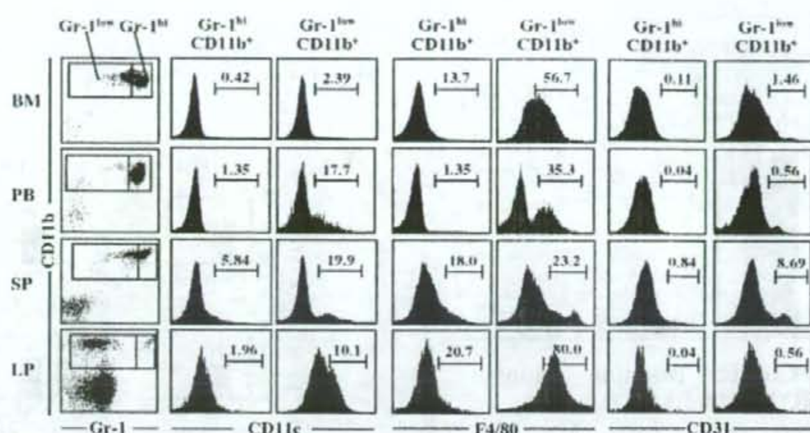

FIGURE 4. BM Gr-1^{high}CD11b$^+$ cells are phenotypically granulocytes. BM cells were isolated from colitic SCID mice previously transferred with CD4$^+$CD45RB^{high} T cells. Then cells were stained with mAbs against CD11c, F4/80, or CD31 in addition to mAgs against CD11b and Gr-1. Data represent FACS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

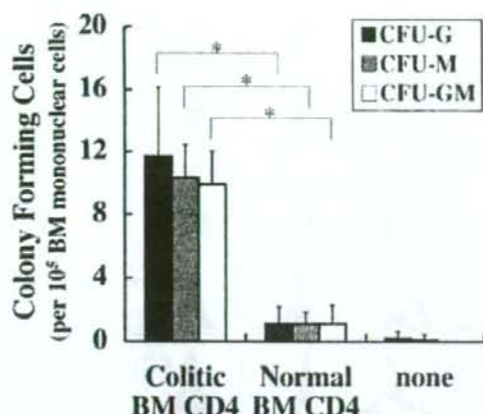

FIGURE 5. Colitic BM CD4$^+$ cells secrete colony-forming factors. BM mononuclear cells from normal BALB/c mice were plated in methylcellulose-containing media with 2000-fold diluted supernatants obtained from anti-CD3/CD28-stimulated colitic or normal BM CD4$^+$ cells. Hematopoietic colonies containing 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).

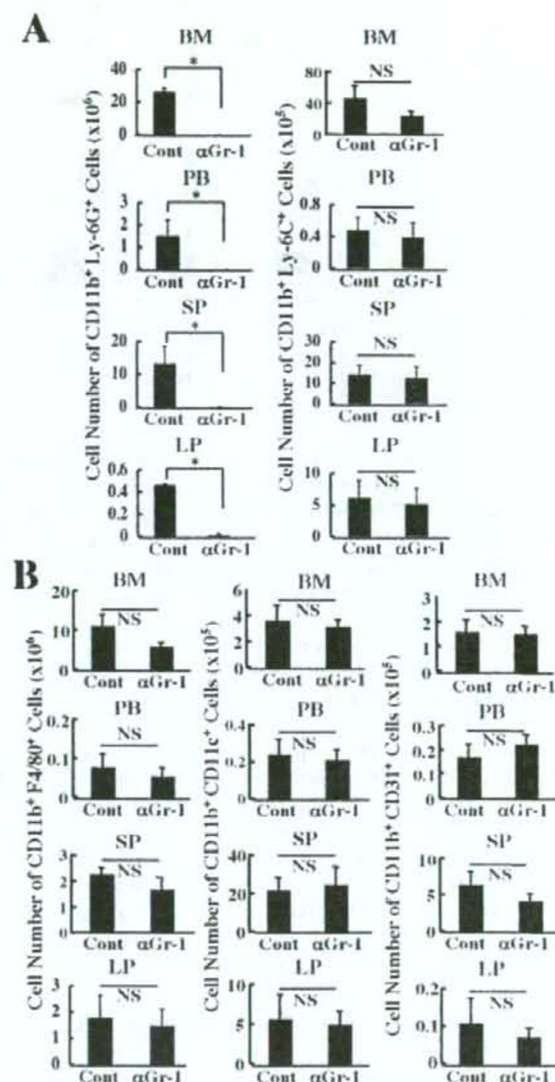

FIGURE 6. Anti-Gr-1 mAb treatment depletes Gr-1^{high}CD11b$^+$Ly-6C$^+$ cells, but not CD11b$^+$F4/80$^+$ macrophages, CD11b$^+$CD11c$^+$ dendritic cells, and CD11b$^+$CD31$^+$ myeloid suppressor cells. Expression of Ly-6G, Ly-6C, F4/80, CD11c, or CD31 in addition to CD11b and Gr-1 on cells obtained 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.

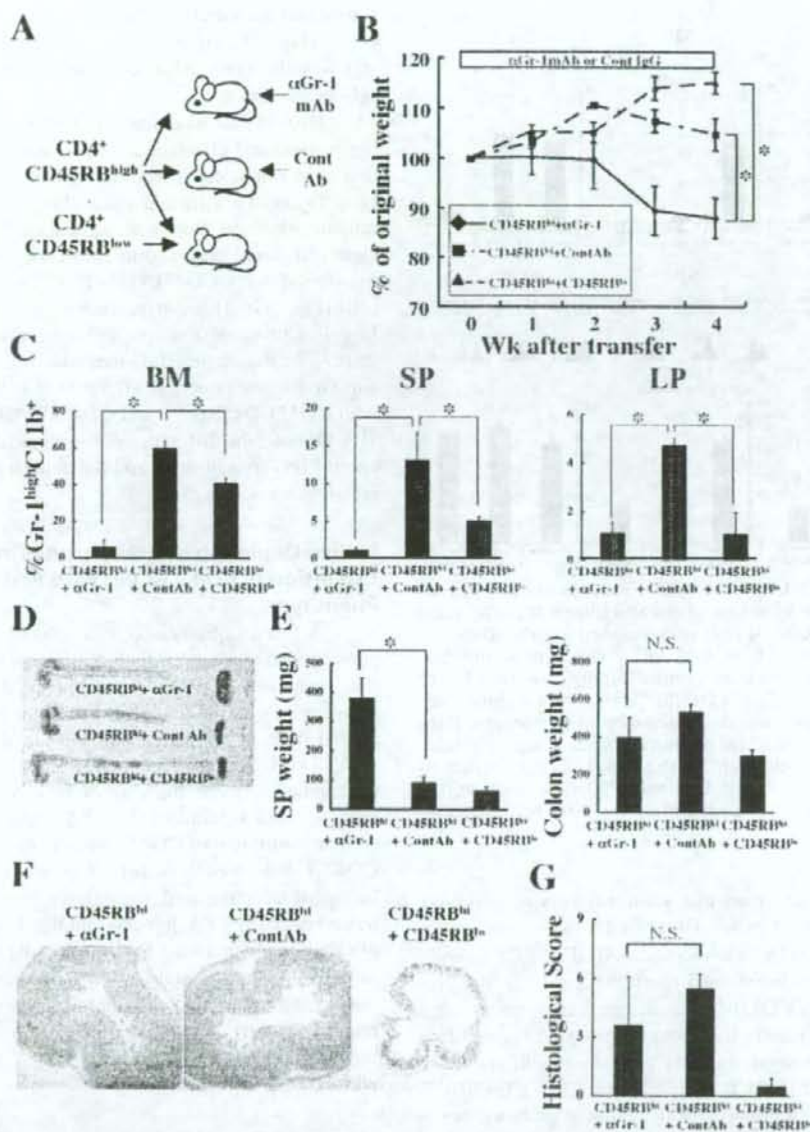

FIGURE 7. Anti-Gr-1 mAb treatment does not ameliorate colitis, but exacerbated wasting disease. A: Recipient SCID mice were administered anti-Gr-1 mAb or control rat IgG for 4 weeks starting from the time of CD4$^+$CD45RB^{high} T-cell transfer. Other SCID control mice were transferred with CD4$^+$CD45RB^{high} 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-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.

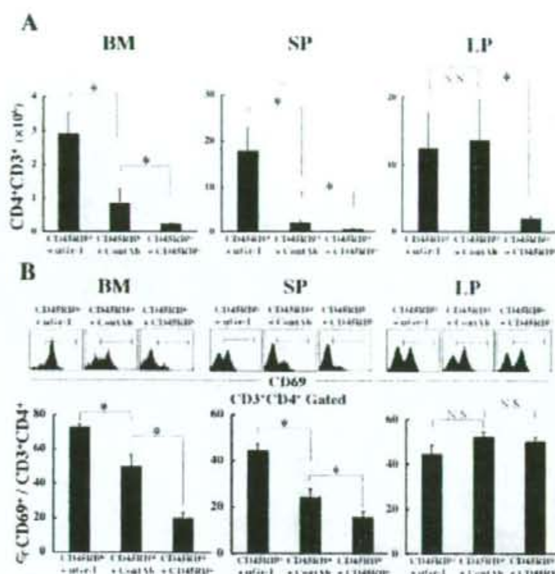

FIGURE 8. Anti-Gr-1 mAb treatment induces marked expansion of CD4$^+$ T cells in bone marrow and spleen of colitic mice. A: BM, SP, and LP CD4$^+$ T cells were isolated from SCID mice 4 weeks after transfer of CD4$^+$CD45RB^{high} cells and administration with anti-Gr-1 mAb or control rat IgG, or transfer 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 $\pm$ 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 $\pm$ 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-1^{high}CD11b$^+$ granulocytes, but not Gr-1^{low}CD11b$^+$ monocytes in SP (Fig. 7C). Consistent with the above-mentioned results (Figs. 1, 2), the proportion of Gr-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-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 $\pm$ 0.71 in the control IgG-treated mice, 3.6 $\pm$ 2.46 in the anti-Gr-1-treated mice, and 0.5 $\pm$ 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

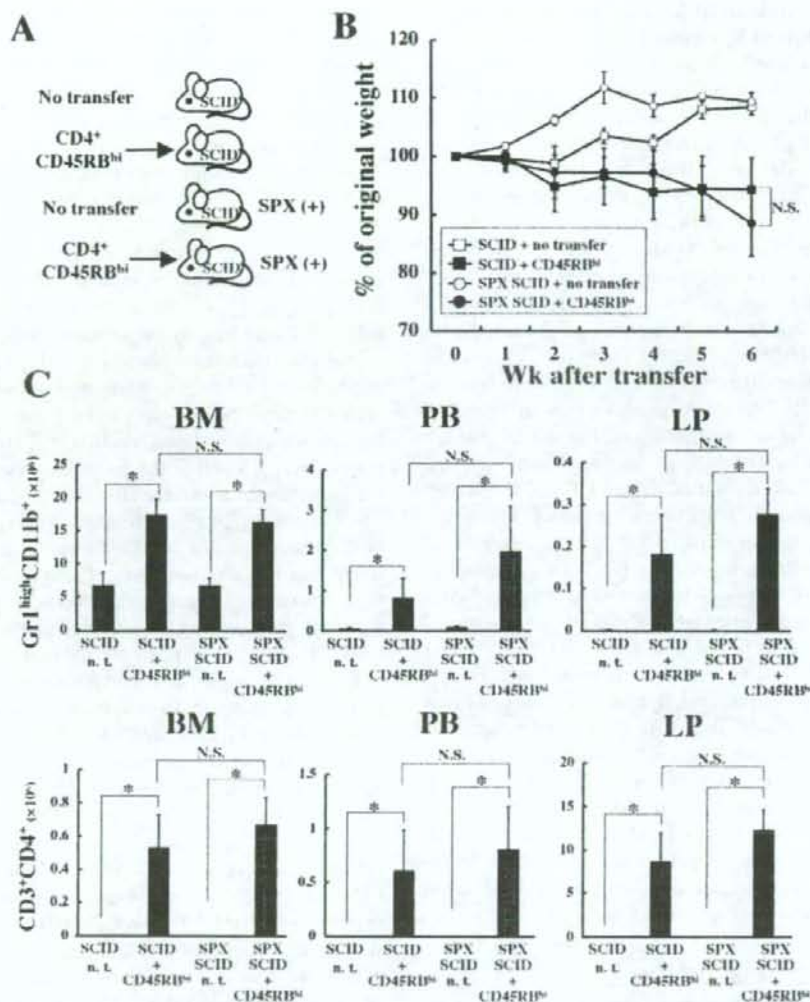

FIGURE 9. Splenectomized SCID mice transferred with CD4$^+$CD45RB^{high} T cells develop marked granulopoiesis. A: SCID mice were splenectomized 2 weeks before CD4$^+$CD45RB^{high} T-cell transfer. Mice were divided into 4 groups (each, $n = 5$) as follows; Group 1, SCID mice without splenectomy and 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-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-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.

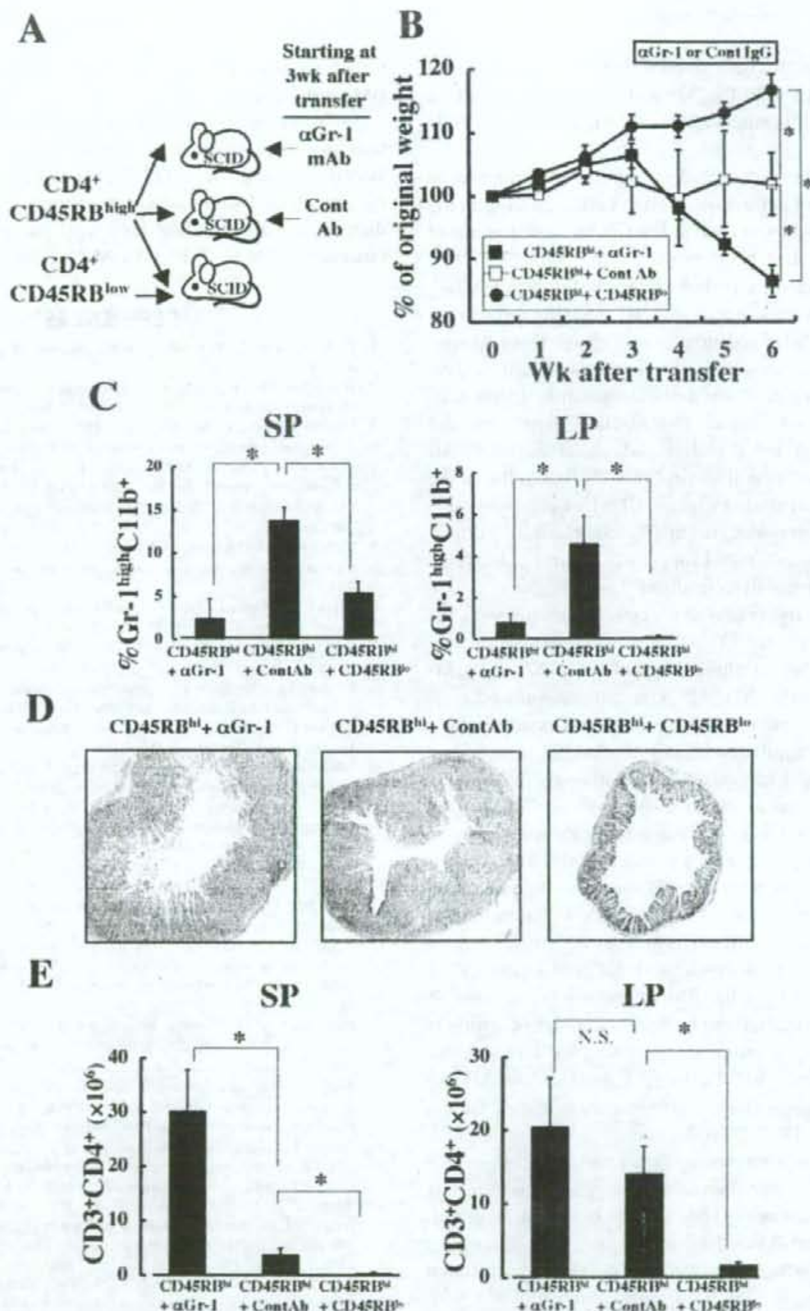

FIGURE 10. Delayed anti-Gr-1 mAb treatment does not ameliorate colitis, but increased systemic CD4$^+$ T cells. A: Recipient SCID mice were administered anti-Gr-1 mAb or control rat IgG for 3 weeks starting from 3 weeks after transfer. Other SCID control mice were transferred with CD4$^+$CD45RB^{high} 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-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.

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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Continuous generation of colitogenic CD4$^+$ T cells in persistent colitis

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Inflammatory bowel diseases take chronic courses due to the expansion of colitogenic CD4$^+$ cells. However, it is unclear whether the persistent disease is driven by continuous reactivation of colitogenic memory CD4$^+$ cells to generate effector CD4$^+$ cells or by continuous generation of effector CD4$^+$ cells from naïve cells. To clarify this issue, we performed a series of sequential adoptive transfers of Ly5.2$^+$ and Ly5.1$^+$ CD4$^+$CD45RB^{high} cells into RAG-2$^{-/-}$ mice at different time points. We show here that the secondarily transferred CD4$^+$CD45RB^{high} cells can be converted to CD4$^+$CD44^{high}CD62L$^-$IL-7Rα^{high} effector-memory T cells even in the presence of pre-existing effector-memory CD4$^+$ cells. Although the total cell numbers of CD4$^+$ 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$^+$ cells produced significantly lower amounts of IFN-γ and IL-17 than CD4$^+$ cells arising from secondary transfer. In conclusion, the continuous generation of colitogenic CD4$^+$ cells that compensate for exhausted CD4$^+$ 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$^+$ 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$^+$ effector and memory T cells. Nevertheless, the nature of the colitogenic CD4$^+$ 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$^+$ effector T cells, several possible mechanisms may be involved. One possibility is that the persistency is driven by the initial colitogenic CD4$^+$ memory T clones acting like memory stem cells [10] throughout the entire course of disease. In this case, colitogenic CD4$^+$ effector T cells would be generated from colitogenic CD4$^+$ memory T cells that are established at the initial onset, but are presumably suppressed by regulatory CD4$^+$ 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$^+$ 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$^+$ memory T cells could actually be established in such a situation. A second possibility is that the same or different epitope-specific colitogenic CD4$^+$ effector T cells are generated and expand after priming from newly recruited naïve CD4$^+$ 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$^+$ effector or memory T cells prevent or ignore the priming, expansion, and phenotypic and functional conversion of newly recruited naïve CD4$^+$ T cells to effector or memory CD4$^+$ T cells. To evaluate these unsolved, but critical, issues, we performed sequential adoptive transfers of Ly5.1$^+$ and Ly5.2$^+$ CD4$^+$CD45RB^{high} T cells into immunodeficient RAG-2$^{-/-}$ mice at different time points to induce chronic colitis.

Results

Newly transferred CD4$^+$CD45RB^{high} cells are converted into effector-memory T cells in colitic mice

To assess the possibility that continuous generation of new colitogenic CD4$^+$ T cells from naïve CD4$^+$ T cells occurs routinely in colitic mice, we performed sequential adoptive transfers of Ly5.2$^+$ and Ly5.1$^+$ CD4$^+$CD45RB^{high} T cells into RAG-2$^{-/-}$ mice at different time points. To do this, we divided RAG-2$^{-/-}$ recipient mice into three groups (Fig. 1A): Group 1, RAG-2$^{-/-}$ mice transferred with Ly5.2$^+$CD4$^+$CD45RB^{high} T cells at 0 wk; Group 2, RAG-2$^{-/-}$ mice transferred with Ly5.2$^+$CD4$^+$CD45RB^{high} T cells at 0 wk and with Ly5.1$^+$CD4$^+$CD45RB^{high} T cells at 5 wk; and Group 3, RAG-2$^{-/-}$ mice transferred with Ly5.1$^+$CD4$^+$CD45RB^{high} T cells at 5 wk. Mice were observed for 10 wk after the first transfer of Ly5.2$^+$CD4$^+$CD45RB^{high} T cells. In Group 1 and Group 2, mice developed colitis over time after the first transfer of Ly5.2$^+$CD4$^+$CD45RB^{high} 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$^+$CD4$^+$CD45RB^{high} 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$^+$ T cell accumulation was made by isolating CD4$^+$ 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$^+$ and Ly5.1$^+$ CD4$^+$ 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$^+$CD45RB^{high} T cells, whether Ly5.1$^+$ or Ly5.2$^+$, were converted to CD4$^+$CD44^{high} effector or memory cells both in SP and LP (Fig. 1F). In addition, substantial numbers of CD4$^+$ 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$^+$CD4$^+$CD45RB^{high} naïve T cells could be converted to CD4$^+$CD44^{high} effector or memory cells even in the presence of pre-existing Ly5.2$^+$ effector or memory cells that had previously expanded in colitic mice. Second, newly (secondarily transferred) and previously (primarily transferred) established CD4$^+$CD44^{high} effector or memory cells compete with each other and the already present CD4$^+$ T cells to occupy the space available to a constant number of CD4$^+$ T cells in established chronic colitis at 10 wk.

Competition between colitic CD4$^+$ 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$^+$ T cells in colitic mice, we next divided RAG-2$^{-/-}$ mice into three groups according to the number of Ly5.2$^+$CD4$^+$CD45RB^{high} T cells transferred at the first transfer (Fig. 2A): Group 1, 3×10^4 cells (named "10 ^ 4"), Group 2, 3×10^5 cells ("10 ^ 5"), and Group 3, 3×10^6 cells ("10 ^ 6"). All groups of mice were secondarily transferred with the same number (3×10^5) of Ly5.1$^+$CD4$^+$CD45RB^{high} 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 ^ 5") and Group 3 ("10 ^ 6") 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 ^ 4") 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

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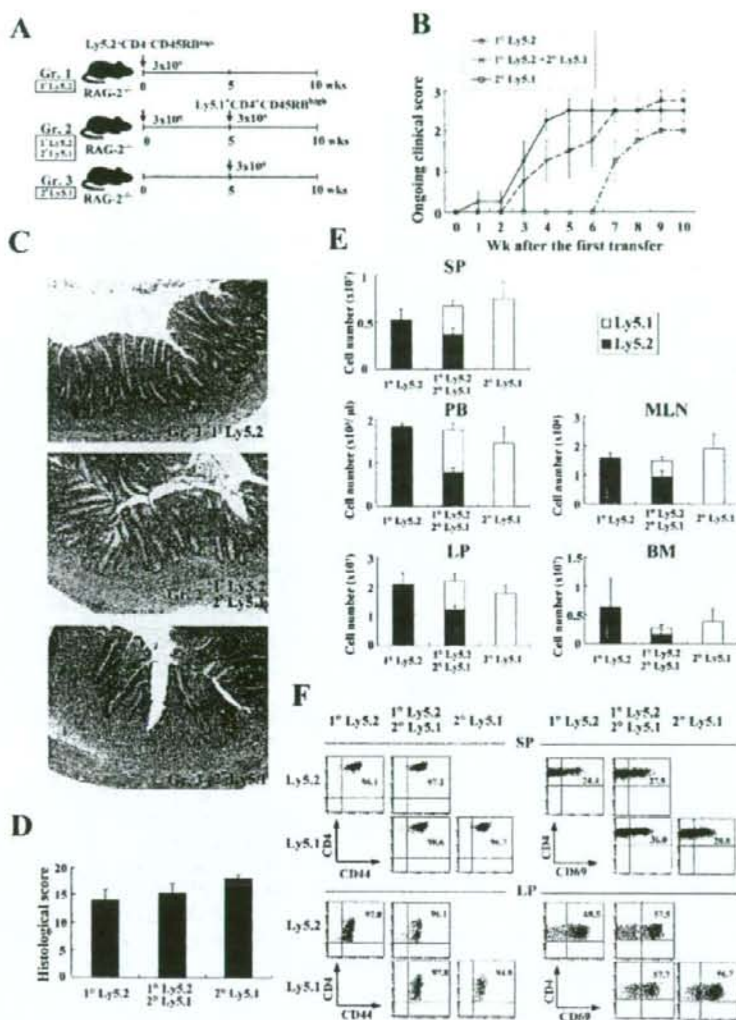

Figure 1. Newly recruited CD4+CD45RB^{high} T cells are primed and converted to CD4+CD44^{high} T cells. (A) Experimental design. C57BL/6-Ly5.2-RAG-2$^{-/-}$ mice were divided into three groups as described in the *Materials and methods*. (B) Ongoing clinical scores for the three groups were determined 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+CD4+ T cells from SP, PB, LP, MLN, and BM. (F) Phenotypic characterization of SP and LP CD4+ T cells after the transfer of CD4+CD45RB^{high} 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+ T cells in each tissue of established colitic mice is equivalent. Furthermore, the ratio of Ly5.2+ cells to Ly5.1+ 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 ("10^4") mice and greater than unity in Group 3 ("10^6"), whereas in Group 2 ("10^5") mice transferred with the same numbers of Ly5.2+ cells and Ly5.1+ 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+CD45RB^{high} T cells could be differentiated into effector or memory CD4+ T cells in this sequential transfer experiment. As shown in Fig. 3, almost all CD4+ T cells in any tissues, whether Ly5.1+ or Ly5.2+ cells, had

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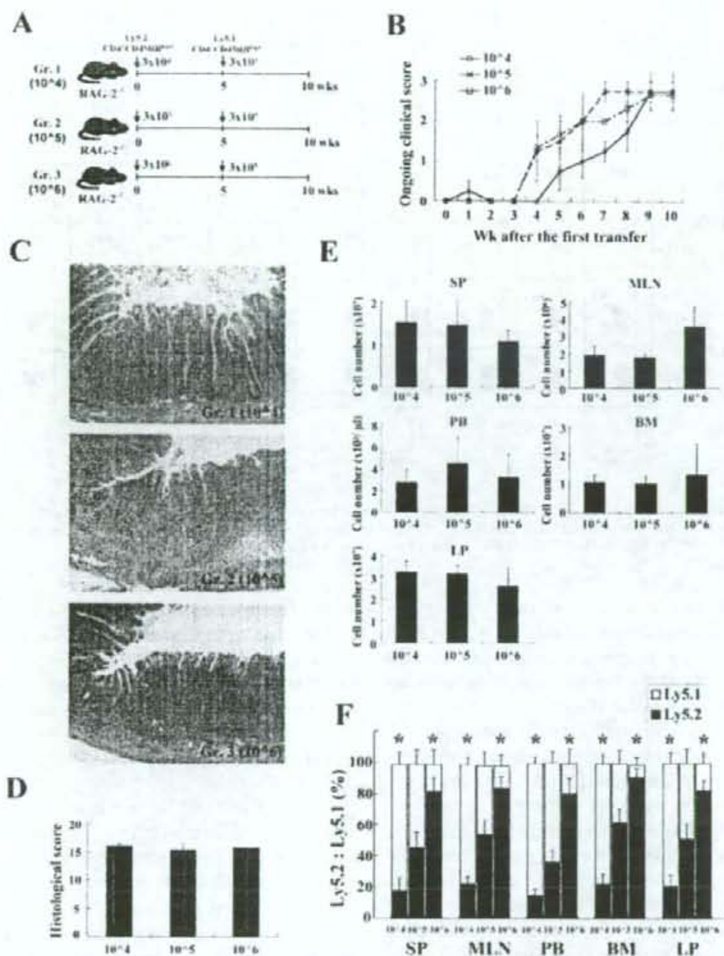

Figure 2. Newly generated CD4$^+$CD44^{high} T cells compete with pre-existing CD4$^+$CD44^{high} T cells depending on the transferred cell numbers. (A) Experimental design. C57BL/6-Ly5.2-RAG-2$^{-/-}$ mice were divided into three groups as described in the *Materials and methods*. (B) Ongoing clinical 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$^+$CD4$^+$ T cells from SP, PB, LP, MLN, and BM. Cell numbers of Ly5.1$^+$ or Ly5.2$^+$ CD3$^+$CD4$^+$ T cells were determined by four-color flow cytometry. (F) Phenotypic characterization of SP, MLN, PB, BM and LP CD4$^+$ T cells after the transfer of CD4$^+$CD45RB^{high} T cells. Results shown are from six mice per group. *$p<0.05$ *vs.* Ly5.2 ratio in same group.

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

TCR Vβ repertoire is constant between first and second transferred cells

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

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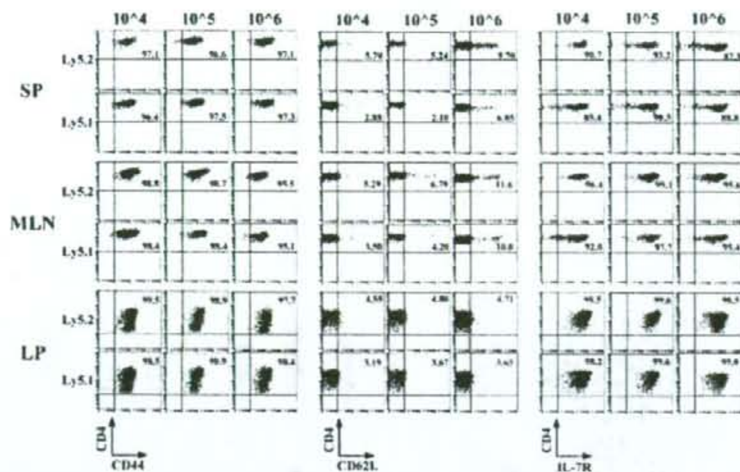

Figure 3. Phenotypic characterization of SP, MLN and LP CD4+ T cells after a sequential adoptive transfer as described in Fig. 2. Flow cytometric analysis shows that most of the transferred CD4+CD45RB^{high} T cells in Group 1 (10^4), Group 2 (10^5), and Group 3 (10^6) mice have the characteristics of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

the first transfer. To clarify this issue, splenic Ly5.1+ and Ly5.2+ CD4+ T cells from three groups were analyzed for their TCR Vβ repertoire by three-color flow cytometry. It is reasonable to use SP CD4+ T cells in place of LP CD4+ T cells for this assay, since we previously demonstrated that colitic SP CD4+ T cells had similar characteristics in that they were CD4+CD44^{high}CD62L−IL-7Rα^{high} T_{EM}-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 Vβ repertoire with dominant Vβ2, Vβ4, Vβ8.1/8.2, and Vβ14 was almost constant between previously and newly transferred cells, regardless of the different numbers of the first transfer, indicating that colitogenic CD4+ T cells recognizing the same or similar antigenic epitopes developed in accordance with the frequency or number of colitogenic antigen-specific naïve CD4+ T cells. In addition, the pattern of TCR Vβ repertoire in colitic mice, whether with older Ly5.2+ and newer Ly5.1+ CD4+ T cells, was similar to that of originally transferred CD4+CD45RB^{high} T cells (Fig. 4).

Previously generated, older CD4+ T cells produce less IFN-γ and IL-17

It has recently been demonstrated that, during chronic viral infection, the functions of virus-specific CD8+ T cells often become impaired and exhausted in the persistent presence of viral antigens, in contrast to the highly functional effector and memory CD8+ T cells generated after virus clearance in acute infection [11, 16]. Since colitogenic CD4+ T cells in colitic RAG-2$^{-/-}$ mice induced by an adoptive transfer of CD4+CD45RB^{high} 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+ T_{EM}-like cells gradually become exhausted over time after the transfer. To assess whether newly recruited T_{EM}-like cells that are differentiated from the secondarily transferred CD4+CD45RB^{high} T cells can compete and prevail against the previously established and possibly exhausted T_{EM}-like cells in colitic mice, we next divided RAG-2$^{-/-}$ mice into two groups (Fig. 5A): Group 1, RAG-2$^{-/-}$ mice transferred with Ly5.2+CD4+CD45RB^{high} T cells at 0 wk, and Group 2, RAG-2$^{-/-}$ mice transferred with Ly5.2+CD4+CD45RB^{high} T cells at 0 wk, and secondarily transferred with Ly5.1+CD4+CD45RB^{high} 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+ T cells in each colitic mouse is equivalent. As shown in Fig. 5F, almost all LP CD4+ T cells in any tissues, whether old Ly5.2+ cells or new Ly5.1+ cells, had a phenotype of CD4+CD44^{high}CD62L−IL-7Rα+ T_{EM}-like cells.

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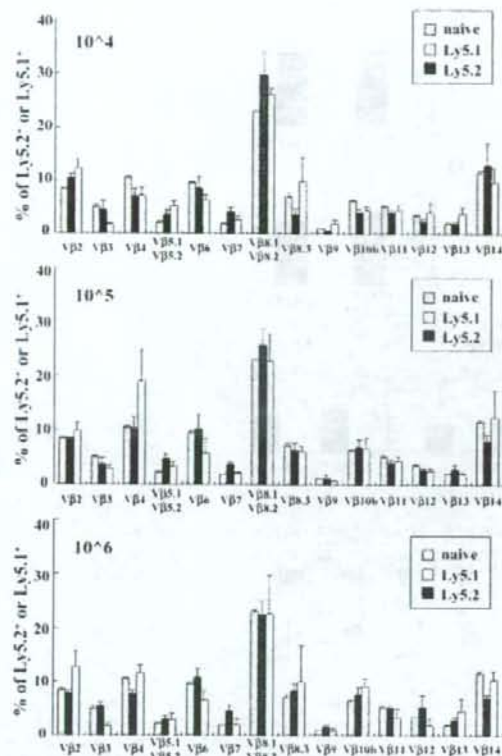

Figure 4. Flow cytometric analysis of Vβ families on the surface of the splenic $CD4^+$ T cells in the Group 1 (10^4), Group 2 (10^5), and Group 3 (10^6) mice described in Fig. 2. To analyze the TCR Vβ family repertoire, splenic cells were four-color-stained with PerCP-conjugated anti-CD3mAb, allophycocyanin-conjugated anti-CD4 mAb, PE-conjugate anti-Ly5.1 or Ly5.2 mAb, and the indicated mAb of a panel of 14 FITC-conjugated Vβ mAb. Each percentage value indicates the frequency of each Vβ ($n=6$). Naive, $CD4^+CD45RB^{high}$ T cells serves as a control.

Of note, we found that the old LP $Ly5.2^+$ cells produced significantly less IFN-γ (Th1) and IL-17 (Th17) cytokines than the new LP $Ly5.1^+$ cells (Fig. 6A and B), indicating that the old LP $Ly5.2^+$ cells long after the first transfer had fallen into exhaustion. In addition, the ratios of $IL\text{-}17^+$, but not $IFN\text{-}\gamma^+$, cells in the old MLN and SP $Ly5.2^+CD4^+$ cells were significantly decreased as compared with those of new $Ly5.1^+$ cells (Fig. 6A and B), suggesting that colitogenic $CD4^+$ 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-γ. Since it has been reported that PD-1 is one of markers for exhausted $CD8^+$ T cells in chronic LMCV infection in mice and HIV infection in human [17–19], we also checked this molecule in old $Ly5.2^+$ cells and new $Ly5.1^+$ cells. Contrary to the above data of cytokine production and previous reports of exhausted PD-1-expressing $CD8^+$ T cells [17–19], no difference in PD-1 expression was found between old $Ly5.2^+$ cells and new $Ly5.1^+$ cells at 17 wk (Fig. 5F), indicating that PD-1 is not an appropriate marker for the exhaustion for murine $CD4^+$ 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 $CD4^+CD45RB^{high}$ T cells can be primed, expand and differentiate into $CD4^+CD44^{high}CD62L^-IL\text{-}7R\alpha^{high}$ T_{EM}-like cells in colitic RAG-$2^{-/-}$ mice induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells in competition with previously established colitogenic T_{EM}-like $CD4^+$ T cells. Of note, the patterns of TCR Vβ repertoire were constantly similar between the first and the second transfers, indicating that a set of colitogenic polyclonal $CD4^+$ 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 $CD4^+$ T_{EM}-like cells produced less IFN-γ and IL-17 than newly developed LP $CD4^+$ T_{EM}-like cells, suggesting that old colitic LP $CD4^+$ T cells showed immunological exhaustion over time. These findings suggest that continuous generation of colitogenic $CD4^+$ T cells from newly recruited naive $CD4^+$ T cells to compensate for the older exhausted $CD4^+$ T cells is one of the mechanisms sustaining chronic colitis.

Although IBD is thought to be caused by colitogenic effector $CD4^+$ T cells, which probably respond to intestinal bacterial antigens and damage the target intestine, the nature of the colitogenic $CD4^+$ T cells over time remains largely unclear. How are these colitogenic $CD4^+$ T cells generated and maintained in the body of patients with IBD? Are these colitogenic $CD4^+$ T cells just effector $CD4^+$ T cells, which are generated from naive $CD4^+$ T cells or from colitogenic memory $CD4^+$ 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 $CD8^+$ T cells in animal models of acute viral infection and vaccination [22–25]. According to this scenario, it seems that colitogenic 'true' memory $CD4^+$ 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 $CD8^+$ and $CD4^+$ 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 $CD4^+$ T cells of this transfer model strongly expressed CD44 and IL-7Rα, which is a reliable marker for memory, but not effector, $CD4^+$ T cells; and also we recently demonstrated that IL-7, which is an important factor for survival of memory $CD4^+$ T cells [21], is essential for the persistence of colitis by showing that $IL\text{-}7^{-/-}$ × $RAG\text{-}1^{-/-}$ mice transferred with colitogenic LP

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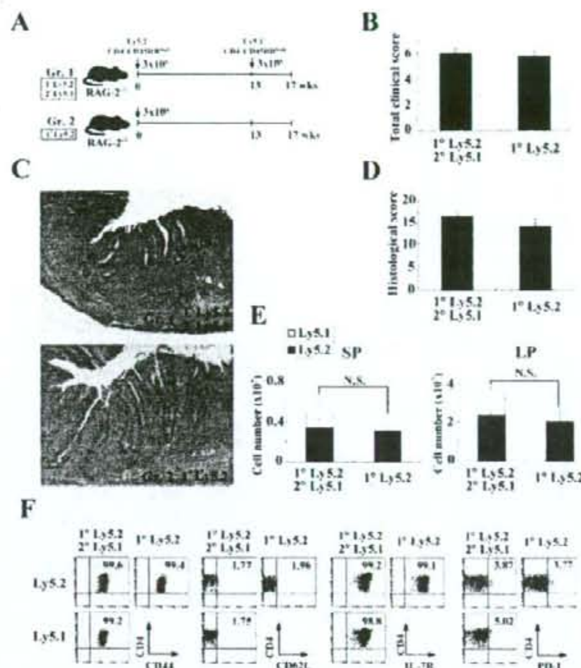

Figure 5. Pre-existing CD4⁺CD44^{high} T cells are gradually exhausted. (A) Experimental design. C57BL/6-Ly5.2-RAG-2$^{-/-}$ mice were divided into two groups: Group 1, RAG-2$^{-/-}$ mice transferred with Ly5.2-derived CD4$^+$CD45RB^{high} T cells at 0 wk (n=6), and Group 2, RAG-2$^{-/-}$ mice transferred with Ly5.2-derived CD4$^+$CD45RB^{high} T cells at 0 wk, and again transferred with Ly5.1-derived CD4$^+$CD45RB^{high} 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$^+$CD4$^+$ T cells from SP and LP. The cell number of Ly5.1$^+$ or Ly5.2$^+$ CD3$^+$CD4$^+$ 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$^+$ T cells after the transfer of CD4$^+$CD45RB^{high} T cells. Representative results shown are from one mice per group (six mice per group were analysed).

CD4$^+$CD44^{high}IL-7Rα^{high} cells did not develop colitis [28]. Since the survival of memory, but not effector, CD4$^+$ T cells is believed to be dependent on IL-7 [21], our results suggest that colitogenic CD4$^+$CD44^{high}CD62L$^-$IL-7Rα^{high} T_{EM} 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$^+$CD44^{high}CD62L$^-$IL-7Rα^{high} T_{EM}-like cells reside in colitic BM, which is believed to lack intestinal bacterial antigens but produce IL-7 [28].

Naïve CD4$^+$ T cells are known to proliferate extensively, probably in response to foreign or self antigens, and are converted to memory-like CD4$^+$CD44^{high} T cells in lymphopenic immunodeficient mice, such as SCID and RAG-2$^{-/-}$ mice, in the process of 'lymphopenia-driven proliferation' [29]. Thus, it is likely that CD4$^+$CD44^{high}IL-7Rα^{high} cells in CD4$^+$CD45RB^{high} 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$^+$ 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$^+$ and CD4$^+$ T cells defined mainly by studies of models of acute viral infection. Further studies will be needed to address this issue.

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

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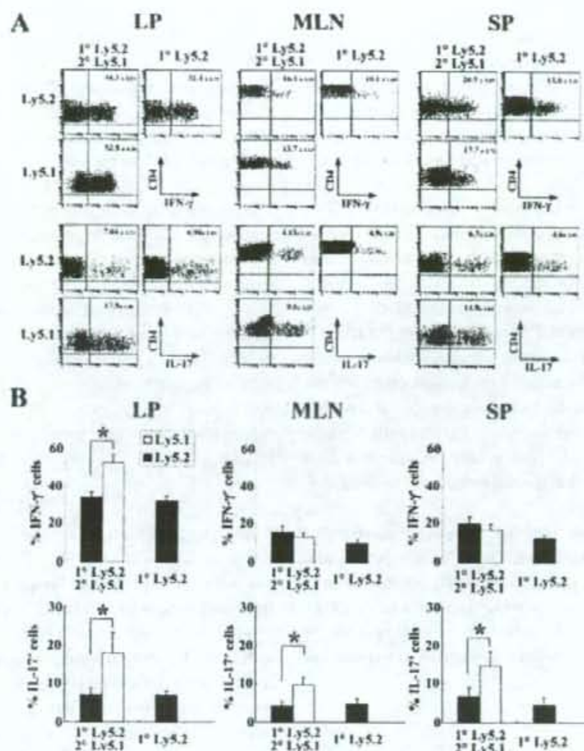

Figure 6. Preexisting $CD4^+CD44^{high}$ T cells are gradually exhausted. (A) Expression of IFN-γ and IL-17 on freshly isolated cells from LP, MLN, and SP in the Group 1 and Group 2 mice described in Fig. 5. Cells were labeled for Ly5.1, Ly5.2, CD4, and intracellular IFN-γ or IL-17. $Ly5.2^+$ and $Ly5.1^+$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

demonstrated that pre-existing 'true' memory $CD4^+$ T cells in RAG-$2^{-/-}$ mice after adoptive transfer prevent the proliferation and conversion of newly transferred naïve $CD4^+$ T cells to the memory-like phenotype [30]. Furthermore, the same group previously demonstrated that $RAG^{-/-}$ mice transferred with a large number of naïve $CD4^+CD45RB^{high}$ T cells (12×10^6 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 naïve T cells in the lymphopenic condition. Nevertheless, the fact that newly recruited naïve $CD4^+CD45RB^{high}$ 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 $CD4^+$ T cells, may suggest the older colitogenic effector or memory $CD4^+$ T cells are exhausted and permit the expansion of newly recruited colitogenic $CD4^+$ T cells in our model. By contrast, surprisingly, we found that newly recruited $CD4^+CD45RB^{high}$ T cells can be primed and expand extensively even in established colitic mice in which pre-existing colitogenic $CD4^+$ T cells were fully expanded and occupied the space of $CD4^+$ T cells. Thus, our present results may link the frequent recurrence in natural history of IBD to the continuous generation of colitogenic $CD4^+$ effector and memory T cells, because the frequency of recurrence gradually decreases in accordance with immunosenescence in patients with a decreased supply of naïve T cells by thymic involution. Interestingly, in addition, the total cell number of $CD4^+$ T cells was constant, and the ratio of pre-existing and newly recruited $CD4^+CD44^{high}$ T cells in the body was dependent on the ratio of the transferred cell numbers, indicating that both old and new $CD4^+$ T cells compete for space and a possibly constant amount of IL-7 depending on the frequency of transferred colitogenic naïve $CD4^+$ T cells.

Of note, we also demonstrated that production of IFN-γ and IL-17 by older LP $CD4^+CD44^{high}$ T cells was significantly less as compared with newly developed LP $CD4^+CD44^{high}$ T cells (Fig. 5), indicating that these LP $CD4^+CD44^{high}IL\text{-}7R\alpha^{high}$ cells become

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exhausted over time, as seen in CD8$^+$ T cells in persistent virus infection [17, 18]. Thus, it appears that colitogenic LP CD4$^+$ T cells in colitic mice are a mixture of IL-7-dependent CD4$^+$ T_{EM}-like cells and exhaustion-facing effector cells derived from these CD4$^+$ T_{EM}-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$^+$CD45RB^{high} 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$^+$ cells obtained from colitic CD4$^+$CD45RB^{high} cell-transferred SCID mice into new SCID mice. Although SCID mice transferred with colitic LP CD4$^+$ 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$^+$ 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$^+$ T cells from naïve CD4$^+$ 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$^+$ T cells but also to suppress and control the new generation of colitogenic CD4$^+$ 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$^{-/-}$) 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-7Rα (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^{5}-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-γ; TC11–18H10, PE-conjugated anti-mouse IL-17; B20.6, FITC-conjugated anti-mouse Vβ2; KJ25, FITC-conjugated anti-mouse Vβ3; KT4, FITC-conjugated anti-mouse Vβ4; MR9–4, FITC-conjugated anti-mouse Vβ5.1/2; RR4–7, FITC-conjugated anti-mouse Vβ6; TR310, FITC-conjugated anti-mouse Vβ7; MR5–2, FITC-conjugated anti-mouse Vβ8.1/2; B21.14, FITC-conjugated anti-mouse Vβ8.3; MR10–2, FITC-conjugated anti-mouse Vβ9; B21.5, FITC-conjugated anti-mouse Vβ10^b; RR3–15, FITC-conjugated anti-mouse Vβ11; MR11–1, FITC-conjugated anti-mouse Vβ12; IN12.3, FITC-conjugated anti-mouse Vβ13; 14.2, FITC-conjugated anti-mouse Vβ14. Biotinylated antibodies were detected with PE-streptavidin (BD PharMingen).

T cell preparation

For isolation of peripheral lymphocytes, 600 μL PB was collected from each mouse and diluted 1:1 with PBS. The diluted blood was layered over Lymphosepar II (IBL, Gunma, Japan) and centrifuged at 400 × g for 30 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^{2+} Mg^{2+}-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$^+$ T cells were obtained by positive selection using anti-CD4 (L3T4) MACS magnetic beads. The resultant cells contained >94% CD4$^+$ cells when analyzed by FACSCalibur.

Adoptive transfer protocols

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

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Group 3, RAG-2$^{-/-}$ mice transferred with 3×10^5 Ly5.1-derived CD4$^+$CD45RB^{high} T cells at 5 wk after the starting point ($n=6$). 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 anti-mouse CD4 mAb and FITC-conjugated anti-CD45RB mAb, then sorted to yield the CD45RB^{high} (highest staining 30%) fraction on a FACS Aria (Becton Dickinson, Sunnyvale, CA). Each mouse was injected intraperitoneally with 3×10^5 CD4$^+$CD45RB^{high} 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$^{-/-}$ mice into three groups: Group 1, RAG-2$^{-/-}$ mice transferred with 3×10^4 Ly5.2$^+$CD4$^+$CD45RB^{high} T cells at 0 wk ("10 ^ 4", $n=6$); Group 2, RAG-2$^{-/-}$ mice transferred with 3×10^5 Ly5.2$^+$CD4$^+$CD45RB^{high} T cells at 0 wk ("10 ^ 5", $n=6$); and Group 3, RAG-2$^{-/-}$ mice transferred with 3×10^6 Ly5.2$^+$CD4$^+$CD45RB^{high} T cells at 0 wk ("10 ^ 6", $n=6$). All groups of mice were transferred with 3×10^5 Ly5.1$^+$CD4$^+$CD45RB^{high} 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$^{-/-}$ mice into two groups: Group 1, RAG-2$^{-/-}$ mice transferred with 3×10^5 Ly5.2$^+$CD4$^+$CD45RB^{high} T cells at 0 wk of the starting point ($n=6$); and Group 2, RAG-2$^{-/-}$ mice transferred with 3×10^5 Ly5.2$^+$CD4$^+$CD45RB^{high} T cells at 0 wk and with 3×10^5 Ly5.1$^+$CD4$^+$CD45RB^{high} T cells at 13 wk after the first transfer ($n=6$). Mice were skilled 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 μm) 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 FcγR-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-γ 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 μg/mL brefeldin A (GolgiPlug; BD PharMingen) was added. Cells were first preincubated with FcγR-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 Cytofix/Cytoperm™ (BD PharMingen) at 4°C for 30 min. Staining and washing were performed in Perm/Wash Buffer™ (BD PharMingen), and cells were stained with PE-conjugated anti-IFN-γ or anti-IL-17 mAb.

Statistical analysis

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

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Abbreviations: **IBD:** inflammatory bowel disease · **LP:** lamina propria · **MLN:** mesenteric LN · **PB:** peripheral blood · **SP:** spleen · **T_{CM}:** central-memory T · **T_{EM}:** effector-memory T

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