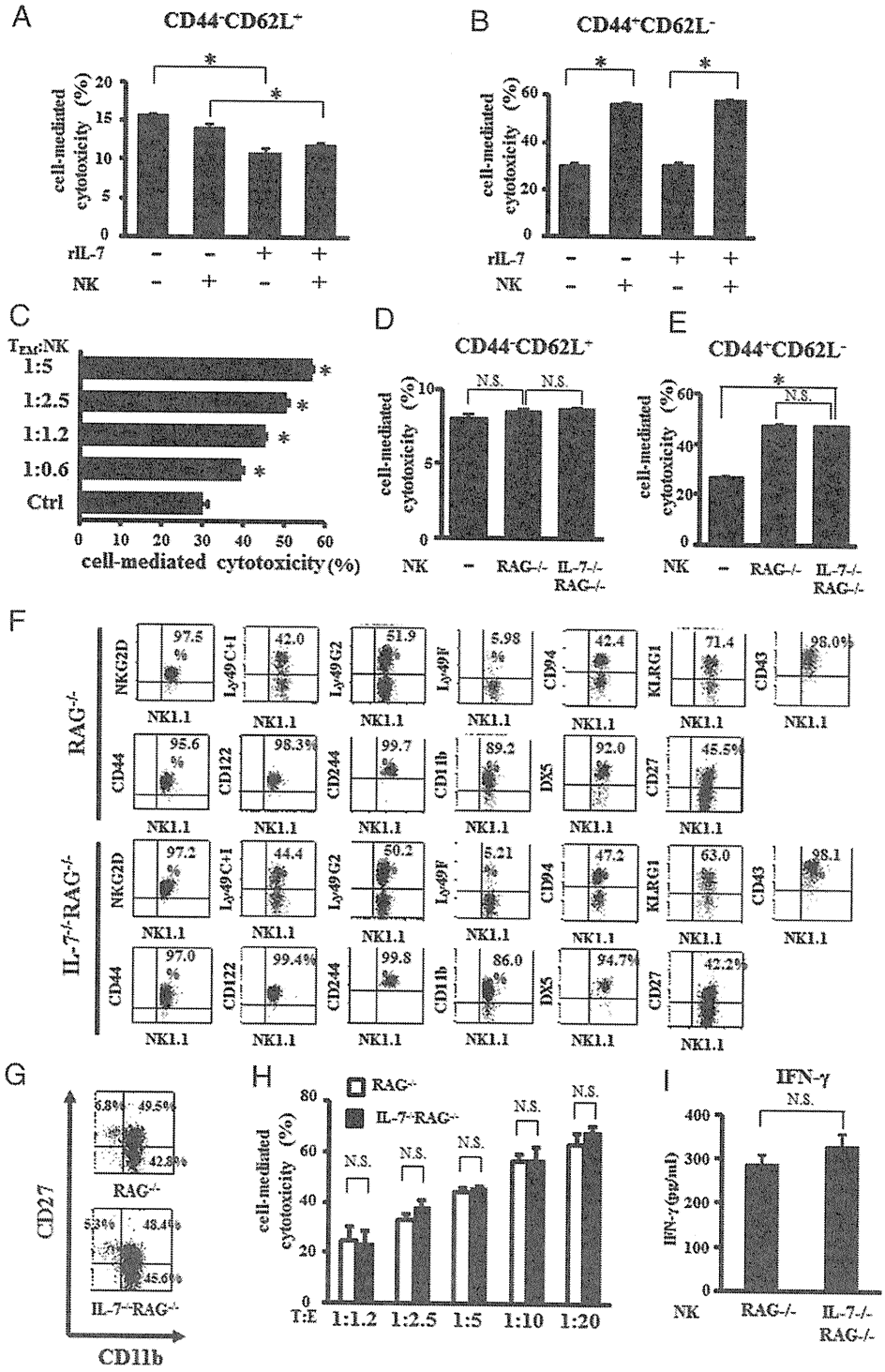

FIGURE 5. Cytotoxic activity of NK cells is not affected in the presence or absence of IL-7. (**A–C**) Splenic NK cells were isolated from WT mice by FACS sorting. Either $CD4^+CD62L^+CD44^-$ naive T cells isolated from WT SP (A) or $CD4^+CD62L^-CD44^+$ T_{EM} from colonic LP in $RAG^{-/-}$ mice that 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 $IL\text{-}7^{-/-}$ $RAG1^{-/-}$ 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 $IL\text{-}7^{-/-}RAG1^{-/-}$ 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 $IL\text{-}7^{-/-}$ $RAG^{-/-}$ 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 $IL\text{-}7^{-/-}RAG^{-/-}$ 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 $IL\text{-}7^{-/-}$ $RAG^{-/-}$ (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)*

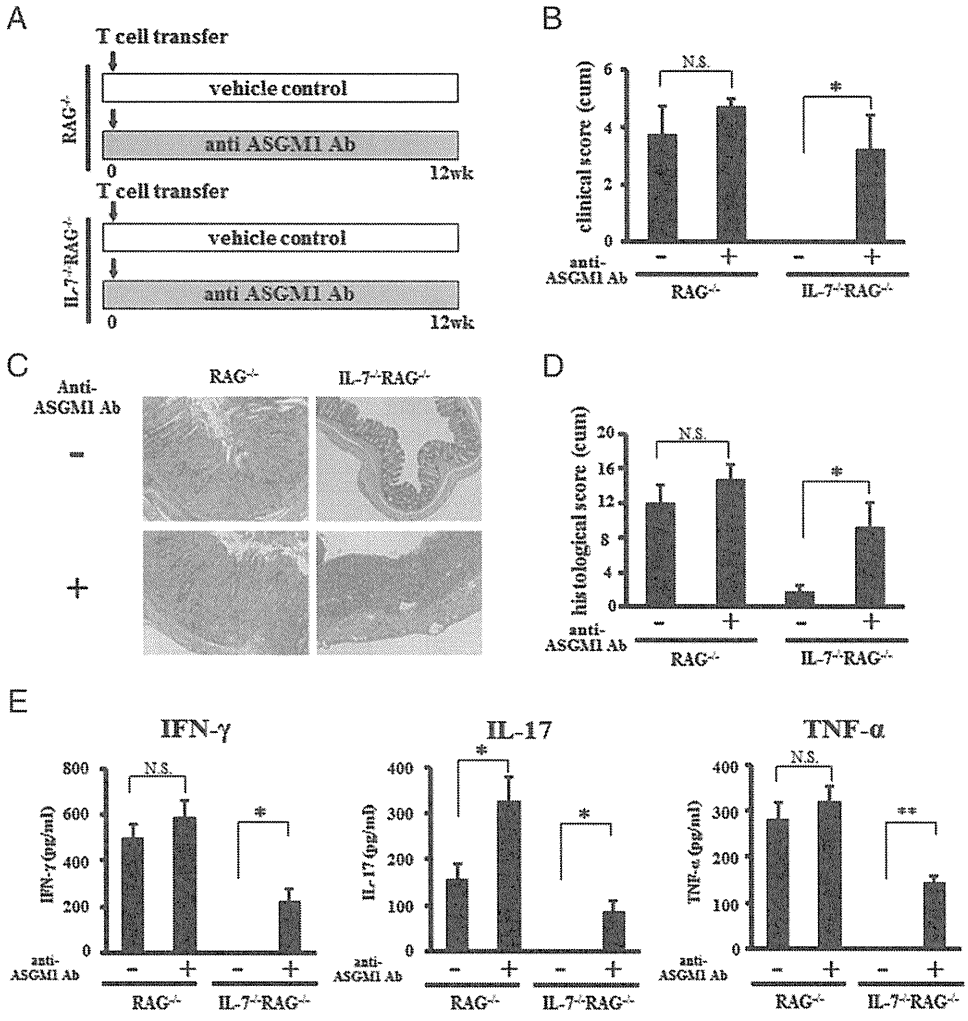

FIGURE 6. NK cell depletion with anti-ASGM1 Ab in naive T cell-receiving IL-7$^{-/-}$RAG$^{-/-}$ mice, as well as in RAG$^{-/-}$ recipients, results in the development of colitis. (**A**) Protocol for NK cell depletion in a colitis setting. Naive T cell-receiving RAG$^{-/-}$ and IL-7$^{-/-}$RAG$^{-/-}$ 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.

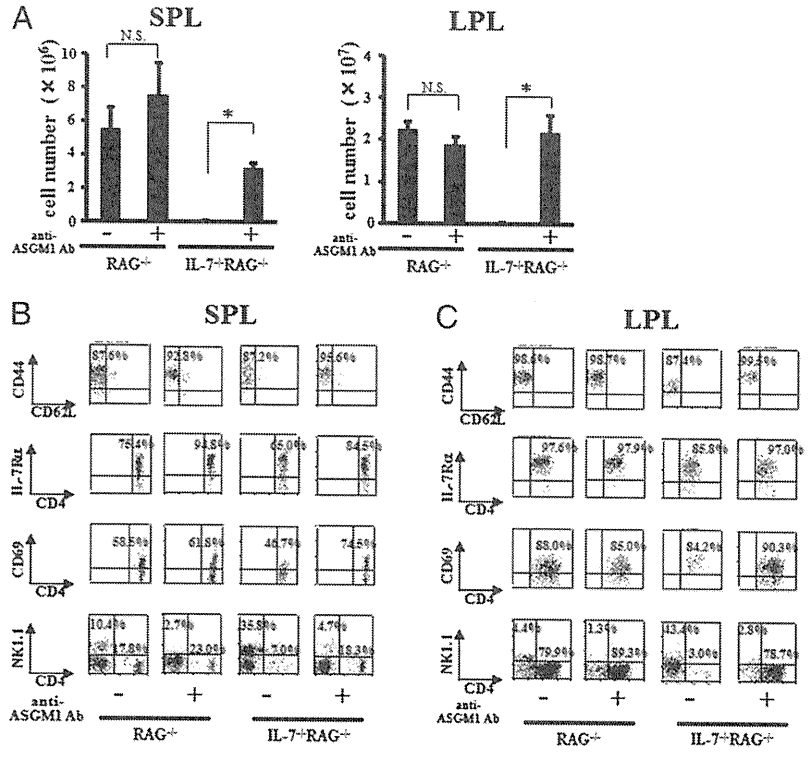

FIGURE 7. Colitogenic T_{EM} are induced in naive T cell-receiving IL-$7^{-/-}$RAG$^{-/-}$ by NK cell depletion. (**A**) Absolute numbers of $CD4^+$ T cells are shown. $CD4^+$ SPL (*left*) or colonic LPL (*right*) were isolated from naive T cell-receiving RAG$^{-/-}$ and IL-$7^{-/-}$ RAG$^{-/-}$ mice 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

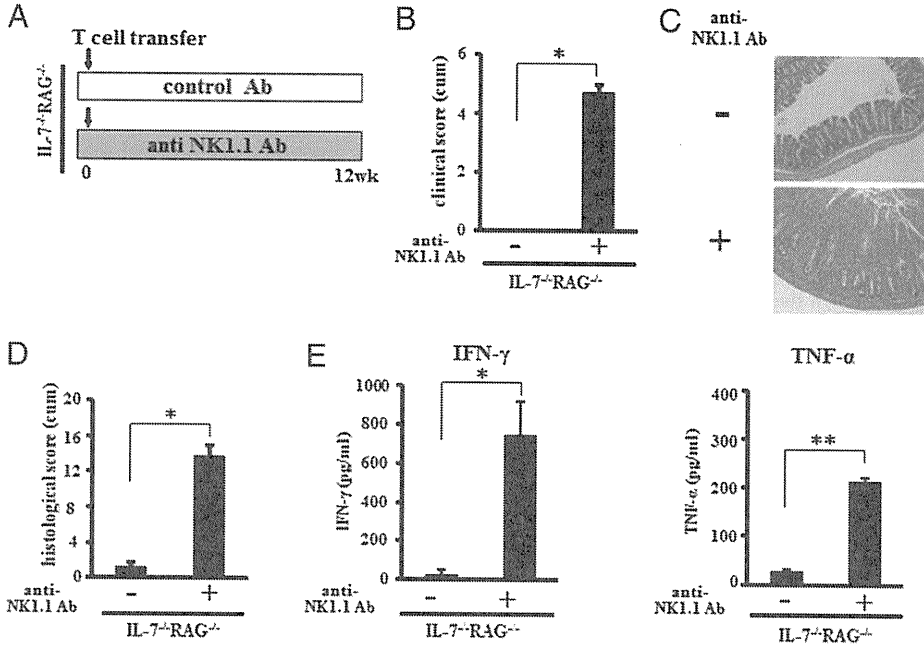

FIGURE 8. NK cell depletion with anti-NK1.1 Ab in naive T cell-receiving IL-$7^{-/-}$RAG$^{-/-}$ mice results in the elicitaion of colitis. (**A**) Protocol for NK cell depletion in a chronic colitis setting. IL-$7^{-/-}$RAG$^{-/-}$ mice receiving naive T cells were injected with either 0.5 mg/mouse 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. *$p < 0.05$, **$p < 0.001$.

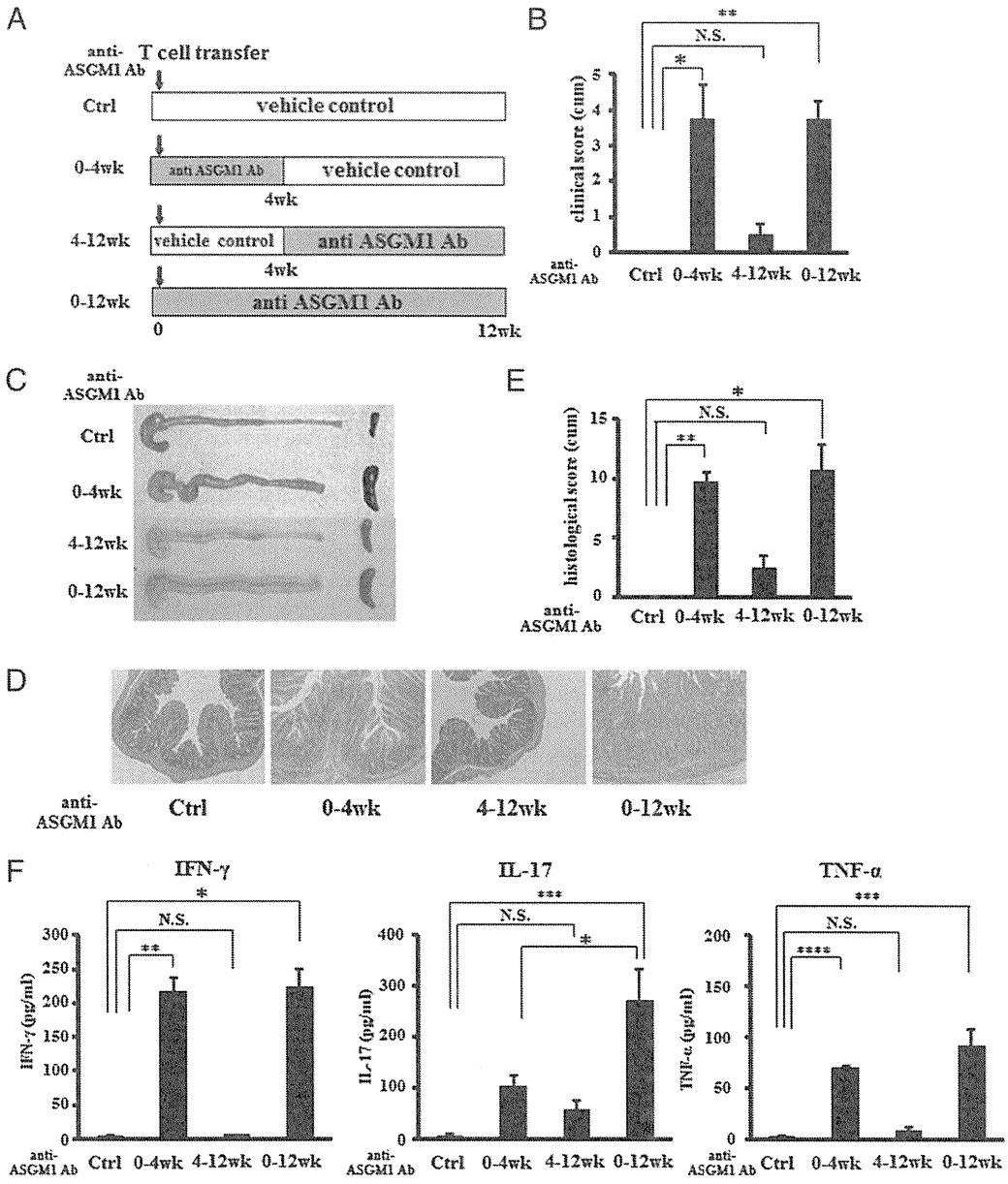

FIGURE 9. NK cell depletion at the early stage, but not at a late stage, in naive T cell-receiving IL-$7^{-/-}$RAG$^{-/-}$ mice results in the elicitation of massive colitis. (**A**) Protocol for NK cell depletion in a setting of chronic colitis. IL-$7^{-/-}$RAG$^{-/-}$ mice were injected with either 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-

stitution into the $RAG^{-/-}$ recipients (Fig. 3). The number of $CD4^+$ T cells in SPL and MLN was significantly increased 5–7 d after the transfer when NK cells were depleted compared with the control (Fig. 3A, 3B). Additionally, the significant increase of the $CD44^+CD62L^-$ T_{EM} subset was observed at this point when NK cells were depleted. $CD4^+CD44^+CD62L^-$ colitogenic T_{EM} are suggested to be susceptible to cell death when they are activated. We therefore analyzed the expression of several markers characteristic of NK cell targets on the $CD44^+CD62L^-$ T_{EM} subset, such as Fas, DR5, and Qa-1, which are the specific receptors or ligand for Fas ligand, TRAIL, and NKG2A, respectively (Fig. 4). As expected, this T cell subset expresses high levels of Fas and DR5, thereby making them susceptible to apoptosis (20). Additionally, these T cells also express some but not a significant level of Qa-1, which induces inhibitory signaling in NK cells via NKG2A. These data indicate that NK cells may suppress $CD4^+CD44^+CD62L^-$ colitogenic T_{EM} via apoptosis, and consistent with our previous observation of downregulated Bcl-2 and upregulated annexin V in $CD4^+$ T cells by the lack of IL-7 in vivo (10).

Furthermore, we also observed an increased unique T cell subset, $CD44^-CD62L^-$, when NK cells were depleted (Fig. 3C–E). We were able to observe these cells in the SPL and MLN within 2 wk after T cell transfer into RAG mice, and subsequently they were not detectable afterward (Fig. 7B, 7C). The fact that the $CD44^-$ $CD62L^-$ T cell subset was only observed at the beginning of colitogenic T cell development would suggest that this interesting population may be associated with the importance of early stage at the pathogenic T cell development in this chronic colitis model. This T cell subset, which is distinct from $CD44^+CD62L^-$ T_{EM}, is likely to be a second target of NK cells. However, the expressions of Fas and DR5 are lower on these cells compared with those of the $CD44^+CD62L^-$ T_{EM} (Fig. 4). The expression of Qa-1 in $CD44^-CD62L^-$ is not greatly different from that of the $CD44^+$ $CD62L^-$ subset. This phenotype of the $CD44^-CD62L^-$ subset does not suggest that it is a target of NK cells. However, a recent report showed that CD44 expression on Th1 cells is required to prevent apoptosis via Fas signaling (35). Thus, the $CD44^-$ $CD62L^-$ subset may be susceptible to apoptosis, since these cells still express some level of Fas on their surface. This may be one of the reasons why early stage of T cell development in this colitis model is targeted by NK cells. Additionally, this possibility may be a potential reason why Th1 cells fail to survive when transferred into $IL\text{-}7^{-/-}RAG^{-/-}$ mice. It is also possible that NK cells may regulate $CD44^+CD62L^-$ and $CD44^-CD62L^-$ cells by different mechanisms. Analysis of IL-7R expression levels of the $CD44^-CD62L^-$ subset revealed two distinct populations: $IL\text{-}7R^{hi}$ and $IL\text{-}7R^{lo}$ (indicated with an arrow in Fig. 4). The $IL\text{-}7R^{lo}$ population in this subset could potentially arise due to transient downregulation of IL-7R expression during differentiation. Unfortunately, the scarcity of these cells prohibited their further analysis and characterization. However, these cells still need to be further studied.

Our recent studies suggested that $IL\text{-}7^{-/-}RAG^{-/-}$ mice were able to induce colitis when parabiosed with colitic $RAG^{-/-}$ recipient mice that had received naive T cells 6 wk previously (15). Moreover, deparabiosed $IL\text{-}7^{-/-}RAG^{-/-}$mice, which were surgically separated from T cell-receiving $RAG^{-/-}$–$IL\text{-}7^{-/-}RAG^{-/-}$ parabionts 6 wk after the initial surgery, still maintained chronic colitis for at least another 12 wk (16). The latter finding is similar to our present observation that $IL\text{-}7^{-/-}RAG^{-/-}$ recipient mice, which had been depleted of NK cells at an early stage during induction, showed elicited colitis, even after completion of the anti-ASGM1 Ab treatment (Fig. 9). However, the mechanism by which the colitogenic T cells are maintained in the $IL\text{-}7^{-/-}RAG^{-/-}$ mice after the establishment of massive colitis is still unclear. One potential interpretation is that the pathogenic T cells can continue to proliferate, resulting in induction of colitis when the T cell number exceeds the capacity of the NK cells to suppress the T cells. A second possibility is based on the recent report that IL-17 inhibits NK cell-suppressive ability (36). It has been suggested that the increased IL-17 production from T cells that occurs when the severity of the colitis increases may affect NK cell function. The latter possibility is supported by one of our observations that NK cell depletion starting at the late stage of colitis development failed to exacerbate colitis (Supplemental Fig. 1).

We observed that the characteristics of NK cells are not modified by the lack of IL-7 in $RAG^{-/-}$ mice (Fig. 5F). This observation is consistent with a previous report by Vosshenrich et al. (37) showing that the lack of IL-7 does not affect the growth, phenotype, or effector functions of NK cells in vivo, although IL-7 had been reported to influence NK cell differentiation. Consistent with this, we also observed that the differentiation of NK cells, which is characterized by the expression of CD11b and CD27 (31), is not altered in the same mice (Fig. 5G). Additionally, there is no significant difference between NK cells derived from $RAG^{-/-}$ and $IL\text{-}7^{-/-}RAG^{-/-}$ mice in terms of their cytotoxic activities against the target cells such as T cells and YAC-1 cells (Fig. 5D, 5E, 5H) as well as the production of IFN-γ (Fig. 5I). These data indicate that the dramatic difference in the severity of colitis between $IL\text{-}7^{-/-}RAG^{-/-}$ and $RAG^{-/-}$ recipients following NK depletion is not caused by a difference in NK function between NK cells derived from $RAG^{-/-}$ and $IL\text{-}7^{-/-}RAG^{-/-}$ mice.

The $IL\text{-}7^{-/-}RAG^{-/-}$ recipient mice that received naive T cells failed to induce colitis even though the cytotoxicity of NK cells was not altered. One potential explanation of this result is that the susceptibility of T cells to apoptosis is increased in these mice. It has been reported by others that the expression of Bcl-2, an anti-apoptotic molecule, in T cells is downregulated in $IL\text{-}7^{-/-}$ mice (38, 39). We have also reported that Bcl-2 expression is downregulated in T cells injected into $IL\text{-}7^{-/-}RAG^{-/-}$ recipient mice (10). A second explanation is based on our previous report that IL-7 contributes to the expansion of colitogenic T cells (39). Thus, these data suggest that colitogenic T cells are not able to survive in the mice due to their reduced expansion and increased susceptibility to apoptosis at the early stage of colitis development.

In this study, we demonstrate NK cell-mediated regulation of T cell development, which is associated with the pathogenesis of chronic colitis. Although the detailed mechanism still remains to be elucidated, an insight into such a mechanism is significant for understanding the regulation of mucosal immune responses.

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Disclosures

The authors have no financial conflicts of interest.

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Upregulated IL-7 Receptor α Expression on Colitogenic Memory CD4$^+$ T Cells May Participate in the Development and Persistence of Chronic Colitis

Tamako Shinohara,*[,1] Yasuhiro Nemoto,*[,1] Takanori Kanai,[†] Kaori Kameyama,* Ryuichi Okamoto,* Kiichiro Tsuchiya,* Tetsuya Nakamura,* Teruji Totsuka,* Koichi Ikuta,[‡] and Mamoru Watanabe*

We have previously demonstrated that IL-7 is essential for the persistence of colitis as a survival factor of colitogenic IL-7Rα–expressing memory CD4$^+$ T cells. Because IL-7Rα is broadly expressed on various immune cells, it is possible that the persistence of colitogenic CD4$^+$ T cells is affected by other IL-7Rα–expressing non-T cells. To test this hypothesis, we conducted two adoptive transfer colitis experiments using IL-7Rα$^{-/-}$ CD4$^+$CD25$^-$ donor cells and IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ recipient mice, respectively. First, IL-7Rα expression on colitic lamina propria (LP) CD4$^+$ T cells was significantly higher than on normal LP CD4$^+$ T cells, whereas expression on other colitic LP immune cells, (e.g., NK cells, macrophages, myeloid dendritic cells) was conversely lower than that of paired LP cells in normal mice, resulting in predominantly higher expression of IL-7Rα on colitogenic LP CD4$^+$ cells, which allows them to exclusively use IL-7. Furthermore, RAG-2$^{-/-}$ mice transferred with IL-7Rα$^{-/-}$ CD4$^+$CD25$^-$ T cells did not develop colitis, although LP CD4$^+$ T cells from mice transferred with IL-7Rα$^{-/-}$ CD4$^+$CD25$^-$ T cells were differentiated to CD4$^+$ CD44^{high}CD62L$^-$ effector-memory T cells. Finally, IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice transferred with CD4$^+$CD25$^-$ T cells developed colitis similar to RAG-2$^{-/-}$ mice transferred with CD4$^+$CD25$^-$ T cells. These results suggest that IL-7Rα expression on colitogenic CD4$^+$ T cells, but not on other cells, is essential for the development of chronic colitis. Therefore, therapeutic approaches targeting the IL-7/IL-7R signaling pathway in colitogenic CD4$^+$ T cells may be feasible for the treatment of inflammatory bowel diseases. *The Journal of Immunology*, **2011, 186: 2623–2632.**

Inflammatory bowel disease (IBD) is characterized by idiopathic chronic intestinal inflammation, which commonly takes a persistent course with lifelong recurrence (1–4). According to current understanding, IBD is caused by inappropriate responses of the activated immune system to intestinal commensal bacteria in patients with a genetically susceptible background. Above all, effector CD4$^+$ T cells including Th1, Th2, and Th17 are highlighted in the pathogenesis of IBD, because some groups have reported the association between genes involved in the Th17/IL-23 pathway and IBD (5, 6). Alternatively, we have investigated the possibility that long-lived memory CD4$^+$ T cells are the main cause of the persistence of IBD and have proved the importance of IL-7 for the maintenance system of memory CD4$^+$ T cells in chronic colitis (7).

IL-7 is a stromal cell-derived cytokine that is secreted by fetal liver cells, stromal cells in the bone marrow, and the thymus and other epithelial cells, including intestinal goblet cells (8, 9). Recently, IL-7 has emerged as a critical key cytokine involved in controlling the survival of peripheral resting CD4$^+$ T cells, including naive and memory cells, but not effector cells, and their homeostatic turnover proliferation (8–15). The effect of IL-7 on CD4$^+$ T cells is controlled by the expression of the specific receptors for IL-7, the state of differentiation of the T cells, the available concentration of IL-7, and whether there is concomitant TCR signaling (16, 17).

In contrast to the role of IL-7 in naive and memory CD4$^+$ T cells in the resting state, the pathologic role of IL-7 in chronic immune-mediated diseases, such as autoimmune diseases and IBD, remains largely unclear. We have previously demonstrated that 1) IL-7 is constitutively produced by intestinal epithelial cells, especially by goblet cells (18); 2) IL-7 transgenic mice developed chronic colitis that mimicked histopathologic characteristics of human IBD (19); 3) colonic lamina propria (LP) CD4$^+$IL-7Rα^{high} T cells in RAG-2$^{-/-}$ mice in which colitis was induced by adoptive transfer of CD4$^+$CD45RB^{high} T cells have characteristics of colitogenic memory T cells (20); 4) the selective elimination of CD4$^+$IL-7Rα^{high} T cells by administrating toxin-conjugated anti–IL-7Rα mAb completely ameliorated ongoing colitis in TCR-α–deficient mice (21); and 5) IL-7 is essential for the persistence of colitis by showing that IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice transferred with colitogenic LP CD4$^+$ T cells did not develop colitis (22).

*Department of Gastroenterology and Hepatology, Graduate School, Tokyo Medical and Dental University, Tokyo 113-8519, Japan; [†]Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo 160-8852, Japan; and [‡]Laboratory of Biological Protection, Department of Biological Responses, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan

[1]T.S. and Y.N. contributed equally to this work.

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Address correspondence to Dr. Mamoru Watanabe, Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. E-mail address: mamoru.gast@tmd.ac.jp

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Abbreviations used in this article: IBD, inflammatory bowel disease; LP, lamina propria; MFI, mean fluorescence intensity; SP, spleen; T_{EM}, effector-memory T; Treg, regulatory T cell; TSLP, thymic stromal lymphopoietin; WT, wild type.

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We hypothesize that the dysregulated IL-7/IL-7Rα pathway is critically involved in the pathogenesis of animal models of chronic colitis and human IBD, although IL-7 seems to be strictly regulated at a constant level as a homeostatic cytokine to maintain the number of $CD4^+$ memory T cells in the body.

IL-7R consists of the α-chain (CD127) and the cytokine receptor γ-chain (IL-2Rγ; CD132), which is shared by the common γ-chain family cytokines (IL-2, IL-4, IL-9, IL-15, and IL-21) (14, 15). Because IL-7Rα is broadly expressed on $CD4^+$ T and NK cells, macrophages, dendritic cells, fibroblasts, and epithelial cells (14, 15), the persistence of colitogenic memory $CD4^+$ T cells may be affected by those cells in the form of "IL-7 competition". To assess this possibility, we attempted to clarify the link between the expression of IL-7Rα on various cells in the whole body in normal and colitic conditions and the pathogenesis of chronic colitis. In this study, we prove that IL-7Rα expression on $CD4^+$ T cells, but not on other cells (NK cells, granulocytes, macrophages, and dendritic cells), is essential for the development of colitis by use of an adoptive transfer colitis model using IL-7Rα$^{-/-}$ donor cells and IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ recipient mice.

Materials and Methods

Animals

Female C57BL/6 mice were purchased from Japan CLEA (Tokyo, Japan). C57BL/6-background RAG-2$^{-/-}$ mice were obtained from Taconic Farms (Hudson, NY). C57BL/6-background IL-7Rα$^{-/-}$ mice have been described previously (23). IL-7Rα$^{-/-}$ mice were intercrossed with RAG-2$^{-/-}$ mice to generate IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice in the Animal Care Facility of Tokyo Medical and Dental University. Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of Tokyo Medical and Dental University. Female donors and recipients were used at 6-12 wk of age. All experiments were approved by the regional animal study committees and were performed according to institutional guidelines and home office regulations.

Purification of T cell subsets

$CD4^+$ T cells were isolated from spleen cells of IL-7Rα$^{-/-}$ or C57BL/6 mice using the anti-CD4 (L3T4)-MACS system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Enriched $CD4^+$ T cells (96–97% pure, as estimated by FACSCalibur [Becton Dickinson, Sunnyvale, CA]) were then labeled with PE-conjugated anti-mouse CD4 (RM4-5; BD Pharmingen, San Diego, CA) and FITC-conjugated anti-CD25 (7D4; BD Pharmingen). Subpopulations of $CD4^+$ cells were generated by two-color sorting on a FACSAria (Becton Dickinson). All populations were >97.0% pure on reanalysis. To isolate LP $CD4^+$ T cells, the entire colon was opened longitudinally, washed with PBS, and cut into small pieces. The dissected mucosa was incubated with Ca^{2+}, Mg^{2+}-free HBSS containing 1 mM DTT (Sigma-Aldrich) for 45 min to remove mucus, then treated with 3.0 mg/ml collagenase (Worthington Biomedical, Freehold, NJ) for 2 to 3 h. The cells were subjected to Ficoll-Hypaque density gradient centrifugation (40%/75%). Enriched LP $CD4^+$ T cells were obtained by positive selection using anti-CD4 (L3T4) MACS magnetic beads. The resultant cells when analyzed by FACSCalibur contained >95% $CD4^+$ cells.

In vivo experimental design

The role of IL-7Rα in the development and persistence of murine chronic colitis was investigated through a series of in vivo experiments.

Experiment 1. To assess the necessity of IL-7Rα on donor $CD4^+$ cells in the development of colitis, we performed cell transfer experiments using wild type (WT) and IL-7Rα$^{-/-}$ mice as donors. RAG-2$^{-/-}$ mice were injected i.p. with 3×10^5 splenic $CD4^+CD25^-$ T cells obtained from normal 8-wk-old WT and IL-7Rα$^{-/-}$ mice. As a negative control, RAG-2$^{-/-}$ mice were transferred with $CD4^+CD25^-$ T cells (3×10^5) and $CD4^+CD25^+$ regulatory T cells (Tregs; 1×10^5).

Experiment 2. To assess the necessity of IL-7Rα expression on cells of recipient mice in the development of colitis, we transferred $CD4^+CD25^-$ T cells (3×10^5) obtained from WT mice into RAG-2$^{-/-}$ mice and IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice as recipients. The recipient mice were weighed immediately after transfer and then three times per week. They were also observed for clinical signs such as hunched posture, piloerection, diarrhea, and blood in the stool. Mice were sacrificed 11 wk after transfer for experiment 1 and 8 wk after transfer for experiment 2 and assessed for a clinical score (24) that is the sum of four parameters as follows: hunching and wasting, 0 or 1; colon thickening, 0–3 (0, no colon thickening; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); and stool consistency, 0–3 (0, normal beaded stool; 1, soft stool; 2, diarrhea; 3, bloody stool) (24). To monitor the clinical signs during the observation period, the disease activity index is defined as the sum (0–5 points) of the parameters other than colon thickening.

Histologic examination

Tissue samples were fixed in PBS containing 10% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with H&E. Two tissue samples from the proximal and distal parts of the colon were prepared. The sections were analyzed without prior knowledge of the type of T cell reconstitution or treatment. The area most affected was graded by the number and severity of lesions. The mean degree of inflammation in the colon was calculated using a modification of a previously described scoring system (25) as the sum of three parameters: crypt elongation, 0–3; mononuclear cell infiltration, 0–3; and frequency of crypt abscesses.

Cytokine ELISA

To measure cytokine production, 1×10^5 LP $CD4^+$ T cells were cultured in 200 μl of culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in 96-well plates (Costar, Cambridge, MA) precoated with 5 μg/ml hamster anti-mouse CD3ε mAb (145-2C11; BD Pharmingen) and 2 μg/ml hamster anti-mouse CD28 mAb (37.51; BD Pharmingen) in PBS overnight at 4°C (24). Culture supernatants were removed after 48 h and assayed for cytokine production. Cytokine concentrations were determined by specific ELISA per the manufacturer's recommendation (R&D Systems, Minneapolis, MN).

Flow cytometry

To detect the surface expression of various molecules, isolated splenocytes or LP mononuclear cells were preincubated with an FcγR-blocking mAb (CD16/32; 2.4G2; BD Pharmingen) for 20 min followed by incubation with specific FITC-, PE-, PECy5-, or biotin-labeled Abs for 30 min on ice. The following mAbs, other than biotin-conjugated anti-mouse IL-7Rα (A7R34; Immuno-Biological Laboratories (Takasaki Japan), were obtained from BD Pharmingen: anti-CD4 mAb (RM4-5), anti-CD25 mAb (7D4), anti-CD45RB mAb (16A), anti-CD62L (MEL-14), anti-CD44 mAb (IM7), anti-CD69 mAb (H1.2F3), and anti–Bcl-2 mAb (3F11). Biotinylated Abs were detected with PE-streptavidin. Standard two- or three-color flow cytometric analyses were obtained using the FACSCalibur with CellQuest software. Background fluorescence was assessed by staining with control-irrelevant isotype-matched mAbs. To analyze the TCR Vβ family repertoire, splenic cells were double-stained with PE-conjugated anti-CD4 mAb (RM4-5) and the following FITC-conjugated mAbs: Vβ2; KJ25, Vβ3; KT4, Vβ4; MR9-4, Vβ5; RR4-7, Vβ6; TR310, Vβ7; MR5-2, Vβ8.1/2; B21.14, Vβ8.3; MR10-2, Vβ9; B21.5, Vβ10; RR3-15, Vβ11; MR11-1, Vβ12; IN12.3, Vβ13; 14.2, Vβ14; and KJ23, Vβ17. All Abs were purchased from BD Pharmingen.

For intracellular staining of cytokines, $CD4^+$ T cells were cultured for 12 h with ionomycin (500 ng/ml), PMA (50 ng/ml), and BD GolgiPlug (1 μl/ml BD Pharmingen). After the stimulation, cells were collected and their surface molecules were stained. Cells were fixed using Cytofix/Cytoperm Kit (BD Pharmingen) and then stained with PE-conjugated anti–IL-17A mAb (TC11-18H10; BD Pharmingen) or FITC-conjugated anti–IFN-γ mAb (XMG1.2; BD Pharmingen) for 20 min (26).

Statistical analysis

We examined the normality of each group. If either group was not normally distributed, we assessed the difference between two groups using the Mann-Whitney *U* test. If both groups were normally distributed, we assessed the variance of population within each group using *F* test. With homoscedasticity of both populations, we assessed the difference between two groups using the Student *t* test. Without homoscedasticity, we assessed the difference using Welch's *t* test. We used the program Statcell for all statistical analysis Differences were considered to be statistically significant when $p < 0.05$.

Results

IL-7Rα is expressed on various immune cells in WT and colitic mice

To first assess the role of the IL-7/IL-7R signaling pathway in the development of chronic colitis, we analyzed the expression of

IL-7Rα on various immune compartments in colonic LP of normal C57BL/6 mice (normal mice) and colitic C57BL/6-RAG-2$^{-/-}$ mice previously transferred with WT CD4$^+$CD25$^-$ T cells (colitic mice). First, both normal and colitic LP CD3$^+$CD4$^+$ T cells highly expressed IL-7Rα, but the mean fluorescence intensity (MFI) of IL-7Rα expression on LP CD3$^+$CD4$^+$ T cells from colitic mice was significantly higher than in normal mice (Fig. 1*A*, 1*B*). Conversely, the MFIs of IL-7Rα expression on colitic LP CD3$^-$NK1.1$^+$ NK cells, CD11b$^+$Gr^{high} granulocytes, CD11b$^+$Gr$^{low/-}$ macrophages, and CD11b$^+$CD11c$^+$ myeloid dendritic cells were significantly downregulated compared with those from normal mice (Fig. 1*A*, 1*B*). In addition, there were no differences in the expression of IL-7Rα on CD3$^-$NKp46$^+$ NK22-like cells (27–29) and CD11b$^-$CD11c$^+$ lymphoid dendritic cells (Fig. 1*A*, 1*B*). These changes of IL-7Rα expression in LP cells of colitic mice resulted in the highest expression of IL-7Rα on CD3$^+$CD4$^+$ T cells as compared with that on other compartments (Fig. 1*B*), suggesting preferential use of IL-7 by CD3$^+$CD4$^+$ T cells in colitic conditions.

Naive CD4$^+$ T cells are retained in substantial numbers in spleens of IL-7Rα$^{-/-}$ mice

Given the evidence that various immune compartments constitutively express IL-7Rα, we next attempted to assess the role of IL-7Rα expression in the development of chronic colitis induced by adoptive transfer of CD4$^+$CD25$^-$ T cells obtained from age-matched WT or IL-7Rα$^{-/-}$ mice into RAG-2$^{-/-}$ mice. It was particularly interesting that the expression level of IL-7Rα on colitic LP CD3$^+$CD4$^+$ T cells was significantly higher than that of other compartments in colitic conditions (Fig. 1). Because it is also known that IL-7/IL-7R signaling is critically involved in T cell development in thymus and the periphery (9, 10), we first assessed phenotypic characteristics of splenic CD4$^+$ T cells in age-matched WT and IL-7Rα$^{-/-}$ mice before starting a series of adoptive transfer experiments. Consistent with previous reports (23, 30), the absolute cell number of CD3$^+$CD4$^+$ T cells recovered from spleen (SP) of IL-7Rα$^{-/-}$ mice was significantly lower than that of WT mice (data not shown). Although the ratio of naive (CD44$^{low/-}$CD62L$^+$) versus memory (CD44^{high}CD62L$^-$) T cells in SP of IL-7Rα$^{-/-}$ mice was markedly decreased compared with that of WT mice, a substantial number of naive CD4$^+$ T cells were retained in SP of IL-7Rα$^{-/-}$ mice (Fig. 2*A*). In addition, we confirmed that SP CD4$^+$ T cells of IL-7Rα$^{-/-}$ mice did not express IL-7Rα, and no differences in the expression of CD69, Foxp3, and CD25 were found between two groups (Fig. 2*A*). Of note, Bcl-2 expression in SP CD4$^+$ T cells of IL-7Rα$^{-/-}$ mice was significantly lower than that of WT mice ($p < 0.05$; Fig. 2*A*), which seemed to be consistent with previous reports that IL-7 is essential for survival of CD4$^+$ T cells (24). It was also possible that CD4$^+$CD25$^-$ donor T cells in SPs of IL-7Rα$^{-/-}$ mice retain restricted clonality of CD4$^+$ T cells because of the dysregulated differentiation of CD4$^+$ T cells in the thymus as compared with that in WT mice. To test this possibility, we compared TCR Vβ repertoires of SP CD4$^+$CD25$^-$ T cells from age-matched IL-7Rα$^{-/-}$ and WT mice. Flow cytometric analysis of these SP CD4$^+$ cells using a panel of 15 anti-Vβ mAbs showed that the major Vβ population was Vβ8.1/8.2 in both groups, and the only significant difference in Vβ repertoires between the groups was Vβ8.3 (Fig. 2*B*).

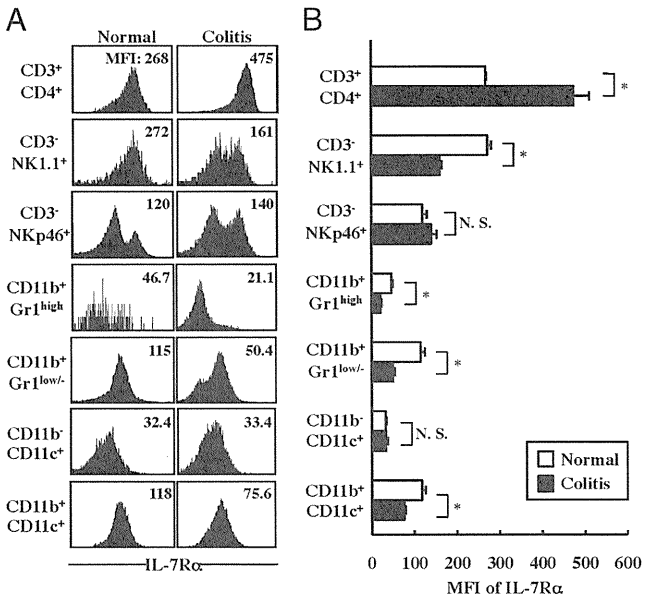

FIGURE 1. IL-7Rα expression on various immune compartments obtained from colonic LP of normal and colitic mice. *A*, Dot plot analysis shows the IL-7α expression on each fraction of immune cells from colonic LP of normal and colitic mice. Numerical values on the dot plots and histograms express the mean percentage of each fraction. *B*, The bar graphs show the MFI of IL-7Rα on each immune compartment obtained from colonic LP of normal and colitic mice. The graph data are the mean ± SEM. *$p < 0.05$. N.S., not significant.

RAG-2$^{-/-}$ mice transferred with IL-7Rα$^{-/-}$ CD4$^+$CD25$^-$ T cells did not develop mild colitis

To then assess the role of the IL-7R signaling pathway in the development of chronic colitis, we used a chronic colitis model induced by adoptive transfer of SP CD4$^+$CD25$^-$ T cells from IL-7Rα$^{-/-}$ or control WT mice into RAG-2$^{-/-}$ recipients (Fig. 3*A*).

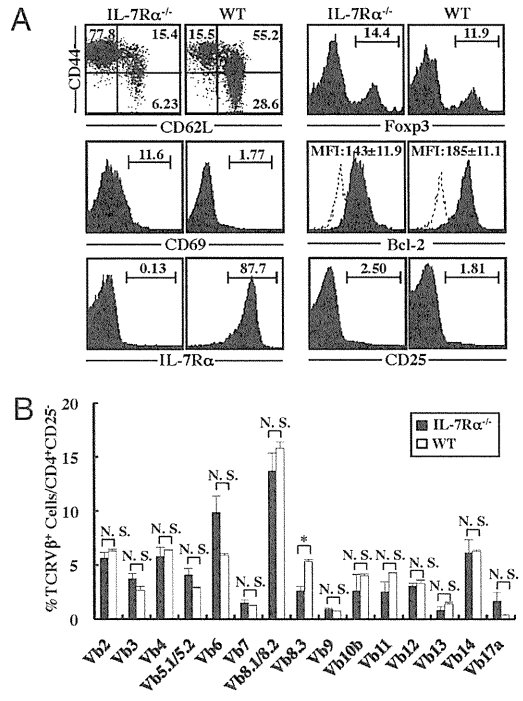

FIGURE 2. Phenotypic characterization of splenic CD4$^+$ T cells obtained from age-matched WT and IL-7Rα$^{-/-}$ mice. *A*, FACS analysis shows the expression of CD44/CD62L, IL-7Rα, Foxp3, and Bcl-2 on/in splenic CD4$^+$ T cells. The dotted line in the Bcl-2 histogram shows the baseline of isotype control. *B*, Flow cytometric analysis of Vβ families on the surface of the splenic CD4$^+$ T cells. To analyze the TCR Vβ family repertoire, splenic cells were double-stained with PE-conjugated anti-CD4 mAb (RM4-5) and a panel of 15 FITC-conjugated Vβ mAbs. The percentage value of each Vβ is the frequency pooled from three independent experiments ($n = 6$). The data are the mean ± SEM. *$p < 0.05$. N.S., not significant.

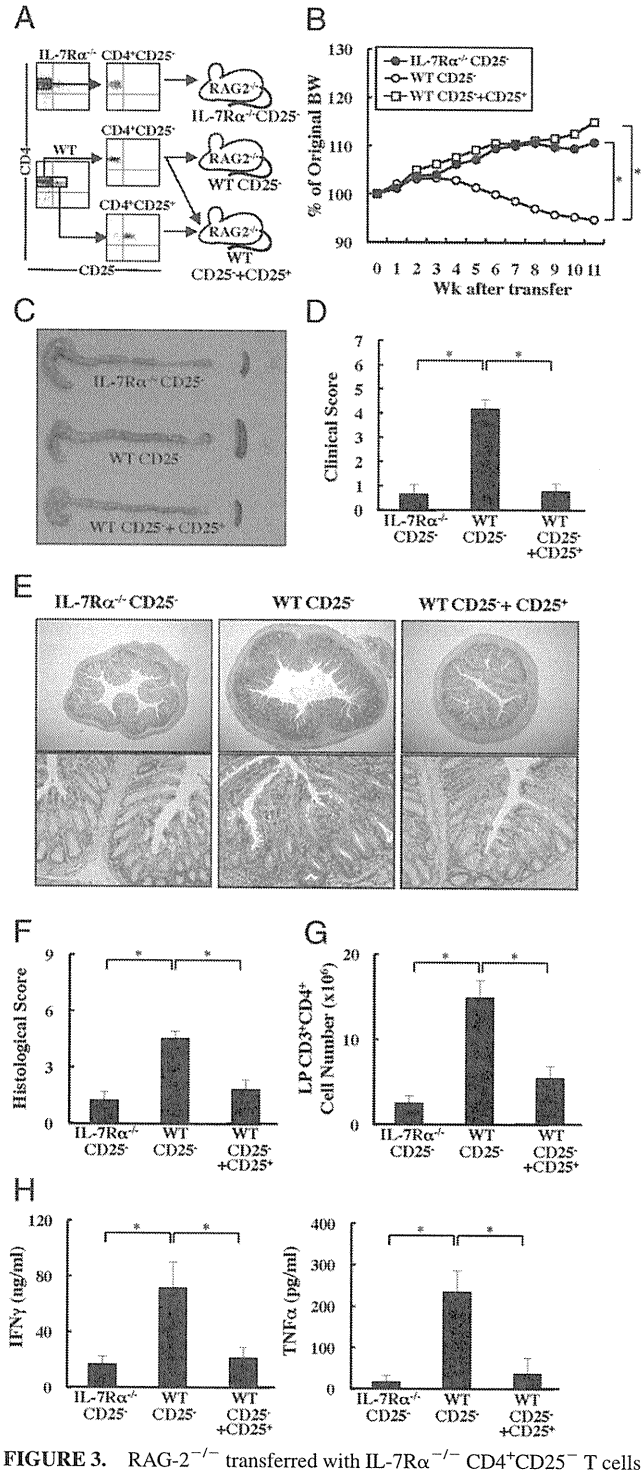

FIGURE 3. RAG-$2^{-/-}$ transferred with IL-7R$\alpha^{-/-}$ $CD4^+CD25^-$ T cells did not develop chronic colitis. *A*, RAG-$2^{-/-}$ mice were transferred with splenic $CD4^+CD25^-$ T cells obtained from age-matched WT or IL-7R$\alpha^{-/-}$ mice (3×10^5 cells per mouse). As a negative control, RAG-$2^{-/-}$ mice were transferred with splenic WT $CD4^+CD45RB^{high}$ T cells (3×10^5 cells per mouse) and $CD4^+CD25^+$ Tregs (1×10^5 cells per mouse). *B*, Change in body weight over time is expressed as a percentage of the original weight. Data are represented as the mean ± SEM of nine mice in each group. *$p < 0.05$, compared with colitic RAG-$2^{-/-}$ mice transferred with $CD4^+CD25^-$ T cells. *C*, Gross appearance of the colon, SP, and mesenteric lymph nodes from RAG-$2^{-/-}$ mice transferred with IL-7R$\alpha^{-/-}$ $CD4^+CD25^-$ T cells (*top*), RAG-$2^{-/-}$ mice transferred with WT $CD4^+CD25^-$ T cells (*middle*), and RAG-$1^{-/-}$ transferred with WT $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Tregs (*bottom*). *D*, Clinical scores were determined at 8 wk after the transfer as described in *Materials and Methods*. Data are indicated as the mean ± SEM of seven mice in each group. *$p < 0.001$. *E*, Histologic examination of the colon from RAG-$2^{-/-}$ mice transferred with IL-7R$\alpha^{-/-}$ $CD4^+CD25^-$ T cells (*left*), RAG-$2^{-/-}$ mice transferred with WT $CD4^+CD25^-$ T cells (*middle*), and RAG-$1^{-/-}$ transferred with WT $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Tregs (*right*) at 11 wk after the transfer. Original magnification ×40 (*upper*) and ×100 (*lower*). *F*, Histologic scoring at 11 wk after transfer. Data are indicated as the mean ± SEM of seven mice in each group. *$p < 0.05$. *G*, LP $CD3^+CD4^+$ T cells were isolated at 11 wk after transfer, and the number was determined by flow cytometry. Data are indicated as the mean ± SEM of seven mice in each group. *$p < 0.05$. *H*, Cytokine production by LP $CD4^+$ T cells. LP $CD4^+$ T cells were isolated at 11 wk after transfer and stimulated with anti-CD3 and anti-CD28 mAbs for 48 h. IFN-γ and TNF-α concentrations in culture supernatants were measured by ELISA. Data are indicated as the mean ± SD of seven mice in each group. *$p < 0.05$.

As a negative control, RAG-$2^{-/-}$ mice were transferred with a mixture of SP $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Tregs obtained from WT mice. As depicted in Fig. 3*B*, RAG-$2^{-/-}$ mice transferred with WT $CD4^+CD25^-$ T cells manifested progressive weight loss from 4 wk after transfer (Fig. 3*B*). In contrast, RAG-$2^{-/-}$ mice transferred with IL-7R$\alpha^{-/-}$ $CD4^+CD25^-$ T cells as well as RAG-$2^{-/-}$ mice transferred with a mixture of $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Tregs appeared healthy and showed a gradual increase of body weight (Fig. 3*B*). To check the possibility that mice transferred with IL-7R$\alpha^{-/-}$ $CD4^+CD25^-$ T cells develop colitis with delayed kinetics, we observed all groups of mice until 11 wk after transfer. Eleven weeks after transfer, RAG-$2^{-/-}$ mice transferred with WT $CD4^+CD25^-$ T cells, but not those transferred with IL-7R$\alpha^{-/-}$ $CD4^+CD25^-$ T cells or WT $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Tregs, had enlarged colons with greatly thickened walls (Fig. 3*C*). The same mice also showed the enlargement of SP and mesenteric lymph nodes (Fig. 3*C*). The assessment of colitis by clinical scores showed a clear difference between RAG-$2^{-/-}$ mice transferred with WT $CD4^+CD25^-$ T cells and the other two groups (Fig. 3*D*).

Histologic examination showed prominent epithelial hyperplasia with glandular elongation and massive infiltration of mononuclear cells in LP of RAG-$2^{-/-}$ mice transferred with WT $CD4^+CD25^-$ T cells (Fig. 3*E*, *middle panels*). In contrast, these inflammatory changes were mostly abrogated, and only a few mononuclear cells were observed in the LP of the colon from RAG-$2^{-/-}$ mice transferred with IL-7R$\alpha^{-/-}$ $CD4^+CD25^-$ T cells (Fig. 3*E*, *left panels*) or with a mixture of SP $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Tregs (Fig. 3*E*, *right panels*). This difference was also confirmed by the histologic scores of multiple colon sections: 5.35 ± 0.40 in RAG-$2^{-/-}$ mice transferred with WT $CD4^+CD25^-$ T cells versus 1.65 ± 0.57 in RAG-$2^{-/-}$ mice transferred with IL-7R$\alpha^{-/-}$ $CD4^+CD25^-$ T cells and 2.00 ± 0.74 in RAG-$2^{-/-}$ mice transferred with a mixture of SP $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Tregs ($p < 0.001$; Fig. 3*F*). Further quantitative evaluation of $CD4^+$ T cell infiltration was made by calculating the absolute cell number of LP $CD3^+CD4^+$ T cells recovered from the resected bowels. Significantly fewer $CD4^+$ T cells were recovered from the colonic tissue of RAG-$2^{-/-}$ mice transferred with IL-7R$\alpha^{-/-}$ $CD4^+CD25^-$ T cells or a mixture of SP $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Tregs as compared with colitic RAG-$2^{-/-}$ mice transferred with WT $CD4^+CD25^-$ T cells (Fig. 3*G*). We also examined the cytokine production by LP $CD4^+$ T cells. LP $CD4^+$ T cells from RAG-$2^{-/-}$ mice transferred with IL-7R$\alpha^{-/-}$ $CD4^+CD25^-$ T cells or a mixture of SP $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Tregs produced significantly lower amounts of IFN-γ and TNF-α than did colitic RAG-$2^{-/-}$ mice transferred with WT $CD4^+CD25^-$ T cells upon in vitro stimulation (Fig. 3*H*).

Importantly, further flow cytometric analysis revealed that almost all the SP and LP $CD3^+CD4^+$ T cells isolated from all three groups of mice at 11 wk after transfer were $CD44^{high}CD62L^-$ $CD69^+$ effector-memory T (T_{EM}) cells (Supplemental Fig. 1), indicating that the transferred $CD4^+CD25^-$ T cells could differentiate to activated T_{EM} cells regardless of the expression of IL-7Rα or the presence or absence of Tregs. These results suggest that the lack of IL-7Rα prevented the development of colitis primarily by inhibiting the expansion or survival of colitogenic $CD4^+$ T_{EM} cells in the colon in accordance with the lower expression of Bcl-2 (Fig. 2*A*). We found that SP and LP $CD4^+$ T cells isolated from all groups of mice at 11 wk after transfer did not express IL-15Rβ, which is a critical receptor for IL-15 signaling, and thymic stromal lymphopoietin (TSLP) receptor, which is critical for TSLP signaling via TSLPR/IL-7Rα complex receptors (Supplemental Fig. 1), indicating that IL-15 and TSLP may not be involved in this colitis model.

To further assess whether IL-$7R\alpha^{-/-}$ $CD4^+$ T cells are unable to produce inflammatory cytokines intrinsically or as the result of a secondary effect from disorder of cell proliferation or maintenance, we performed the following experiments. First, we accessed ex vivo cytokine production of IL-$7R\alpha^{-/-}$ or WT SP $CD4^+$ T cells under Th1 polarizing conditions (Supplemental Fig. 2*A*). As shown in Supplemental Fig. 2*B*, IL-$7R\alpha^{-/-}$ SP $CD4^+$ T cells expressed lower levels of IFN-γ than did WT SP $CD4^+$ T cells under the Th1 polarizing ex vivo conditions. This finding was confirmed by the statistical analysis (Supplemental Fig. 2*C*). Next, we examined the ability of the IL-$7R\alpha^{-/-}$ $CD4^+$ T cells to produce inflammatory cytokines under the same inflammatory conditions as the WT $CD4^+$ T cells. For this purpose, the same number (3×10^5 cells per mouse) of $Ly5.2^+$ IL-$7R\alpha^{-/-}$ SP $CD4^+$ $CD25^-$ T cells and $Ly5.1^+$ WT SP $CD4^+CD25^-$ T cells were cotransferred to RAG-$2^{-/-}$ recipients (Fig. 4*A*). The percentage of $Ly5.2^+$-derived IL-$7R\alpha^{-/-}$ T cells in peripheral blood was gradually decreased after transfer, while that of $Ly5.1^+$-derived WT T cells in peripheral blood was conversely increased, and the difference was significant 2 wk after transfer (Fig. 4*B*). Six weeks after transfer, all mice developed colitis (data not shown). Although the recovered cell number of $Ly5.2^+$ SP or LP $CD4^+$ T cells derived from IL-$7R\alpha^{-/-}$ donors at 6 wk after transfer was

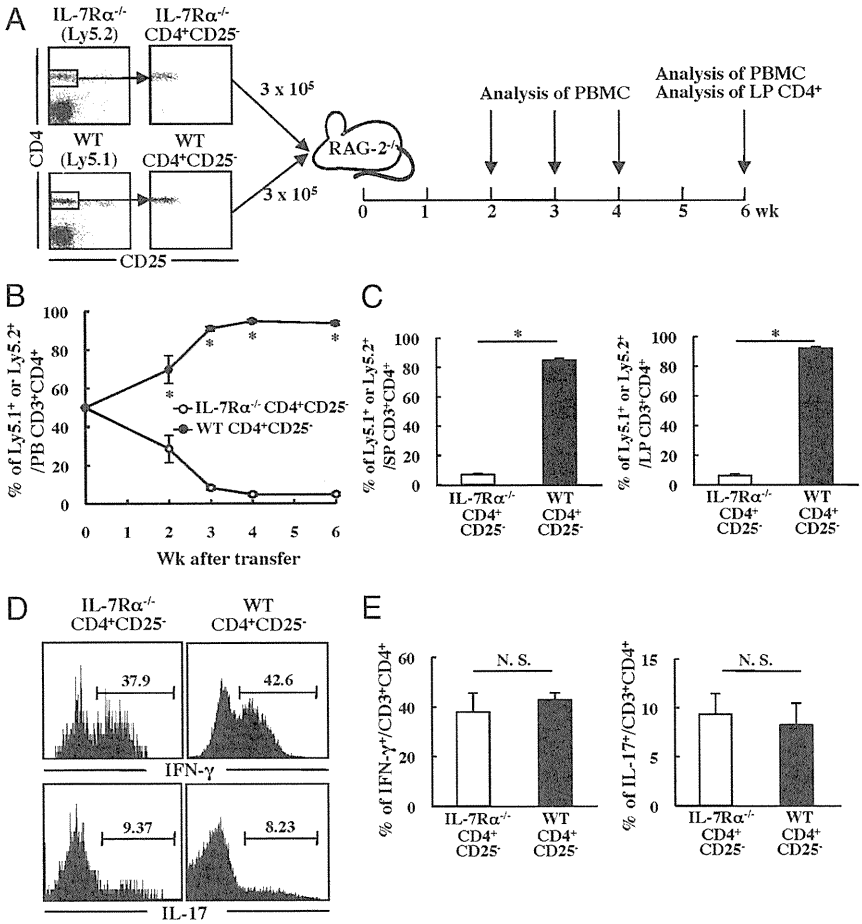

FIGURE 4. IL-$7R\alpha^{-/-}$ $CD4^+CD25^-$ T cells cotransferred with WT $CD4^+CD25^-$ T cells to RAG-$2^{-/-}$ mice could produce IFN-γ and IL-17, but could not survive. *A*, To discern why IL-$7R\alpha^{-/-}$ $CD4^+CD25^-$ T cells could not induce colitis, we cotransferred the same number (3×10^5) of $Ly5.2^+$ IL-$7R\alpha^{-/-}$ $CD4^+CD25^-$ T cells and $Ly5.1^+$ WT $CD4^+CD25^-$ T cells to $Ly5.2^+$ RAG-$2^{-/-}$ mice, and we compared the cell number and ability to produce Th1/Th17 cytokines between transferred IL-$7R\alpha^{-/-}$ and WT cells. PBMCs were collected 1, 2, 3, and 4 wk after the transfer. All mice were sacrificed and analyzed 6 wk after the transfer. *B*, Percentage of $Ly5.1^+$ or $Ly5.2^+$ cells in peripheral blood $CD3^+CD4^+$ cells at each time point were determined by flow cytometry. *C*, Percentage of $Ly5.1^+$ or $Ly5.2^+$ cells in SP and LP $CD3^+CD4^+$ cells 6 wk after the transfer. *D*, IFN-γ and IL-17 expression in recovered LP $CD4^+$ T cells from IL-$7R\alpha^{-/-}$ or WT donor mice. LP $CD4^+$ T cells were collected from RAG-$2^{-/-}$ recipients 6 wk after the transfer; they were cultured with ionomycin, PMA, and GolgiPlug for 12 h as mentioned in *Materials and Methods*. IFN-γ and IL-17 expression of them were determined by flow cytometry using intracellular staining methods. $CD3^+CD4^+Ly5.1^+$ cells were considered as $CD4^+$ T cells from WT donor mice, while $CD3^+CD4^+Ly5.1^-$ cells were considered as $CD4^+$ T cells from IL-$7R\alpha^{-/-}$ donor mice. Numerical values on the histograms express the mean percentage of each fraction. *E*, Percentage of IFN-γ^+ cells and IL-17^+ cells in LP $CD3^+CD4^+$ T cells from IL-$7R\alpha^{-/-}$ or WT donor mice. Data are indicated as the mean ± SEM of five mice in each group. $^*p < 0.05$.

significantly lower than that from Ly5.1+ WT donors (Fig. 4*C*), both WT and IL-7Rα$^{-/-}$ donor-derived CD4+ T cells could similarly express IFN-γ and IL-17 in the colitic condition (Fig. 4*D*). These results indicate that IL-7Rα$^{-/-}$ cells in the absence of the neighboring WT cells cannot produce Th1 or Th17 cytokines as a result of suppression of colitis through a disorder of proliferation or maintenance, rather than intrinsically impaired ability.

As shown in Fig. 2*A*, the ratio of naive T cells in SP of IL-7Rα$^{-/-}$ mice was significantly lower than that of WT mice. Therefore, the possibility remains that this different ratio of naive-memory phenotypes of transferred cells might influence the strength of colitis. To rule out this possibility, we next performed another transfer experiment using the same number of naive CD4+ T cells (3×10^5 cells per mouse) as donor cells. RAG-2$^{-/-}$ mice were transferred with SP CD3+CD4+CD62L+CD44− naive T cells obtained from age-matched WT or IL-7Rα$^{-/-}$ mice (Fig. 5*A*). As a negative control, RAG-2$^{-/-}$ mice were transferred with SP WT naive T cells and CD4+CD25+ Tregs (Fig. 5*A*). As expected, neither mice transferred with IL-7Rα$^{-/-}$ naive T cells nor mice transferred with naive T cells and Tregs developed colitis as assessed by gross appearance of the colon (Fig. 5*B*), clinical (Fig. 5*C*) and histologic scorings (Fig. 5*D*, 5*E*), and the absolute cell number of LP CD3+CD4+ T cells (Fig. 5*F*) in sharp contrast to the diseased mice transferred with WT naive T cells, confirming that IL-7Rα expression on CD4+ T cells is essential for the development of colitis, regardless of the different ratio of naive and memory cells in SP of IL-7Rα$^{-/-}$ mice and WT mice. We further performed an apoptosis assay using annexin V/PI staining in this

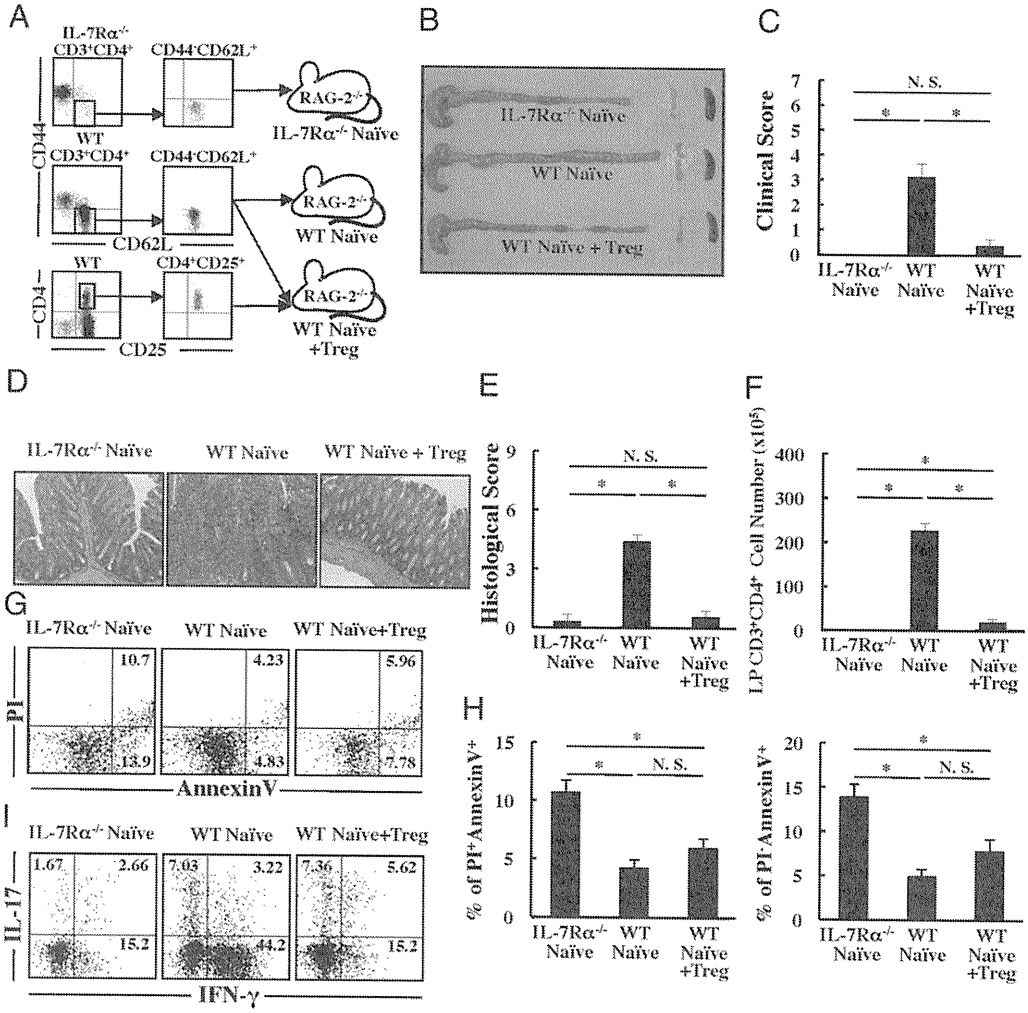

FIGURE 5. RAG-2$^{-/-}$ transferred with IL-7Rα$^{-/-}$ CD3+CD4+CD62L+CD44− T cells did not develop chronic colitis. *A*, RAG-2$^{-/-}$ mice were transferred with splenic CD3+CD4+CD62L+CD44− T cells obtained from age-matched WT or IL-7Rα$^{-/-}$ mice (3×10^5 cells per mouse). As a negative control, RAG-2$^{-/-}$ mice were transferred with splenic WT CD3+CD4+CD62L+CD44− T cells (3×10^5 cells per mouse) and CD4+CD25+ Tregs (1×10^5 cells per mouse). *B*, Gross appearance of the colon, SP, and mesenteric lymph nodes from RAG-2$^{-/-}$ mice transferred with IL-7Rα$^{-/-}$ CD3+CD4+CD62L+CD44− (*top*), RAG-2$^{-/-}$ mice transferred with WT CD3+CD4+CD62L+CD44− T cells (*middle*), and RAG-1$^{-/-}$ transferred with WT CD3+CD4+CD62L+CD44− T cells and CD4+CD25+ Tregs (*bottom*). *C*, Clinical scores were determined at 8 wk after the transfer as described in *Materials and Methods*. Data are indicated as the mean ± SEM of each group. *$p < 0.05$. *D*, Histologic examination of the colon from RAG-2$^{-/-}$ mice transferred with IL-7Rα$^{-/-}$ CD3+CD4+CD62L+CD44− T cells (*left*), RAG-2$^{-/-}$ mice transferred with WT CD3+CD4+CD62L+CD44− T cells (*middle*), and RAG-1$^{-/-}$ transferred with WT CD3+CD4+CD62L+CD44− T cells and CD4+CD25+ Tregs (*right*) at 8 wk after the transfer. Original magnification ×40 (*upper*) and ×100 (*lower*). *E*, Histologic scoring at 8 wk after transfer. Data are indicated as the mean ± SEM of each group. *$p < 0.05$. *F*, LP CD3+CD4+ T cells were isolated at 8 wk after transfer, and the number was determined by flow cytometry. Data are indicated as the mean ± SEM of each group. *$p < 0.05$. *G*, The expression of propidium iodide (PI) and annexin V in SP CD4+ T cells from RAG-2$^{-/-}$ mice transferred with IL-7Rα$^{-/-}$ CD3+CD4+CD62L+CD44− T cells, RAG-2$^{-/-}$ mice transferred with WT CD3+CD4+CD62L+CD44− T cells, and RAG-2$^{-/-}$ transferred with WT CD3+CD4+CD62L+CD44− T cells and CD4+CD25+ Tregs at 8 wk after the transfer. *H*, The percentage of early apoptotic cells (annexin V+PI−) and late apoptotic cells (annexin V+PI−). *I*, Intracellular staining of cytokines (IL-17/IFN-γ) in the colonic LP CD4+ T cells. Numerical values on the dot plots and histograms express the mean percentage of each fraction.

setting. IL-7Rα$^{-/-}$ SP CD4$^+$ T cells underwent apoptosis more frequently than WT SP CD4$^+$ T cells (Fig. 5*G*, 5*H*), which supports the hypothesis that expression of IL-7Rα on CD4$^+$ T cells is important for their survival. Furthermore, the expression of IL-17 and IFN-γ in IL-7Rα$^{-/-}$ LP CD4$^+$ T cells was markedly decreased compared with that in WT LP CD4$^+$ T cells (Fig. 5*I*).

IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice transferred with CD4$^+$CD25$^-$ T cells developed colitis

To further assess the role of IL-7/IL-7R signaling in the development of chronic colitis, we next focused on IL-7Rα expression on non-T cells, such as APCs and NK cells that reside in RAG-2$^{-/-}$ recipients, because it is possible that IL-7 is competitively used by various IL-7Rα–expressing immune compartments, and the competition may affect the development of chronic colitis. To test this hypothesis, WT CD4$^+$CD25$^-$ T cells were transferred into RAG-2$^{-/-}$ or IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice (Fig. 6*A*). As a negative control, a mixture of WT CD4$^+$CD25$^-$ T cells and CD4$^+$CD25$^+$ Tregs was transferred into RAG-2$^{-/-}$ mice (Fig. 6*A*). When CD4$^+$CD25$^-$ T cells were transferred into the control RAG-2$^{-/-}$ mice, the recipients, as expected, rapidly developed severe wasting disease associated with clinical signs of severe colitis, in particular, weight loss, persistent diarrhea and occasionally bloody stool and anal prolapses, in sharp contrast to healthy RAG-2$^{-/-}$ mice transferred with a mixture of CD4$^+$ CD25$^-$ T cells and CD4$^+$CD25$^+$ Tregs (Fig. 6*B*). When CD4$^+$ CD25$^-$ T cells were transferred into the IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice, the recipients also developed severe wasting chronic colitis (Fig. 6*B*). These RAG-2$^{-/-}$ and IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice transferred with CD4$^+$CD25$^-$ T cells, but not RAG-2$^{-/-}$ mice transferred with a mixture of CD4$^+$CD25$^-$ T cells and CD4$^+$ CD25$^+$ Tregs, had enlarged colons with significantly thickened walls accompanied with enlarged SPs and mesenteric lymph nodes 8 wk after transfer (Fig. 6*C*). Consistent with this finding, clinical scores of RAG-2$^{-/-}$ and IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice transferred with CD4$^+$CD25$^-$ T cells were significantly higher than those of RAG-2$^{-/-}$ mice transferred with a mixture of CD4$^+$ CD25$^-$ T cells and CD4$^+$CD25$^+$ Tregs (Fig. 6*D*). No significant difference in clinical scores was found between RAG-2$^{-/-}$ and IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice transferred with CD4$^+$CD25$^-$ T cells, although the score of IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice tended to be higher than that of RAG-2$^{-/-}$ mice transferred with CD4$^+$CD25$^-$ T cells (Fig. 6*D*).

Histologic examination showed that tissue sections from RAG-2$^{-/-}$ and IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice transferred with CD4$^+$ CD25$^-$ T cells were characterized by inflammatory infiltrate, epithelial hyperplasia, crypt cell damage, and goblet cell depletion, in contrast to RAG-2$^{-/-}$ mice transferred with a mixture of CD4$^+$CD25$^-$ T cells and CD4$^+$CD25$^+$ Tregs, which showed no features of colitis (Fig. 6*E*). This difference was also confirmed by histologic scoring of multiple colon sections (Fig. 6*F*). Consistent with the histologic assessment, the numbers of LP CD4$^+$ T cells recovered from RAG-2$^{-/-}$ and IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice transferred with CD4$^+$CD25$^-$ T cells were similar to each other but significantly higher than that from noncolitic RAG-2$^{-/-}$ mice transferred with a mixture of CD4$^+$CD25$^-$ T cells and CD4$^+$ CD25$^+$ Tregs (Fig. 6*G*). Cytokine production by LP CD4$^+$ T cells is depicted in Fig. 6*H*. LP CD4$^+$ T cells from RAG-2$^{-/-}$ and IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice transferred with CD4$^+$CD25$^-$ T cells produced significantly higher levels of IFN-γ and TNF-α than did those from the control mice transferred with a mixture of CD4$^+$ CD25$^-$ T cells and CD4$^+$CD25$^+$ Tregs (Fig. 6*H*).

Flow cytometric analysis revealed that the LP CD4$^+$ T cells isolated from all groups of mice at 8 wk after transfer were CD44^{high}CD62L$^-$CD69$^+$ T_{EM} cells (Supplemental Fig. 3*A*), indicating that the transferred CD4$^+$CD25$^-$ T cells could differentiate to activated T_{EM} cells regardless of the presence or absence of IL-7Rα on non-T cells in the RAG-2$^{-/-}$ recipient mice. Intracellular analysis further showed that almost the same fraction of LP CD4$^+$ T cells from both RAG-2$^{-/-}$ and IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice transferred with CD4$^+$CD25$^-$ T cells had differentiated to IFN-γ–producing Th1 or IL-17–producing Th17 (Supplemental Fig. 3*B*). In contrast, the expression of IFN-γ in LP CD4$^+$ T cells from RAG-2$^{-/-}$ mice transferred with a mixture of CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells was markedly reduced as compared with the groups with colitis (Supplemental Fig. 3*B*).

To further clarify whether the lower number of CD4$^+$CD25$^-$ T cells in the transfer experiment makes this difference significant, RAG-2$^{-/-}$ mice and IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice were transferred with 3 × 10^5 or 1 × 10^5 WT SP CD4$^+$CD25$^-$ T cells. As a negative control, RAG-2$^{-/-}$ mice were transferred with splenic WT CD4$^+$CD25$^-$ T cells (3 × 10^5 cells per mouse) and CD4$^+$ CD25$^+$ Tregs (3 × 10^5 cells per mouse; Supplemental Fig. 4*A*). However, no differences were found in clinical and histologic colitis scores or the absolute number of LP CD3$^+$CD4$^+$ T cells between IL-7Rα$^{-/-}$ and WT transferred groups, irrespective of lower or higher number of donor T cells (Supplemental Fig. 4*B*–*E*). Although we also checked the expression of MHC class II on CD11b$^-$CD11c$^+$ classical dendritic cells and CD11b$^+$CD11c$^+$ myeloid dendritic cells in this experiment (Supplemental Fig. 4*F*), no differences were detected between any groups. Diminished expression of MHC class II on dendritic cells in RAG-2$^{-/-}$ mice, which is caused by elevated level of IL-7 with lymphopenia, may recover after transferred CD4$^+$ T cells consume IL-7.

Discussion

This study has demonstrated that the high expression of IL-7Rα on colitic CD4$^+$ T cells, but not on non-T cells, is essential for the development and persistence of colitis. This finding is supported by the findings that 1) the MFI of IL-7Rα expression of LP CD4$^+$ T cells is significantly higher than that of other non-CD4$^+$ T cells in colitic conditions, 2) the MFI of IL-7Rα expression of colitic LP CD4$^+$ T cells is significantly higher than that of normal LP CD4$^+$ T cells, 3) RAG-2$^{-/-}$ mice transferred with IL-7Rα$^{-/-}$ CD4$^+$CD25$^-$ T cells do not develop colitis, and 4) IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD25$^-$ T cells develop colitis similar to that in transferred IL-7Rα$^{+/+}$ × RAG-2$^{-/-}$ mice. Collectively, IL-7Rα expression on colitic CD4$^+$ T, but not on other cells, is essential for the development and persistence of chronic colitis.

It was originally reported that IL-7Rα is highly expressed on lymphocytes such as T cells (16). Consistent with this report, we have previously reported that the IL-7/IL-7R signaling pathway is critical for the maintenance of IL-7Rα^{high} colitogenic CD4$^+$ memory T cells (18, 20). Furthermore, we showed that treatment with neutralizing anti–IL-7Rα mAb ameliorated ongoing chronic colitis (18). More recently, several reports have proved the importance of the IL-7/IL-7R signal in nonlymphocytes. Guimond et al. (31) have reported that IL-7Rα is expressed on some types of dendritic cells, and that in the lymphopenic environment the IL-7/IL-7R signal of dendritic cells leads to depression of its MHC class II molecule, which results in the suppression of the proliferation of CD4$^+$ T cells. Other recent reports that IL-7Rα is broadly expressed on NK cells, dendritic cells, and macrophages in normal conditions (16, 17), suggesting the need for us to further investigate the importance of the IL-7/IL-7R signaling pathway in non-T cells for the development and persistence of chronic colitis. Although IL-7Rα expression on

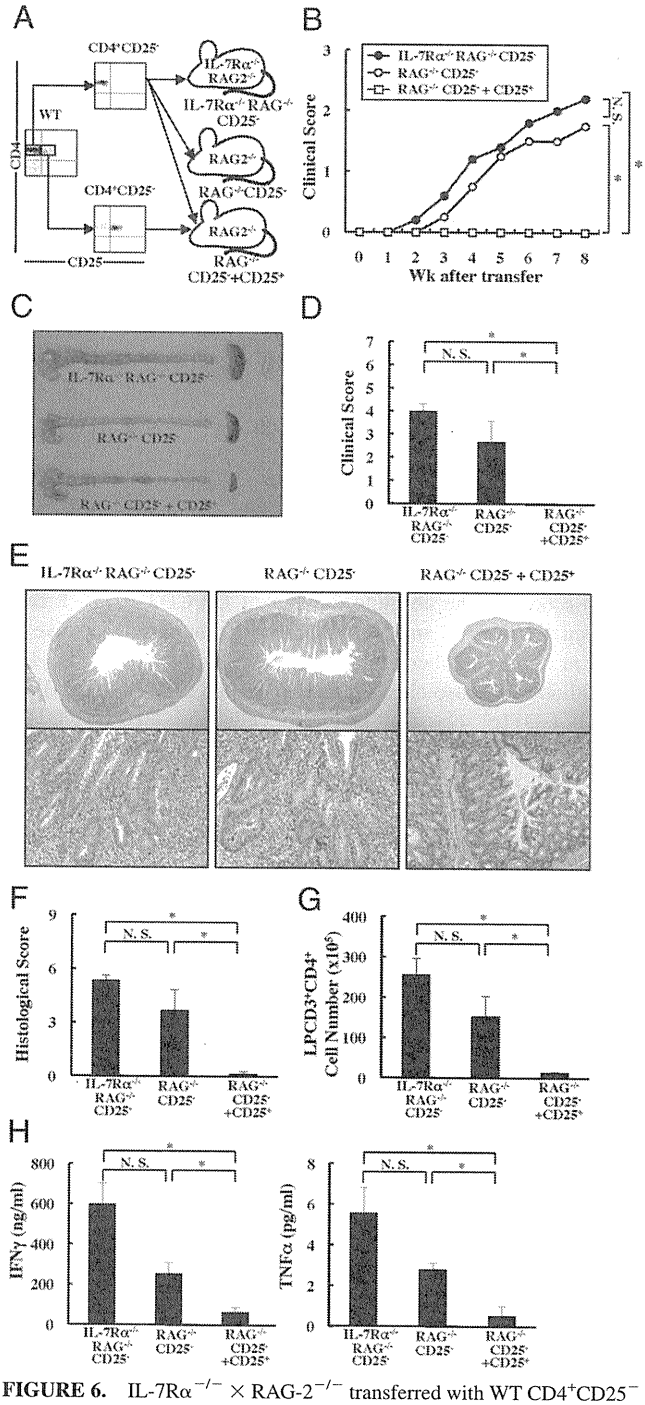

FIGURE 6. IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ transferred with WT CD4$^+$CD25$^-$ T cells developed chronic colitis. *A*, RAG-2$^{-/-}$ mice and IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice were transferred with splenic WT CD4$^+$CD25$^-$ T cells (3×10^5 cells per mouse). As a negative control, RAG-2$^{-/-}$ mice were transferred with splenic WT CD4$^+$CD25$^-$ T cells (3×10^5 cells per mouse) and CD4$^+$CD25$^+$ Tregs (1×10^5 cells per mouse). *B*, Disease activity index during 8 wk after transfer. *$p < 0.05$. *C*, Gross appearance of the colon, SP, and mesenteric lymph nodes from IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice transferred with CD4$^+$CD25$^-$ T cells (*top*), RAG-2$^{-/-}$ mice transferred with CD4$^+$CD25$^-$ T cells (*middle*), and RAG-2$^{-/-}$ mice transferred with CD4$^+$CD25$^-$ T cells and CD4$^+$CD25$^+$ Tregs. *D*, Clinical scores were determined at 8 wk after the transfer as described in *Materials and Methods*. Data are indicated as the mean ± SEM of seven mice in each group. *$p < 0.001$. *E*, Histologic examination of the colon from IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice transferred with CD4$^+$CD25$^-$ T cells (*left*), RAG-2$^{-/-}$ mice transferred with CD4$^+$CD25$^-$ T cells (*middle*), and RAG-2$^{-/-}$ mice transferred with CD4$^+$CD25$^-$ T cells and CD4$^+$CD25$^+$ Tregs (*right*). Original magnification ×40 (*upper*) and ×100 (*lower*). *F*, Histologic scoring 8 wk after transfer.

some LP populations, such as NK cells, granulocytes, macrophages, and CD11b$^+$CD11c$^+$ myeloid dendritic cells, in colitic mice was significantly downregulated compared with that in normal mice, the expression level of IL-7Rα on colitic CD4$^+$ T cells was conversely high, with the result that colitogenic memory CD4$^+$ T cells sustain the highest expression of IL-7Rα in inflammatory conditions.

IL-7Rα$^{-/-}$ mice are originally lymphopenic, because of the loss of IL-7/IL-7R signaling pathway in lymphocytes, which is a critical factor for their development in the thymus and their maintenance in the periphery. Comparison of the surface phenotypes of SP CD4$^+$ T cells in IL-7Rα$^{-/-}$ and WT mice by flow cytometric analysis revealed no significant differences in the expression of CD69, CD25, and Foxp3 (Fig. 2*A*). Manifestation of an antiapoptosis molecule Bcl-2 of CD4$^+$ T cells from IL-7Rα$^{-/-}$ mice was lower than that of CD4$^+$ T cells from WT mice, which corresponds to the previous reports that the IL-7/IL-7R signal maintains T cells, upregulating the antiapoptosis molecule. Nevertheless, we detected a substantial number of CD44^{low}CD62L$^+$ naive CD4$^+$ T cells resident in the SPs of IL-7Rα$^{-/-}$ mice, although their relative number in IL-7Rα$^{-/-}$ mice was significantly lower than that in WT mice. Because of the scarcity of naive CD4$^+$ T cells in IL-7Rα$^{-/-}$ mice, it was possible that the failure of some part of naive T cells to develop might occur in the thymus, which would lead to the loss of some TCR repertoires needed for the onset of colitis. Thus, we compared the TCR Vβ repertoires of SP CD4$^+$ T cells in IL-7Rα$^{-/-}$ mice to those in WT mice. However, except in the ratio of Vβ8.3, no evidence was found of skewed development in TCR Vβ repertoires between age-matched IL-7Rα$^{-/-}$ and WT mice.

As expected, RAG2$^{-/-}$ mice transferred with SP IL-7Rα$^{-/-}$ CD4$^+$CD25$^-$ T cells did not develop colitis, in sharp contrast to colitic RAG2$^{-/-}$ mice transferred with WT CD4$^+$CD25$^-$ T cells. Nevertheless, flow cytometric analysis revealed that SP and LP CD4$^+$ T cells from RAG2$^{-/-}$ mice transferred with IL-7Rα$^{-/-}$ CD4$^+$CD25$^-$ T cells differentiated to CD44^{high}CD62L$^-$ T_{EM} cells as well as those from colitic RAG2$^{-/-}$ mice transferred with WT CD4$^+$CD25$^-$ T cells. This result suggests that IL-7R deficiency in CD4$^+$ T cells causes the disorder of cell proliferation or maintenance rather than the impaired development of memory CD4$^+$ T cells, in accordance with the downmodulated Bcl-2 expression of IL-7Rα$^{-/-}$ CD4$^+$ T cells. As shown in Fig. 3*H*, production of Th1 cytokines from recovered LP CD4$^+$ T cells of the IL-7Rα$^{-/-}$ CD25$^-$ group was significantly lower than that of the WT CD25$^-$ group. However, IL-7Rα$^{-/-}$ CD4$^+$ T cells could express Th1 and Th17 cytokines to an extent similar to that in WT CD4$^+$ T cells in the colitic condition (Fig. 4). Therefore, we conclude that disorder of IL-7Rα$^{-/-}$ CD4$^+$ T cells to proliferate and survive is the main mechanism underlying their inability to induce colitis, whereas their reduced inflammatory cytokine production is a secondary effect. Furthermore, we also analyzed other common γ-receptor–associated receptor IL-15Rβ to determine whether it was upregulated to compensate for the lack of IL-7Rα. However, no dif-

Data are indicated as the mean ± SEM of seven mice in each group. *$p < 0.05$. *G*, LP CD3$^+$CD4$^+$ T cells were isolated at 11 wk after transfer, and the number was determined by flow cytometry. Data are indicated as the mean ± SEM of seven mice in each group. *$p < 0.05$. *H*, Cytokine production by LP CD4$^+$ T cells. LP CD4$^+$ T cells were isolated at 11 wk after transfer and stimulated with anti-CD3 and anti-CD28 mAbs for 48 h. IFN-γ and TNF-α concentrations in culture supernatants were measured by ELISA. Data are indicated as the mean ± SD of seven mice in each group. *$p < 0.05$.

ference was found in the expression of IL-15Rβ on SP or LP $CD4^+$ T cells from each group. These results suggest that IL-7Rα expression on colitogenic $CD4^+$ T cells is essential for the development and persistence of colitis.

Next, we used IL-7Rα$^{-/-}$ × RAG2$^{-/-}$ mice to access the importance of the IL-7/IL-7Rα signaling pathway in non-T cells. At the start of this project, we hypothesized that IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice transferred with $CD4^+CD25^-$ T cells would develop more severe colitis than the control transferred RAG-2$^{-/-}$ recipient mice by considering two points. First, we thought that the availability of IL-7 for colitogenic $CD4^+$ T cells might increase in IL-7Rα$^{-/-}$ × RAG2$^{-/-}$ mice as a result of the loss of IL-7 consumption by IL-7Rα–lacking non-T cells. Actually, IL-7 concentration in serum from IL-7Rα$^{-/-}$ mice is reported to be higher than that from WT mice (31). Thus, it was possible that the persistence of colitogenic memory $CD4^+$ T cells is affected by those cells in the form of IL-7 competition. Second, we had to consider the presence of newly identified RORγt$^+$ IL-22–producing NK cells (so called NK-22 cells) (27–29, 32, 33) for the development of chronic colitis, because it has been shown that these NK-22 cells constitutively express IL-7Rα. Importantly, it has been reported recently that IL-22 is protective in murine DSS-induced colitis model using IL-22$^{-/-}$ × RAG-2$^{-/-}$ mice (33), leading to speculation that these NK-22 cells reside in intestinal LP of RAG-2$^{-/-}$ mice and may be regulated by the IL-7/IL-7R signaling pathway. Unexpectedly, we could not detect any significant differences regarding the severity of colitis between RAG-2$^{-/-}$ and IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ recipient mice. This finding was also confirmed by the experiment using a smaller number of $CD4^+CD25^-$ T cells as donor cells. Instead, we found that the expression of IL-7Rα on colitic LP $CD4^+$ T cells was significantly higher than that on normal LP $CD4^+$ T cells (Fig. 1), suggesting a mechanism for exclusive use of IL-7 by highly IL-7Rα–expressing colitic $CD4^+$ T cells.

Previously, we showed that IL-7Rα expression on LP $CD4^+$ T cells in $CD4^+CD45RB^{high}$ T cell-transferred RAG-2$^{-/-}$ mice is downmodulated at the early effector phase of colitogenic $CD4^+$ T cell differentiation (1–2 wk after transfer) and is again upregulated at the memory phase when colitis is established (>4 wk after transfer) (22). Thus, it is possible that the competition for IL-7 between colitogenic $CD4^+$ T cells and other non-T cells occurs during such an early phase of colitis development. Otherwise, IL-7 competition between T cells versus non-T cells may occur at more acute immune responses, such as acute bacterial infections, which is mainly regulated by IL-7Rα–downmodulating effector T cells (10).

Finally, it is important to discuss the therapeutic strategies for the treatment of IBD. Because IL-7 is the most important cytokine for the maintenance of homeostasis of all the resting memory $CD4^+$ T cells, it seems to be unsafe to adopt the blockade of IL-7/IL7R signaling pathway for the treatment of IBD. As shown in this study, however, it should be emphasized that the highest expression of IL-7Rα is found in colitogenic memory LP $CD4^+$ T cells as compared with non-$CD4^+$ T cell compartments and normal $CD4^+$ T cells. In such a situation, it is possible that a neutralizing or depleting anti–IL-7Rα mAb would preferentially target colitogenic memory $CD4^+$ T cells with the highest expression of IL-7Rα. Consistent with this notion, a recent report has shown that targeted depletion of pathogenic Th1 and Th17 cells, which express high levels of lymphotoxin-α, inhibits autoimmune diseases (34). In addition, it may be necessary to develop a molecular targeting therapy against the IL-7Rα molecule that is more specific for the target organ, rather than a systemic therapy, using effective drug delivery to inflamed mucosa of IBD.

Collectively, we have shown that IL-7Rα expression on $CD4^+$ T cells is essential for the development of colitis in this model. This finding suggests that IL-7Rα on colitogenic memory LP $CD4^+$ T cells is one of the important targets in IL-7/IL-7R signal blocking therapy.

Disclosures

The authors have no financial conflicts of interest.

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Suppression of *Hath1* Gene Expression Directly Regulated by Hes1 Via Notch Signaling Is Associated with Goblet Cell Depletion in Ulcerative Colitis

Xiu Zheng, MD, Kiichiro Tsuchiya, MD, PhD, Ryuichi Okamoto, MD, PhD, Michiko Iwasaki, MD, PhD, Yoshihito Kano, MD, Naoya Sakamoto, MD, PhD, Tetsuya Nakamura, MD, PhD, and Mamoru Watanabe, MD, PhD

Background: The transcription factor *Atoh1/Hath1* plays crucial roles in the differentiation program of human intestinal epithelium cells (IECs). Although previous studies have indicated that the Notch signal suppresses the differentiation program of IEC, the mechanism by which it does so remains unknown. This study shows that the undifferentiated state is maintained by the suppression of the *Hath1* gene in human intestine.

Methods: To assess the effect of Notch signaling, doxycycline-induced expression of Notch intracellular domain (NICD) and Hes1 cells were generated in LS174T. *Hath1* gene expression was analyzed by quantitative reverse-transcription polymerase chain reaction (RT-PCR). Hath1 promoter region targeted by HES1 was determined by both reporter analysis and ChIP assay. Expression of Hath1 protein in ulcerative colitis (UC) was examined by immunohistochemistry.

Results: Hath1 mRNA expression was increased by Notch signal inhibition. However, Hath1 expression was suppressed by ectopic HES1 expression alone even under Notch signal inhibition. Suppression of the *Hath1* gene by Hes1, which binds to the 5′ promoter region of Hath1, resulted in suppression of the phenotypic gene expression for goblet cells. In UC, the cooperation of aberrant expression of HES1 and the disappearance of caudal type homeobox 2 (CDX2) caused Hath1 suppression, resulting in goblet cell depletion.

Conclusions: The present study suggests that Hes1 is essential for *Hath1* gene suppression via Notch signaling. Moreover, the suppression of Hath1 is associated with goblet cell depletion in UC. Understanding the regulation of goblet cell depletion may lead to the development of new therapy for UC.

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Key Words: ulcerative colitis, Hath1, Hes1, Notch signaling

The gut epithelium undergoes continual renewal throughout adult life, maintaining the proper architecture and function of the intestinal crypts. This process involves highly coordinated regulation of the induction of cellular differentiation and the cessation of proliferation, and vice versa.[1–3] Many studies of the regulation of intestinal differentiation have shown that cellular formation of the villi in small and large intestine is affected by various intracellular signaling pathways such as Notch, Wnt, and BMP.[4–7] Moreover, recent studies have also shown that dysregulation of the differentiation system for prompt intestinal epithelial cell formation induces the pathology of such intestinal diseases as colon cancer, Crohn's disease and ulcerative colitis (UC).[8] Then it was suggested that crucial genes for the differentiation of intestinal epithelium cells (IECs) become corrupt by aberrant cell signaling on the pathogenesis of intestinal diseases.

One of the most important genes for cell formation is a basic helix-loop-helix (bHLH) transcription factor, *Atoh1*, and its human homolog, *Hath1*, which is essential for the differentiation toward secretory lineages in small and large intestine.[9] Using a ubiquitin proteasomal system, we demonstrated that regulation of Hath1 protein in colon carcinogenesis is regulated by glycogen synthase kinase 3β (GSK3β) via Wnt signaling. Moreover, Hath1 and β-catenin protein are reciprocally regulated by GSK3β in Wnt signaling for the coordination between cell differentiation and

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From the Department of Gastroenterology and Hepatology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan.

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The first two authors contributed equally to this work.

Reprints: Mamoru Watanabe, MD, PhD, MD, PhD, Professor and Chairman, Department of Gastroenterology and Hepatology, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan (e-mail: mamoru.gast@tmd.ac.jp)

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proliferation. These findings together indicate that the deletion of adenomatous polyposis coli (APC) in colon carcinogenesis causes Hath1 protein degradation by switching the target of GSK3β from β-catenin to Hath1, resulting in maintenance of the undifferentiated state.[10] The dysregulation of prompt differentiation of IEC thus causes major intestinal diseases, and elucidation of the roles of various cell-signaling pathways in intestine is therefore important in understanding the pathogenesis of intestinal diseases.

We have also recently reported aberrant expression of Notch intracellular domain (NICD) in lesions showing goblet cell depletion in UC patients.[8] Moreover, forced expression of NICD caused the suppression of phenotypic genes for goblet cells in human intestinal epithelial cells. It has also been reported that forced expression of NICD in murine intestinal epithelial cells caused the depletion of goblet cells with the decrease of Atoh1 expression.[5] Thus, it is likely that *Atoh1* gene expression is regulated by Notch signaling, leading to subsequent control of intestinal epithelial cell lineage decision of the crypt cells.

The regulation of Hath1, however, is less well understood in human intestine. In previous reports, regulation of *Atoh1* gene expression was assessed using the mouse or chicken promoter region,[11,12] but the critical domains of the mouse and chicken sequences are not completely conserved in the Hath1 promoter region and enhancer region. To date, the regulation of *Hath1* gene expression has not been assessed using the human sequence. In particular, it remains unknown how *Hath1* gene expression is suppressed by Notch signaling in the intestine. It also remains unknown whether goblet cell depletion in UC is affected by Hath1 expression in intestinal epithelial cells.

In this study we demonstrated that Hes1 expression via Notch signaling is enough to suppress the *Hath1* gene by directly binding to the 5′ promoter region of Hath1. In UC, the cooperation of Hes1 and caudal type homeobox 2 (CDX2) caused the suppression of Hath1, resulting in the goblet cell depletion.

MATERIALS AND METHODS

Cell Culture

Human colon carcinoma-derived LS174T cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, 4 mM L-glutamine. Except where indicated otherwise, cells were seeded at a density of 5×10^5 cells/mL in each experiment. Cell cultures and transfections of plasmid DNA were performed as previously described.[6] A cell line expressing Notch1 intracellular domain (NICD), Hes1, HeyL (Tet-On NICD, Tet-On Hes1, Tet-On HeyL cells) under the control of doxycycline (DOX, 100 ng/mL, ClonTech, Palo Alto, CA) was generated as previously described.[8] The cell lines were supplemented with Blastcidin (7.5 μg/mL, Invitrogen, La Jolla, CA) and Zeocin (750 μg/mL, Invitrogen) for maintenance. The inhibition of Notch signaling was achieved by the addition of LY411,575 (1 μM).

Quantitative Real-time Polymerase Chain Reaction (PCR)

Total RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Aliquots of 1 μg of total RNA were used for cDNA synthesis in 20 μL of reaction volume. One microliter of cDNA was amplified with Cyber Green in a 20-μL reaction as previously described.[6] The primer sequences in this study are summarized in Supporting Information Table S1.

Plasmids

5′ Hath1 reporter plasmid was generated by cloning a 1031-bp sequence 5′ of the human *Hath1* gene (corresponding to −1,029 to +2 of the promoter region) into a pGL4 basic vector (Promega, Madison, WI). Hath1 reporter plasmid containing the 3′ region was generated by cloning a 4811-bp sequence 3′ of the human *Hath1* gene (corresponding to +1401 to +6211 of the Hath1 genome) into the 5′ Hath1 reporter plasmid. Internal deletion mutants of the 5′ Hath1 reporter plasmid in which three Hes1 binding sites CACGCG (−305 to −300, −269 to −264, −159 to −154) were replaced with GTCGAC were constructed by PCR-mediated mutagenesis.[13] Doxycycline-dependent expression of NICD was achieved by cloning the gene encoding the intracellular portion of the mouse Notch1 into the pcDNA4/TO/myc-his vector (Invitrogen).[8] Doxycycline-dependent expression of Hes1 was achieved by cloning the gene encoding rat Hes1 into the pcDNA4/TO/myc-his vector (Invitrogen). Doxycycline-dependent expression of HeyL was achieved by cloning the gene encoding human HeyL into the pcDNA4/TO/myc-his vector (Invitrogen). All constructs were confirmed by DNA sequencing.

Luciferase Assays

LS174T cell seeded in a 6-well plate culture dish were transfected with 4 μg of reporter plasmid along with 10 ng of pRL-tk plasmid (Promega). Cells were harvested 36 hours after transfection, lysed by three cycles of freezing and thawing, and the luciferase activities in each sample as indicated by arbitrary unit were normalized against Renilla luciferase activities as previously described.[10]

Chromatin Immunoprecipitation Assay

A chromatin immunoprecipitation (ChIP) assay was performed essentially as previously described with some modifications.[6] LS174T/Hes1 cells were seeded onto a 150-mm dish, then stimulated with DOX or left untreated for 12 hours. Immunoprecipitation was performed overnight at 4°C with 10 μg of an anti-Hes1 (a kind gift from Dr. T. Sudo), normal mouse immunoglobulin G (sc-2025, Santa Cruz Biotechnology, Santa Cruz, CA), or an anti-histone H3 antibody (Abcam,

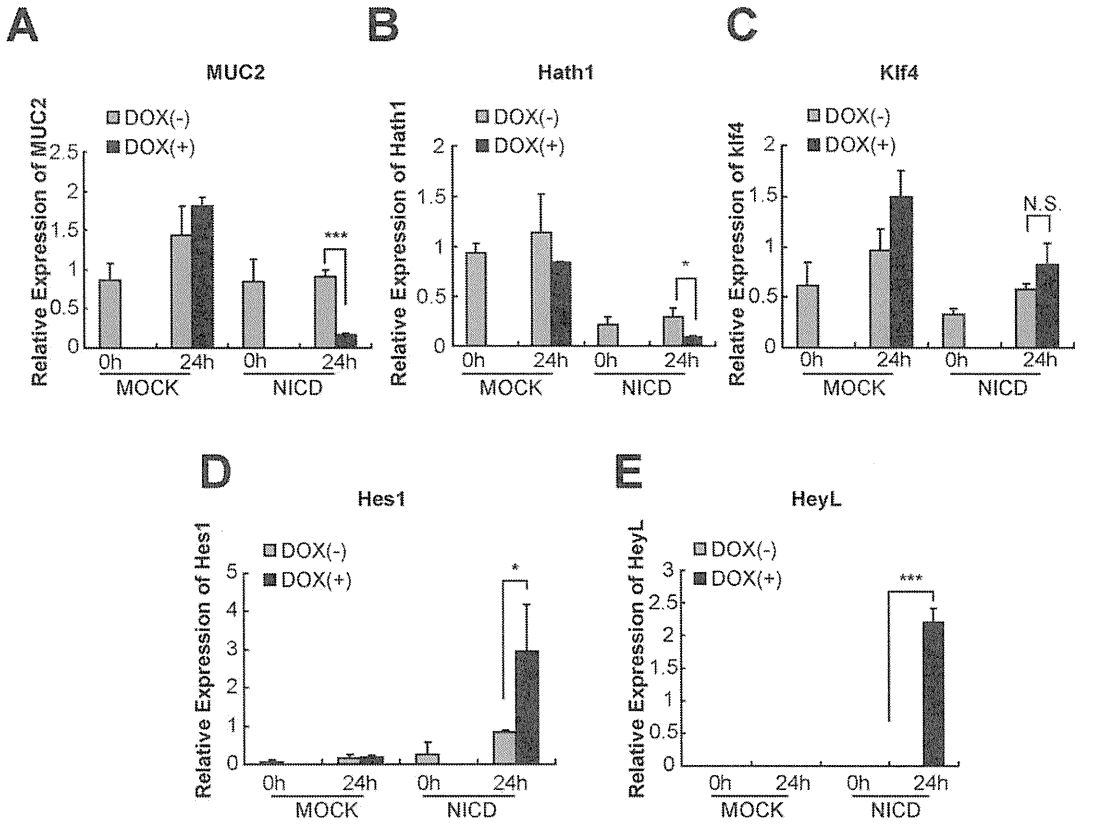

FIGURE 1. Gene alternation in LS174T cells by the expression of NICD. NICD is induced by DOX using the Tet-on system to mimic the acceleration of the Notch signal in LS174T cells. NICD expression by DOX decreased the expression of MUC2 (A) and Hath1 (B) genes. Klf4 gene expression was not affected (C). NICD also induced expression of Hes family genes such as Hes1 (D) and HeyL (E). ($^{*}P < 0.05$, $^{***}P < 0.001$, $n = 3$).

Cambridge, MA). The genomic DNA fragments in the immunoprecipitated samples were analyzed by PCR using primers indicating the positions on the genomic DNA relative to the translation start site (Supporting Information Table 1). The same amounts of DNA samples were analyzed by conventional PCR in parallel with the following parameters: denaturation at 94°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 68°C for 60 seconds for 45 cycles. The products were resolved by agarose gel electrophoresis, stained with ethidium bromide, and visualized using an ImageQuant TL system (GE Healthcare, Milwaukee, WI).[6] The primer sequences in this study are summarized in Supporting Information Table S1.

Human Intestinal Tissue Specimens

Human tissue specimens were obtained from patients who underwent endoscopic examination or surgery at Yokohama Municipal General Hospital or Tokyo Medical and Dental University Hospital. Normal colonic mucosa was obtained from patients with colorectal cancer who underwent colectomy. Each of three patients with UC and colon cancer were examined. Written informed consent was obtained from each patient and the study was approved by the Ethics Committee of both Yokohama Municipal General Hospital and Tokyo Medical and Dental University.

Immunohistochemistry

Hath1 antibody (1:5000) was originally generated as previously described. Hes1 antibody (1:10,000) was the same as in the ChIP assay. Fresh frozen tissue was used after microwave treatment (500W, 10 minutes) in 10 mM citrate buffer for Hath1 and Hes1. The standard ABC method (Vectastain; Vector Laboratories, Burlingame, CA) was used, and staining was developed by addition of diaminobenzidine (Vector Laboratories).

Statistical Analyses

Quantitative real-time PCR analyses were statistically analyzed with Student's *t*-test. *P* less than 0.05 was considered statistically significant.

RESULTS

Notch Signaling Suppresses *Hath1* Gene Expression But Not Kuppel-like Factor 4 (Klf4) Gene in Human IECs

Expression of Atoh1 seems to be regulated at its transcriptional level, as forced expression of NICD in murine IECs causes the decrease of Atoh1 mRNA expression and subsequent depletion of goblet cells in vivo.[5] We therefore assessed the effect of the Notch signal on the expression of