Figure 1. RORγt$^+$ Th1-like cells and RORγt$^-$ Th1 cells co-reside in inflamed mucosa of colitis. (*A*) Transfer protocol A. RAG-2$^{-/-}$ mice were transferred with RORγt$^{gfp/+}$ CD4$^+$CD25$^-$ T cells (Gr.2^A, n = 6). Age-matched RORγt$^{gfp/+}$ mice were used as a negative control (Gr.1^A, n = 6). Mice were sacrificed 6 weeks after transfer. (*B*) Expression of IL-17A and IFN-γ in colonic CD3$^+$CD4$^+$ T cells. (*C*) Mean percentages of Th17, Th17/Th1, and Th1 cells (including Th1-like cells) in colonic LP CD3$^+$CD4$^+$ T cells. (*D*) Expression of IL-17A and IFN-γ in colonic GFP$^+$ and GFP$^-$ CD3$^+$CD4$^+$ T cells of Gr.2^A mice. (*E*) Mean percentages of colonic Th17, Th17/Th1, and Th1 cells in the GFP$^+$ and GFP$^-$ CD3$^+$CD4$^+$ T cells of Gr.2^A mice. (*F*) Expression of the indicated mRNA in the colonic CD3$^+$CD4$^+$ cells, normalized to Act-b expression. Statistical data (*C, E, F*) show mean ± standard error of mean (n = 6/group). *P < .05. NS, not significant.

cent protein (GFP) reporter complementary DNA knocked-in at the site for initiation of RORγt translation on the C57BL/6 (Ly5.2) background were described previously.[15] Mice were maintained under specific pathogen-free conditions. All experiments were approved by the regional animal study committees. (See Supplementary Materials and Methods for full details).

Results

RORγt$^+$ Th1-Like and RORγt$^-$ Th1 Cells Co-Reside in the Inflamed Colitis Mucosa

We first assessed the presence of Th17 and Th1 cells in the inflammatory conditions of colitis following adoptive transfer of CD4$^+$CD25$^-$ T cells obtained from RORγt$^{gfp/+}$ mice into RAG-2$^{-/-}$ mice (Group 2 in protocol A [Gr.2^A]) (Figure 1*A*). We confirmed that the transferred splenic CD4$^+$CD25$^-$ T cells did not express GFP (data not shown). As expected, Gr.2^A mice developed severe colitis as assessed by weight loss (Supplementary Figure 1*A*), gross colon appearance (Supplementary Figure 1*B*), clinical score (Supplementary Figure 1*C*), histology (Supplementary Figure 1*D*), and the absolute number of colonic lamina propria (LP) CD4$^+$ T cells (Supplementary Figure 1*E*), in contrast to age-matched RORγt$^{gfp/+}$ mice (Gr.1^A). The percentages of Th1 (IL-17A$^-$IFN-γ$^+$) and Th17/Th1

(IL-17A$^+$IFN-γ^+) cells in Gr.2^A mice were significantly higher than those in Gr.1^A, while the percentage of Th17 (IL-17A$^+$IFN-γ^-) cells in Gr.2^A mice was lower than that in Gr.1^A mice (Figure 1B and C). Notably, significant numbers of Th17/Th1 cells were only found in colitic Gr.2^A mice, but not in RORγt$^{gfp/+}$ Gr.1^A mice (Figure 1B and C). To further investigate the distinct differences between LP RORγt$^+$ and RORγt$^-$ CD3$^+$CD4$^+$ T cells in Gr.2^A mice with colitis, GFP$^+$ and GFP$^-$ CD3$^+$CD4$^+$ cells were sorted (Figure 1D), and approximately 10% of LP CD3$^+$CD4$^+$ T cells in colitic Gr.2^A mice were found to be GFP-positive. As expected, almost no LP GFP$^-$CD3$^+$ CD4$^+$ cells in Gr.2^A mice expressed IL-17A, while half of them expressed IFN-γ. Surprisingly, however, almost half of the GFP$^+$CD3$^+$CD4$^+$ T cells expressed IFN-γ without retaining IL-17A expression, while a small but substantial percentage of those cells expressed IL-17A as Th17 (IL-17A$^+$IFN-γ^-) and Th17/Th1 (IL-17A$^+$IFN-γ^+) cells (Figure 1D). Although the percentages of Th17 and Th17/Th1 cells in GFP$^+$CD3$^+$CD4$^+$ T cells were small, they were significantly higher than those in GFP$^-$CD3$^+$CD4$^+$ T cells, while the percentage of dominant Th1 cells in GFP$^+$CD3$^+$CD4$^+$ T cells was comparable to that in GFP$^-$CD3$^+$CD4$^+$ T cells (Figure 1E). Control GFP$^+$ CD3$^+$CD4$^+$ T cells obtained from the colon and small intestine of normal RORγt$^{gfp/+}$ Gr.1A mice did not express IFN-γ, while GFP$^-$ CD3$^+$CD4$^+$ T cells obtained from the colon and small intestine of Gr.1A mice express significant levels of IFN-γ (Supplementary Figure 1F). Additionally, in vitro–stimulated (transforming growth factor [TGF]–β + IL-6) GFP$^+$ Th17 cells expressed IL-17A, but not IFN-γ (Supplementary Figure 1G).

To further characterize GFP$^+$ and GFP$^-$ CD3$^+$CD4$^+$ T cells obtained from colitic Gr.2^A mice, we compared Th1 and Th17 marker messenger RNA (mRNA) expression. As expected, the expression levels of Th17 markers, such as Rorc, IL-17A, and IL-17F, in GFP$^+$CD3$^+$CD4$^+$ T cells in Gr.2^A mice were significantly higher than in GFP$^-$CD3$^+$CD4$^+$ T cells in Gr.2^A mice or CD3$^+$CD4$^+$ T cells in Gr.1^A mice, while expression of IL-22 and IL-21 in GFP$^+$CD3$^+$CD4$^+$ T cells tended to be reduced and increased when compared to that in GFP$^-$CD3$^+$CD4$^+$ T cells, respectively, although not significantly (Figure 1F). This suggests that IL-22 and IL-21 are not solely produced by Th17 cells, but possibly also by Th22, Th1, and T$_{FH}$ cells, and not by NK-22 (CD3$^-$) cells. Surprisingly, however, GFP$^+$ T cells also expressed Th1 markers, such as Tbx21 (gene for T-bet)[16] and IFN-γ to a comparable level as GFP$^-$ cells (Figure 1F), suggesting that a portion of RORγt$^+$ CD3$^+$CD4$^+$ T cells are able to express T-bet, and produce IFN-γ but not IL-17A.
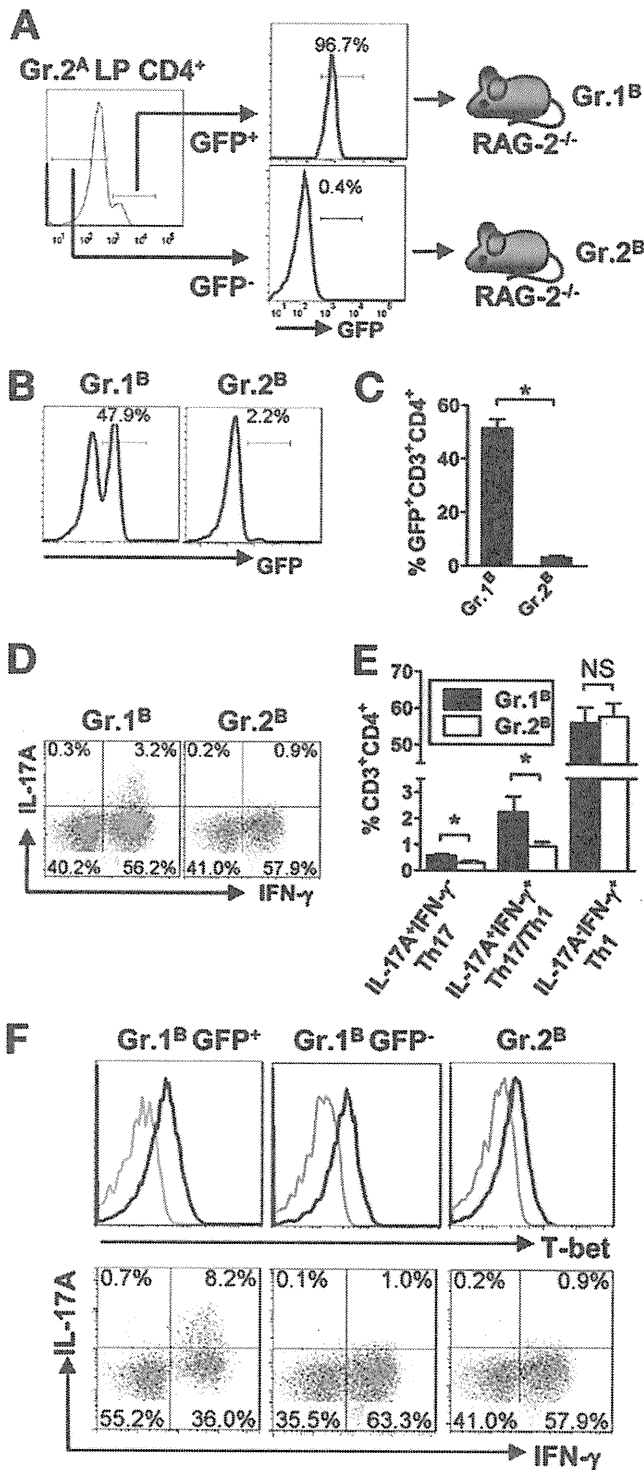
RORγt$^+$T-bet$^+$ Th1-Like Cells and RORγt$^-$T-bet$^+$ Th1 Cells in Colitic Mice Are Colitogenic

Given the possibility that there are at least 2 types of IFN-γ–expressing cells, Th1 (RORγt$^-$) and Th1-like (RORγt$^+$), in the inflamed mucosa of colitic mice, we asked whether each population is colitogenic after re-transfer into RAG-2$^{-/-}$ mice. To this end, new RAG-2$^{-/-}$ mice were re-transferred with LP GFP$^+$ (Gr.1^B) or GFP$^-$ (Gr.2^B) CD3$^+$CD4$^+$ T cells isolated from colitic RAG-2$^{-/-}$ mice previously transferred with CD4$^+$CD25$^-$ T cells obtained from RORγt$^{gfp/+}$ mice (Gr.2^A mice [Figure 1A]) (Figure 2A). Both groups of mice similarly developed colitis as assessed by weight loss (Supplementary Figure 2A), colon appearance (Supplementary Figure 2B), clinical score (Supplementary Figure 2C), histological score (Supplementary Figure 2D), and absolute number of infiltrating LP CD3$^+$CD4$^+$ T cells (Supplementary Figure 2E). Although GFP expression was retained in approximately half of the LP CD3$^+$CD4$^+$ T cells in Gr.1^B mice, but not induced in those cells in Gr.2^B mice (Figure 2B and C), the ratio of IL-17A$^-$IFN-γ^+ Th1 cells was comparable in the 2 groups (Figure 2D and E). In contrast, the ratio of Th17 and Th17/Th1 cells in Gr.1^B mice was significantly higher than that in Gr.2^B mice (Figure 2D and E). Notably, almost all GFP$^+$ CD3$^+$CD4$^+$ T cells in Gr.1^B mice expressed T-bet, because the histogram showed 1 peak shift compared to the isotype control (Figure 2F, upper). Therefore, these RORγt$^+$T-bet$^+$ CD3$^+$CD4$^+$ T cells in Gr.1^B mice were composed of 3 subpopulations; IL-17A$^+$IFN-γ^+ Th17/Th1 cells, IL-17A$^-$IFN-γ^+ Th1-like cells, and IL-17A$^-$IFN-γ^- double-negative cells (Figure 2F, lower), while almost all GFP$^-$ CD3$^+$CD4$^+$ T cells in Gr.1^B mice expressed T-bet, and these RORγt$^-$T-bet$^+$ CD3$^+$CD4$^+$ T cells were composed of 2 subpopulations; Th1 cells and double-negative cells (Figure 2F). Like GFP$^-$ CD3$^+$CD4$^+$ T cells in Gr.1^B mice, almost all GFP$^-$ CD3$^+$CD4$^+$ T cells in Gr.2^B mice expressed T-bet, and were composed of 2 subpopulations; Th1 cells and double-negative cells (Figure 2F). As we could not detect GFP expression in cells in Gr.2^B mice during this experiment, these cells included classical Th1 cells and "alternative Th1 cells" via RORγt$^+$ CD3$^+$CD4$^+$ T cells.

T$_R$ Cells Not Only Suppress Development of Colitis, But Also Induce Accumulation of Th17 and Th17/Th1 Cells

We next investigated whether T$_R$ cells affect the developmental pathway of Th17, Th17/Th1, Th1-like, and Th1 cells (Figure 3A). We confirmed that RAG-2$^{-/-}$ mice transferred with CD4$^+$CD45RB^{high} T cells (Ly5.1$^+$) and CD4$^+$CD25$^+$ T$_R$ cells (Ly5.2$^+$) (Gr.2^C) did not develop colitis, as assessed by weight loss (Supplementary Figure 3A), colon appearance (Supplementary Figure 3B), clinical score (Supplementary Figure 3C), histological appearance and its score (Supplementary Figure 3D), and the absolute number of LP CD3$^+$CD4$^+$ T cells (Supplementary Figure 3E), in contrast to mice transferred with CD4$^+$CD45RB^{high} T cells alone (Gr.1^C), which developed colitis. Surprisingly, however, the percentages of not only Th17 cells, but also Th17/Th1 cells derived from the Ly5.1$^+$CD4$^+$CD45RB^{high} T-cell population in Gr.2^C mice were significantly higher than those in Gr.1^C mice, while the percentage of Th1 cells in Gr.2^C mice was significantly

Figure 2. RORγt$^+$T-bet$^+$ Th1-like cells and RORγt$^-$T-bet$^+$ Th1 cells in colitic mice are colitogenic. (A) Transfer protocol B. RAG-2$^{-/-}$ mice were transferred with GFP$^+$ (Gr.1^B, n = 6) or GFP$^-$ (Gr.2^B, n = 6) CD3$^+$CD4$^+$ T cells obtained from colitic Gr.2^A mice in transfer protocol A (see Figure 1A). Mice were sacrificed 10 weeks after transfer. (B) Expression of GFP in colonic CD3$^+$CD4$^+$ T cells. (C) Percentage of GFP$^+$ cells in colonic CD3$^+$CD4$^+$ T cells. (D) Expression of IL-17A and IFN-γ in colonic CD3$^+$CD4$^+$ T cells. Data are representative of six mice in each group. (E) Mean percentages of Th17, Th17/Th1, and Th1 cells (including Th1-like cells) in the colonic CD3$^+$CD4$^+$ T cells. (F) Expression of T-bet and IL-17A/IFN-γ in GFP$^+$ and GFP$^-$ CD3$^+$CD4$^+$ T cells (T-bet, black; Isotype, gray). Statistical data (C, E) show mean ± standard error of mean (n = 6/group). *P < .05. NS, not significant.

lower than that in Gr.1^C mice (Figure 3B-a and b). We further assessed the expression of tumor necrosis factor (TNF)−α and cytotoxic T lymphocyte−associated antigen (CTLA-4) molecules, because TNF-α is one of the representative pathological molecules for the pathogenesis of IBD,[17] and CTLA-4 is a member of the inhibitory CD28 family, which are preferentially expressed in cells that have regulatory function, such as T$_R$ cells.[18] Like expression pattern of IFN-γ, the percentages of Ly5.1-derived IL-17A$^+$TNF-α^- and IL-17A$^+$TNF-α^+ cells in noncolitic Gr.2^C mice were significantly higher than those in Gr.1^C mice, while the percentage of IL-17A$^-$TNF-α^+ cells in noncolitic Gr.2^C mice was significantly lower than that in Gr.1^C mice (Figure 3C-a and b). In contrast, the percentages of all 3 IL-17A$^+$CTLA-4$^-$, IL-17A$^+$CTLA-4$^+$, and IL-17A$^-$CTLA-4$^+$ subpopulations in noncolitic Gr.2^C mice were significantly higher than those in Gr.1^C mice (Figure 3D-a and b). Real-time quantitative polymerase chain reaction analysis confirmed that expression levels of Rorc, IL-17A, IL-17F, and IL-22 in Ly5.1$^+$CD4$^+$CD45RB^{high} T-cell−derived CD3$^+$CD4$^+$ T cells in Gr.2^C mice were significantly higher than those in Gr.1^C mice, whereas those of Tbx21 and IFN-γ in Ly5.1$^+$CD3$^+$CD4$^+$ T cells in Gr.2^C mice were significantly lower than those cells in Gr.1^C mice (Figure 3E). In contrast, Foxp3 mRNA was solely expressed in Ly5.2-derived T$_R$ cells in Gr.2^C mice, but not in Ly5.1$^+$CD3$^+$CD4$^+$ T cells (Figure 3E).

T$_R$ Cells Suppress Development of Colitis With the Increase of RORγt$^+$ Th17 and Th17/Th1 Cells

To precisely determine whether T$_R$ cells suppress the differentiation pathway of Th17, Th17/Th1, Th1-like, and Th1 cells in mice in which the development of colitis is prevented, we used CD4$^+$CD45RB^{high} T cells obtained from RORγt$^{gfp/+}$ mice. To this end, RAG-2$^{-/-}$ mice were transferred with RORγt$^{gfp/+}$ CD4$^+$CD45RB^{high} T cells (Ly5.2$^+$) alone (Gr.1^D) or RORγt$^{gfp/+}$ CD4$^+$CD45RB^{high} T cells (Ly5.2$^+$) plus CD4$^+$CD25$^+$ T$_R$ cells (Ly5.1$^+$) (Gr.2^D) (Figure 4A). As expected, Gr.1^D mice developed colitis, whereas Gr.2^D mice did not, as assessed by weight loss (Supplementary Figure 4A), clinical score (data not shown), histological appearance (data not shown), and absolute number of LP CD3$^+$CD4$^+$ T cells (Supplementary Figure 4B), in contrast to mice transferred with RORγt$^{gfp/+}$ CD4$^+$CD45RB^{high} T cells alone (Gr.1^D), which developed colitis. Similarly to the previous experiments (Figure 3), the percentages of Th17 and Th17/Th1 cells in Gr.2^D mice were significantly higher than those in Gr.1^D mice, while the ratio of Th1 cells, including Th1-like cells, in Gr.2^D mice was significantly lower than in Gr.1^D mice (Supplementary Figure 4C-a and b). The percentages of IL-17A$^+$CTLA-4$^-$, IL-17A$^+$CTLA-4$^+$, and IL-17A$^-$CTLA-4$^+$ subpopulations in noncolitic Gr.2^D mice were significantly higher than those in Gr.1^D mice (Supplementary Figure 4D-a and b). The percentage of GFP$^+$ Ly5.2$^+$CD3$^+$CD4$^+$ T cells in Gr.2^D mice was markedly up-regulated (Figure 4B), and was statistically higher
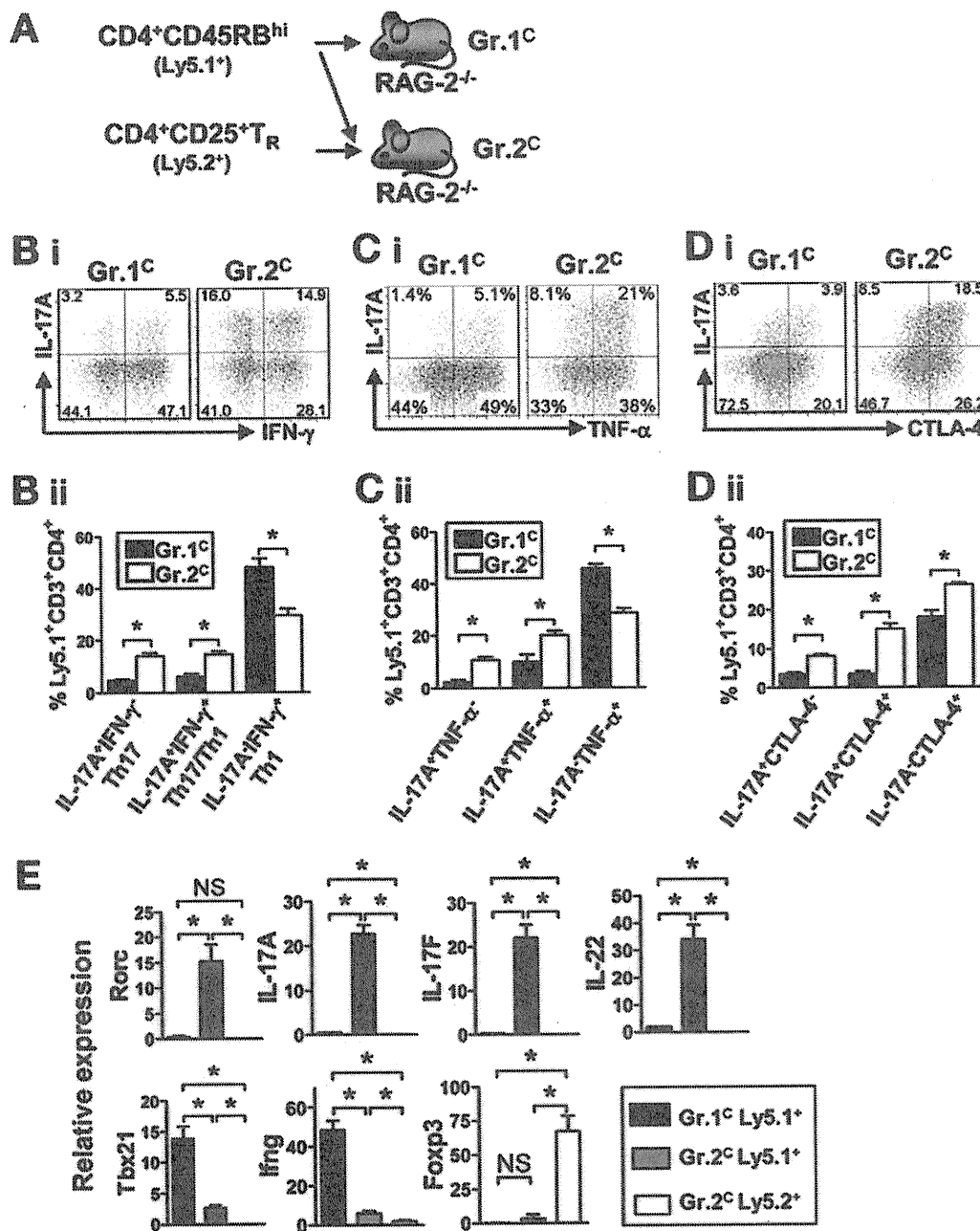
Figure 3. T$_R$ cells not only suppress the development of colitis but also induce the accumulation of Th17 and Th17/Th1 cells in noncolitic mice. (A) Transfer protocol C. RAG-2$^{-/-}$ mice were transferred with Ly5.1$^+$CD4$^+$CD45RB^{high} T cells alone (Gr.1^C, n = 6) or Ly5.1$^+$CD4$^+$CD45RB^{high} T cells plus Ly5.2$^+$CD4$^+$CD25$^+$ T$_R$ cells (Gr.2^C, n = 6). Mice were sacrificed 7 weeks after transfer. (B-i) Expression of IL-17A and IFN-γ in colonic Ly5.1$^+$CD3$^+$CD4$^+$ T cells. Data are representative of 6 mice in each group. (B-ii) Mean percentages of Th17, Th17/Th1, and Th1 cells (including Th1-like cells) in colonic CD3$^+$CD4$^+$ T cells. (C-i) Expression of IL-17A and TNF-α in colonic Ly5.1$^+$CD3$^+$CD4$^+$ T cells. Data are representative of six mice in each group. (C-ii) Mean percentages of IL-17A$^+$TNF-α$^-$, IL-17A$^+$TNF-α$^+$, and IL-17A$^-$TNF-α$^+$ in colonic CD3$^+$CD4$^+$ T cells. (D-i) Expression of IL-17A and CTLA-4 in colonic Ly5.1$^+$CD3$^+$CD4$^+$ T cells. Data are representative of 6 mice in each group. (D-ii) Mean percentages of IL-17A$^+$CTLA-4$^-$, IL-17A$^+$CTLA-4$^+$, and IL-17A$^-$CTLA-4$^+$ in colonic CD3$^+$CD4$^+$ T cells. (E) Expression of the indicated mRNA in colonic Ly.5.1$^+$CD3$^+$CD4$^+$ T cells, normalized to Act-b expression. Statistical data (B-ii, C-ii, D-ii, E) show mean ± standard error of mean (n = 6/group). *P < .05. NS, not significant.

when compared to Gr.1^D mice (Figure 4C). However, the absolute number of GFP$^+$Ly5.2$^+$CD3$^+$CD4$^+$ T cells in Gr.2^D mice was comparable to that in Gr.1^D mice (Supplementary Figure 4F), because the absolute number of LP CD3$^+$CD4$^+$ T cells in Gr.1^D mice with colitis was significantly increased as compared to that in noncolitic Gr.2^D mice (Supplementary Figure 4B).

Real-time quantitative polymerase chain reaction analysis revealed that GFP$^-$ CD3$^+$CD4$^+$ T cells in colitic Gr.1^D mice dominantly express Tbx21, but not Rorc, while GFP$^+$ CD3$^+$CD4$^+$ T cells in those mice express not only Rorc, but also Tbx21 (Figure 4D), suggesting that RORγt$^+$T-bet$^+$ CD3$^+$CD4$^+$ T cells reside in the GFP$^+$CD3$^+$CD4$^+$ T cells of colitic mice. In noncolitic

Gr.2^D mice, GFP$^+$Ly5.2$^+$CD3$^+$CD4$^+$ T cells dominantly expressed Rorc, but not Tbx21, while GFP$^-$Ly5.2$^+$ CD3$^+$CD4$^+$ T cells did not express Rorc, but slightly expressed Tbx21 (Figure 4D). Importantly, T-bet protein was slightly, but significantly, detected in all LP

CD3$^+$CD4$^+$ T cells in both GFP$^+$ and GFP$^-$ CD3$^+$CD4$^+$ populations from colitic Gr.1^D mice, whereas its expression was blocked in LP GFP$^+$ and GFP$^-$ CD3$^+$CD4$^+$ populations from noncolitic Gr.2^D mice (Figure 4E). The histogram shows 1 peak shift compared to the isotype control, suggesting that all cells are T-bet−positive, but we cannot rule out a possibility that there may be cells within these populations that do not express T-bet. Interestingly, CTLA-4 expression in Th17, Th17/Th1, and Th1 cells obtained from the LP of noncolitic Gr.2^D mice was significantly higher than the corresponding cells obtained from the LP of colitic Gr.1^D mice (Figure 4F, Supplementary Figure 4G). Furthermore, CTLA-4 expression in Th17 and Th17/Th1 was significantly higher than the paired Th1 cells in both colitic Gr.1^D mice and noncolitic Gr.2^D mice (Figure 4F, Supplementary Figure 4G). In addition, we also found that a portion of T_R cells in Gr.2^D mice converted into IFN-γ−expressing T cells (Supplementary Figure 4E).
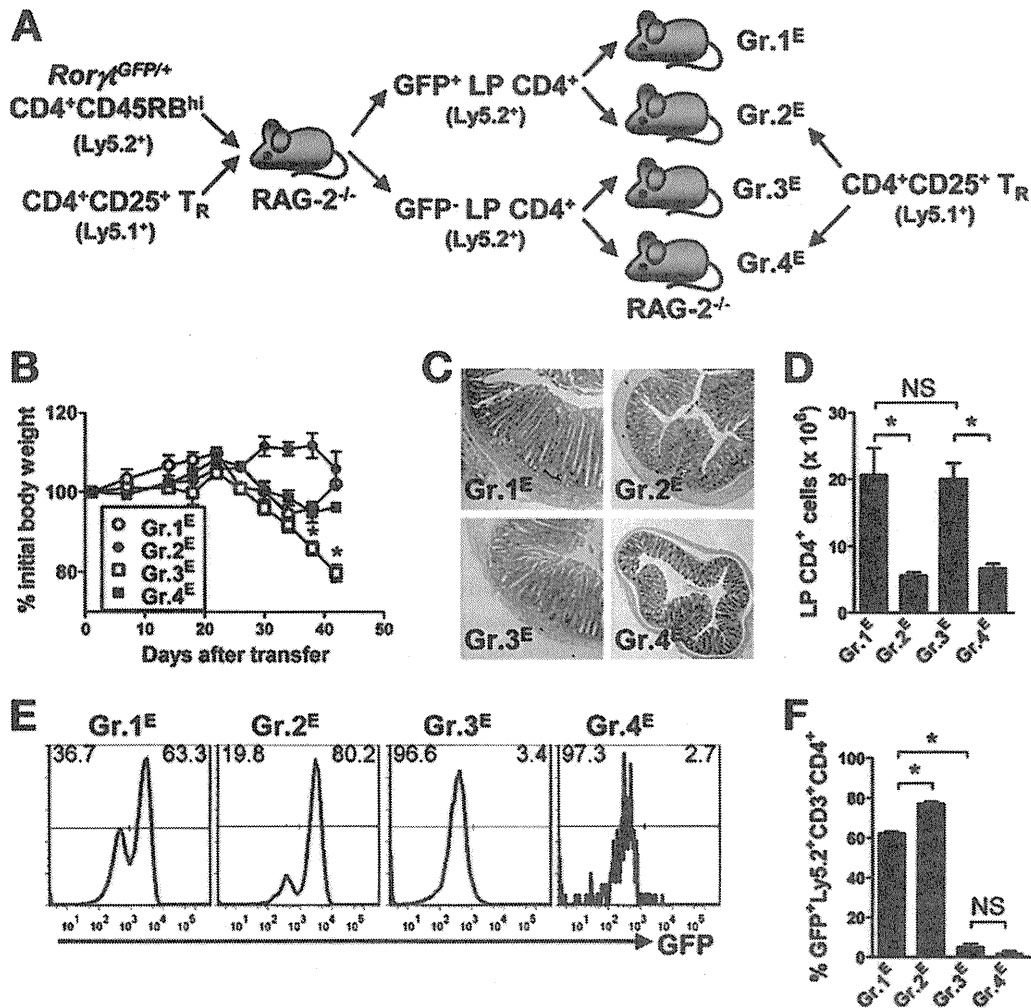
T_R Cells Suppress Development of Colitis Induced by Colitogenic RORγt$^+$ or RORγt$^-$ CD4$^+$ T Cells Resided in Noncolitic Mice

We next investigated whether RORγt$^{gfp/+}$ CD4$^+$ CD45RB^{high} T-cell−derived RORγt$^+$ (GFP$^+$) or RORγt$^-$ (GFP$^-$) CD4$^+$ T cells residing in noncolitic RAG-2$^{-/-}$ mice in which the development of colitis is prevented by cotransfer of CD4$^+$CD25$^+$ T_R cells (Ly5.1$^+$) with RORγt$^{gfp/+}$ CD4$^+$CD45RB^{high} T cells (Ly5.2$^+$) have the potential to induce colitis when they are separated from the T_R population and retransferred to new RAG-2$^{-/-}$ mice as depicted in Figure 5A. Although Gr.3^E mice, which were transferred with non-Th17 RORγt$^-$ (GFP$^-$) cells, showed marked weight loss, Gr.1^E mice, which were transferred with a mixture of Th17 cells and Th17/Th1 RORγt$^+$ (GFP$^+$) cells, did not, indicating that the weight loss in Gr. 3^E mice was significantly lower than in other groups (Figure 5B). This suggests that wasting disease is mediated by non-Th17 cells (possibly Th1 cells). Nevertheless, the colons of both Gr.1^E and Gr.3^E mice, but not Gr.2^E and Gr.4^E mice, showed similarly thickened walls (data not shown). Total clinical scores of Gr.1^E and Gr.3^E mice were comparably high and
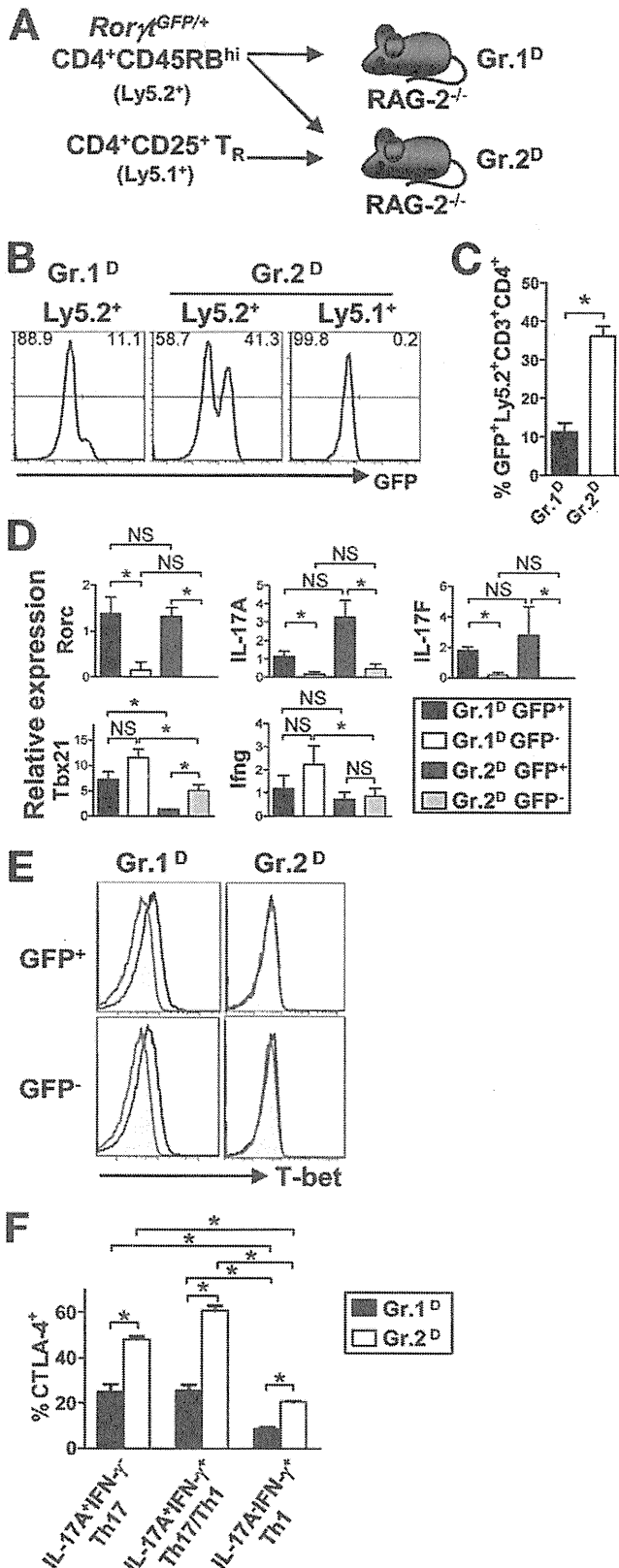
Figure 4. T_R cells suppress the development of colitis with the increase of RORγt$^+$ cells. (A) Transfer protocol D. RAG-2$^{-/-}$ mice were transferred with Ly5.2$^+$ RORγt$^{gfp/+}$ CD4$^+$CD45RB^{high} T cells alone (Gr.1^D, n = 6) or Ly5.2$^+$ RORγt$^{gfp/+}$ CD4$^+$CD45RB^{high} T cells plus Ly5.1$^+$CD4$^+$CD25$^+$ T_R cells (Gr.2^D, n = 6). Mice were sacrificed 6 weeks after transfer. (B) Expression of GFP in colonic Ly5.2$^+$ or Ly5.1$^+$ CD3$^+$CD4$^+$ T cells. Data are representative of 6 mice in each group. (C) Percentage of GFP$^+$ cells in colonic Ly5.2$^+$ CD3$^+$CD4$^+$ T cells. (D) Expression of the indicated mRNA in GFP$^+$ or GFP$^-$ CD3$^+$CD4$^+$ cells obtained from the colonic Ly.5.2$^+$ CD3$^+$CD4$^+$ T cells of Gr.1^D and Gr.2^D mice, normalized to Act-b expression. (E) Expression of T-bet in GFP$^+$ or GFP$^-$ colonic Ly5.2$^+$CD3$^+$CD4$^+$ T cells of each group. Data are representative of 6 mice in each group. (F) Mean percentages of CTLA-4 in colonic IL-17A$^+$IFN-γ$^-$ Th17, IL-17A$^+$IFN-γ$^+$ Th17/Th1, and IL-17A$^-$IFN-γ$^+$ Th1 cells. Statistical data (C, D, F) show mean ± standard error of mean (n = 6/group). *P < .05. NS, not significant.

Figure 5. T_R cells suppress the development of colitis induced by RORγt$^+$ or RORγt$^-$ CD4$^+$ T cells resided in noncolitic mice. (A) Transfer protocol E. RAG-2$^{-/-}$ mice were transferred with GFP$^+$ or GFP$^-$ Ly5.2$^+$CD4$^+$ T cells obtained from noncolitic mice previously transferred with Ly5.2$^+$CD4$^+$CD45RB^{high} T cells and Ly5.1$^+$CD4$^+$CD25$^+$ T_R cells with or without cotransfer of freshly isolated Ly5.1$^+$CD4$^+$CD25$^+$ T cells from normal mice (Gr.1^E-Gr.4^E, each n = 6). Mice were sacrificed 6 weeks after transfer. (B) Change in body weight. *$P < .05$. (C) Histopathology of distal colon at 6 weeks after transfer. Original magnification, $\times$40. (D) Absolute cell number of recovered colonic LP CD3$^+$CD4$^+$ T cells at 6 weeks after transfer. (E) Expression of GFP in colonic Ly5.2$^+$CD3$^+$CD4$^+$ T cells of each group. Data are representative of 6 mice in each group. (F) Mean percentages of GFP expression in colonic Ly5.2$^+$CD3$^+$CD4$^+$ T cells of each group. Data (D, F) show mean $\pm$ standard error of mean (n = 6/group). *$P < .05$. NS, not significant.

higher than the paired Gr. 2^E and Gr.4^E mice, respectively (Supplementary Figure 5A). Consistent with this, Gr.1^E and Gr.3^E mice, but not Gr.2^E and Gr.4^E mice, microscopically developed severe colitis with a marked increase of infiltrating mononuclear cells in the colon, in sharp contrast to Gr.2^E and Gr.4^E mice with no colitis (Figure 5C). The histological scores of colitic Gr.1^E and Gr.3^E mice were comparable, but were significantly higher than the paired controls, Gr.2^E and Gr.4^E, respectively (Supplementary Figure 5B). Although the absolute cell number of LP CD3$^+$CD4$^+$ T cells in Gr.1^E and Gr.3^E was equivalent, significantly higher numbers of CD3$^+$CD4$^+$ T cells were recovered from the LP of Gr.1^E and Gr.3^E mice than from the paired Gr.2^E and Gr.4^E mice, respectively (Figure 5D).

Given the evidence that both LP GFP$^+$ and GFP$^-$ CD3$^+$CD4$^+$ T cells in noncolitic mice previously transferred with CD4$^+$CD45RB^{high} T cells and T_R cells are colitogenic if they are separated from T_R cells and transferred to new

RAG-2$^{-/-}$ mice, we next examined the plasticity of those cells after the second transfer. Although LP Ly5.2$^+$GFP$^-$ CD3$^+$CD4$^+$ T cells in Gr.3^E and Gr.4^E mice did not gain GFP expression after retransfer, regardless of whether colitis developed, approximately one third of LP CD3$^+$CD4$^+$ T cells derived from Ly5.2$^+$GFP$^+$ T cells in Gr.1^E with colitis lost the expression of GFP, and this loss in LP Ly5.2$^+$CD3$^+$CD4$^+$ T cells of Gr.2^E mice was blocked by cotransfer of T_R cells (Figure 5E and F), suggesting that T_R cells suppress the differentiation from RORγt$^+$ Th17 and Th17/Th1 cells to RORγt$^-$ Th1 cells during colitis development.

T_R Cells Modulate the Balance Between Th17 and Th1 Cells

We finally assessed if T_R cells remodulate the balance of Th17, Th17/Th1, and Th1 cells after colitis is established. To this end, we prepared colitic RAG-2$^{-/-}$ mice previously transferred with Ly5.1$^+$CD4$^+$CD45RB^{high}

T cells. Eight weeks after transfer, RAG-2$^{-/-}$ mice were transferred with LP Ly.5.1$^+$CD3$^+$CD4$^+$ T cells obtained from the established colitic mice with (Gr.2^F) or without (Gr.1^F) Ly5.2-derived T_R cells (Figure 6A). First, we confirmed that Gr.1^F mice developed colitis, while Gr.2^F mice cotransferred with T_R cells developed significantly milder colitis as compared to Gr.1^F mice, as assessed by weight loss (Supplementary Figure 6A), clinical score (Supplementary Figure 6B), colon appearance (data not shown), histology (Supplementary Figure 6C), and the absolute number of LP CD3$^+$CD4$^+$ T cells recovered (Supplemen-

tary Figure 6D). Furthermore, the percentage of Th17 cells in the LP CD3$^+$CD4$^+$ T cells of colitic Gr.1^F mice was significantly reduced compared to the original mice with colitis, while conversely, the percentages of Th17/Th1 and Th1 cells in Gr.1^F mice were significantly higher when compared to those of the original mice with colitis, suggesting a sequential developmental pathway from Th17 to Th17/Th1 and Th1 cells after retransfer. In contrast, the percentage of Th1 cells in the LP CD3$^+$CD4$^+$ T cells of Gr.2^F mice was significantly lower than in Gr.1^F mice, while the percentages of Th17 cells in the LP CD3$^+$CD4$^+$ T cells of Gr.2^F mice were significantly higher than in Gr.1^F mice (Figure 6B and C). Therefore, the ratio of Th17 to Th1 cells in Gr.2^F mice was markedly higher than that in Gr.1^F mice (Figure 6D), indicating that cotransfer of T_R cells skews the balance of Th17 and Th1 cells from Th1 to Th17 cells. The percentage of Th17/Th1 cells in Gr.2^F mice tended to be higher than that in Gr.1^F mice, but the difference was not significant (Figure 6C). The CTLA-4 expression of both IL-17A$^+$ and IL-17A$^-$ CD4$^+$ T cells in Gr.2^F mice was significantly increased as compared to the paired percentages in Gr.1^F mice (Figure 6E and F). These results suggest that T_R cells suppress the maintenance of Th1 cells, or induce the re-differentiation from Th1 to Th17 cells.
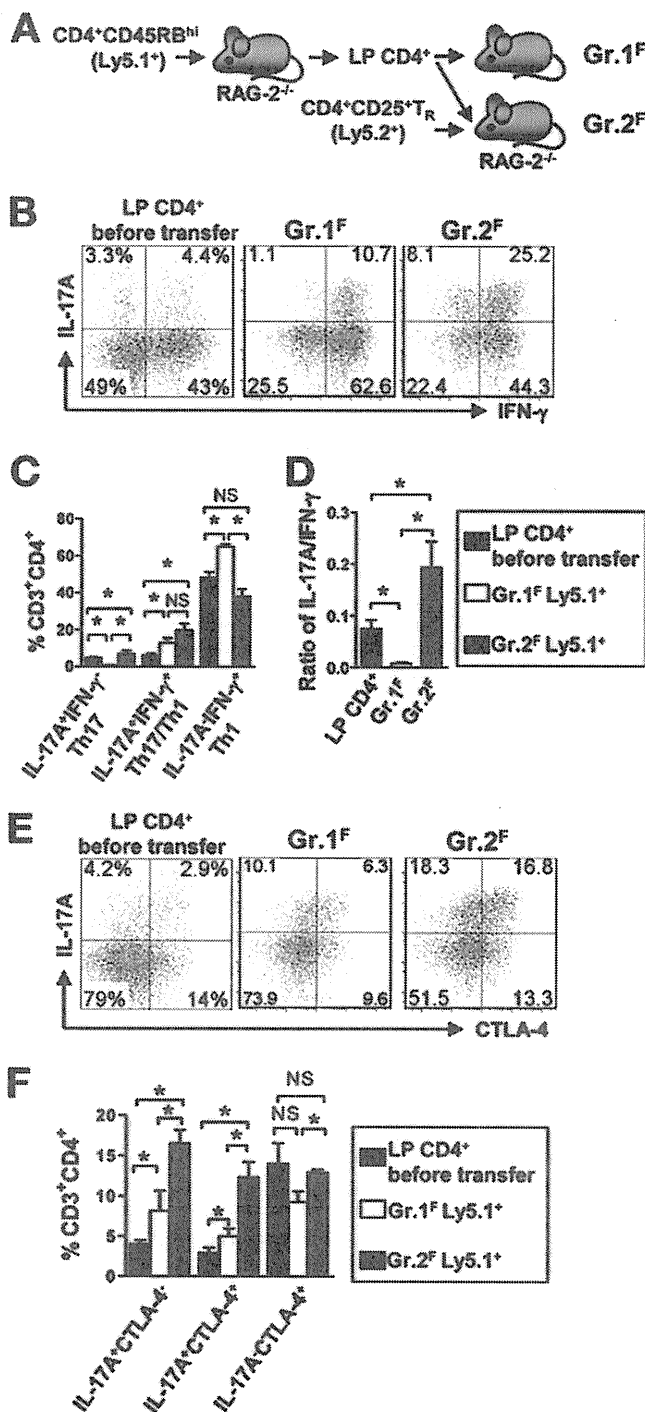
Discussion

In the present study, we demonstrated that colitogenic RORγt$^+$ CD4$^+$ T cells reside in inflamed mucosa dominantly as Th1-like cells that exclusively express IFN-γ, rather than Th17/Th1 cells expressing both IFN-γ and IL-17A; these Th1-like cells developmentally lose RORγt expression and terminally differentiate into RORγt$^-$T-bet$^+$ Th1 cells that exclusively express IFN-γ, which we designate "alternative Th1 cells"; and as a highlight finding, T_R cells suppress development of colitis by blocking the developmental pathway from Th17 to alternative Th1 cells via Th17/Th1 and Th1-like cells.

Figure 6. T_R cells modulate the balance between Th17 and Th1 cells. (A) Transfer protocol F. RAG-2$^{-/-}$ mice were transferred with colitogenic CD4$^+$ T cells obtained from colitic mice previously transferred with Ly5.1$^+$CD4$^+$CD45RB^{high} T cells with (Gr.2^F, n = 6) or without (Gr.1^F, n = 6) Ly5.2$^+$CD4$^+$CD25$^+$ T_R cells. Mice were sacrificed 6 weeks after transfer. (B) Expression of IL-17A and IFN-γ in the originally transferred LP Ly5.1$^+$CD3$^+$CD4$^+$ T cells and Ly5.1$^+$CD3$^+$CD4$^+$ T cells isolated from the colon. Data are representative of 6 mice in each group. (C) Mean percentages of Th17, Th17/Th1, and Th1 cells (including Th1-like cells) in the originally transferred LP Ly5.1$^+$CD3$^+$CD4$^+$ T cells and Ly5.1$^+$CD3$^+$CD4$^+$ T cells isolated from the colon of Gr.1^F or Gr.2^F mice. (D) Ratio of Th17 to Th1 cells in LP Ly5.1$^+$CD3$^+$CD4$^+$ T cells. (E) Expression of IL-17A and CTLA-4 in colonic Ly5.1$^+$CD3$^+$CD4$^+$ T cells. Data are representative of six mice in each group. (F) Mean percentages of IL-17A$^+$CTLA-4$^-$, IL-17A$^+$CTLA-4$^+$, and IL-17A$^-$CTLA-4$^+$ in Ly5.1$^+$CD3$^+$CD4$^+$ T cells isolated from the originally transferred LP Ly5.1$^+$CD3$^+$CD4$^+$ T cells and Ly5.1$^+$CD3$^+$CD4$^+$ T cells isolated from the colon of Gr.1^F or Gr.2^F mice. Statistical data (C, D, F) show mean ± standard error of mean (n = 6/group). $^*P < .05$.

Recent reports demonstrated that Th17 cells have an ability to divert to IFN-γ—producing cells in vitro, or via a combined system using sequential in vitro and in vivo experiments and vice versa.[10,19,20] The current study proposes a linear developmental pathway of alternative Th1 cells in the development of colitis from naïve to Th17, Th17/Th1, and Th1-like cells and then to alternative Th1 cells, in addition to a developmental pathway of classical Th1 cells (Supplementary Figure 7). However, it still remains to be determined whether the sequential path from naïve to Th17 to Th17/Th1 to Th1-like to alternative Th1 cells occurs during the development of colitis, as we did not analyze the data using an IL-17A/F, or IFN-γ/T-bet reporter in this project. Further studies will be needed to address this issue.

Results of our current project show some similarities to the findings reported by Weaver's group,[10] but the experimental protocols and interpretations of the 2 studies are quite different. Weaver's group used in vitro—induced IL-17F+ Th17 cells sorted from naïve IL-17F reporter CD4+ T cells that had been stimulated with TGF-β and IL-6 as donor cells, and then transferred those cells into RAG-1−/− mice. In this setting, mice not only developed colitis, but also retained IL-17A−IFN-γ+ CD4+ T cells in the inflamed mucosa. They concluded that committed Th17 cells give rise to progeny that lost IL-17A expression and up-regulated IFN-γ expression at the late stage of colitis development. They called such cells "Th1-like" cells. However, they did not characterize the expression of RORγt and T-bet in IL-17A−IFN-γ+ CD4+ T cells in the inflamed mucosa of colitic RAG-1−/− mice transferred with in vitro—induced Th17 cells, but did demonstrate that in vitro—induced Th17 cells with RORγt, but not T-bet, expression convert to "Th1-like" cells with T-bet, but not RORγt, expression in all in vitro stimulation systems. In our project, however, we performed a series of studies using a consecutive in vivo adoptive transfer system, including the induction stage of Th17 cells and the late stage. Therefore, unlike Weaver's group, we characterized in vivo pathologically occurring RORγt+ Th17 or Th17/Th1 cells in colitic mice, and used those cells rather than the in vitro—stimulated Th17 cells as donor cells. In this setting, the majority of RORγt+ CD3+CD4+ T cells in the colitic mice were composed of IL-17A−IFN-γ+ CD4+ T cells that express both Rorc and Tbx21 and a few IL-17A+IFN-γ+ Th17/Th1 and IL-17A+IFN-γ− Th17 cells. Importantly, additional adoptive transfer of RORγt+ CD3+CD4+ T cells into RAG-2−/− mice demonstrated that approximately half of the transferred RORγt+ cells lose their RORγt expression and gained T-bet, while the other half retain it up to 6 weeks after the retransfer, suggesting that RORγt+ Th17, Th17/Th1, and Th1-like cells in colitic mice act as colitogenic "memory stem-like" cells for the generation of terminally differentiated Th1 cells.

In such a linear in vivo developmental pathway of Th17 and Th17/Th1 to Th1-like and alternative Th1 cells, we strikingly demonstrated that T_R cells suppress the transi-

tion of Th17/Th1 into Th1-like cells (Figure 3 and 4, Supplementary Figure 6), as paradoxically the ratio of Th17 and Th17/Th1 cells is significantly higher in non-colitic RAG-2−/− mice transferred with CD4+CD45RBhigh T and T_R cells than RAG-2−/− mice transferred with CD4+CD45RBhigh T cells alone. Indeed, although almost all previous reports conclude that T_R cells suppress production of both IFN-γ and IL-17A in this model[21,22] and other animal models of chronic inflammation,[23] the conclusion is based on the assessment of the production of IL-17A by anti-CD3 monoclonal antibody stimulation or expression of IL-17A mRNA in the intestinal mucosa. The current intracellular staining method that precisely discriminates effector and T_R cells using the Ly5.1/Ly5.2 congenic system may make it possible to draw a surprising conclusion. The finding that cotransfer of T_R cells resulted in an increased ratio of Th17 and Th17/Th1, but a decreased ratio of Th1, indicates that T_R cells block the transition of Th17/Th1 cells into Th1-like cells.

Furthermore, the role of T_R cell—mediated accumulation of Th17 and Th17/Th1 cells should be discussed in the context of a recently published article by Ghoreschi et al.[24] They emphasize that the presence and absence of TGF-β determines the fate of the subsequent development of regulatory and pathological Th17 cells, respectively, in a model of experimental autoimmune encephalomyelitis. According to this article and the previous finding that T_R cells produce TGF-β,[25] our data suggest that T_R cells instruct development of regulatory Th17 cells in noncolitic mice cotransferred with T_R cells. Consistently, regardless of the increased Th17 and Th17/Th1 cells or decreased Th1 cells, expression of CTLA-4 in those cells of noncolitic mice cotransferred with T_R cells was significantly up-regulated, indicating that T_R cells suppress colitis by not only suppressing development of Th1 cells, but also inducing the inhibitory molecule, CTLA-4, in effector T cells. Given the finding that T_R cells require their CTLA-4 expression to protect from colitis,[26] CTLA-4 not only in T_R but also in effector cells may play an important role in inducing inhibitory signaling through CD80/CD86 in noncolitic conditions. However, this scenario seems to be unlikely because we showed that the ratio of IL-17A+TNF-α+ cells in noncolitic mice cotransferred with T_R cells was significantly higher than that of paired cells in colitic mice; and Th17 and Th17/Th1 cells in noncolitic mice cotransferred with T_R cells are precolitogenic (Figure 5). Therefore, the mechanism of the developmental pathways of Th17 cells in the colitis model and encephalomyelitis model may be distinct.

Collectively, the current study solves the riddle of whether Th1 or Th17 cells are essential for development of colitis. Our answer is both, because Th17 cells may be precursor cells for alternative Th1 cells. Therefore, T-bet, which is involved in the functionality of both classical and alternative Th1 cells, may be a feasible target for the treatment of IBD.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.05.052.

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The Development of Colitogenic CD4$^+$ T Cells Is Regulated by IL-7 in Collaboration with NK Cell Function in a Murine Model of Colitis

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We previously reported that IL-7$^{-/-}$RAG$^{-/-}$ mice receiving naive T cells failed to induce colitis. Such abrogation of colitis may be associated with not only incomplete T cell maintenance due to the lack of IL-7, but also with the induction of colitogenic CD4$^+$ T cell apoptosis at an early stage of colitis development. Moreover, NK cells may be associated with the suppression of pathogenic T cells in vivo, and they may induce apoptosis of CD4$^+$ T cells. To further investigate these roles of NK cells, RAG$^{-/-}$ and IL-7$^{-/-}$RAG$^{-/-}$ mice that had received naive T cells were depleted of NK cells using anti-asialo GM1 and anti-NK1.1 Abs. NK cell depletion at an early stage, but not at a later stage during colitogenic effector memory T cell (T$_{EM}$) development, resulted in exacerbated colitis in recipient mice even in the absence of IL-7. Increased CD44$^+$CD62L$^-$ T$_{EM}$ and unique CD44$^-$CD62L$^-$ T cell subsets were observed in the T cell-reconstituted RAG$^{-/-}$ recipients when NK cells were depleted, although Fas, DR5, and IL-7R expressions in this subset differed from those in the CD44$^+$CD62L$^-$ T$_{EM}$ subset. NK cell characteristics were the same in the presence or absence of IL-7 in vitro and in vivo. These results suggest that NK cells suppress colitis severity in T cell-reconstituted RAG$^{-/-}$ and IL-7$^{-/-}$RAG$^{-/-}$ recipient mice through targeting of colitogenic CD4$^+$CD44$^+$CD62L$^-$ T$_{EM}$ and, possibly, of the newly observed CD4$^+$CD44$^-$CD62L$^-$ subset present at the early stage of T cell development. *The Journal of Immunology*, 2012, 188: 000–000.

The pathogenesis of inflammatory bowel diseases (IBD), such as Crohn's disease and ulcerative colitis in humans, is known to be associated with dysregulated immune responses to luminal contents including Ags derived from commensal bacteria in gut. In patients with Crohn's disease, for example, excessive amounts of proinflammatory cytokines, such as IFN-γ, TNF, and IL-17 (1), are secreted predominantly by CD4$^+$ T cells infiltrating colonic tissues. The activities of these cells are thought to reflect the severity of IBD. Additionally, it is known that adoptive transfer of CD4$^+$ naive T cells into lymphopenic immune-deficient animals, such as SCID and RAG$^{-/-}$ mice, induces chronic inflammation in the colon and is considered an animal model of IBD (2, 3).

IL-7 is an important cytokine that is associated with the proliferation of immature B and T cells (4) as well as with homeostatic maintenance of peripheral T cells in vivo (5, 6). We have previously reported that IL-7 is secreted by intestinal epithelia, especially goblet cells (7), and that spontaneous colitis that is similar to IBD in humans is induced in transgenic mice overexpressing IL-7 (8). Additionally, we have shown that the IL-7R^{high}CD4$^+$ T cell subset is pathogenic (9) when the cells are transferred into RAG$^{-/-}$ mice (10, 11). Moreover, we have also shown that adoptive transfer of naive T cells in RAG and IL-7 double-deficient (IL-7$^{-/-}$RAG$^{-/-}$) mice fails to induce colitis (10). Therefore, IL-7 was initially considered to be essential for the induction of colitis. However, it is known that IL-7 is not required for the in vitro differentiation from naive T cells into Th1 or Th17 cells (12). It is also known that the spontaneous proliferation, which is dependent on Ag ligation to the CD3/TCR complex, can be observed even in the T cell-reconstituted IL-7$^{-/-}$RAG$^{-/-}$ re-

cipient mice (13, 14). Additionally, our recent studies suggested that effector $CD4^+$ T cells were able to induce colitis even in IL-$7^{-/-}RAG^{-/-}$ mice that were parabiosed with colitic $RAG^{-/-}$ mice that had been injected with naive T cells 6 wk previously (15). Moreover, a deparabiosed IL-$7^{-/-}RAG^{-/-}$ mouse, which was surgically separated from T cell-receiving $RAG^{-/-}$–IL-$7^{-/-}$ $RAG^{-/-}$ parabionts 6 wk after the initial surgery, still maintained chronic colitis for at least another 12 wk (16). These results suggested that the abrogation of colitis in the T cell-reconstituted IL-$7^{-/-}RAG^{-/-}$ mice may be associated with not only incomplete T cell maintenance due to the lack of IL-7, but also with another mechanism by which the colitogenic $CD4^+$ T cell development is suppressed.

It is known that NK cells are responsible for innate immune responses, including the depletion of tumor cells or cells infected with various kinds of viruses (17). Additionally, NK cells induce inflammation in tissues by the production of proinflammatory cytokines such as IFN-γ (18, 19). On the other hand, NK cells are also known to be critical for anti-inflammatory effects in the context of autoimmune diseases (20, 21). It has been reported that NK cells abrogate disease severity of experimental autoimmune encephalomyelitis (EAE) due to the suppression of pathogenic T cells (22, 23). It has also been reported that depletion of NK cells results in enhanced severity of a chronic colitis model (24). However, the mechanisms by which NK cells regulate inflammation in this colitis model have not been well described. In this regard, we hypothesized that the abrogation of colitogenic T cell development that we observed in naive T cell-receiving IL-$7^{-/-}$ $RAG^{-/-}$ mice is associated with the effect of NK cells. We therefore focused our analysis of this phenomenon on NK cells.

Materials and Methods
Animals

Wild-type (WT) C57BL/6 mice were purchased from Japan CLEA (Tokyo, Japan). Rag-deficient ($RAG^{-/-}$) mice on a C57BL/6 background were obtained from Taconic (Hudson, NY) and the Central Laboratories for Experimental Animals (Kanagawa, Japan). IL-$7^{-/-}$ mice were provided by Dr. R. Zamoyska (National Institute for Medical Research, London, U.K.) and were intercrossed with $RAG^{-/-}$ to generate IL-$7^{-/-}RAG^{-/-}$ mice. Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of Tokyo Medical and Dental University. Donors and recipients were used between 8 and 16 wk age. All animal experimentations were approved by the Animal Review Board of Tokyo Medical and Dental University and were performed in accordance with institutional guidelines.

Abs

The following mAbs and reagents were obtained from BD Pharmingen (San Jose, CA): anti-CD3ε (145-2C11), anti-CD4 (RM4-5), anti-CD11b (M1/70), anti-CD11c (HL3), anti-CD27 (LG.3A10), anti-CD28 (37.51), anti-CD43 (S7), anti-CD44 (IM7), anti-CD45RB (16A), anti-CD51 (RMV-7), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-CD94 (18d3), anti-CD95, anti-CD178 (MFL4), anti-CD244.2 (2B4 B6 alloantigen), anti-Ly49C,I (5E6), anti-Ly49D (4E5), anti-Ly49F (HBF-719), anti-Ly49G2 (4D11), anti–IL-7Rα (A7R34), anti-NK1.1 (PK136), and streptavidin. Biotin-conjugated anti-mouse NKG2A/C/E, biotin-conjugated anti-mouse IL-7Rα (A7R34), FITC-conjugated anti-mouse pan-NK cells (CD49b), and FITC-conjugated anti-mouse CD3ε (145-2C11) mAbs were purchased from eBioscience (San Diego, CA).

Flow cytometry (FACS)

To detect the cell surface expression of a variety of molecules, isolated mononuclear cells from individual organs including spleen (SPL), mesenteric lymph node (MLN), and colonic lamina propria (LP) were analyzed by FACS using standard staining methods. Briefly, the cells were suspended in PBS containing 2% FBS, which was used as the suspension fluid for subsequent staining, preincubated with an FcγR-blocking mAb (anti-CD16/32; 2.4G2; BD Biosciences) for 15 min to prevent nonspecific binding by the secondary Ab, and washed with suspension fluid followed by staining

with specific FITC-, PE-, PerCP-, allophycocyanin-, or biotin-labeled Abs for 20 min on ice. Standard two-, three-, or four-color flow cytometric analyses were performed using the FACSCalibur (Becton Dickinson, Sunnyvale, CA) with appropriate software (CellQuest; BD Biosciences). Background fluorescence was also assessed by staining with control irrelevant isotype-matched mAbs.

NK cell depletion in vivo

The anti-asialo GM1 (ASGM1) polyclonal Ab was obtained from Wako Chemicals (Osaka, Japan) and reconstituted according to the manufacturer's specifications. The anti-NK1.1 mAb was affinity purified from the culture supernatant of a hybridoma clone, PK136, obtained from the American Type Culture Collection (Manassas, VA). For effective depletion of NK cells in vivo, either the anti-ASGM1 polyclonal Ab (0.25 mg/mouse) or anti-NK1.1 mAb (0.5 mg/mouse) was injected i.p. into mice (25) at the indicated time points in each experiment. The same amount of rabbit Ig (Rockland Immunochemicals, Gilbertsville, PA) or mouse IgG2a (Medical & Biological Laboratories, Nagoya, Japan) were used as the controls, respectively, for some of experiments. Effective (~95%) depletion of NK cells in vivo was confirmed by FACS analysis of single cells derived from individual organs such as SPL, MLN, and colonic LP.

Purification of naive T cell subsets and induction of colitis

For naive T cell purification, splenic mononuclear cells were obtained from WT mice and $CD4^+$ T cells were isolated using anti-CD4 (L3T4) MACS magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Enriched $CD4^+$ T cells (94–97% pure as estimated by FACS) were then labeled with PerCP- or allophycocyanin-conjugated anti-CD4, PE- or allophycocyanin-conjugated anti-CD44, and FITC-conjugated anti-CD62L. Subpopulations of $CD4^+$ cells were generated by three-color sorting on a FACSAria (Becton Dickinson). All populations were 98.0% pure on reanalysis. To induce an animal model of chronic colitis, 5×10^5 $CD4^+CD44^-CD62L^+$ (naive) T cells were adoptively transferred i.p. into 8- to 12-wk-old $RAG^{-/-}$ or IL-$7^{-/-}RAG^{-/-}$ recipient mice as previously described (2, 3, 10).

Isolation of LP lymphocytes

LP lymphocytes (LPL) were isolated from healthy or colitic mice as previously described (10). Briefly, $RAG^{-/-}$ or IL-$7^{-/-}RAG^{-/-}$ recipient mice were sacrificed 6–12 wk after injection of naive T cells to induce colitis. The entire length of the colon was removed, opened longitudinally, washed with PBS, and cut into small pieces. The dissected tissues were incubated with Ca^{2+}-, Mg^{2+}-free HBSS containing 1 mM DTT (Sigma-Aldrich, St. Louis, MO) for 45 min to remove mucus, and the epithelial layer was then treated with 3.0 mg/ml collagenase (Roche Diagnostics, Mannheim, Germany) and 0.01% DNase (Worthington Biomedical, Freehold, NJ) for 2 h. The cells were pelleted, washed twice with PBS, and were then subjected to density gradient centrifugation using 40–75% isotonic Percoll (Amersham Biotech, Piscataway, NJ) solution diluted with HBSS. Isolated whole LP mononuclear cells were subjected to FACS to analyze each lymphocyte subset. In some experiments, such LP mononuclear cells were further labeled with allophycocyanin-conjugated anti-CD4 and FITC-conjugated anti-CD3 to isolate colitogenic $CD4^+$ T cell subsets by FACSAria. All populations were 98.0% pure on reanalysis. Isolated LP $CD4^+$ T cells were subjected to cytokine production and cytotoxicity assays.

Determination of clinical score of colitis

The clinical score of colitis was determined using previously described methods (26) with minor modifications and was assessed by trained individuals blinded to the treatment group. Briefly, initial body weight and wasting, hunching over, piloerection, diarrhea, and blood in the stool or per rectum of the T cell-receiving $RAG^{-/-}$ or IL-$7^{-/-}RAG^{-/-}$ recipient mice were assessed when sacrificed. For wasting, weight loss of <20% from baseline was assigned 0 points and weight loss of >20% was assigned 1 point. For hunched over appearance, no obvious hunching was assigned 0 point, and extensive hunching was assigned as 1 point. For colon thickening, normal features were assigned 0 points, mild thickening was assigned 1 point, moderate thickening was assigned 2 points, and severe thickening was assigned 3 points. For stool consistency, 0 points were assigned to well-formed pellets, 1 point to pasty and semiformed stools that did not adhere to the anus, and 2 points to liquid stools that did adhere to the anus. An additional point was added if gross blood was noted. The scores of these parameters were added, resulting in a total clinical score ranging from 0 (healthy) to 8 (maximal colitis activity).

Histopathological examination of colitis

Mice receiving naive T cells were sacrificed 6 or 12 wk after the T cell transfer, and colonic specimens taken from proximal, middle, and distal colons were subjected to histopathological assessment. For this assessment, tissue samples were fixed in 10% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with H&E. The H&E-stained sections were analyzed without prior knowledge of the type of donors, recipients, and treatments. The degree of inflammation in the colon was graded according to a modification of the previously described scoring system (26, 27). Briefly, for mucosal damage, 0 points were assigned to normal appearance, 1 point to discrete lymphoepithelial lesions, 2 points to diffuse crypt elongation, and 3 points to extensive crypt elongation or mucosal erosion/ulceration. For cell infiltration the points assigned were: 0, to normal, or presence of occasional leukocytes; 1, to widely scattered leukocytes or focal aggregates of leukocytes; 2, to confluence of leukocytes extending into the submucosa with focal effacement of the muscularis; 3, to transmural extension of leukocyte infiltration. For crypt abscess, the assigned points were: 0, to no crypt abscess; 1, to the presence of crypt abscess. The cumulative degree of these parameters was calculated as a total histological score ranging from 0 (no change) to 21 (extensive cell infiltration and tissue damage).

ELISA

To measure cytokine production, 1×10^5 LP $CD4^+$ T cells were cultured in 200 μl RPMI 1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS, 500 U/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich), 10 mM HEPES, 1% nonessential amino acids, and 50 μM 2-ME (Life Technologies Invitrogen, Carlsbad, CA), termed complete RPMI 1640, in the presence of 5 μg/ml plate-bound anti-CD3ε (145-2C11) and 2 μg/ml soluble anti-CD28 (37.51) mAbs on flat-bottom 96-well plates (Costar, Cambridge, MA) at 37°C in a humidified atmosphere incubator containing 5% CO_2 for 48 h. Culture supernatants were removed and analyzed for the production of cytokines such as IFN-γ, TNF, and IL-17. Cytokine concentrations were determined using specific ELISAs (R&D Systems, Minneapolis, MN) as per the manufacturer's recommendations.

Isolation of NK cells and cytotoxicity assay

Spleen cell suspensions were prepared from $RAG^{-/-}$ or $IL-7^{-/-}RAG^{-/-}$ mice and treated with NH_4Cl buffer to remove erythrocytes. The NK cell population was then labeled with FITC-conjugated anti-DX5 (CD49b) and isolated for use as effector cells in the cytotoxicity assay by sorting on a FACSAria. The purity of isolated NK cells was 98.0% on reanalysis. To measure cytokine production, 5×10^4 NK cells were cultured in 200 μl RPMI 1640 supplemented with 10% FBS, 500 U/ml penicillin, and 100 μg/ml streptomycin in the presence of 100 ng/ml rIL-2, 100 ng/ml rIL-12, and 100 ng/ml rIL-18 on flat-bottom 96-well plates at 37°C in a humidified atmosphere incubator containing 5% CO_2. Culture supernatants were removed after 24 h and analyzed for IFN-γ production. Cytotoxicity assays were performed using the flow cytometric method reported by Xu et al. (28; see also Ref. 29). Briefly, isolated naive T cells from WT mice or LP effector memory T cells (T_{EM}) from colitic mice were labeled with a lipophilic green fluorescent cell linker, PKH2 (Sigma-Aldrich), which is incorporated into the plasma membrane. Uniform labeling of cells was confirmed by flow cytometry. Labeled 5×10^4 target T cells were coincubated in round-bottom 96-well plates (Costar) with effector NK cells (T:E ratio, 1:5 to 1:0.6) in complete RPMI 1640 supplemented with 100 ng/ml rIL-2 (PeproTech, London, U.K.) with or without 50 ng/ml rIL-7 (PeproTech) at 37°C in humidified air containing 5% CO_2 for 4 h. Naive T cells or LP colitogenic T cells that were incubated under the same conditions but without effector NK cells were also prepared as controls. Cells were then collected, stained with propidium iodide (PI), and analyzed by FACS. Cytotoxic activity was determined by calculating the percentage of the double-positive population for both PI (FL2) and PKH2 (FL1). In some experiments, a mouse lymphoma cell line, YAC-1, obtained from the American Type Culture Collection, was used as target cells for a $[^{51}Cr]$ release assay with the standard protocol. Briefly, target cells were labeled with 3.7 MBq of $Na_2[^{51}Cr]O_4$ for 1 h at 37°C and washed three times with PBS before mixing (1×10^4/well) with effector cells in round-bottomed 96-well plates at different E:T ratios (1.25:1, 2.5:1, 5:1, 10:1, 20:1) in triplicates. After 4 h incubation, cell-free supernatants were collected and radioactivity measured by MicroBeta counter (Wallac). The percentage of lysis is calculated by (sample release − spontaneous release)/(maximum release − spontaneous release).

Statistical analysis

The results are expressed as the means ± SEM. Statistical significance was determined using the nonparametric Mann–Whitney U test, and differences were considered to be statistically significant when $p < 0.05$.

Results

NK cell depletion induces the early onset of colitis in naive T cell-transferred $RAG^{-/-}$ mice

It has been reported that NK cells suppress the severity of inflammatory diseases such as EAE and colitis (22, 24). NK cells were depleted in the latter colitis study by injection of anti-NK1.1 or anti-ASGM1 Abs, or by the use of a perforin-deficient animal. That study suggested that NK cells may possibly have cytolytic activity for colitogenic $CD4^+$ T_{EM} in this model since knockout of the perforin gene resulted in exacerbation of disease severity. However, it is unclear which stage in the development of colitis is affected by NK cells. Therefore, we first assessed the effect of NK cell depletion at different time points in the development of chronic colitis.

To examine the effect of NK cells in the development of chronic inflammation in the colon, an animal model of colitis was induced by adoptive transfer of $CD4^+CD62L^+D44^-$ (naive) T cells derived from WT SP into $RAG^{-/-}$ recipient mice (2, 3). NK cells were depleted by i.p. injection of the anti-ASGM1 Ab (or vehicle control [PBS]) every other day for 12 wk starting from the day before naive T cell transfer (Fig. 1A). Additionally, some groups were injected with the anti-ASGM1 Ab for 4 wk followed by vehicle control for 8 wk (Fig. 1A), or with the vehicle control for 4 wk followed by 8 wk anti-ASGM1 Ab (Supplemental Fig. 1). Mice injected with the anti-ASGM1 Ab for 12 wk, or for just the first 4 wk, started to show wasting earlier than the vehicle control group that was injected for 12 wk (Fig. 1B). Alternatively, mice injected with vehicle control for 4 wk followed by 8 wk anti-ASGM1 Ab showed a similar wasting curve to that of mice injected with vehicle control for 12 wk (data not shown), suggesting that NK cell depletion at the later stage of colitis induction does not affect the severity of colitis.

However, there was no significant difference in clinical scores between these groups 12 wk after the T cell transfer (Fig. 1C), and all mouse groups showed a similar degree of colitis with thickening and shortening of the colon as well as splenomegaly when sacrificed (Fig. 1D). Consistent with this finding, microscopic evaluation of each group showed similar histopathological features such as wall thickening of the colon, infiltration mainly by mononuclear cells, crypt abscesses, crypt elongation, a decrease in goblet cells, and epithelial damage (Fig. 1E, 1F). Moreover, the production of proinflammatory cytokines by colonic LP T cells isolated from each group was similar (Fig. 1G).

However, there was concern that anti-ASGM1 Ab treatment at an early stage may affect the colitis severity in the $RAG^{-/-}$ mice receiving naive T cells, since the groups with the Ab treatment at an early stage for 4 wk and 12 wk started to exhibit wasting earlier than the control group without the Ab treatment (Fig. 1B). Therefore, we examined these mice at a relatively early time and, interestingly, we found that the Ab-treated group showed significantly more severe colitis in clinical and histological scores compared with the control group 6 wk after T cell transfer (Fig. 2). These data indicate that NK cell depletion affects the early stage of colitis development.

$CD62L^- CD44^+$ and $CD62L^- CD44^-$ T cell subsets are increased by NK cell depletion in naive T cell-reconstituted $RAG^{-/-}$ recipient mice

Because the exacerbation at an early stage of colitis development was observed following NK cell depletion, we assessed the number of $CD4^+$ T cells in SPL and MLN of naive T cell-receiving $RAG^{-/-}$ mice treated with or without the anti-ASGM1 Ab. As seen in Fig. 3A and 3B, increased numbers of T cells were detected, especially
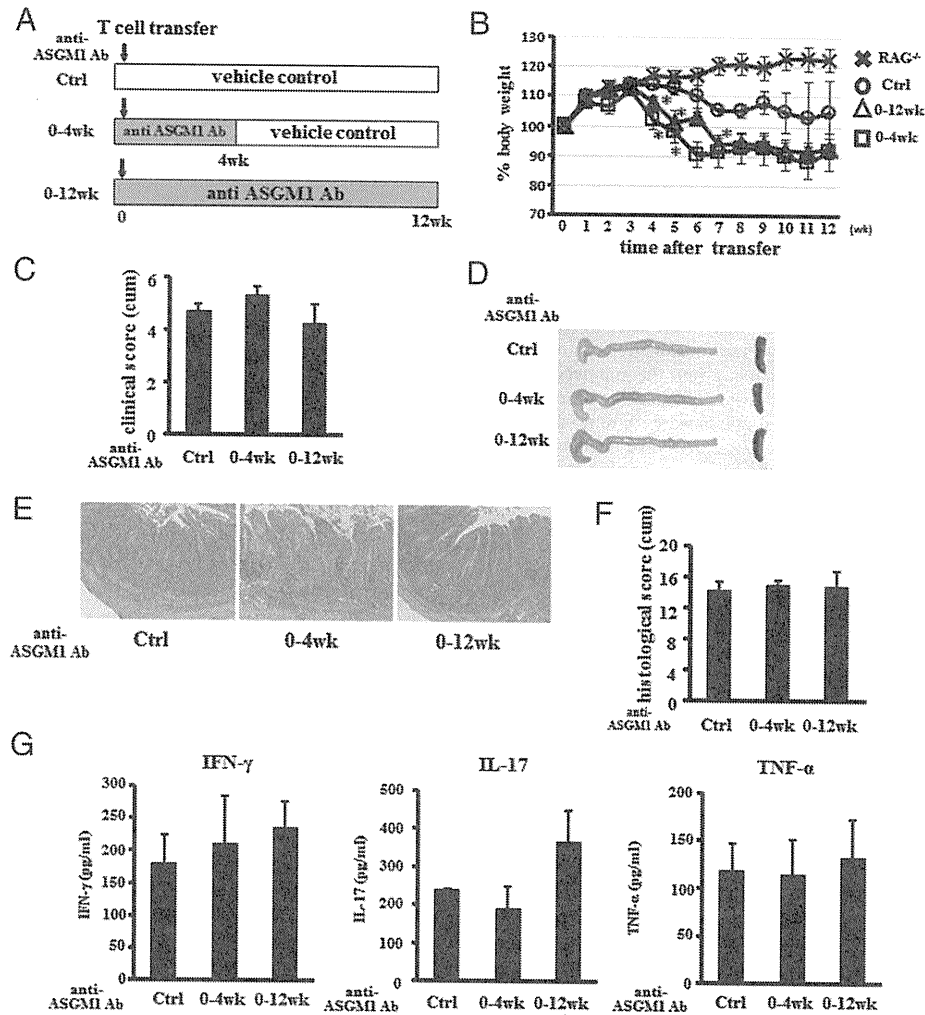
FIGURE 1. NK cell depletion at the early stage of colitis induction in RAG$^{-/-}$ mice results in wasting disease. **(A)** Protocol for NK cell depletion in a chronic colitis setting. RAG$^{-/-}$ mice were injected with either 0.25 mg/mouse anti-ASGM1 Ab (0–12 wk) or vehicle control (Ctrl) every second day for 12 wk from the day before adoptive transfer of naive T cells, or were injected with anti-ASGM1 Ab for 4 wk followed by vehicle control injection for 8 wk (0–4 wk). **(B)** Wasting, as defined by percentage of initial body weight, in RAG$^{-/-}$ mice induced colitis. Mice were injected with naive T cells and either vehicle control for 12 wk (Ctrl, O), anti-ASGM1 Ab for 12 wk (0–12 wk, △), or anti-ASGM1 Ab for 4 wk followed by 8 wk vehicle control (0–4 wk, □). The non–T cell-injected control group is also shown (RAG$^{-/-}$, cross). Data are expressed as means ± SEM from four mice. *p < 0.05. **(C)** Clinical scores of each group are shown. Data are expressed as means ± SEM from four mice. **(D)** Gross appearance of colons (left) and SP (right) from naive T cell-transferred RAG$^{-/-}$ recipients injected with either vehicle control for 12 wk (Ctrl, top), anti-ASGM1 Ab for 4 wk and then control for 8 wk (0–4 wk, middle), or anti-ASGM1 Ab for 12 wk (0–12 wk, bottom). Representative features from four experiments are shown. **(E)** Histological feature of colons from naive T cell-transferred RAG$^{-/-}$ recipients injected with control for 12 wk (Ctrl, left), anti-ASGM1 Ab for 4 wk and then control for 8 wk (0–4 wk, middle), or anti-ASGM1 Ab for 12 wk (0–12 wk, right). Representative features from each group are shown. **(F)** Histological scores of each group are shown. Data are expressed as means ± SEM from four mice. **(G)** Cytokine production by LP T cells from each group is shown. Concentrations of IFN-γ (left), TNF (middle), and IL-17 (right) in the culture supernatant were measured using ELISA. Data are indicated as means ± SEM from four samples.

in the SPL, within a week after naive T cell injection. Moreover, treatment with the anti-ASGM1 Ab revealed a significantly increased number of T cells in SPL and MLN (Fig. 3A, 3B). Thus, we next determined the development of T$_{EM}$ in these mice by assessment of the expression levels of CD62L and CD44 on T cells. From day 1 to day 3, most T cells still expressed CD62L, but not CD44, regardless of anti-ASGM1 Ab treatment. Interestingly, a CD62L$^-$CD44$^-$ subset had appeared in both SPL and MLN by day 5 after treatment with anti-ASGM1 Ab (Fig. 3C–F). This unique T cell subset was significantly increased in the naive T cell-receiving RAG$^{-/-}$ mice treated with anti-ASGM1 Ab, especially in MLN, on days 5 and 7 (Fig. 3F), suggesting that NK cells target this CD62L$^-$CD44$^-$ T cell subset upon development of colitogenic CD62L$^-$CD44$^+$ T$_{EM}$.

It is thought that the T$_{EM}$, but not a naive T cell subset, is targeted by NK cells to regulate excessive immune responses (23,

28). However, our observation indicated that a CD62L$^-$CD44$^-$ T cell subset is increased in the absence of NK cells. Therefore, we next assessed the expression levels of several markers, which are associated with NK cell function, on each of the T cell subsets. Splenic CD62L$^+$CD44$^-$ (naive, R1; Fig. 4, left panel), CD62L$^-$CD44$^-$ (R2), and CD62L$^-$CD44$^+$ (effector memory, R3) T cell subsets were isolated for FACS analysis from RAG$^{-/-}$ mice that had received naive T cells 5 d previously with anti-ASGM1 Ab treatment the day before T cell reconstitution. The expression of Fas and DR5 in CD62L$^-$CD44$^+$ cells was higher than that in CD62L$^+$CD44$^-$ T cells (Fig. 4). Interestingly, the expression levels of Fas and DR5 in CD62L$^-$CD44$^-$ cells were similar to those of CD62L$^+$CD44$^-$, but not of CD62L$^-$CD44$^+$. Additionally, the expression level of Qa-1 was similar for all of these T cell subsets (Fig. 4). Furthermore, the expression level of IL-7R/CD127 in CD62L$^-$CD44$^+$ cells was similar to that of CD62L$^+$
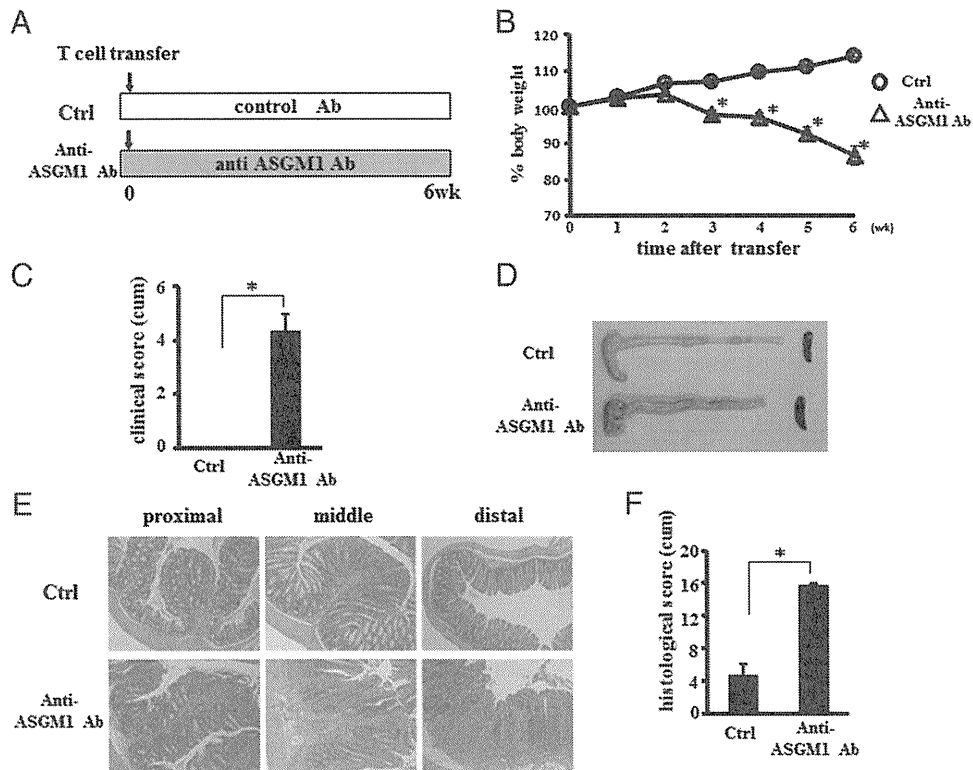
FIGURE 2. NK cell depletion in RAG$^{-/-}$ recipients results in early onset colitis development. (**A**) Protocol for NK cell depletion in a chronic colitis setting. RAG$^{-/-}$ mice were injected with either 0.25 mg/mouse anti-ASGM1 Ab or control IgG every second day for 6 wk from the day before adoptive transfer of naive T cells. (**B**) Wasting, as defined by percentage of initial body weight, in RAG$^{-/-}$ mice induced colitis. Mice were injected with naive T cells with either control IgG (O) or anti-ASGM1 Ab (△) for 6 wk. Data are expressed as means ± SEM from four mice. *$p < 0.05$. (**C**) Clinical scores of each group are shown. Data are expressed as means ± SEM from four mice. *$p < 0.05$. (**D**) Gross appearance of colons (*left*) and SP (*right*) from naive T cell-transferred RAG$^{-/-}$ recipients injected with either control IgG (*top*) or anti-ASGM1 Ab for 6 wk (*bottom*). Representative features from four experiments are shown. (**E**) Histological feature of proximal (*left*), middle (*middle*), and distal (*right*) colons from naive T cell-transferred RAG$^{-/-}$ recipients injected with either control IgG (*top*) or anti-ASGM1 Ab for 6 wk (*botttom*). Representative features from each group are shown. (**F**) Histological scores of each group are shown. Data are expressed as means ± SEM from four mice. *$p < 0.005$.

CD44$^-$ cells. Most CD62L$^-$CD44$^-$ cells showed a similar IL-7R/CD127 expression level to the other subsets; however, some cells within this subset showed a lower expression of the IL-7R as seen in Fig. 4 (arrow). These results indicate that the mechanism by which NK cells suppress CD62L$^-$CD44$^-$ T cells may be different from that by which they suppress T$_{EM}$, which is due to NK cell-induced apoptosis via Fas and/or DR5.

*The lack of IL-7 does not affect the cytotoxic activity of NK cells*

Because we have previously observed the upregulated annexin V and downregulated Bcl-2 expressions in the CD4$^+$ T cells transferred into IL-7$^{-/-}$RAG$^{-/-}$ recipients (10), we speculated that the ability of NK cells to suppress the T cells could be affected by the presence or absence of IL-7. We therefore performed a cytotoxicity assay to test this hypothesis. As expected, NK cells (effector) had negligible cytotoxicity toward CD62L$^+$CD44$^-$ naive T cells (target) derived from WT SP (T:E ratio, 1:5) regardless of whether rIL-7 was present (Fig. 5A). When CD62L$^-$CD44$^+$ T$_{EM}$ derived from colonic LP of RAG$^{-/-}$ mice, which had been injected with naive T cells 12 wk previously, were coincubated with the NK cells (T:E ratio, 1:5), the mortality of the target cells was elevated but this cell-mediated cytotoxicity did not change in the presence of rIL-7 (Fig. 5B). These results suggested that the cytotoxic ability of NK cells derived from WT mice was not affected by IL-7 and further suggested that the susceptibility of T cells to the cytotoxic activity of NK cells was not changed by the presence of IL-7 using this assay. When increasing the ratio of CD62L$^-$

CD44$^+$ T$_{EM}$ (T:E ratio, 1:5 to 1:0.6), the mortality was decreased (Fig. 5C). These data suggest that the cytotoxicity is decreased when the number of target T cells exceeds the capacities of effector NK cells to suppress T cells. However, it was still unclear whether the cytotoxic ability of NK cells could be modulated during its development in vivo in the presence or absence of IL-7. Therefore, the cytotoxic ability of NK cells derived from RAG$^{-/-}$ and IL-7$^{-/-}$ RAG$^{-/-}$ mice was examined. As seen in Fig. 5D, there was little mortality of CD62L$^+$ CD44$^-$ naive T cells alone, and this mortality was unaffected even if coincubated with NK cells derived from either RAG$^{-/-}$ or IL-7$^{-/-}$RAG$^{-/-}$ mice (T:E ratio, 1:5). The mortality of CD62L$^-$CD44$^+$ T$_{EM}$ was elevated compared with that of T$_{EM}$ alone when coincubated with NK cells derived from RAG$^{-/-}$ mice and was similar to that following coincubation with NK cells derived from IL-7$^{-/-}$RAG$^{-/-}$ mice (T:E ratio, 1:5; Fig. 5E).

Additionally, the expression levels of NK receptors (30) that reflect the function of NK cells (Fig. 5F), as well as the levels of CD11b and CD27 that determine the differentiation status of NK cells (31) (Fig. 5G), were not altered in NK cells derived from IL-7$^{-/-}$RAG$^{-/-}$ mice, compared with those from RAG$^{-/-}$ mice.

To further demonstrate that there are no differences of NK cell functions between RAG$^{-/-}$ and IL-7$^{-/-}$RAG$^{-/-}$, we also measured the cytotoxic activities of these cells against YAC-1 cells using the [^{51}Cr] release assay, as well as the production of IFN-γ from these cells. As seen in Fig. 5H and 5I, neither the cytotoxicities against YAC-1 cells nor IFN-γ production of NK cells was modified in the IL-7$^{-/-}$RAG$^{-/-}$ mice when compared with
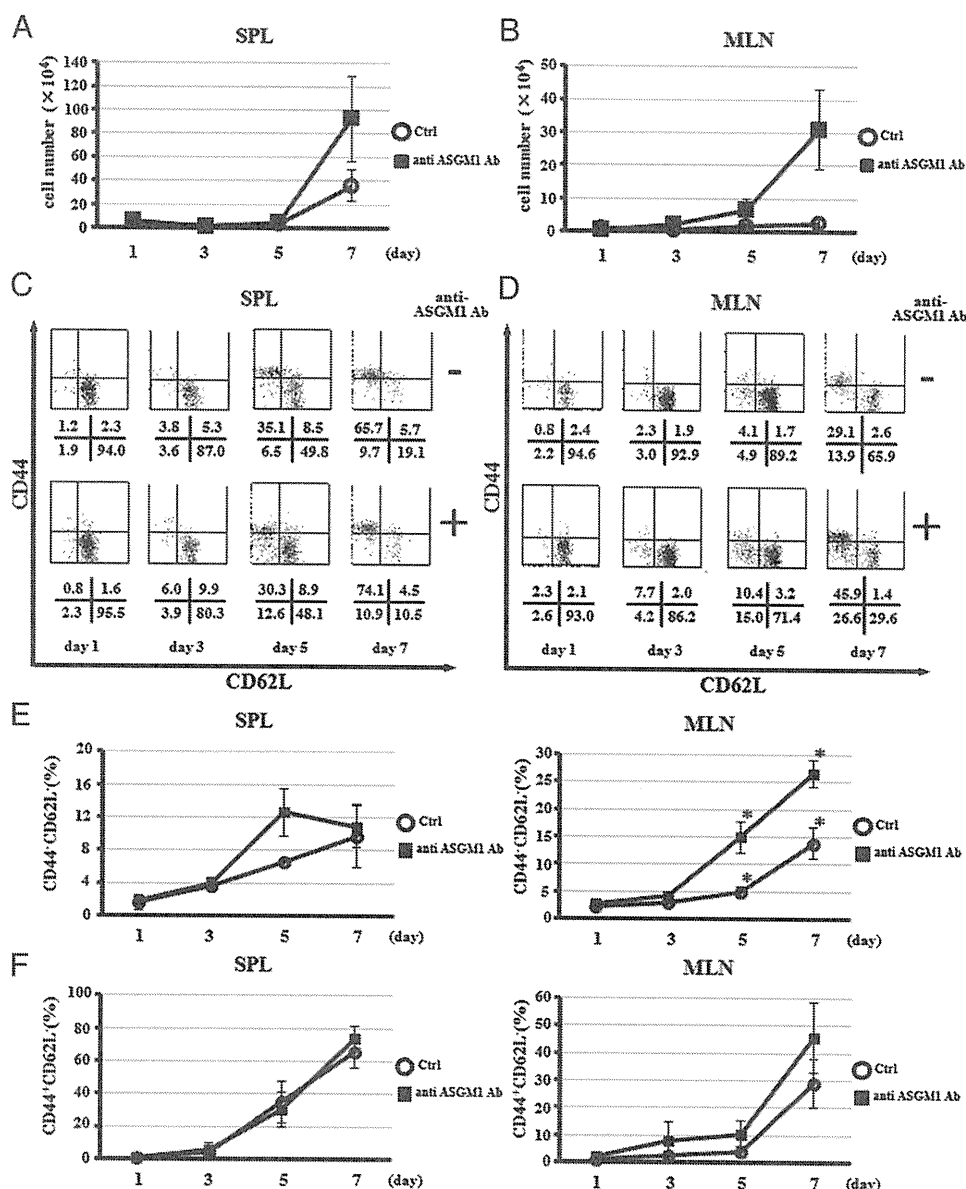
FIGURE 3. NK cell depletion results in the increase in CD44$^+$CD62L$^-$ and CD44$^-$CD62L$^-$ subsets in naive T cell-transferred RAG$^{-/-}$ mice. (**A** and **B**) Naive T cells derived from WT SP were adoptively transferred into RAG$^{-/-}$ mice that were preinjected with either vehicle control (O) or anti-ASGM1 Ab (■). Mice were sacrificed and the total number of CD4$^+$ T cells isolated from SP (A) or MLN (B) was counted. Cells were stained with PerCP-conjugated anti-CD3 and allophycocyanin-conjugated anti-CD4 Abs, and were then subjected to FACS to calculate the number of T cells in each sample. The number of CD4$^+$ T cells at the indicated time points is shown. Data are expressed as means ± SEM ($n = 4$). (**C** and **D**) The naive T cell-receiving RAG$^{-/-}$ mice that had been preinjected with either vehicle control or anti-ASGM1 Ab were sacrificed at the indicated time points after naive T cell transfer. The isolated lymphocytes from SP (C) or MLN (D) were stained with allophycocyanin-conjugated anti-CD4, PerCP-conjugated anti-CD3, FITC-conjugated anti-CD62L, and PE-conjugated anti-CD44 Abs and were subjected to FACS. Representative data from four experiments are shown. The numbers in each data quadrant indicate percentage of gated populations. (**E** and **F**) The percentage of CD44$^-$CD62L$^-$ cells in RAG$^{-/-}$ mice that received naive T cells with or without anti-ASGM1 Ab injection. Mice were sacrificed at the indicated time points, lymphocytes isolated from SP (E) or MLN (F) were stained with anti-CD3, anti-CD4, anti-CD62L, and anti-CD44 Abs and were then subjected to FACS to analyze the percentage of the subset. Data are expressed as means ± SEM from five experiments. *$p < 0.05$.

RAG$^{-/-}$ mice. These results confirm that a lack of IL-7 does not affect the cytotoxic activity of NK cells either in vitro or in vivo.

K cell depletion elicits severe colitis in naive T cell-transferred IL-7$^{-/-}$ RAG$^{-/-}$ recipient mice

We previously reported that the development of colitis is abrogated by a lack of IL-7. Given that NK cells can suppress T cells in vitro and in vivo independently of IL-7, we next assessed the influence of NK cells on colitis in the context of IL-7 deficiency in vivo. IL-7$^{-/-}$ RAG$^{-/-}$ mice were injected i.p. with naive T cells with or without anti-ASGM1 Ab treatment, and colitis was monitored after 12 wk

(Fig. 6A). As previously observed, the induction of colitis was completely abrogated in vehicle control-injected IL-7$^{-/-}$RAG$^{-/-}$ mice, as shown by clinical and histological scores and cytokine production from colonic LP lymphocytes, although the presence of occasional leukocytes was observed in colonic tissues (Fig. 6B–E). However, when anti-ASGM1 Ab was injected, IL-7$^{-/-}$RAG$^{-/-}$ mice showed elicitation of colitis and similar severity of clinical phenotypes, such as wasting and diarrhea, as did the groups of RAG$^{-/-}$ recipients with or without anti-ASGM1 Ab treatment (Fig. 6B). Consistent with these findings, a significant deterioration in histological findings, such as mucosal damage, cell infil-
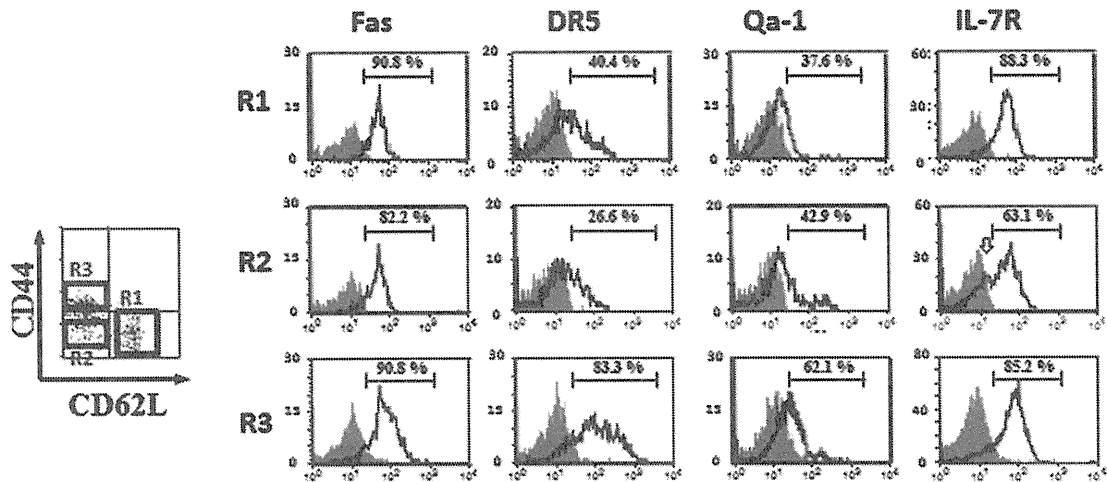
FIGURE 4. NK cells may target the CD62L⁻CD44⁻ subset by a different mechanism from that by which they target the CD62L⁻CD44⁺ subset. RAG⁻ᐟ⁻ mice preinjected with anti-ASGM1 Ab were sacrificed 5 d after naive T cell transfer. Isolated splenocytes were stained with anti-CD62L, anti-CD44, and either anti-Fas, anti-DR5, anti-Qa1, or anti-CD127 Abs (open histograms) or isotype-matched control (filled histograms) and were then subjected to FACS. The populations within the appropriate gate on forward scatter and side scatter and either CD62L⁺CD44⁻ (naive, R1), CD62L⁻CD44⁻ (R2) or CD62L⁻CD44⁺ (T$_{EM}$, R3) were analyzed. Representative data from three experiments are shown.

tration, and crypt abscesses, was also observed in IL-7⁻ᐟ⁻RAG⁻ᐟ⁻ mice when treated with anti-ASGM1 Ab (Fig. 6C, 6D) in association with the exacerbation in the clinical scores of these mice. Moreover, the production of cytokines such as IFN-γ, TNF-α, and IL-17 by colonic LPL from anti-ASGM1 Ab-treated IL-7⁻ᐟ⁻ RAG⁻ᐟ⁻ mice was significantly upregulated when compared with the vehicle control-treated group, despite the fact that their production was relatively lower than that of RAG⁻ᐟ⁻ groups with or without anti-ASGM1 Ab treatment (Fig. 6E). These results suggest that severe inflammation occurs in colonic tissues following NK cell depletion even in IL-7⁻ᐟ⁻RAG⁻ᐟ⁻ mice.

Additionally, to confirm the activities of cells that had infiltrated the tissues, absolute numbers of splenic and colonic LP CD4⁺ T cells isolated from these colitic mice were calculated (Fig. 7A) and analyzed by FACS (Fig. 7B, 7C). As seen in Fig. 7B and 7C, the percentage of NK1.1⁺ cells in both SP and colonic LP was greatly decreased in T cell-reconstituted mice treated with anti-ASGM1 Ab. Note that the percentages of NK1.1⁺ populations in both SP and colonic LP from T cell-reconstituted IL-7⁻ᐟ⁻RAG⁻ᐟ⁻ mice not treated with the anti-ASGM1 Ab were dramatically increased, because there were less CD4⁺ T cells in the tissues (Fig. 7A). Additionally, CD4⁺ T cells with a CD44⁺CD62L⁻ phenotype were observed in all mouse groups (Fig. 7B, 7C). However, the percentage of these cells was lower, especially in colonic LP, in IL-7⁻ᐟ⁻RAG⁻ᐟ⁻ recipient mice not treated with the anti-ASGM1 Ab relative to the other groups. Associated with this finding, the expression levels of IL-7R and CD69 in both splenic and colonic LP CD4⁺ T cells from IL-7⁻ᐟ⁻RAG⁻ᐟ⁻ recipient mice not treated with the anti-ASGM1 Ab were downregulated relative to the other groups (Fig. 7B, 7C). However, treatment with the anti-ASGM1 Ab resulted in an increase in CD4⁺CD44⁺CD62L⁻ T cells in both splenic and colonic LP, as well as upregulation of the expression of IL-7R and CD69 in IL-7⁻ᐟ⁻RAG⁻ᐟ⁻ recipient mice. These results indicate that the T cells reconstituted into IL-7⁻ᐟ⁻RAG⁻ᐟ⁻ recipient mice are still able to survive even 12 wk after injection, but that they somehow fail to differentiate sufficiently to induce colitis. Moreover, these data suggest that the depletion of NK cells in this context may assist the T cells to establish themselves as pathogenic T cells.

To further confirm whether such elicitation of pathogenic T cells in IL-7⁻ᐟ⁻RAG⁻ᐟ⁻ recipients was induced by NK cell depletion,

anti-NK1.1 Ab was used for the same model. IL-7⁻ᐟ⁻RAG⁻ᐟ⁻ mice were injected i.p. with naive T cells with or without anti-NK1.1 Ab treatment, and colitis was monitored after 12 wk (Fig. 8A). The IL-7⁻ᐟ⁻RAG⁻ᐟ⁻ recipients injected with anti-NK1.1 Ab showed severe colitis (Fig. 8B–D) with increased production of proinflammatory cytokines by the colonic LPL when compared with the isotype control-injected mice (Fig. 8E). These results suggested that the phenotypes shown in IL-7⁻ᐟ⁻RAG⁻ᐟ⁻ recipients may reflect NK cell regulation of T cell development in this model.

*NK cell depletion at an early stage is critical for the induction of colitis in naive T cell-transferred IL-7⁻ᐟ⁻RAG⁻ᐟ⁻ recipient mice*

Because NK cell depletion resulted in the exacerbation of colitis even in IL-7⁻ᐟ⁻RAG⁻ᐟ⁻ recipient mice, we finally examined the effect of NK cell depletion at early and late stages of colitis development in IL-7⁻ᐟ⁻RAG⁻ᐟ⁻ recipient mice. Mice receiving naive T cells were also injected every 48 h with either the vehicle control for 12 wk (Ctrl), anti-ASGM1 Ab for 12 wk (0–12 wk), anti-ASGM1 Ab for 4 wk followed by vehicle control for 8 wk (0–4 wk), or vehicle control for 4 wk followed by 8 wk anti-ASGM1 Ab (4–12 wk), and colitis was monitored after 12 wk (Fig. 9A). Mice injected with anti-ASGM1 Ab for the first 4 wk, or for the entire 12 wk, showed significantly more severe clinical phenotypes of colitis than did the other groups (Fig. 9B), which was associated with thickening and shortening of the colon and splenomegaly (Fig. 9C). Severe inflammation of the colon, as judged by histological analysis, was also noticeably induced in these two groups (Fig. 9D, 9E). However, mice injected with the anti-ASGM1 Ab at a later stage failed to induce colitis, although minor clinical symptoms and infiltration of a few cells into the colon were occasionally observed (Fig. 9B–E). Moreover, these degrees of severity of colitis were consistent with cytokine production from colitic LP T cells, since significantly upregulated IFN-γ and TNF-α production was observed in the groups treated with the anti-ASGM1 Ab either at the beginning or throughout the entire period, but not in the group treated with the Ab only at the later stage (Fig. 9F). Note that the level of IL-17 production in mice treated for the entire period with anti-ASGM1 Ab was significantly higher than that of mice treated with the Ab only at the
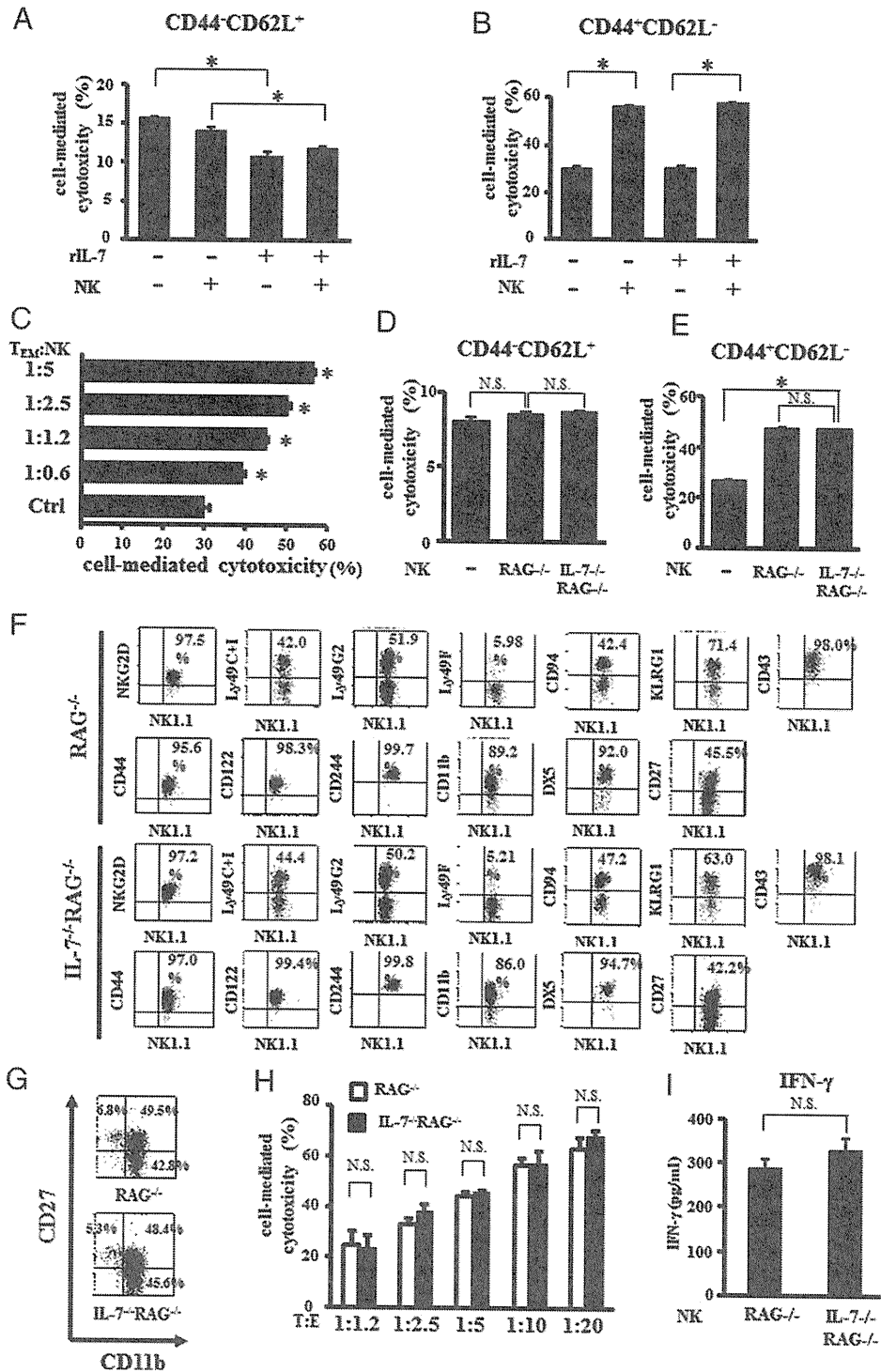
FIGURE 5. Cytotoxic activity of NK cells is not affected in the presence or absence of IL-7. (A–C) Splenic NK cells were isolated from WT mice by FACS sorting. Either CD4$^+$CD62L$^+$CD44$^-$ naive T cells isolated from WT SP (A) or CD4$^+$CD62L$^-$CD44$^+$ T$_{EM}$ from colonic LP in RAG$^{-/-}$ mice that received naive T cells 12 wk previously (B and C) were stained with PKH2 and cocultured as target (T) cells with the isolated NK cells as effector (E) cells, in the presence or absence of IL-7 for 4 h. Cells were then harvested and stained with PI. The PKH2 and PI double-positive population is assumed to represent dead target cells (28). The mortality of target cells was calculated as the ratio of dead PKH2$^+$ cells. (A) T:E ratio, 1:5, with or without rIL-7; (B) T:E ratio, 1:5, with or without rIL-7; (C) T:E ratio, 1:5, 1:2.5, 1:1.25, or 1:0.625, without rIL-7. Control (CD4$^+$ T cells alone) is also shown as a negative control. Data are expressed as means ± SEM from three experiments. *$p < 0.001$. (D and E) Splenic NK cells were isolated from either RAG$^{-/-}$ or IL-7$^{-/-}$ RAG1$^{-/-}$ mice by FACS sorting. Either the CD62L$^+$CD44$^-$ naive T (D) or the CD62L$^-$CD44$^+$ T$_{EM}$ (E) subset was stained with PKH2 and cocultured for 4 h with splenic NK cells derived from either RAG$^{-/-}$ or IL-7$^{-/-}$RAG1$^{-/-}$ mice. Cells were then stained with PI and subjected to the cytotoxic assay described above. Data are expressed as means ± SEM from three experiments. *$p < 0.001$. (F) Splenic NK cells were isolated from RAG$^{-/-}$ and IL-7$^{-/-}$ RAG$^{-/-}$ mice, and the expression of each NK receptor on these cells was assessed by FACS. The numbers indicate the percentage of cells positive for each NK receptor in the NK1.1-positive population. (G) Splenic NK cells isolated from either RAG$^{-/-}$ or IL-7$^{-/-}$RAG$^{-/-}$ mice were stained with anti-CD11b and anti-CD27 Abs and were then subjected to FACS to evaluate their differentiation status. The numbers indicate the quadrant percentages of each differentiation status in the NK1.1-positive population. (H) Splenic NK cells were isolated from RAG$^{-/-}$ (open) and IL-7$^{-/-}$ RAG$^{-/-}$ (filled) mice by FACS sorting. YAC-1 cells were labeled with Na$_2$[^{51}Cr]O$_4$ and cocultured as target (T) cells with the isolated NK cells as (*Figure legend continues*)

FIGURE 6. NK cell depletion with anti-ASGM1 Ab in naive T cell-receiving IL-7$^{-/-}$RAG$^{-/-}$ mice, as well as in RAG$^{-/-}$ recipients, results in the development of colitis. (**A**) Protocol for NK cell depletion in a colitis setting. Naive T cell-receiving RAG$^{-/-}$ and IL-7$^{-/-}$RAG$^{-/-}$ mice were injected with either anti-ASGM1 Ab (0–12 wk) or vehicle control (Ctrl) every second day for 12 wk starting from the day before adoptive transfer of naive T cells. (**B**) Clinical scores of each group are shown. Data are expressed as means ± SEM from four mice. *$p <$ 0.05. (**C**) Histological features of colons from naive T cell-transferred RAG$^{-/-}$ and IL-7$^{-/-}$RAG$^{-/-}$ recipients injected with either vehicle control (Ctrl) or anti-ASGM1 for 12 wk (0–12 wk). Representative features from four experiments are shown. (**D**) Histological scores of each group are shown. Data are expressed as means ± SEM from four mice. *$p <$ 0.05. (**E**) Cytokine production by LP T cells from each group is shown. Concentrations of IFN-γ (*left*), TNF (*middle*), and IL-17 (*right*) in the culture supernatant are measured by ELISA. Data are indicated as means ± SEM from four samples. *$p <$ 0.05, **$p <$ 0.01.
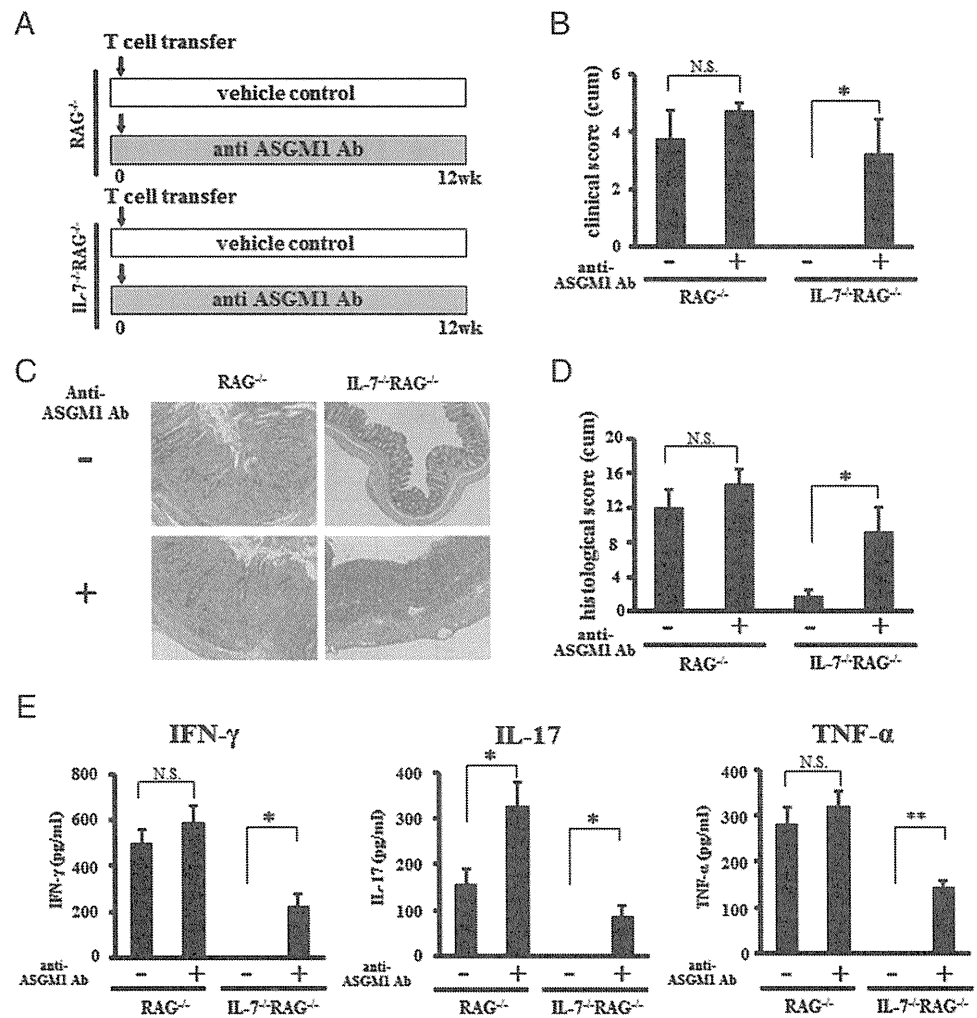
beginning (Fig. 9F), although there was no significant difference in either clinical or histological scores between these groups (Fig. 9B, 9E). These results suggest that NK cell depletion at the early stage, but not the late stage, of T_{EM} development is critical for the induction of colitis in IL-7$^{-/-}$RAG$^{-/-}$ recipient mice.

Discussion

We previously reported that adoptively transferred WT naive T cells injected into IL-7$^{-/-}$RAG$^{-/-}$ mice interestingly failed to induce colitis (10). However, it is known that IL-7 is not required for the in vitro differentiation of naive T cells into Th1 or Th17 cells (12). We therefore speculated that the reason why the IL-7$^{-/-}$RAG$^{-/-}$ mice that received naive T cells failed to maintain colitogenic CD4$^+$ T_{EM} may be associated not only with a lack of IL-7, but also with another mechanism that involves suppression of the primary stage of T_{EM} development in the recipients. We previously reported that apoptosis is preferentially induced in CD4$^+$ T cells when IL-7 is lacking in vivo. Thus, increased numbers of annexin V$^+$CD4$^+$ T cells were observed in IL-7$^{-/-}$RAG$^{-/-}$ recipient mice, into which these T cells had been adoptively transferred, compared with CD4$^+$ T cells in RAG$^{-/-}$ recipient mice (10). These data suggested that T cell suppression via apoptosis is

a mechanism by which colitis is abrogated in IL-7$^{-/-}$RAG$^{-/-}$ recipient mice. We therefore determined whether NK cells, which are known to induce apoptosis in CD4$^+$ T cells, may play a role in such T cell suppression.

Several reports have suggested that NK cells suppress the inflammation caused by autoimmune responses not only in animal models such as EAE and collagen-induced arthritis, but also in clinical samples from patients with multiple sclerosis and systemic lupus erythematosus in humans (20–22, 28, 32, 33). For example, depletion of NK cells using Abs against NK1.1 or ASGM1 results in disease exacerbation in the EAE model (22, 28). Additionally, it has also been reported that NK cell depletion exacerbates an animal model of colitis, although the details underlying the mechanism have not been elucidated (24).

In the present study, NK cells were depleted in the naive T cell adoptively transferred colitis model to analyze the role of NK cells in this model. RAG$^{-/-}$ and IL-7$^{-/-}$RAG$^{-/-}$ mice that had received naive T cells were depleted of NK cells using an anti-ASGM1 (Figs. 1, 2, 6, 9, Supplemental Figs. 1, 2). However, it was of concern that ASGM1 may be expressed not only in NK cells but also in some subsets of T cells and macrophages when activated (34). Therefore, we also administered anti-NK1.1 Ab

effector (E) cells for 4 h. T:E ratio, 1:20, 1:10, 1:5, 1:2.5, or 1:1.25. Data are expressed as means ± SEM from three experiments. (I) Cytokine production by NK cells from each group is shown. Concentrations of IFN-γ in the culture supernatant are measured by ELISA. Data are indicated as means ± SEM from four samples.

FIGURE 7. Colitogenic T_{EM} are induced in naive T cell-receiving IL-7$^{-/-}$RAG$^{-/-}$ by NK cell depletion. (**A**) Absolute numbers of CD4$^+$ T cells are shown. CD4$^+$ SPL (*left*) or colonic LPL (*right*) were isolated from naive T cell-receiving RAG$^{-/-}$ and IL-7$^{-/-}$ RAG$^{-/-}$ mice injected with either vehicle control (−) or anti-ASGM1 Ab (+) for 12 wk. Data are expressed as means ± SEM from five mice. *$p < 0.001$. (**B** and **C**) Isolated SPL (B) or colonic LPL (C) were stained with anti-CD4 and either anti-CD44, anti-CD127/IL-7Rα, anti-CD69, or anti-NK1.1 Abs and were then subjected to FACS analysis. Representative data from four experiments are shown.
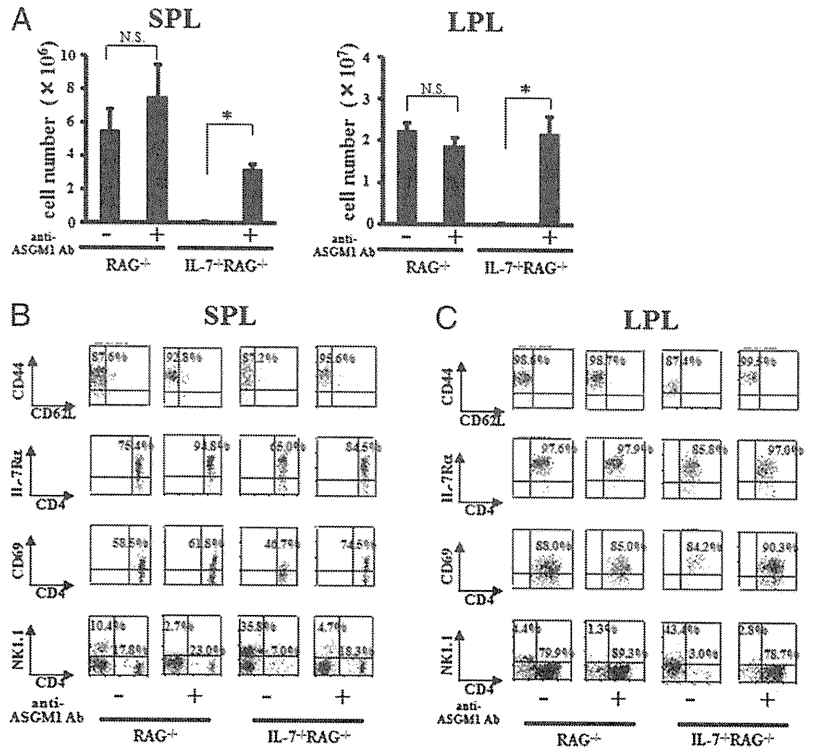
using another experimental approach to confirm that the phenotypes shown in this model were induced by NK cell depletion (Fig. 8). Note that administration of anti-ASGM1 without T cell reconstitution to the IL-7$^{-/-}$RAG$^{-/-}$ mice does not trigger any inflammation in the colon (Supplemental Fig. 2). Also note that the appropriate controls, such as the same amount of rabbit Ig as a control for anti-ASGM1 polyclonal Ab and mouse IgG2a as an

isotype-matched control for anti-NK1.1 (PK136), respectively, do not induce colitis in the recipients either (Figs. 2, 8, Supplemental Fig. 2). Interestingly, NK cell depletion at an early stage during colitis induction resulted in exacerbated colitis in the recipient, even in IL-7$^{-/-}$ RAG$^{-/-}$ recipient mice, in association with increased clinical and histological scores as well as upregulated cytokine production by colonic LP T cells. We observed strong
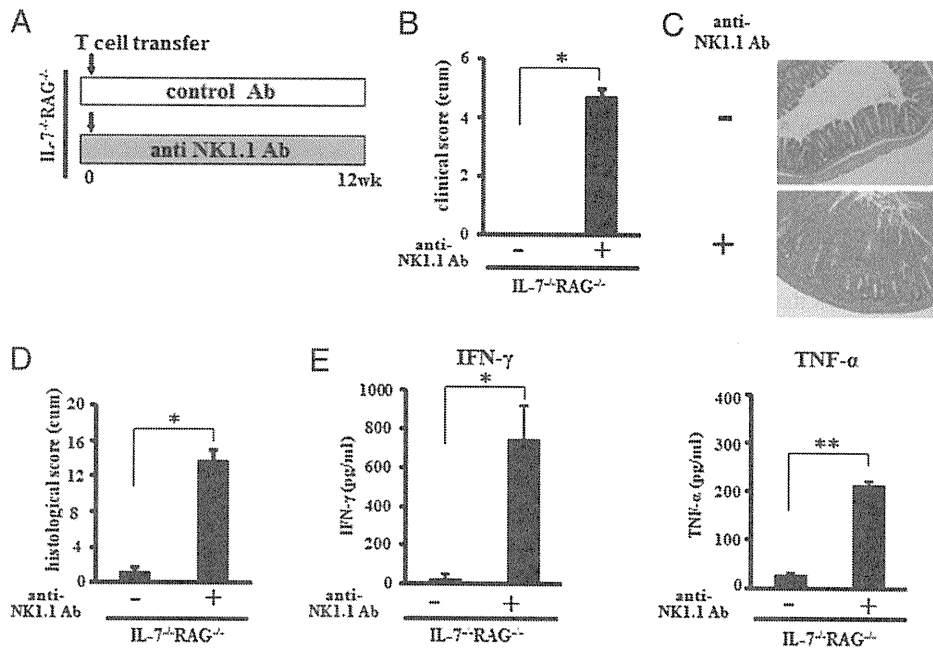
FIGURE 8. NK cell depletion with anti-NK1.1 Ab in naive T cell-receiving IL-7$^{-/-}$RAG$^{-/-}$ mice results in the elicitaion of colitis. (**A**) Protocol for NK cell depletion in a chronic colitis setting. IL-7$^{-/-}$RAG$^{-/-}$ mice receiving naive T cells were injected with either 0.5 mg/mouse anti-NK1.1 Ab or isotype control every second day for 12 wk. (**B**) Clinical scores of each group are shown. Data are expressed as means ± SEM from five mice. *$p < 0.001$. (**C**) Histological feature of colons from naive T cell-transferred IL-7$^{-/-}$RAG$^{-/-}$ recipients injected with isotype control (−, *top*) or anti-NK1.1 Ab (+, *bottom*). Representative features from each group are shown. (**D**) Histological scores of each group are shown. Data are expressed as means ± SEM from five mice. *$p < 0.001$. (**E**) Cytokine production by LP T cells from each group is shown. Concentrations of IFN-γ (*left*) and TNF-α (*right*) in the culture supernatant were measured by ELISA. Data are indicated as means ± SEM from five samples. *$p < 0.05$, **$p < 0.001$.
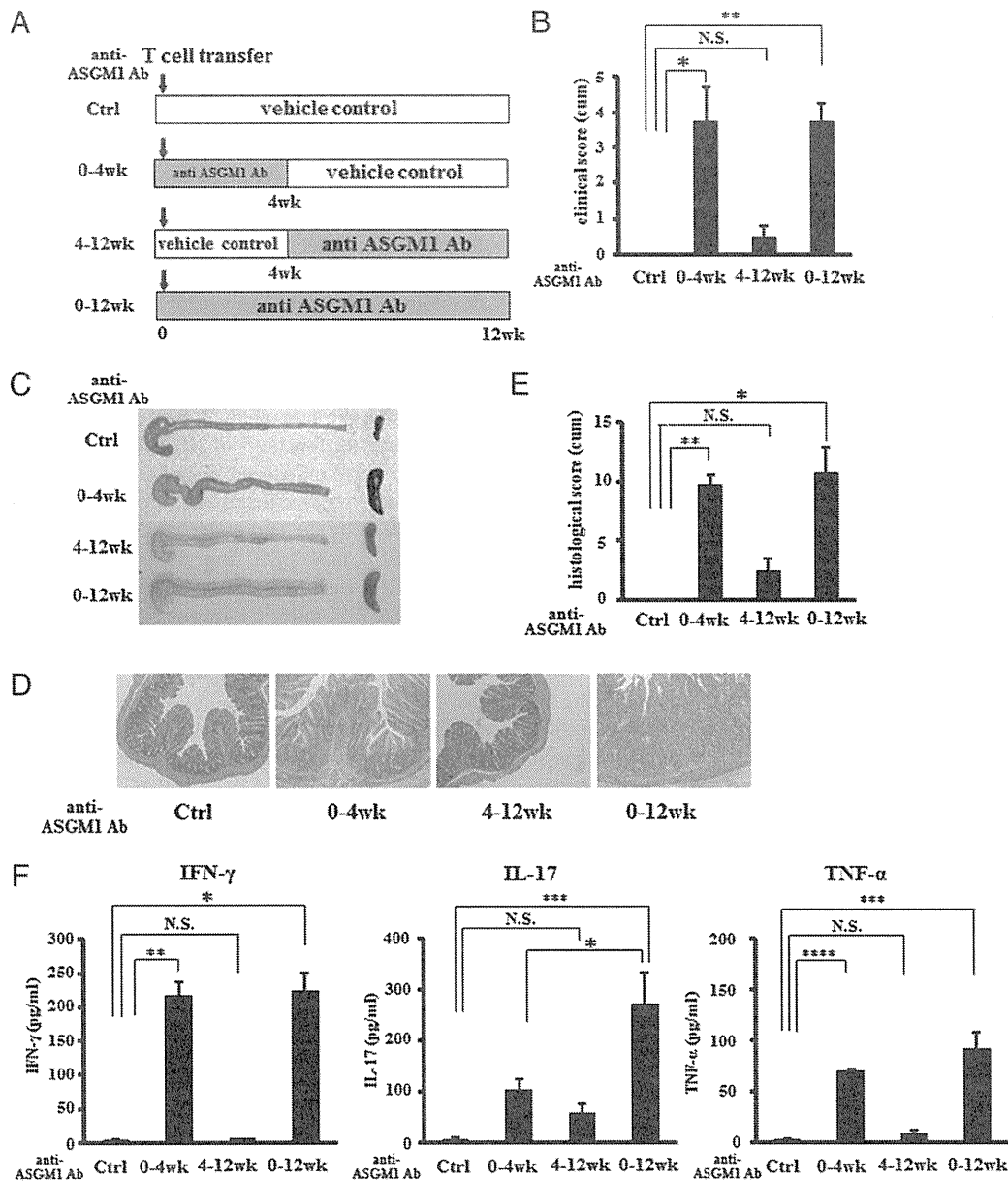
FIGURE 9. NK cell depletion at the early stage, but not at a late stage, in naive T cell-receiving IL-7$^{-/-}$RAG$^{-/-}$ mice results in the elicitation of massive colitis. (**A**) Protocol for NK cell depletion in a setting of chronic colitis. IL-7$^{-/-}$RAG$^{-/-}$ mice were injected with either vehicle control (Ctrl) or anti-ASGM1 Ab (0–12 wk) for 12 wk, anti-ASGM1 Ab for 4 wk followed by vehicle control for 8 wk (0–4 wk), or vehicle control for 4 wk followed by anti-ASGM1 Ab for 8 wk (4–12 wk). (**B**) Clinical scores of each group are shown. Data are expressed as means ± SEM from four mice. *p < 0.05, **p < 0.005. (**C**) Gross appearance of colons (*left*) and SP (*right*) from naive T cell-transferred IL-7$^{-/-}$RAG$^{-/-}$ recipients injected with either vehicle control for 12 wk (Ctrl), anti-ASGM1 for 4 wk and then vehicle control for 8 wk (0–4 wk), vehicle control for 4 wk and then anti-ASGM1 Ab for 8 wk (4–12 wk), or anti-ASGM1 Ab for 12 wk (0–12 wk). Representative features from four experiments are shown. (**D**) Histological feature of colons from naive T cell-transferred IL-7$^{-/-}$ RAG$^{-/-}$ recipients injected with either control for 12 wk (Ctrl), anti-ASGM1 for 4 wk and then control for 8 wk (0–4 wk), vehicle control for 4 wk and then anti-ASGM1 Ab for 8 wk (4–12 wk), or anti-ASGM1 for 12 wk (0–12 wk). Representative features of each group are shown. (**E**) Histological scores of each group are shown. Data are expressed as means ± SEM from four mice. *p < 0.05, **p < 0.01. (**F**) Cytokine production by LP T cells from each group is shown. Concentrations of IFN-γ (*left*), TNF (*middle*), and IL-17 (*right*) in the culture supernatant were measured by ELISA. Data are indicated as means ± SEM from four samples. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001.

infiltration in colonic tissues ~4 wk after the adoptive transfer into RAG$^{-/-}$ recipients (10). We therefore compared the effect of NK cell depletion by treatment with an anti-ASGM1 Ab at early (0–4 wk) or late stages (4–12 wk) after naive T cell transfer to treatment over the entire 12-wk period (0–12 wk) after transfer. Ab treatment at the early stage and over the entire 12 wk resulted in a similar degree of colitis exacerbation whereas Ab treatment at the late stage did not exacerbate colitis (Figs. 1, 9). Such exacerbation of colitis occurred relatively latent in the presence of IL-7 in the RAG$^{-/-}$ compared with the IL-7$^{-/-}$RAG$^{-/-}$ recipients

when sacrificed at 12 wk after T cell transfer (Figs. 6, 7). However, the difference of colitis severity in the RAG$^{-/-}$ recipients with or without Ab treatment was interestingly remarkable when sacrificed at 6 wk after T cell receiving (Fig. 2). These results imply that NK cell function is critical for colitogenic T cell suppression at the early stage of colitis development.

Because the CD4$^+$CD44$^+$CD62L$^-$ colitogenic T$_{EM}$ in the recipients were suggested to be suppressed at the early stage by NK cells (Figs. 1, 2, 9), we further analyzed the effect of NK cells on the development of CD4$^+$ T cells within a week after recon-