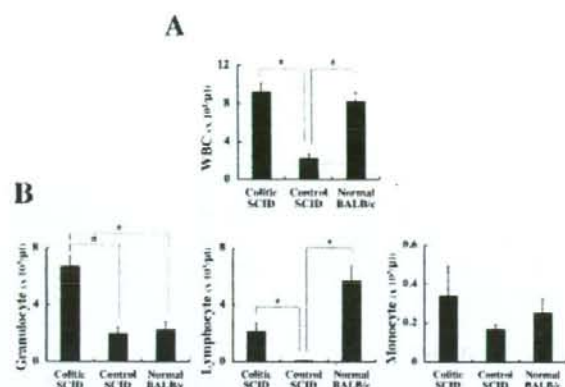
### Immunohistochemistry

Consecutive cryostat bone marrow sections (6  $\mu$ m) 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  $5 \times 10^5$  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. \* $P < 0.05$ .

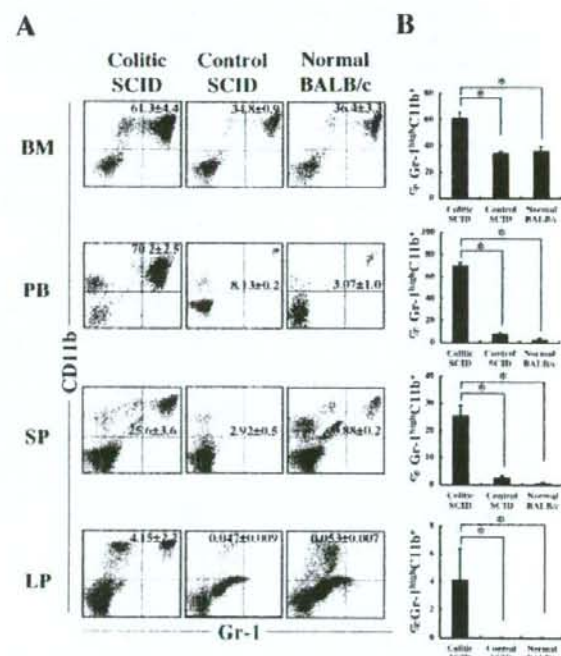
anti-CD3/CD28 mAbs-stimulated colitic or normal BM or LP CD4<sup>+</sup> T cells for 48 hours. Colonies were scored 7 days after incubation of cells at 5% CO<sub>2</sub> in a humidified atmosphere.

### In Vivo Experimental Design

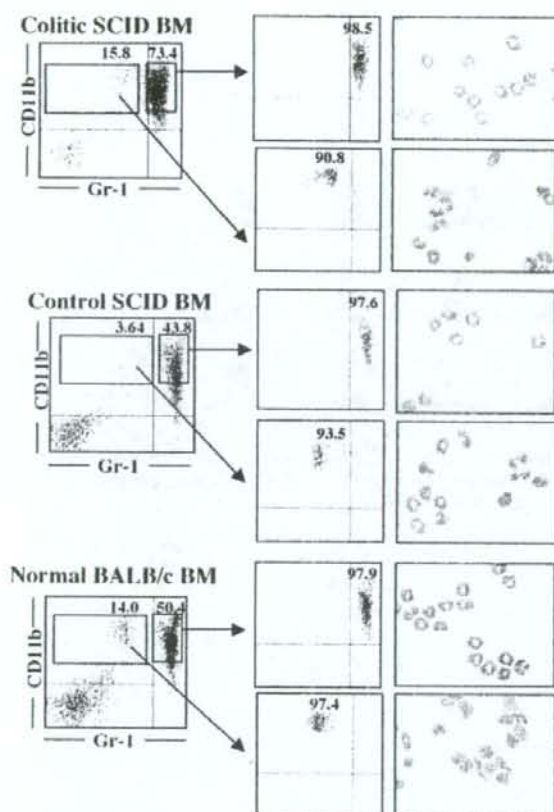
We first checked whether in vivo anti-Gr-1 treatment preferentially depletes granulocytes, but not dendritic cells and macrophages, and myeloid suppressor cells by assessing the expression of Ly-6G molecule that is a more specific marker for granulocytes, CD11c, F4/80, and CD31 in addition to CD11b and 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)<sup>19</sup> on CD11b<sup>+</sup>Gr-1<sup>high</sup> or CD11b<sup>+</sup>Gr-1<sup>low</sup> 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  $3 \times 10^5$  CD4<sup>+</sup>CD45RB<sup>high</sup> 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  $3 \times 10^5$  CD4<sup>+</sup>CD45RB<sup>high</sup> and  $3 \times 10^5$  CD4<sup>+</sup>CD45RB<sup>low</sup> 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.<sup>20</sup> 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-1<sup>high</sup>CD11b<sup>+</sup> mature granulocytes. Error bar indicates SEM for 10 mice of each group. \* $P < 0.05$ .



**FIGURE 3.** BM Gr-1<sup>high</sup>CD11b<sup>+</sup> cells are morphologically granulocytes. Cytopsin staining with Wright was performed using isolated BM Gr-1<sup>high</sup>CD11b<sup>+</sup> and Gr-1<sup>low</sup>CD11b cells. BM cells were isolated from colitic SCID mice, age-matched normal SCID mice, and normal BALB/c mice. Then Gr-1<sup>high</sup>CD11b<sup>+</sup> and Gr-1<sup>low</sup>CD11b cells isolated using FACS Vantage were stained with Wright after cytopsin procedures. Original,  $\times 40$ . Data represent 3 independent experiments.

the colon was calculated using a modification of a previously described scoring system.<sup>19</sup>

### Statistical Analysis

The results are expressed as the mean  $\pm$  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

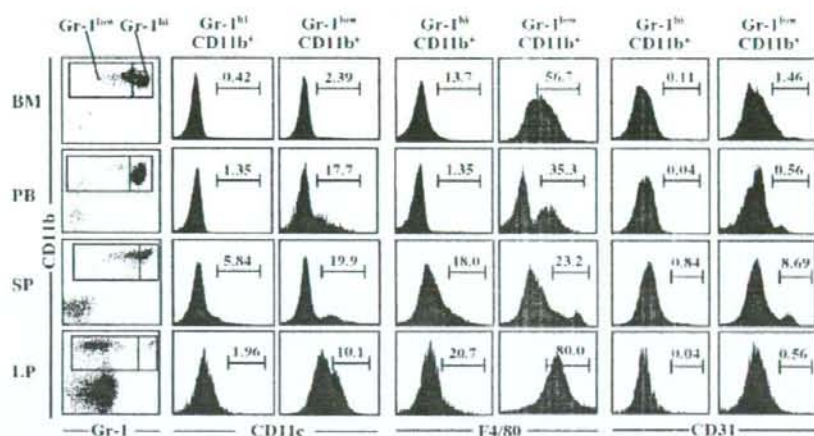
Although SCID-transfer model of colitis induced by adoptive transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells is one of the

representative CD4<sup>+</sup> T-cell-mediated colitis models accompanied by the activation of antigen-presenting cells and CD4<sup>+</sup> T cells,<sup>3,16</sup> 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<sup>+</sup>Gr-1<sup>high</sup> 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<sup>+</sup>Gr-1<sup>high</sup> cells was morphologically categorized as granulocyte by showing that almost all the isolating BM CD11b<sup>+</sup>Gr-1<sup>high</sup> cells from colitic SCID, control SCID, and normal BALB/c mice were preferentially polymorphonuclear-shaped (Fig. 3), while isolated BM CD11b<sup>+</sup>Gr-1<sup>low</sup> cells from 3 groups of mice were a mixture of mononuclear and polynuclear cells (Fig. 3), suggesting that the increase of CD11b<sup>+</sup>Gr-1<sup>high</sup> granulopoiesis stimulated by the infiltrating colitogenic CD4<sup>+</sup> 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<sup>+</sup> T cells in various sites of colitic mice, we assessed the numbers of CD11b<sup>+</sup>Gr-1<sup>high</sup> granulocytes and CD3<sup>+</sup>CD4<sup>+</sup> T cells in various sites at 4 and 10 weeks after transfer. During this course the clinical score



**FIGURE 4.** BM Gr-1<sup>high</sup>CD11b<sup>-</sup> cells are phenotypically granulocytes. BM cells were isolated from colitic SCID mice previously transferred with CD4<sup>+</sup>CD45RB<sup>high</sup> T cells. Then cells were stained with mAbs against CD11c, F4/80, or CD31 in addition to mAbs 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<sup>+</sup>CD4<sup>+</sup> T cells, the absolute number of Gr-1<sup>high</sup>CD11b<sup>-</sup> 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<sup>19</sup> also express CD11b and/or Gr-1 molecules, we next assessed whether our isolated CD11b<sup>+</sup>Gr-1<sup>high</sup> 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<sup>+</sup>Gr-1<sup>high</sup> cells from BM, PB, SP, or LP did not express F4/80 (macrophage marker), CD11c (dendritic cell), and CD31 (myeloid suppressor cell)<sup>19</sup> in sharp contrast to the paired CD11b<sup>+</sup>Gr-1<sup>low</sup> cells that contained such nongranulocyte cells, suggesting that our CD11b<sup>+</sup>Gr-1<sup>high</sup> cells in colitic mice are mainly granulocytes, but not immature dendritic cells and macrophages, and myeloid suppressor cells.

#### Colitic BM CD4<sup>+</sup> T Cells Produce Granulopoietic Cytokines

Given the evidence that CD11b<sup>+</sup>Gr-1<sup>high</sup> 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<sup>+</sup> 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.<sup>21</sup> Among these cytokines the production of IL-3, IL-17, or GM-CSF, but not IL-6 and G-CSF, by anti-

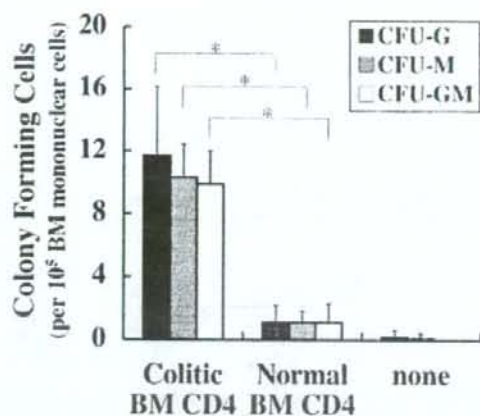
CD3/CD28 mAbs-stimulated colitic BM CD4<sup>+</sup> T cells was significantly increased as compared with that by normal BM and LP CD4<sup>+</sup> T cells, and this was to a similar extent to those by colitic LP CD4<sup>+</sup> T cells (data not shown). Interestingly, the production of Th1 cytokines (IFN- $\gamma$  and TNF- $\alpha$ ) by colitic BM CD4<sup>+</sup> T cells was significantly increased as compared with that by normal BM CD4<sup>+</sup> T cells, but this was significantly lower than that by colitic LP CD4<sup>+</sup> T cells (data not shown).

#### Colitic BM CD4<sup>+</sup> Cells Secrete Colony-forming Factors

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

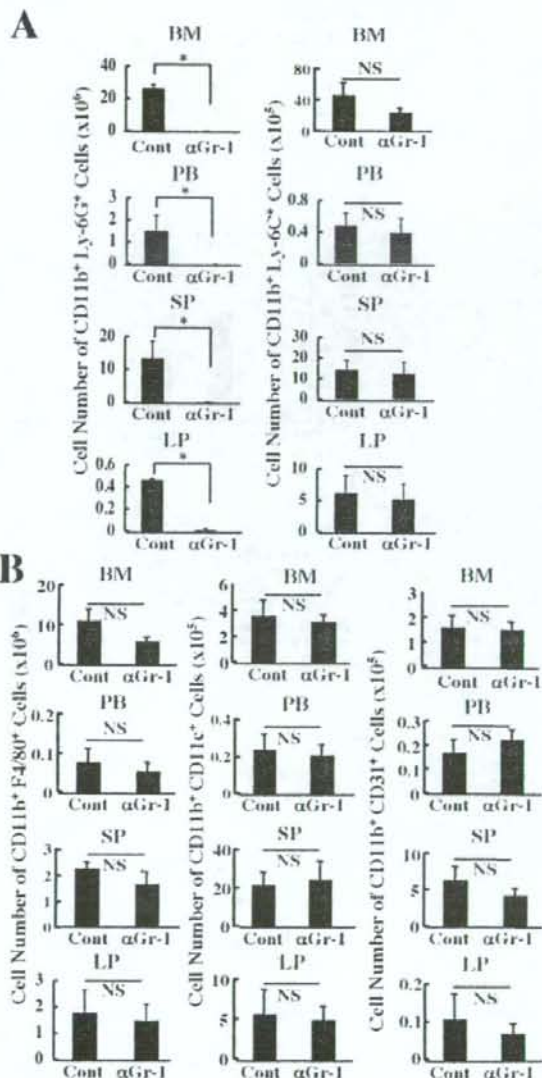


**FIGURE 5.** Colitic BM CD4<sup>+</sup> cells secrete colony-forming factors. BM mononuclear cells from normal BALB/c mice were plated in methylcellulose-containing media with 2000-fold diluted supernatants obtained from anti-CD3/CD28-stimulated colitic or normal BM CD4<sup>+</sup> cells. Hematopoietic colonies containing greater than 30 cells were scored after 7 days. Data are indicated as mean  $\pm$  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<sup>+</sup>CD11b<sup>+</sup> cells, but not Ly-6C<sup>+</sup>CD11b<sup>+</sup> 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<sup>+</sup>F4/80<sup>+</sup> macrophages, CD11b<sup>+</sup>CD11c<sup>+</sup> dendritic cells, and CD11b<sup>+</sup>CD31<sup>+</sup> 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<sup>+</sup>CD45RB<sup>high</sup> 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<sup>+</sup>CD45RB<sup>high</sup> and CD4<sup>+</sup>CD45RB<sup>low</sup> 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  $87.9 \pm 9.29\%$ ) was significantly higher than that of the control IgG-treated mice ( $104.8 \pm 6.53\%$ ) (Fig. 7B). On the contrary, the mice transferred with CD4<sup>+</sup>CD45RB<sup>high</sup>

and CD4<sup>+</sup>CD45RB<sup>low</sup> 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<sup>high</sup>CD11b<sup>+</sup> Ly-6C<sup>+</sup> cells, but not CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages, CD11b<sup>+</sup>CD11c<sup>+</sup> dendritic cells, and CD11b<sup>+</sup>CD31<sup>+</sup> 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  $\pm$  SD of 6 mice in each group. \* $P < 0.05$ . NS, not significantly different.