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.

Acknowledgments

We are grateful to Dr. R. Zamoyska for providing the IL-7–deficient mice and Drs. M. Yamazaki and T. Tomita for critical discussions.

Disclosures

The authors have no financial conflicts of interest.

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J Immunol 2011;186;2623-2632; Prepublished online 7 January 2011;
doi:10.4049/jimmunol.1000057
http://www.jimmunol.org/content/186/4/2623

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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.

Received for publication January 11, 2010. Accepted for publication December 14, 2010.

This work was supported in part by grants-in-aid for Scientific Research, Scientific Research on Priority Areas, Exploratory Research and Creative Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology; the Japanese Ministry of Health, Labor and Welfare; the Japan Medical Association; the Foundation for Advancement of International Science; the Keio Medical Science Foundation; and the Research Fund of Mitsukoshi Health and Welfare Foundation.

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

The online version of this article contains supplemental material.

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.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1000057

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*).

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*).

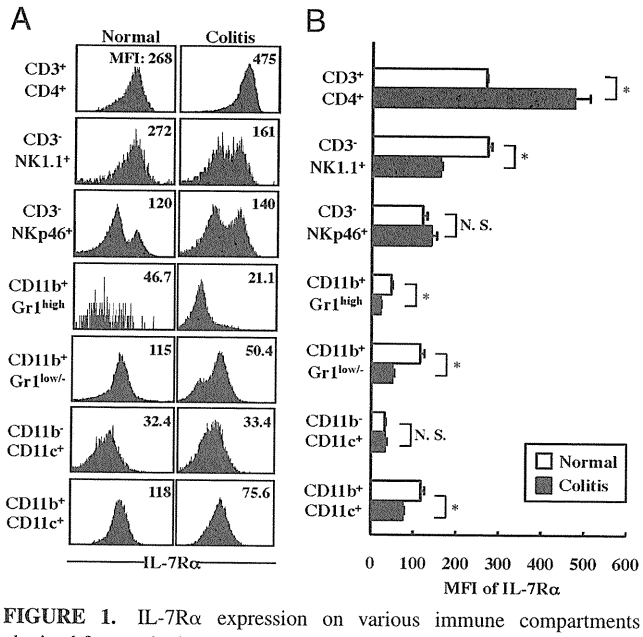

FIGURE 1. IL-7Rα expression on various immune compartments obtained from colonic LP of normal and colitic mice. *A*, Dot plot analysis shows the IL-7α expression on each fraction of immune cells from colonic LP of normal and colitic mice. Numerical values on the dot plots and histograms express the mean 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.

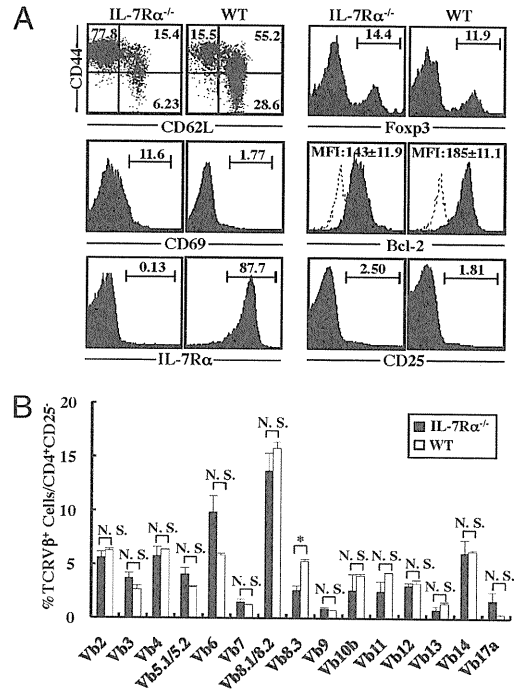

FIGURE 2. Phenotypic characterization of splenic CD4$^+$ T cells obtained from age-matched WT and IL-7Rα$^{-/-}$ mice. *A*, FACS analysis shows the expression of CD44/CD62L, IL-7Rα, Foxp3, and Bcl-2 on/in splenic CD4$^+$ T cells. The dotted line in the Bcl-2 histogram shows the baseline of isotype control. *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.

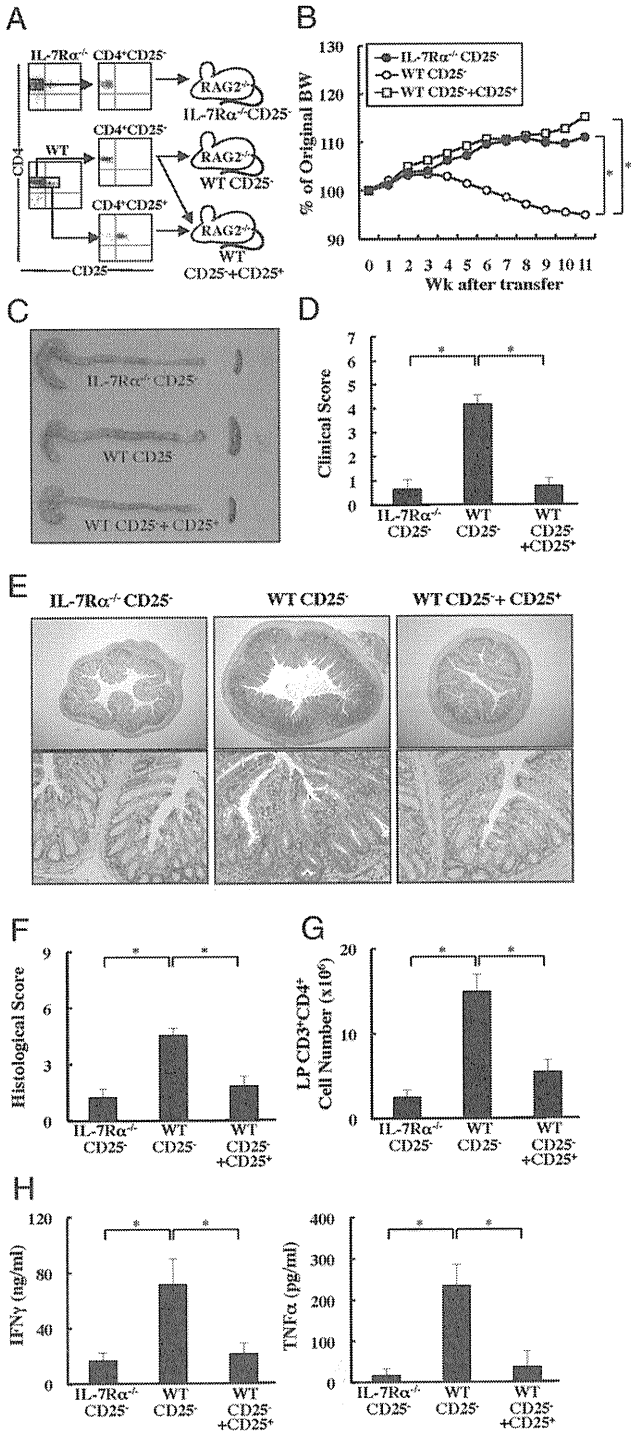

FIGURE 3. RAG-$2^{-/-}$ transferred with IL-7R$\alpha^{-/-}$ $CD4^+CD25^-$ T cells did not develop chronic colitis. *A*, RAG-$2^{-/-}$ mice were transferred with splenic $CD4^+CD25^-$ T cells obtained from age-matched WT or IL-7R$\alpha^{-/-}$ mice (3×10^5 cells per mouse). As a negative control, RAG-$2^{-/-}$ 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-7Rα$^{-/-}$ $CD4^+$ 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-7Rα$^{-/-}$ or WT SP $CD4^+$ T cells under Th1 polarizing conditions (Supplemental Fig. 2*A*). As shown in Supplemental Fig. 2*B*, IL-7Rα$^{-/-}$ 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-7Rα$^{-/-}$ $CD4^+$ 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-7Rα$^{-/-}$ 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-7Rα$^{-/-}$ 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-7Rα$^{-/-}$ donors at 6 wk after transfer was

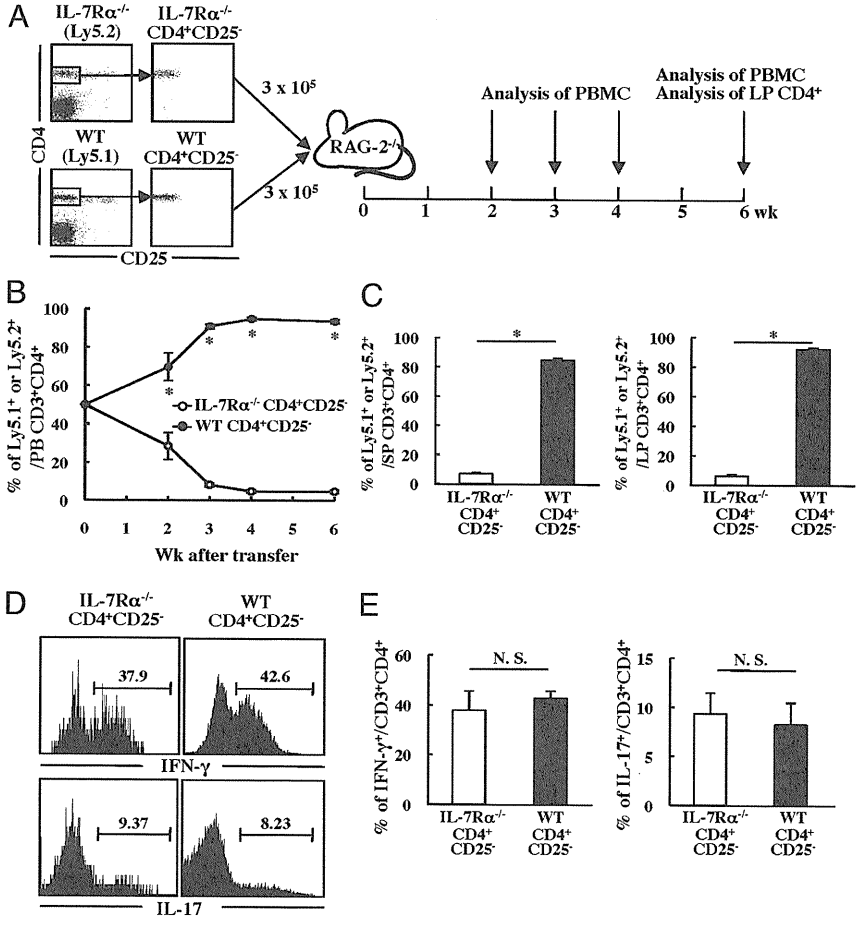

FIGURE 4. IL-7Rα$^{-/-}$ $CD4^+CD25^-$ T cells cotransferred with WT $CD4^+CD25^-$ T cells to RAG-$2^{-/-}$ mice could produce IFN-γ and IL-17, but could not survive. *A*, To discern why IL-7Rα$^{-/-}$ $CD4^+CD25^-$ T cells could not induce colitis, we cotransferred the same number (3×10^5) of $Ly5.2^+$ IL-7Rα$^{-/-}$ $CD4^+CD25^-$ 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-7Rα$^{-/-}$ 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-7Rα$^{-/-}$ 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-7Rα$^{-/-}$ 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-7Rα$^{-/-}$ 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

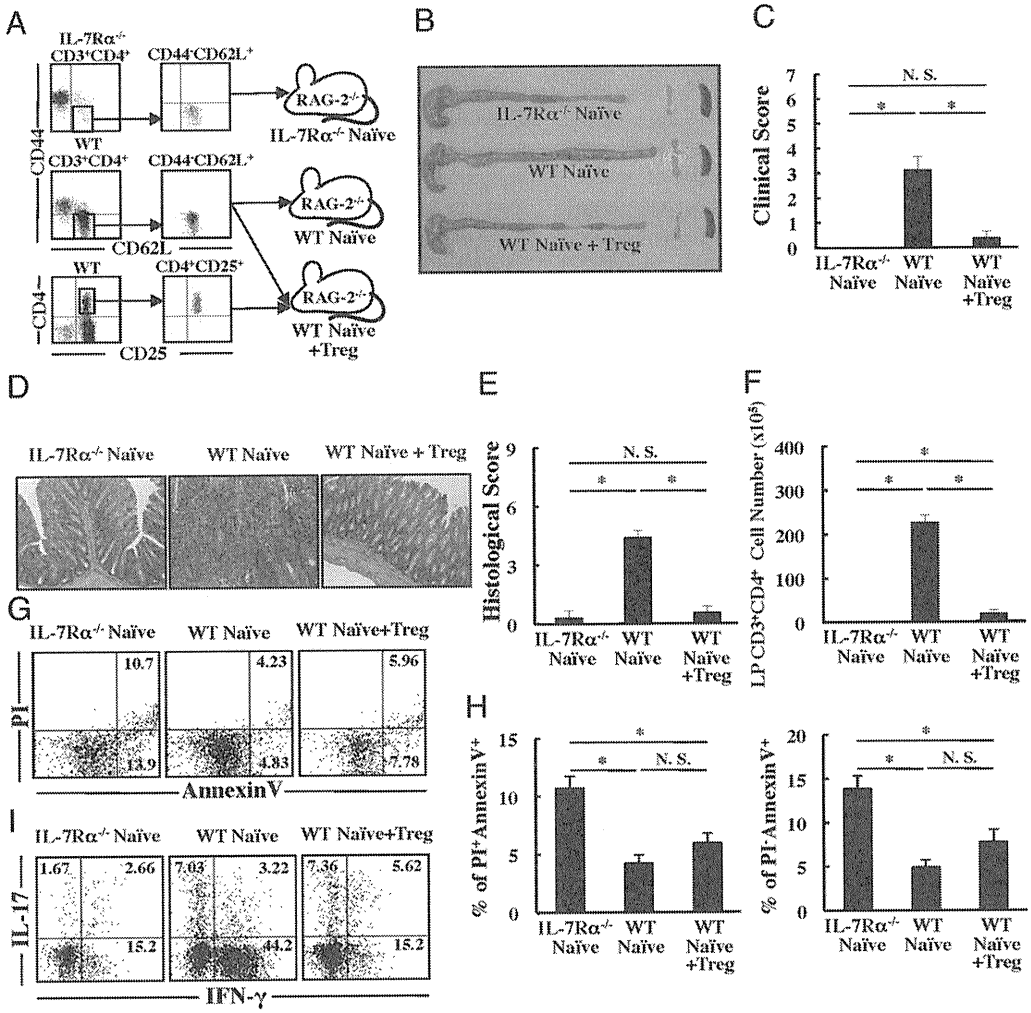

FIGURE 5. RAG-2$^{-/-}$ transferred with IL-7Rα$^{-/-}$ CD3+CD4+CD62L+CD44− T cells did not develop chronic colitis. *A*, RAG-2$^{-/-}$ mice were transferred with splenic CD3+CD4+CD62L+CD44− T cells obtained from age-matched WT or IL-7Rα$^{-/-}$ mice (3×10^5 cells per mouse). As a negative control, RAG-2$^{-/-}$ 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

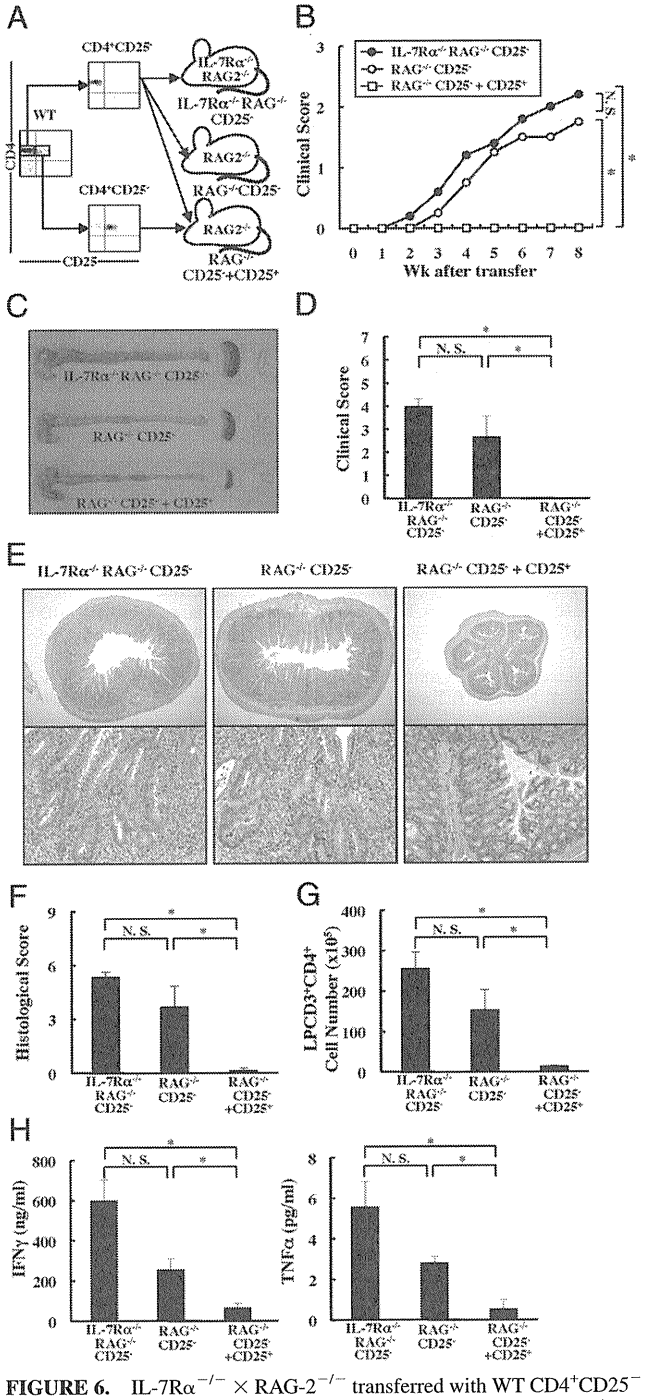

FIGURE 6. IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ transferred with WT CD4$^+$CD25$^-$ T cells developed chronic colitis. *A*, RAG-2$^{-/-}$ mice and IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice were transferred with splenic WT CD4$^+$CD25$^-$ T cells (3×10^5 cells per mouse). As a negative control, RAG-2$^{-/-}$ mice 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. 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. *$p < 0.05$.

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-

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-$7R\alpha^{-/-}$ × $RAG2^{-/-}$ 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-$7R\alpha^{-/-}$ × $RAG\text{-}2^{-/-}$ mice transferred with $CD4^+CD25^-$ T cells would develop more severe colitis than the control transferred $RAG\text{-}2^{-/-}$ recipient mice by considering two points. First, we thought that the availability of IL-7 for colitogenic $CD4^+$ T cells might increase in IL-$7R\alpha^{-/-}$ × $RAG2^{-/-}$ 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-$7R\alpha^{-/-}$ 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 $ROR\gamma t^+$ 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-$22^{-/-}$ × $RAG\text{-}2^{-/-}$ mice (33), leading to speculation that these NK-22 cells reside in intestinal LP of $RAG\text{-}2^{-/-}$ 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 $RAG\text{-}2^{-/-}$ and IL-$7R\alpha^{-/-}$ × $RAG\text{-}2^{-/-}$ 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 $RAG\text{-}2^{-/-}$ 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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ORIGINAL ARTICLE

Human Neutrophil Peptide-1 Aggravates Dextran Sulfate Sodium-induced Colitis

Shinichi Hashimoto, MD, Hirofumi Uto, MD, PhD, Shuji Kanmura, MD, PhD, Toshio Sakiyama, MD, PhD, Manei Oku, MD, Yuji Iwashita, MD, Rie Ibusuki, BA, Fumisato Sasaki, MD, Kazunari Ibusuki, MD, Yoichiro Takami, PhD, Akihiro Moriuchi, MD, PhD, Makoto Oketani, MD, PhD, Akio Ido, MD, PhD, and Hirohito Tsubouchi, MD, PhD

Background: Human neutrophil peptide (HNP)-1, HNP-2, and HNP-3 (HNP-1–3) are useful biomarkers for ulcerative colitis (UC). The precise roles of these peptides in UC are poorly understood, however. The aim of this study was to determine whether HNP-1 affects disease activity in mice with experimental colitis.

Methods: Experimental colitis was induced in BALB/c or severe combined immunodeficiency (SCID) mice using dextran sulfate sodium (DSS). Mice were subsequently treated intraperitoneally with HNP-1 (100 μg/day) or phosphate-buffered saline (PBS) from day 4 to day 6. The severity of colitis was evaluated based on a disease activity index, histologic score, and cytokine expression.

Results: Body weight and colon length significantly decreased and the disease activity index score, histologic score, and myeloperoxidase activity significantly increased in HNP-1-treated BALB/c mice compared with PBS-treated mice. Interferon-γ and tumor necrosis factor-α levels in colon culture supernatants-derived HNP-1-treated mice were also significantly higher, and interleukin (IL)-1β levels tended to increase in response to HNP-1. In addition, treating SCID mice with HNP-1 aggravated DSS-induced colitis and IL-1β levels in colon culture supernatants from these mice were significantly higher than in cultures obtained from control mice. Furthermore, in both BALB/c and SCID mice increased recruitment of F4/80-positive macrophages was observed in the inflamed colonic mucosa following HNP-1 injections.

Conclusions: High concentrations of HNP-1 aggravate DSS-induced colitis, including upregulated expression of such macrophage-derived cytokines as IL-1β. These results indicate that high concentrations of HNP-1–3 in patients with UC may exacerbate disease activity via increased cytokine production.

(Inflamm Bowel Dis 2011;000:000–000)

Key Words: human neutrophil peptide-1, ulcerative colitis, dextran sulfate sodium, macrophage, IL-1β

Inflammatory bowel disease (IBD) primarily manifests as one of two forms: ulcerative colitis (UC) or Crohn's disease (CD). Although the etiology of IBD is not clear, the mucosal immune system plays a central role in intestinal inflammation and injury and cytokines are critical modulators of inflammation. Of note, tumor necrosis factor (TNF)-α and interleukin (IL)-1β are thought to be key cytokines in IBD.[1,2]

IBD is associated with the infiltration of a large number of leukocytes into the bowel mucosa and removing circulating leukocytes—including monocytes, granulocytes, and lymphocytes—is thought to be a promising approach for the treatment of IBD, particularly for UC in Japan.[3,4] The molecular mechanisms underlying the benefits of leukocyte aphaeresis have not been fully elucidated, however.

Defensins, such as the vertebrate α-defensins, are antimicrobial and cytotoxic peptides.[5] α-Defensins include six subfamilies: four human neutrophil peptides (HNP-1 to HNP-4), human defensin (HD)-5, and HD-6.[5,6] HNPs are small cationic peptides that are predominantly expressed in normal bone marrow cells, neutrophils, natural killer cells, and T cells. HNPs are stored in azurophilic granules and are released in response to neutrophil activation.[7–9] The amino acid sequences of HNP-1, HNP-2, and HNP-3 are similar structures, with the peptides consisting of 30, 29, and 30 amino acids, respectively. Twenty-nine of the amino acids are identical among the three peptides and we previously reported that neutrophils infiltrating the bowel

Received for publication July 14, 2011; Accepted July 18, 2011.

From the Department of Digestive and Lifestyle Related Disease, Health Research Course, Human and Environmental Sciences, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan.

Supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Ministry of Health, Labour and Welfare of Japan.

Reprints: Hirofumi Uto, Department of Digestive and Lifestyle Related Disease, Health Research Course, Human and Environmental Sciences, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan (e-mail: hirouto@m2.kufm.kagoshima-u.ac.jp).

DOI 10.1002/ibd.21855

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mucosa in patients with active UC express HNP-1–3.[10] In addition, a proteomic approach revealed that HNP-1–3 levels were elevated in sera from patients with UC compared with samples from patients with CD.[10] Plasma concentrations of HNP-1–3 were also higher in patients with active UC than in healthy subjects or those with inactive UC, CD, or infectious enterocolitis. Furthermore, plasma concentrations of HNP-1–3 in patients with UC who responded to corticosteroid-based therapy decreased after treatment, whereas no change was observed in nonresponders.[10] Interestingly, HNPs are reportedly antimicrobial and regulate IL-8 production and chemotaxis in neutrophils, lymphocytes, and macrophages.[11,12] The relationships between HNP-1–3 and intestinal inflammation or mucosal injury in patients with UC, however, have not been fully investigated. The aim of this study was to determine whether HNP-1 exacerbates disease activity in mice with experimental colitis.

MATERIALS AND METHODS

Reagents

Synthetic HNP-1 was purchased from the Peptide Institute (Osaka, Japan) and dissolved in phosphate-buffered saline (PBS). McCoy's 5A medium, RPMI 1640 medium, fetal bovine serum, penicillin-streptomycin, PBS, L-glutamine, and HEPES buffer were obtained from Invitrogen (La Jolla, CA). TetraColor One was obtained from Seikagaku Biobusiness (Tokyo, Japan). Colorimetric immunoassays to quantify cell proliferation based on bromodeoxyuridine (BrdU) incorporation during DNA synthesis were obtained from Roche Diagnostics (Mannheim, Germany). Annexin V-FITC apoptosis detection kits and tetramethylbenzidine/H_2O_2 substrate solution were obtained from BD Biosciences (Princeton, NJ). Dextran sodium sulfate (DSS; mean molecular weight, 5,000 Da) and cetyltrimethylammonium chloride were obtained from Wako Pure Chemical Industries (Osaka, Japan). Citrate buffer solution was obtained from Sigma-Aldrich Japan (Tokyo, Japan). Interferon (IFN)-γ, IL-1β, and TNF-α ELISA kits were obtained from R&D Systems (Minneapolis, MN).

Animals

Specific pathogen-free male BALB/c mice (7 weeks of age, weighing 25–29 g) were obtained from Kyudo (Saga, Japan). Male severe combined immunodeficiency (SCID) mice (C.B-17/Icr-scid/scid Jcl, 7 weeks of age, weighing 20–24 g) were obtained from CLEA Japan (Tokyo, Japan). Mice were maintained in standard wire cages and allowed free access to food and water for 1 week before entering the study. This study was approved by the Animal Experiment Ethics Committee of the Kagoshima University Graduate School of Medical and Dental Sciences.

Induction of Colitis

Experimental colitis was induced in BALB/c or SCID mice by administering 4.0% or 3.0% DSS in drinking water, respectively, for 7 days. Mice were intraperitoneally injected with HNP-1 (100 μg/day) or PBS from day 4 to day 6. Serum HNP-1 levels were measured using a human HNP 1–3 enzyme-linked immunosorbent assay (ELISA) kit (Hycult Biotechnology, Ude, Netherlands) according to the manufacturer's instructions. Mice were sacrificed and the severity of colitis was evaluated based on body weight, colon length, clinical scoring, and histologic scoring of colitis. Clinical scoring was used to determine values for a disease activity index (DAI), which was based on weight loss, stool consistency, and bleeding, as described previously by Murthy et al.[13] Each clinical parameter was scored on a scale of 0–4 and the parameter values were added together. For histopathologic examination, the cecum and rectum were dissected longitudinally and the length of the entire colon was measured. Sections of colon were fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Histologic scoring was based on the method described previously by Cooper et al.[14] Colon damage was categorized into five groups: grade 0, normal mucosa; grade 1, loss of one-third of the crypts; grade 2, loss of two-thirds of the crypts; grade 3, lamina propria covered with a single epithelial layer with mild inflammatory cell infiltration; grade 4, erosions and marked inflammatory cell infiltration. All scores were obtained in a blinded fashion by two investigators. Colonic tissue myeloperoxidase (MPO) activity, an indicator of the extent of neutrophil infiltration, was assessed as described previously by Qualls et al.[15]

Immunohistochemistry

For macrophage staining, F4/80 antigen was detected using methods similar to those described previously.[16] To assess proliferation of colonic epithelial cells, Ki-67 was stained using methods similar to those described previously.[17] In brief, after DSS was administered to BALB/c or SCID mice with or without HNP-1 for 7 days, deparaffinized sections of colon tissues were incubated with F4/80 monoclonal rat antimouse antibodies (AbD Serotec, Raleigh, NC) or anti-Ki-67 monoclonal antibodies (MIB-5; Dako Cytomation, Copenhagen, Denmark), followed by incubation with Histofine Simple Stain MAX PO (Rat; Nichirei, Japan). Following visualization of F4/80 or Ki-67 antigen, F4/80- or Ki-67-positive cells were counted based on the average obtained from five areas under a microscope at 400× magnification.

Colon Organ Cultures

The colon organ culture conditions have been described previously by Azuma et al.[18] On day 7 after DSS administration the transverse colon was obtained from each mouse. A segment of distal colon was removed from each animal, cut open longitudinally, and washed in PBS containing penicillin and streptomycin. Segments 1 cm in length were placed in 24-well flat-bottom culture plates (Asahi Glass, Tokyo, Japan), containing 1 mL of fresh RPMI 1640 medium supplemented with penicillin and streptomycin, and incubated at 37°C for 24

hours. This experiment was performed using 13 mice from each group. Culture supernatants were stored at −30°C until analysis. Concentrations of IFN-γ, IL-1β, and TNF-α in the supernatants were measured using cytokine ELISA kits according to the manufacturer's protocol and analyzed in duplicate using a microplate reader (Bio-Rad Laboratories, Hercules, CA). Cytokine concentrations in the samples were calculated using standard curves. Protein concentrations in each sample were determined using an RC DC protein assay (Bio-Rad Laboratories).

Cell Culture

Human HT-29 colon cancer cell line (European Collection of Cell Cultures, EC910722201) were obtained from Dainippon Sumitomo Pharma Biomedical (Osaka, Japan) and maintained at 37°C in 5% CO_2 and McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum, streptomycin, penicillin, and L-glutamine.

Stimulation of Cells with HNP-1 and Cell Proliferation

The viability of HT-29 cells was assessed using the TetraColor ONE assay, which examines mitochondrial activity. DNA synthesis was assessed in the cells using a BrdU incorporation assay according to the manufacturer's instructions. Before the experiments cells were trypsinized and washed twice with PBS. Cells were seeded at a concentration of 3×10^4 cells/mL (TetraColor ONE assay) or 1×10^5 cells/mL (BrdU incorporation assay) in 96-well plates containing 90 μL of serum-free medium and incubated for 24 hours. HNP-1 dissolved in PBS or PBS alone were added to the 96-well plates and the cells were incubated in a total volume of 100 μL medium/well with various concentrations of HNP-1 for 12, 24, 48, or 72 hours (n = 6–12 for each condition). For the TetraColor ONE assay, 10 μL of TetraColor ONE solution was added to each well and the cells were incubated at 37°C for 2 hours. Each well was analyzed using a microplate reader (Bio-Rad Laboratories) at 450 nm (reference wavelength, 620 nm). The proliferation rate and BrdU incorporation in HNP-1-treated cells were assessed and absorbance values obtained with untreated cells were defined as 100%.

Annexin V Staining and FACS

An Annexin V-FITC apoptosis detection kit was used to determine the percentage of cells undergoing apoptosis. HT-29 cells were seeded in 12-well plates and grown to ≈80% confluence. Before the experiments, cells were washed twice with PBS and serum-free medium was added. HNP-1 dissolved in PBS or PBS alone was added and the cells were incubated for 6, 12, 18, or 24 hours. The cells were then trypsinized and washed with PBS. The cells were resuspended in 1× binding buffer at a concentration of 1×10^6 cells/mL and Annexin V-FITC (5 μL) and propidium iodide (PI; 10 μL) were added. After incubation in the dark for 15 minutes the samples were analyzed using a FACScan instrument (BD Biosciences). Dead cells were defined as those that were positive for both Annexin V and PI. Data were analyzed using Win MDI software and are shown as means ± SD from three separate experiments.

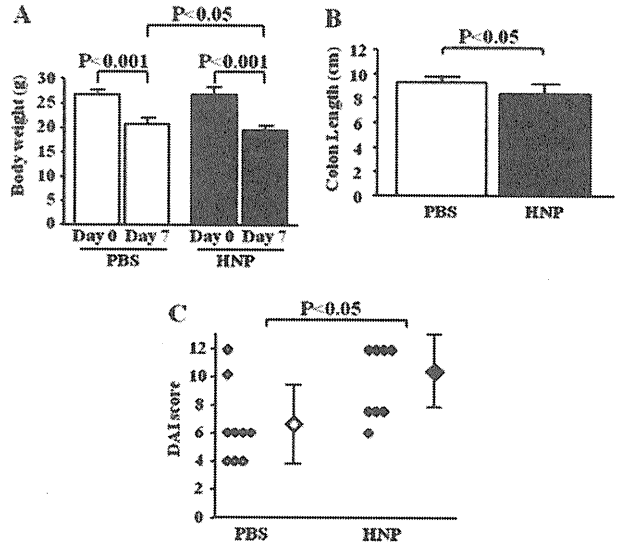

FIGURE 1. Effect of HNP-1 on DSS-induced colitis in BALB/c mice. HNP-1 (100 μg; n = 8) or PBS (n = 9) was intraperitoneally administered to DSS-treated mice on days 4, 5, and 6. (A) Body weights were measured on days 0 and 7. (B) Colon length was assessed on day 7. (C) DAI scores were examined on day 7. Data are shown as means ± SD or dot plots.

Statistical Analysis

Results are expressed as means ± SD or dot plots. Data were analyzed for statistical differences using Mann–Whitney U-tests or Fisher's exact tests. Statistical significance was defined as $P < 0.05$.

RESULTS

Serum Concentrations of HNP-1–3 in Control Mice After HNP-1 Administration

Three BALB/c mice were intraperitoneally injected with single doses of 100 μg of HNP-1 and serum HNP-1 levels were measured using ELISA. After administration of HNP-1, levels of the peptide were 177.33 ± 5.04 ng/mL, 292.40 ± 43.61 ng/mL, 148.27 ± 18.46, and 16.89 ± 14.86 ng/mL at 1, 3, 12, and 24 hours, respectively.

HNP-1 Aggravated DSS-induced Colitis in BALB/c Mice

In BALB/c mice, body weights of HNP-1-treated mice were significantly lower than those of control animals on day 7 (19.58 ± 0.96 g vs. 20.62 ± 1.12 g, P = 0.04; Fig. 1A), and the colons of HNP-1-treated mice were

significantly shorter than those in the PBS-treated group (8.29 ± 0.64 cm vs. 9.26 ± 0.76 cm, $P = 0.02$; Fig. 1B). In addition, DAI scores were significantly higher in the HNP-1-treated mice compared with those in the PBS-treated group (9.33 ± 2.65 vs. 6.20 ± 2.74, $P = 0.02$; percentage of DAI scores greater than 6: 87.5% vs. 22.2%, $P = 0.01$; Fig. 1C).

Histologic examination of the distal colons in the HNP-1-treated mice revealed marked erosion of the mucosal layer, an absence of glandular epithelium, and inflammatory cell infiltration in the submucosal area (Fig. 2A). Histologic scores were significantly higher in the HNP-1-treated group than in the PBS-treated group (3.89 ± 0.33 g vs. 2.90 ± 0.99 g, $P = 0.03$; percentage of scores greater than 3: 87.5% vs. 33.3%, $P < 0.05$; Fig. 2B). Colonic MPO activity, an indicator of neutrophil infiltration, was significantly higher in HNP-1-treated mice compared with control mice (207.03 ± 52.35 U/mg tissue vs. 160.20 ± 43.11 U/mg tissue, $P < 0.05$, $n = 7$ for each group; Fig. 2C). In addition, F4/80-positive macrophages were detected in colonic mucosa that was inflamed in response to DSS with or without HNP-1 administration (Fig. 3A). More F4/80-positive macrophages were detected in sections from HNP-treated mice than in samples from the PBS-treated group (Fig. 3B). In contrast, Ki-67-positive colonic epithelial cells were detected in both the HNP- and PBS-treated mice (Fig. 3C), with no statistical differences between the groups in the number of Ki-67-positive cells (Fig. 3D).

IFN-γ, TNF-α, and IL-1β Levels in Colon Culture Supernatants Derived from BALB/c Mice Treated with DSS and HNP-1

IFN-γ and TNF-α concentrations in colon culture supernatants were significantly higher in samples obtained from HNP-1-treated BALB/c mice compared with samples derived from control mice (Fig. 4). In addition, concentrations of IL-1β tended to be higher in cultures obtained from the HNP-1-treated group compared with those derived from control mice, although the result was not statistically significant.

Aggravation of DSS-induced Colitis by HNP-1 in SCID Mice

To determine whether HNP-1-mediated aggravation of DSS-induced colitis depends on T-helper 1 $CD4^+$ T cells, we investigated the effects of HNP-1 on DSS-induced colitis in SCID mice. Although body weights and colon lengths were not significantly affected by HNP-1 (Fig. 5A,B), DAI scores for DSS-induced colitis in HNP-1-treated SCID mice were significantly higher compared with PBS-treated mice (5.63 ± 2.83 vs. 2.08 ± 2.02, $P < 0.01$; percentage of scores greater than 4: 62.5% vs. 8.3%, $P = 0.02$; Fig. 5C). HNP-1 injection resulted in higher histologic scores

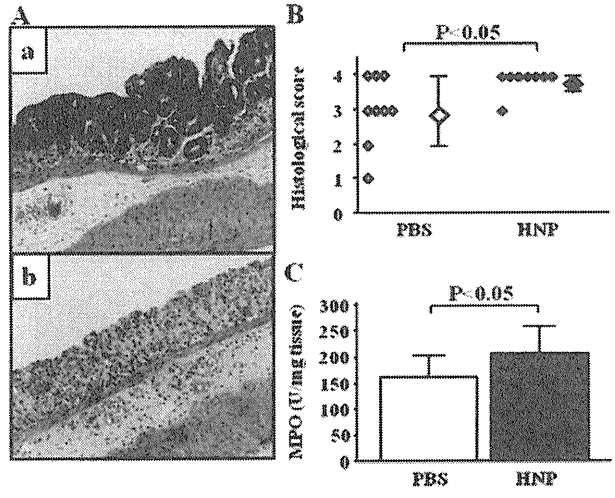

FIGURE 2. Effects of HNP-1 treatment based on histologic changes in BALB/c mice with DSS-induced colitis. (A) Histologic assessment of PBS-treated (a) and HNP-1-treated mice (b) using H&E-stained colonic sections. Original magnification: 100×. (B) Histologic scores for the distal colon. (C) MPO enzymatic activity in the colon.

for DSS-induced colitis in SCID mice (2.38 ± 1.30 vs. 1.25 ± 0.45, $P = 0.04$; percentage of scores greater than 2: 50.0% vs. 0%, $P = 0.01$; Fig. 6A). Colonic MPO activity did not differ in the two groups (Fig. 6B). In addition, more F4/80-positive macrophages were detected in the HNP-1-treated group than in the PBS-treated group (Fig. 6C), whereas no significant difference was observed in the number of Ki-67-positive cells (Fig. 6D). Furthermore, IL-1β concentrations in colon culture supernatants were significantly higher for samples obtained from HNP-1-treated SCID mice with DSS-induced colitis than in samples derived from mice that did not receive HNP-1 (Fig. 6E). IFN-γ and TNF-α were not detected in colon cultures obtained from either HNP-1- or PBS-treated SCID mice.

High Concentrations of HNP-1 Significantly Inhibited HT-29 Cell Viability In Vitro

To investigate the effects of HNP-1 on colon epithelial cell viability, HT-29 cells were treated with a dilution series of HNP-1 from 5 μg/mL to 200 μg/mL for 24 hours. A TetraColor One cell proliferation assay revealed that HT-29 cell proliferation was induced by 5 μg/mL or 10 μg/mL HNP-1, whereas a cytotoxic effect was observed in HT-29 cells at HNP-1 concentrations greater than 100 μg/mL (Fig. 7A). In addition, 100 μg/mL HNP-1 was time-dependently cytotoxic for HT-29 cells (Fig. 7B), and BrdU incorporation in HT-29 cells decreased significantly at HNP-1 concentrations greater than 100 μg/mL (Fig. 7C).

To evaluate the effects of HNP-1 on cytotoxicity and apoptosis in colon epithelial cells, HT-29 cells were incubated with 100 μg/mL HNP-1 and stained for Annexin-V

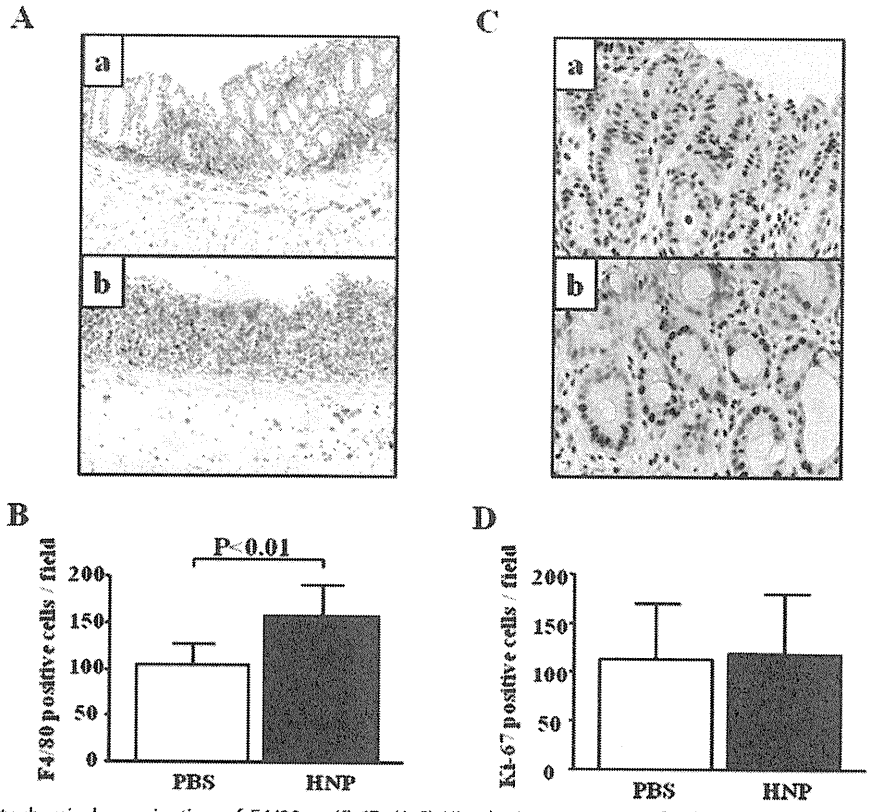

FIGURE 3. Immunohistochemical examination of F4/80 or Ki-67. (A,C) Histologic assessment of colonic sections obtained from PBS-treated (a) and HNP-1-treated mice (b) and stained for F4/80 (A) or Ki-67 (C); brown staining reflects positive macrophage cells or epithelial cell proliferation, respectively. Original magnification: 100× (A) or 400× (C). (B,D) Positively stained cells were counted and averaged using five areas under a microscope at 400× magnification. Data are shown as means ± SD.

and PI. Dead cells were defined as those that were positive for both markers (Fig. 7D). The percentage of cells that were dead in the HT-29 cultures treated with 100 μg/mL HNP-1 significantly increased at 18 hours and 24 hours compared with untreated cultures (Fig. 7E). In contrast, marked numbers of apoptotic cells—defined as those positive for Annexin-V but negative for PI—were not observed in this experiment. This result indicates that HNP-1 caused nonapoptotic cell death.

DISCUSSION

We previously reported that plasma HNP-1–3 are biomarkers for active UC and predictors of treatment efficacy in affected patients.[10] In vitro, HNP-1–3 increase

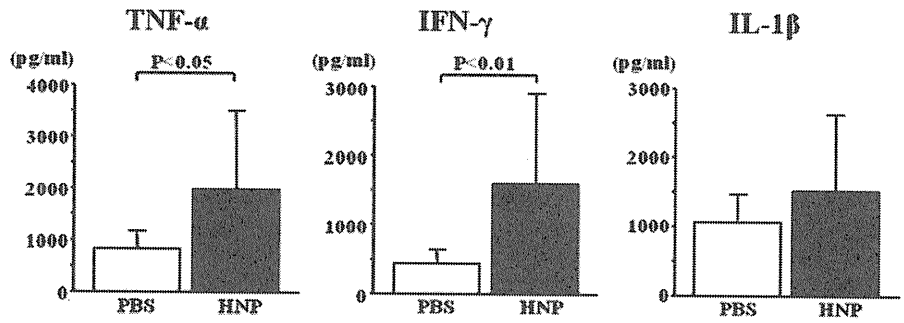

FIGURE 4. Increased cytokine levels in BALB/c mice with DSS-induced colitis. On day 7 after DSS administration, transverse colons were obtained from PBS-treated and HNP-1-treated mice (n = 13 for each group). Colons were cultured for 24 hours in serum-free medium and cytokine levels in the culture supernatants were measured using ELISA. Data are shown as means ± SD.

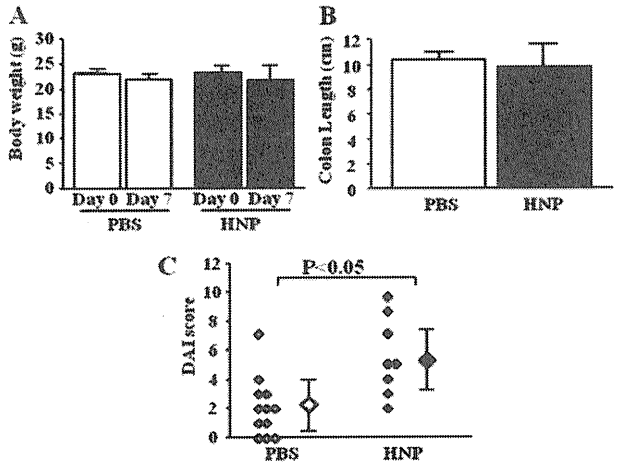

FIGURE 5. Effects of HNP-1 on DSS-induced colitis in SCID mice. HNP-1 (100 μg; $n = 8$) or vehicle (PBS; $n = 12$) was intraperitoneally injected into DSS-treated mice on days 4, 5, and 6. (A) Body weights were measured on days 0 and 7. (B) Colon lengths were assessed on day 7. (C) DAI scores were obtained on day 7. Data are shown as means ± SD or dot plots.

inflammatory cell migration[12,19] and cause these populations to secrete inflammatory cytokines.[20,21] HNP-1 is also cytotoxic to various eukaryotic and tumor cells.[22–25] No studies, however, have addressed whether HNP modulate disease activity and cytokine expression in experimental colitis in vivo or colon epithelial cells in vitro. Here we show that HNP-1 aggravates DSS-induced colitis, an experimental model of UC, in both BALB/c and SCID mice. The enhanced disease was likely mediated, at least in part, by HNP-1-induced cytokine production in macrophages. In contrast, although HNP-1 also inhibited the proliferation of a colon cancer cell line, high concentrations of HNP-1 were required. Thus, HNP-1 appears to modulate DSS-induced colitis primarily via a cytokine-mediated, T-cell-independent mechanism.

Compared with healthy individuals, patients with UC have more mucosal bacteria,[26] which invade the colonic mucosa.[27] These patients have higher neutrophil numbers in the colon mucosa.[28] Interestingly, HNP-1–3 are expressed by infiltrating neutrophils and colon epithelium,[10,29] and

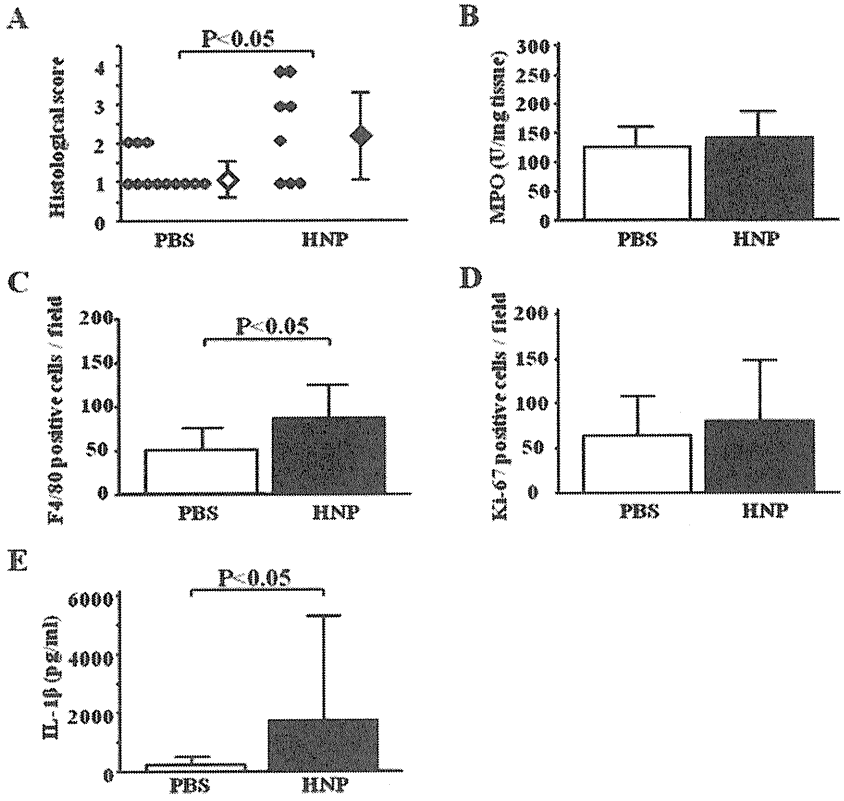

FIGURE 6. Effects of HNP-1 based on histologic changes in SCID mice with DSS-induced colitis. (A) Histologic scores for the distal colons. (B) MPO enzymatic activity in the colons. (C) F4/80-positive cells were counted and averaged using five areas under a microscope at 400× magnification. (D) Ki-67-positive epithelial cells were counted and averaged using five areas under a microscope at 400× magnification. (E) Increased IL-1β levels in SCID mice with DSS-induced colitis. On day 7 after DSS administration, transverse colons were obtained from PBS-treated ($n = 12$) and HNP-1-treated ($n = 8$) mice. Colons were cultured for 24 hours in serum-free medium and cytokine levels in the culture supernatants were measured using ELISAs. Data are shown as means ± SD or dot plots.

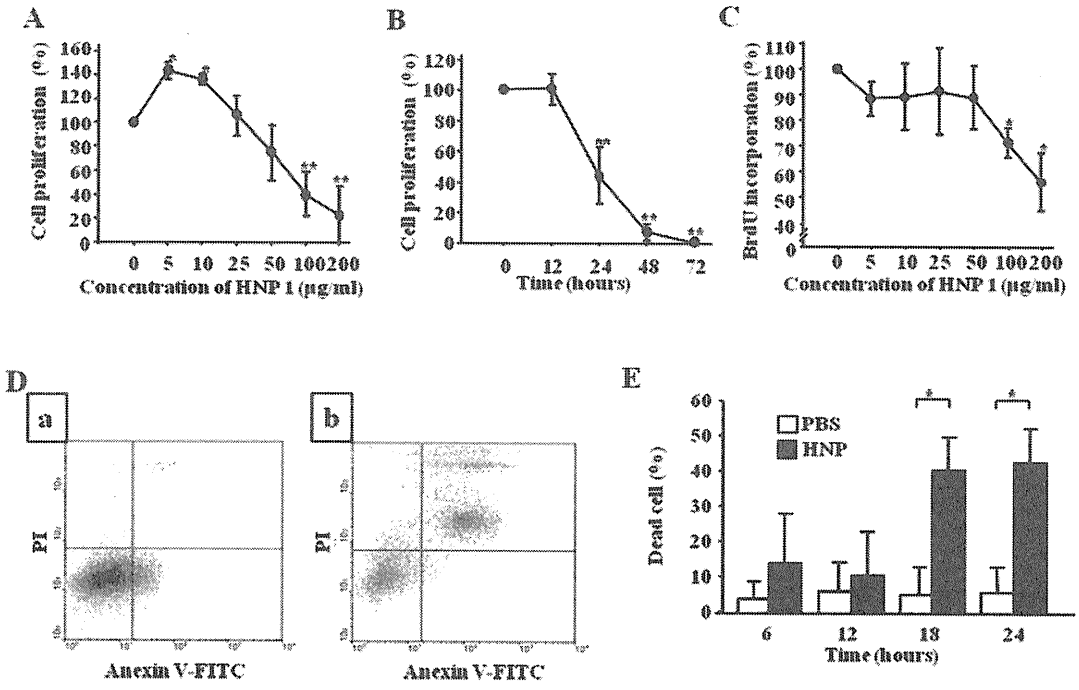

FIGURE 7. Dose- and time-dependent induction of HT-29 cell proliferation by HNP-1 and effects of HNP-1 on HT-29 cell viability. (A) HT-29 cells were incubated with various concentrations of HNP-1 for 24 hours in serum-free medium. Proliferation was assessed using a TetraColor One assay. (B) Cell proliferation was assessed after treatment with 100 μg/mL HNP-1 for 12, 24, 48, or 72 hours. (C) DNA synthesis was assessed based on BrdU incorporation after treatment with various concentrations of HNP-1. Cell proliferation and DNA synthesis were calculated as percentages of the results observed with PBS-treated cells. (D,E) Cell death was measured using FACS analysis, FITC-labeled Annexin V, and PI. Representative flow cytometry analyses of Annexin V-FITC/PI staining are shown. HT-29 cells were untreated for 24 hours (a) or incubated with 100 μg/mL HNP-1 (b). Cells that were Annexin V- and PI-positive were categorized as dead. HT-29 cells were incubated with 100 μg/mL HNP-1 in serum-free medium for 6, 12, 18, or 24 hours (E). Data represent means ± SD from three or six independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ versus PBS-treated cells.

higher levels of these peptides have been detected in patients with active UC than in patients in which the disease is inactive.[10,30] One study showed that HNP-1–3 concentrations in the stool of patients with IBD were ≈14 times higher than in samples obtained from healthy individuals.[31] In addition, HNP-1–3 concentrations are higher at sites of inflammation than in plasma.[32,33] We have shown that HNP-1 exacerbated DSS-induced colitis. Together, these results indicate that neutrophils contribute to the pathogenesis of UC; high concentrations of HNP-1–3 in response to infiltrating bacteria exacerbate the mucosal disorder at inflamed sites in patients with UC.

Although disease markers, including DAI and histologic scores, significantly differed between HNP-1-treated and control mice, the differences may not be physiologically relevant (Figs. 1, 2, 5, 6). The intraperitoneal dosage of HNP-1 was 100 μg, and serum HNP-1 levels were greater than 100 ng/mL during the first 12 hours following a single injection. These serum HNP-1 levels are similar to the plasma HNP-1–3 levels observed in patients with active UC (mean levels: 203.1 ng/mL),[10] although the concentrations were not sustained in our experimental model. Thus, higher and/or more sustained HNP-1 concentrations may induce more severe colitis and larger differences between HNP-1-treated and control groups. Of note, no enterocolitis was detected in BALB/c mice after a single HNP-1 injection without DSS (data not shown). DSS-induced colitis was also primarily observed in the mid to distal colon, with little disease detected in the proximal colon. Histologic scores for the proximal colon were not significantly different between the HNP-1-treated and control groups (data not shown). These results indicate that HNP-1 exacerbates, but does not cause, colitis, and this defensin peptide may be a critical aggravator of colitis in patients with UC.

HNP-1, a 3,442-Da member of the α-defensin family, is an antibiotic peptide containing 30 amino acid residues and three intramolecular disulfide bonds. HNP-1 is produced by human neutrophils, stored in azurophilic granules (30%–50% of the total protein content of these granules), and released in response to inflammation.[7,8] HNP-1 affected the expression of such inflammatory cytokines as TNF-α, IFN-γ, and IL-1β, which aggravated DSS-induced colitis in mice, although the effects on IL-1β levels were not significant (Fig. 4). In addition, IL-1β mRNA