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 $IL-7^{-/-}RAG^{-/-}$ 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: $IL-7R^{hi}$ and $IL-7R^{lo}$ (indicated with an arrow in Fig. 4). The $IL-7R^{lo}$ 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 $IL-7^{-/-}RAG^{-/-}$ 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 $IL-7^{-/-}RAG^{-/-}$ mice, which were surgically separated from T cell-receiving $RAG^{-/-}$–$IL-7^{-/-}RAG^{-/-}$ parabionts 6 wk after the initial surgery, still maintained chronic colitis for at least another 12 wk (16). The latter finding is similar to our present observation that $IL-7^{-/-}RAG^{-/-}$ 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 $IL-7^{-/-}RAG^{-/-}$

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 $IL-7^{-/-}RAG^{-/-}$ 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 $IL-7^{-/-}RAG^{-/-}$ and $RAG^{-/-}$ recipients following NK depletion is not caused by a difference in NK function between NK cells derived from $RAG^{-/-}$ and $IL-7^{-/-}RAG^{-/-}$ mice.

The $IL-7^{-/-}RAG^{-/-}$ 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 $IL-7^{-/-}$ mice (38, 39). We have also reported that Bcl-2 expression is downregulated in T cells injected into $IL-7^{-/-}RAG^{-/-}$ 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

Disclosures

References

1. Fujino, S., A. Andoh, S. Bamba, A. Ogawa, K. Hata, Y. Araki, T. Bamba, and Y. Fujiyama. 2003. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut* 52: 65–70.
2. Powrie, F., M. W. Leach, S. Mauze, L. B. Caddle, and R. L. Coffman. 1993. Phenotypically distinct subsets of CD4$^+$ T cells induce or protect from chronic intestinal inflammation in C. B-17 *scid* mice. *Int. Immunol.* 5: 1461–1471.
3. Ostanin, D. V., J. Bao, I. Koboziev, L. Gray, S. A. Robinson-Jackson, M. Kosloski-Davidson, V. H. Price, and M. B. Grisham. 2009. T cell transfer model of chronic colitis: concepts, considerations, and tricks of the trade. *Am. J. Physiol. Gastrointest. Liver Physiol.* 296: G135–G146.

4. Namen, A. E., S. Lupton, K. Hjerrild, J. Wignall, D. Y. Mochizuki, A. Schmierer, B. Mosley, C. J. March, D. Urdal, and S. Gillis. 1988. Stimulation of B-cell progenitors by cloned murine interleukin-7. *Nature* 333: 571–573.
5. Fry, T. J., and C. L. Mackall. 2005. The many faces of IL-7: from lymphopoiesis to peripheral T cell maintenance. *J. Immunol.* 174: 6571–6576.
6. Surh, C. D., O. Boyman, J. F. Purton, and J. Sprent. 2006. Homeostasis of memory T cells. *Immunol. Rev.* 211: 154–163.
7. Watanabe, M., Y. Ueno, T. Yajima, Y. Iwao, M. Tsuchiya, H. Ishikawa, S. Aiso, T. Hibi, and H. Ishii. 1995. Interleukin 7 is produced by human intestinal epithelial cells and regulates the proliferation of intestinal mucosal lymphocytes. *J. Clin. Invest.* 95: 2945–2953.
8. Watanabe, M., Y. Ueno, T. Yajima, S. Okamoto, T. Hayashi, M. Yamazaki, Y. Iwao, H. Ishii, S. Habu, M. Uehira, et al. 1998. Interleukin 7 transgenic mice develop chronic colitis with decreased interleukin 7 protein accumulation in the colonic mucosa. *J. Exp. Med.* 187: 389–402.
9. Yamazaki, M., T. Yajima, M. Tanabe, K. Fukui, E. Okada, R. Okamoto, S. Oshima, T. Nakamura, T. Kanai, M. Uehira, et al. 2003. Mucosal T cells expressing high levels of IL-7 receptor are potential targets for treatment of chronic colitis. *J. Immunol.* 171: 1556–1563.
10. Totsuka, T., T. Kanai, Y. Nemoto, S. Makita, R. Okamoto, K. Tsuchiya, and M. Watanabe. 2007. IL-7 Is essential for the development and the persistence of chronic colitis. *J. Immunol.* 178: 4737–4748.
11. Fujii, T., T. Tomita, T. Kanai, Y. Nemoto, T. Totsuka, N. Sakamoto, T. Nakamura, K. Tsuchiya, R. Okamoto, and M. Watanabe. 2008. FTY720 suppresses the development of colitis in lymphoid-null mice by modulating the trafficking of colitogenic CD4$^+$ T cells in bone marrow. *Eur. J. Immunol.* 38: 3290–3303.
12. Mangan, P. R., L. E. Harrington, D. B. O'Quinn, W. S. Helms, D. C. Bullard, C. O. Elson, R. D. Hatton, S. M. Wahl, T. R. Schoeb, and C. T. Weaver. 2006. Transforming growth factor-β induces development of the T_H17 lineage. *Nature* 441: 231–234.
13. Min, B., H. Yamane, J. Hu-Li, and W. E. Paul. 2005. Spontaneous and homeostatic proliferation of CD4 T cells are regulated by different mechanisms. *J. Immunol.* 174: 6039–6044.
14. Kieper, W. C., A. Troy, J. T. Burghardt, C. Ramsey, J. Y. Lee, H. Q. Jiang, W. Dummer, H. Shen, J. J. Cebra, and C. D. Surh. 2005. Recent immune status determines the source of antigens that drive homeostatic T cell expansion. *J. Immunol.* 174: 3158–3163.
15. Tomita, T., T. Kanai, Y. Nemoto, T. Totsuka, R. Okamoto, K. Tsuchiya, N. Sakamoto, and M. Watanabe. 2008. Systemic, but not intestinal, IL-7 is essential for the persistence of chronic colitis. *J. Immunol.* 180: 383–390.
16. Tomita, T., T. Kanai, T. Totsuka, Y. Nemoto, R. Okamoto, K. Tsuchiya, N. Sakamoto, T. Ohteki, T. Hibi, and M. Watanabe. 2009. IL-7 is essential for lymphopenia-driven turnover of colitogenic CD4$^+$ memory T cells in chronic colitis. *Eur. J. Immunol.* 39: 2737–2747.
17. Vivier, E., E. Tomasello, M. Baratin, T. Walzer, and S. Ugolini. 2008. Functions of natural killer cells. *Nat. Immunol.* 9: 503–510.
18. Cooper, M. A., T. A. Fehniger, A. Fuchs, M. Colonna, and M. A. Caligiuri. 2004. NK cell and DC interactions. *Trends Immunol.* 25: 47–52.
19. Walzer, T., M. Dalod, S. H. Robbins, L. Zitvogel, and E. Vivier. 2005. Natural-killer cells and dendritic cells: "l'union fait la force". *Blood* 106: 2252–2258.
20. Lunemann, A., J. D. Lunemann, and C. Munz. 2009. Regulatory NK-cell functions in inflammation and autoimmunity. *Mol. Med.* 15: 352–358.
21. Takahashi, K., T. Aranami, M. Endoh, S. Miyake, and T. Yamamura. 2004. The regulatory role of natural killer cells in multiple sclerosis. *Brain* 127: 1917–1927.
22. Zhang, B., T. Yamamura, T. Kondo, M. Fujiwara, and T. Tabira. 1997. Regulation of experimental autoimmune encephalomyelitis by natural killer (NK) cells. *J. Exp. Med.* 186: 1677–1687.

23. Lu, L., K. Ikizawa, D. Hu, M. B. Werneck, K. W. Wucherpfennig, and H. Cantor. 2007. Regulation of activated CD4$^+$ T cells by NK cells via the Qa-1-NKG2A inhibitory pathway. *Immunity* 26: 593–604.
24. Fort, M. M., M. W. Leach, and D. M. Rennick. 1998. A role for NK cells as regulators of CD4$^+$ T cells in a transfer model of colitis. *J. Immunol.* 161: 3256–3261.
25. Kasai, M., T. Yoneda, S. Habu, Y. Maruyama, K. Okumura, and T. Tokunaga. 1981. In vivo effect of anti-asialo GM1 antibody on natural killer activity. *Nature* 291: 334–335.
26. Makita, S., T. Kanai, Y. Nemoto, T. Totsuka, R. Okamoto, K. Tsuchiya, M. Yamamoto, H. Kiyono, and M. Watanabe. 2007. Intestinal lamina propria retaining CD4$^+$CD25$^+$ regulatory T cells is a suppressive site of intestinal inflammation. *J. Immunol.* 178: 4937–4946.
27. Onizawa, M., T. Nagaishi, T. Kanai, K. Nagano, S. Oshima, Y. Nemoto, A. Yoshioka, T. Totsuka, R. Okamoto, T. Nakamura, et al. 2009. Signaling pathway via TNF-α/NF-κB in intestinal epithelial cells may be directly involved in colitis-associated carcinogenesis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 296: G850–G859.
28. Xu, W., G. Fazekas, H. Hara, and T. Tabira. 2005. Mechanism of natural killer (NK) cell regulatory role in experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* 163: 24–30.
29. Slezak, S. E., and P. K. Horan. 1989. Cell-mediated cytotoxicity: a highly sensitive and informative flow cytometric assay. *J. Immunol. Methods* 117: 205–214.
30. Di Santo, J. P. 2006. Natural killer cell developmental pathways: a question of balance. *Annu. Rev. Immunol.* 24: 257–286.
31. Hayakawa, Y., and M. J. Smyth. 2006. CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity. *J. Immunol.* 176: 1517–1524.
32. Takeda, K., and G. Dennert. 1993. The development of autoimmunity in C57BL/6 lpr mice correlates with the disappearance of natural killer type 1-positive cells: evidence for their suppressive action on bone marrow stem cell proliferation, B cell immunoglobulin secretion, and autoimmune symptoms. *J. Exp. Med.* 177: 155–164.
33. Aranami, T., S. Miyake, and T. Yamamura. 2006. Differential expression of CD11c by peripheral blood NK cells reflects temporal activity of multiple sclerosis. *J. Immunol.* 177: 5659–5667.
34. Wiltrout, R. H., A. Santoni, E. S. Peterson, D. C. Knott, W. R. Overton, R. B. Herberman, and H. T. Holden. 1985. Reactivity of anti-asialo GM1 serum with tumoricidal and non-tumoricidal mouse macrophages. *J. Leukoc. Biol.* 37: 597–614.
35. Baaten, B. J., C. R. Li, M. F. Deiro, M. M. Lin, P. J. Linton, and L. M. Bradley. 2010. CD44 regulates survival and memory development in Th1 cells. *Immunity* 32: 104–115.
36. Kawakami, Y., Y. Tomimori, K. Yumoto, S. Hasegawa, T. Ando, Y. Tagaya, S. Crotty, and T. Kawakami. 2009. Inhibition of NK cell activity by IL-17 allows vaccinia virus to induce severe skin lesions in a mouse model of eczema vaccinatum. *J. Exp. Med.* 206: 1219–1225.
37. Vosshenrich, C. A., T. Ranson, S. I. Samson, E. Corcuff, F. Colucci, E. E. Rosmaraki, and J. P. Di Santo. 2005. Roles for common cytokine receptor gamma-chain-dependent cytokines in the generation, differentiation, and maturation of NK cell precursors and peripheral NK cells in vivo. *J. Immunol.* 174: 1213–1221.
38. Kondrack, R. M., J. Harbertson, J. T. Tan, M. E. McBreen, C. D. Surh, and L. M. Bradley. 2003. Interleukin 7 regulates the survival and generation of memory CD4 cells. *J. Exp. Med.* 198: 1797–1806.
39. Okada, E., M. Yamazaki, M. Tanabe, T. Takeuchi, M. Nanno, S. Oshima, R. Okamoto, K. Tsuchiya, T. Nakamura, T. Kanai, et al. 2005. IL-7 exacerbates chronic colitis with expansion of memory IL-7Rhigh CD4+ mucosal T cells in mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* 288: G745–G754.

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

This information is current as of February 16, 2012

Tamako Shinohara, Yasuhiro Nemoto, Takanori Kanai, Kaori Kameyama, Ryuichi Okamoto, Kiichiro Tsuchiya, Tetsuya Nakamura, Teruji Totsuka, Koichi Ikuta and Mamoru Watanabe

---

Supplementary Data	http://www.jimmunol.org/content/suppl/2011/01/07/jimmunol.1000057.DC1.html
References	This article **cites 34 articles**, 9 of which can be accessed free at: http://www.jimmunol.org/content/186/4/2623.full.html#ref-list-1
	Article cited in: **http://www.jimmunol.org/content/186/4/2623.full.html#related-urls**
Subscriptions	Information about subscribing to *The Journal of Immunology* is online at http://www.jimmunol.org/subscriptions
Permissions	Submit copyright permission requests at http://www.aai.org/ji/copyright.html
Email Alerts	Receive free email-alerts when new articles cite this article. Sign up at http://www.jimmunol.org/etoc/subscriptions.shtml/

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).

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 CD3e 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. 1A, 1B). Conversely, the MFIs of IL-7Rα expression on colitic LP CD3$^-$NK1.1$^+$ NK cells, CD11b$^+$Gr1^{high} granulocytes, CD11b$^+$Gr1$^{low/-}$ macrophages, and CD11b$^+$CD11c$^+$ myeloid dendritic cells were significantly downregulated compared with those from normal mice (Fig. 1A, 1B). 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. 1A, 1B). 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. 1B), 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. 2A). 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. 2A). 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. 2A), 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. 2B).

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. 3A).
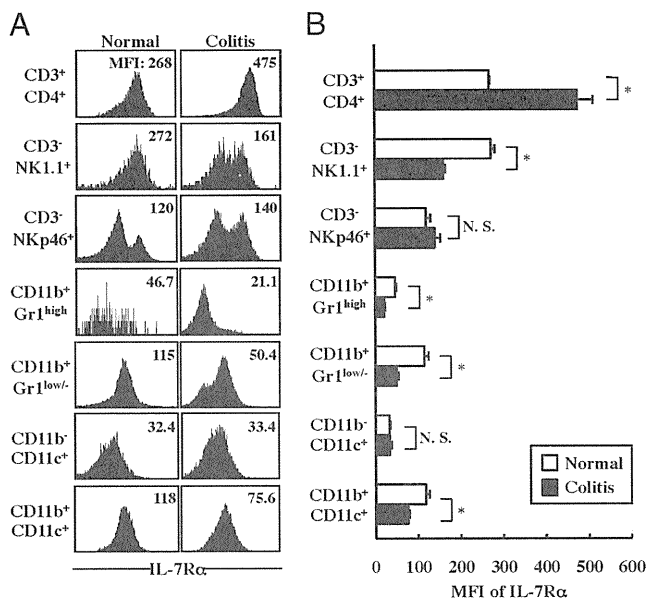

FIGURE 1. IL-7Rα expression on various immune compartments obtained from colonic LP of normal and colitic mice. A, Dot plot analysis shows the IL-7α expression on each fraction of immune cells from colonic LP of normal and colitic mice. Numerical values on the dot plots and histograms express the mean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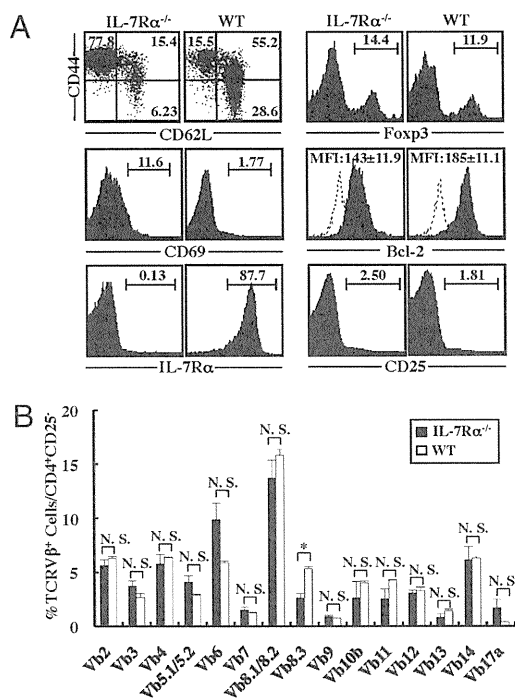

FIGURE 2. Phenotypic characterization of splenic CD4$^+$ T cells obtained from age-matched WT and IL-7Rα$^{-/-}$ mice. A, FACS analysis shows the expression of CD44/CD62L, IL-7Rα, Foxp3, and Bcl-2 on/in splenic CD4$^+$ T cells. The dotted line in the Bcl-2 histogram shows the baseline of isotype control. 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.
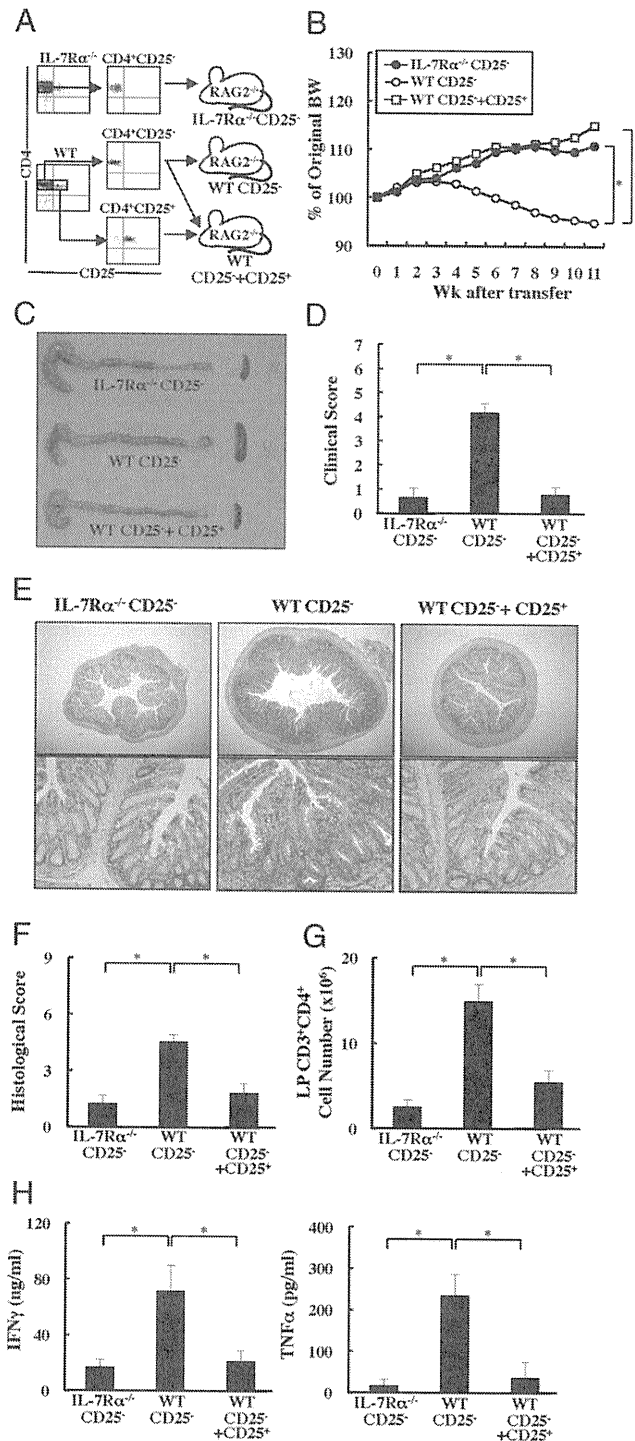
As a negative control, RAG-2$^{-/-}$ mice were transferred with a mixture of SP CD4+CD25$^-$ T cells and CD4+CD25$^+$ Tregs obtained from WT mice. As depicted in Fig. 3B, RAG-2$^{-/-}$ mice transferred with WT CD4+CD25$^-$ T cells manifested progressive weight loss from 4 wk after transfer (Fig. 3B). In contrast, RAG-2$^{-/-}$ mice transferred with IL-7Rα$^{-/-}$ 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. 3B). To check the possibility that mice transferred with IL-7Rα$^{-/-}$ 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-7Rα$^{-/-}$ CD4+CD25$^-$ T cells or WT CD4+CD25$^-$ T cells and CD4+CD25$^+$ Tregs, had enlarged colons with greatly thickened walls (Fig. 3C). The same mice also showed the enlargement of SP and mesenteric lymph nodes (Fig. 3C). 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. 3D).

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. 3E, 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-7Rα$^{-/-}$ CD4+CD25$^-$ T cells (Fig. 3E, left panels) or with a mixture of SP CD4+CD25$^-$ T cells and CD4+ CD25$^+$ Tregs (Fig. 3E, 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-7Rα$^{-/-}$ 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. 3F). 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-7Rα$^{-/-}$ 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. 3G). We also examined the cytokine production by LP CD4$^+$ T cells. LP CD4$^+$ T cells from RAG-2$^{-/-}$ mice transferred with IL-7Rα$^{-/-}$ 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. 3H).

FIGURE 3. RAG-2$^{-/-}$ transferred with IL-7Rα$^{-/-}$ 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-7Rα$^{-/-}$ 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-7Rα$^{-/-}$ 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-7Rα$^{-/-}$ 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. *$p < 0.05$.

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. 2A). 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 mainte-

nance, 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. 2A). As shown in Supplemental Fig. 2B, 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. 2C). 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 × 10⁵ 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. 4A). 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. 4B). 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
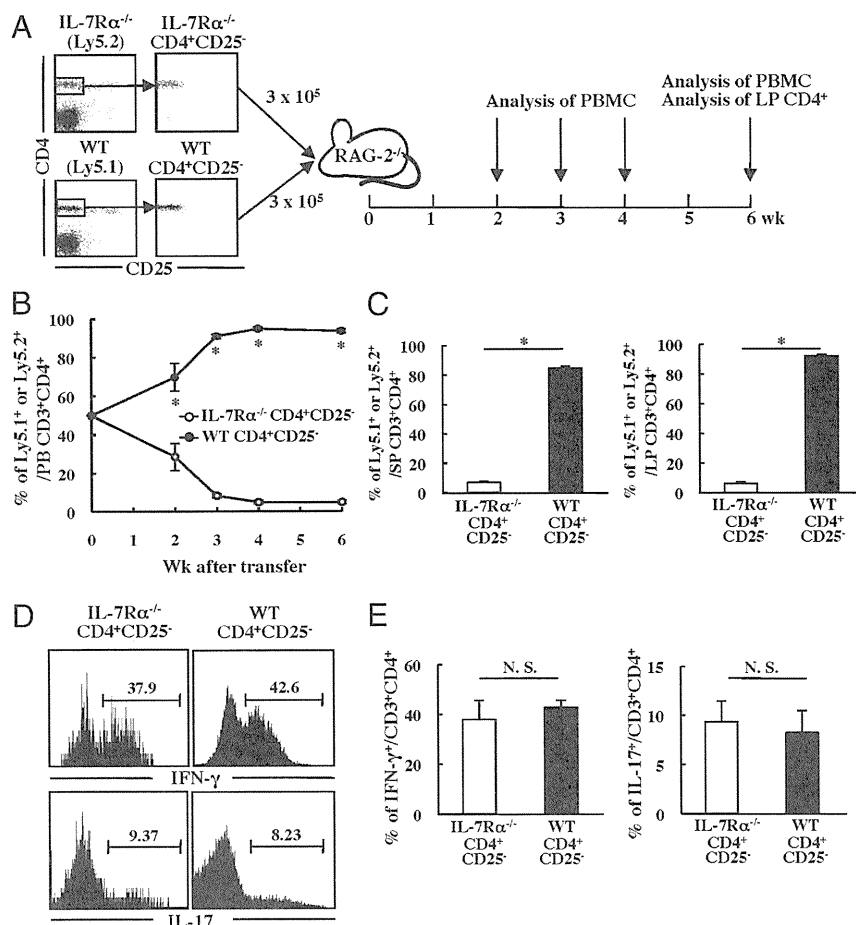

FIGURE 4. IL-7Rα$^{-/-}$ CD4⁺CD25⁻ T cells cotransferred with WT CD4⁺CD25⁻ T cells to RAG-2$^{-/-}$ mice could produce IFN-γ and IL-17, but could not survive. *A*, To discern why IL-7Rα$^{-/-}$ CD4⁺CD25⁻ T cells could not induce colitis, we cotransferred the same number (3 × 10⁵) of Ly5.2⁺ IL-7Rα$^{-/-}$ 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. 4C), both WT and IL-7Rα−/− donor-derived CD4+ T cells could similarly express IFN-γ and IL-17 in the colitic condition (Fig. 4D). 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. 2A, 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 × 10⁵ 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. 5A). As a negative control, RAG-2−/− mice were transferred with SP WT naive T cells and CD4+CD25+ Tregs (Fig. 5A). 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. 5B), clinical (Fig. 5C) and histologic scorings (Fig. 5D, 5E), and the absolute cell number of LP CD3+CD4+ T cells (Fig. 5F) 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
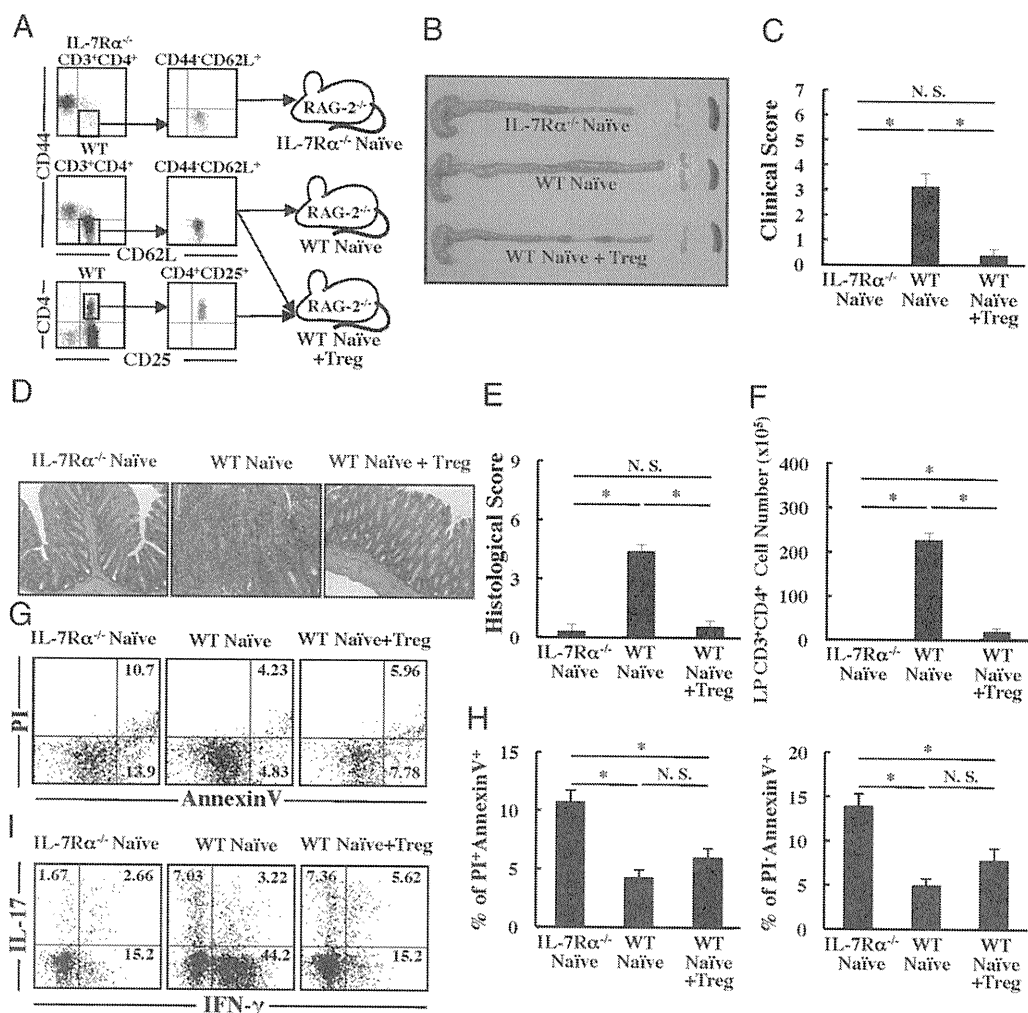

FIGURE 5. RAG-2−/− transferred with IL-7Rα−/− CD3+CD4+CD62L+CD44− T cells did not develop chronic colitis. A, RAG-2−/− mice were transferred with splenic CD3+CD4+CD62L+CD44− T cells obtained from age-matched WT or IL-7Rα−/− mice (3 × 10⁵ cells per mouse). As a negative control, RAG-2−/− mice were transferred with splenic WT CD3+CD4+CD62L+CD44− T cells (3 × 10⁵ cells per mouse) and CD4+CD25+ Tregs (1 × 10⁵ 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. 5G, 5H), 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. 5I).

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. 6A). As a negative control, a mixture of WT CD4$^+$CD25$^-$ T cells and CD4$^+$CD25$^+$ Tregs was transferred into RAG-2$^{-/-}$ mice (Fig. 6A). 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. 6B). When CD4$^+$CD25$^-$ T cells were transferred into the IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice, the recipients also developed severe wasting chronic colitis (Fig. 6B). 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. 6C). 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. 6D). 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. 6D).

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. 6E). This difference was also confirmed by histologic scoring of multiple colon sections (Fig. 6F). 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. 6G). Cytokine production by LP CD4$^+$ T cells is depicted in Fig. 6H. 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. 6H).

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. 3A), 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. 3B). 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. 3B).

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. 4A). 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. 4B–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. 4F), 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
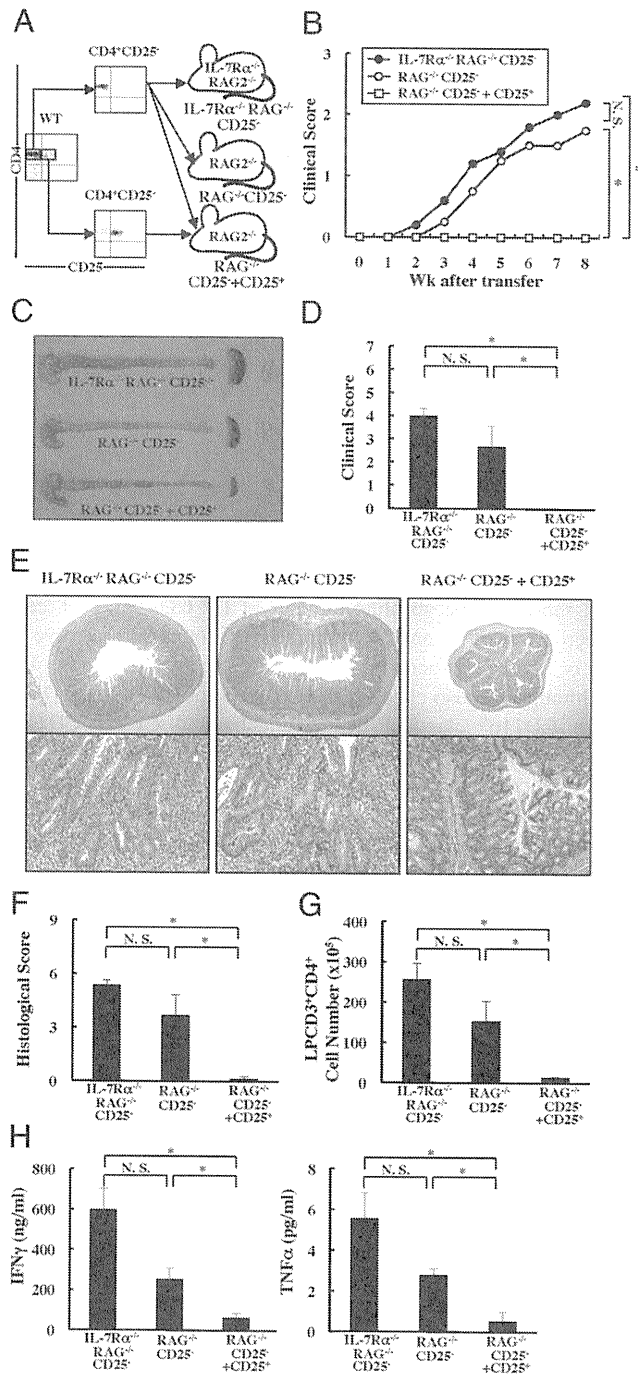
FIGURE 6. IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ transferred with WT CD4$^+$CD25$^-$ T cells developed chronic colitis. *A*, RAG-2$^{-/-}$ mice and IL-7Rα$^{-/-}$ × RAG-2$^{-/-}$ mice were transferred with splenic WT CD4$^+$CD25$^-$ T cells (3 × 10^5 cells per mouse). As a negative control, RAG-2$^{-/-}$ mice 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 (*right*). *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-

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.

References

1. Podolsky, D. K. 2002. Inflammatory bowel disease. *N. Engl. J. Med.* 347: 417–429.
2. Sands, B. E. 2007. Inflammatory bowel disease: past, present, and future. *J. Gastroenterol.* 42: 16–25.
3. Hibi, T., and H. Ogata. 2006. Novel pathophysiological concepts of inflammatory bowel disease. *J. Gastroenterol.* 41: 10–16.
4. Baumgart, D. C., and W. J. Sandborn. 2007. Inflammatory bowel disease: clinical aspects and established and evolving therapies. *Lancet* 369: 1641–1657.
5. Duerr, R. H., K. D. Taylor, S. R. Brant, J. D. Rioux, M. S. Silverberg, M. J. Daly, A. H. Steinhart, C. Abraham, M. Regueiro, A. Griffiths, et al. 2006. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* 314: 1461–1463.
6. Barrett, J. C., S. Hansoul, D. L. Nicolae, J. H. Cho, R. H. Duerr, J. D. Rioux, S. R. Brant, M. S. Silverberg, K. D. Taylor, M. M. Barmada, et al; NIDDK IBD Genetics Consortium; Belgian-French IBD Consortium; Wellcome Trust Case Control Consortium. 2008. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat. Genet.* 40: 955–962.
7. Kanai, T., Y. Nemoto, T. Tomita, T. Totsuka, M. Watanabe, and T. Hibi. 2009. Persistent retention of colitogenic CD4$^+$ memory T cells causes inflammatory bowel diseases to become intractable. *Inflamm. Bowel Dis.* 15: 926–934.
8. Namen, A. E., S. Lupton, K. Hjerrild, J. Wignall, D. Y. Mochizuki, A. Schmierer, B. Mosley, C. J. March, D. Urdal, and S. Gillis. 1988. Stimulation of B-cell progenitors by cloned murine interleukin-7. *Nature* 333: 571–573.
9. Fry, T. J., and C. L. Mackall. 2005. The many faces of IL-7: from lymphopoiesis to peripheral T cell maintenance. *J. Immunol.* 174: 6571–6576.
10. Bradley, L. M., L. Haynes, and S. L. Swain. 2005. IL-7: maintaining T-cell memory and achieving homeostasis. *Trends Immunol.* 26: 172–176.
11. Kanai, T., Y. Nemoto, N. Kamada, T. Totsuka, T. Hisamatsu, M. Watanabe, and T. Hibi. 2009. Homeostatic (IL-7) and effector (IL-17) cytokines as distinct but complementary target for an optimal therapeutic strategy in inflammatory bowel disease. *Curr. Opin. Gastroenterol.* 25: 306–313.
12. Sprent, J., and C. D. Surh. 2002. T cell memory. *Annu. Rev. Immunol.* 20: 551–579.
13. Schluns, K. S., and L. Lefrançois. 2003. Cytokine control of memory T-cell development and survival. *Nat. Rev. Immunol.* 3: 269–279.
14. Surh, C. D., and J. Sprent. 2005. Regulation of mature T cell homeostasis. *Semin. Immunol.* 17: 183–191.
15. Ma, A., R. Koka, and P. Burkett. 2006. Diverse functions of IL-2, IL-15, and IL-7 in lymphoid homeostasis. *Annu. Rev. Immunol.* 24: 657–679.
16. Mazzucchelli, R., and S. K. Durum. 2007. Interleukin-7 receptor expression: intelligent design. *Nat. Rev. Immunol.* 7: 144–154.
17. Palmer, M. J., V. S. Mahajan, L. C. Trajman, D. J. Irvine, D. A. Lauffenburger, and J. Chen. 2008. Interleukin-7 receptor signaling network: an integrated systems perspective. *Cell. Mol. Immunol.* 5: 79–89.
18. Watanabe, M., Y. Ueno, T. Yajima, Y. Iwao, M. Tsuchiya, H. Ishikawa, S. Aiso, T. Hibi, and H. Ishii. 1995. Interleukin 7 is produced by human intestinal epithelial cells and regulates the proliferation of intestinal mucosal lymphocytes. *J. Clin. Invest.* 95: 2945–2953.
19. Watanabe, M., Y. Ueno, T. Yajima, S. Okamoto, T. Hayashi, M. Yamazaki, Y. Iwao, H. Ishii, S. Habu, M. Uehira, et al. 1998. Interleukin 7 transgenic mice develop chronic colitis with decreased interleukin 7 protein accumulation in the colonic mucosa. *J. Exp. Med.* 187: 389–402.
20. Okada, E., M. Yamazaki, M. Tanabe, T. Takeuchi, M. Nanno, S. Oshima, R. Okamoto, K. Tsuchiya, T. Nakamura, T. Kanai, et al. 2005. IL-7 exacerbates chronic colitis with expansion of memory IL-7R^{high} CD4$^+$ mucosal T cells in mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* 288: G745–G754.
21. Yamazaki, M., T. Yajima, M. Tanabe, K. Fukui, E. Okada, R. Okamoto, S. Oshima, T. Nakamura, T. Kanai, M. Uehira, et al. 2003. Mucosal T cells expressing high levels of IL-7 receptor are potential targets for treatment of chronic colitis. *J. Immunol.* 171: 1556–1563.
22. Totsuka, T., T. Kanai, Y. Nemoto, S. Makita, R. Okamoto, K. Tsuchiya, and M. Watanabe. 2007. IL-7 Is essential for the development and the persistence of chronic colitis. *J. Immunol.* 178: 4737–4748.
23. Maki, K., S. Sunaga, Y. Komagata, Y. Kodaira, A. Mabuchi, H. Karasuyama, K. Yokomuro, J. I. Miyazaki, and K. Ikuta. 1996. Interleukin 7 receptor-deficient mice lack gammadelta T cells. *Proc. Natl. Acad. Sci. USA* 93: 7172–7177.
24. Totsuka, T., T. Kanai, R. Iiyama, K. Uraushihara, M. Yamazaki, R. Okamoto, T. Hibi, K. Tezuka, M. Azuma, H. Akiba, et al. 2003. Ameliorating effect of anti-ICOS monoclonal antibody in a murine model of chronic colitis. *Gastroenterology* 124: 410–421.
25. Makita, S., T. Kanai, Y. Nemoto, T. Totsuka, R. Okamoto, K. Tsuchiya, M. Yamamoto, H. Kiyono, and M. Watanabe. 2007. Intestinal lamina propria

retaining CD4+CD25+ regulatory T cells is a suppressive site of intestinal inflammation. *J. Immunol.* 178: 4937–4946.

26. Mikami, Y., T. Kanai, T. Sujino, Y. Ono, A. Hayashi, A. Okazawa, N. Kamada, K. Matsuoka, T. Hisamatsu, S. Okamoto, et al. 2010. Competition between colitogenic Th1 and Th17 cells contributes to the amelioration of colitis. *Eur. J. Immunol.* 40: 2409–2422.

27. Colonna, M. 2009. Interleukin-22-producing natural killer cells and lymphoid tissue inducer-like cells in mucosal immunity. *Immunity* 31: 15–23.

28. Luci, C., A. Reynders, I. I. Ivanov, C. Cognet, L. Chiche, L. Chasson, J. Hardwigsen, E. Anguiano, J. Banchereau, D. Chaussabel, et al. 2009. Influence of the transcription factor RORgammat on the development of NKp46+ cell populations in gut and skin. *Nat. Immunol.* 10: 75–82.

29. Vivier, E., H. Spits, and T. Cupedo. 2009. Interleukin-22-producing innate immune cells: new players in mucosal immunity and tissue repair? *Nat. Rev. Immunol.* 9: 229–234.

30. Peschon, J. J., P. J. Morrissey, K. H. Grabstein, F. J. Ramsdell, E. Maraskovsky, B. C. Gliniak, L. S. Park, S. F. Ziegler, D. E. Williams, C. B. Ware, et al. 1994.

Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J. Exp. Med.* 180: 1955–1960.

31. Guimond, M., R. G. Veenstra, D. J. Grindler, H. Zhang, Y. Cui, R. D. Murphy, S. Y. Kim, R. Na, L. Hennighausen, S. Kurtulus, et al. 2009. Interleukin 7 signaling in dendritic cells regulates the homeostatic proliferation and niche size of CD4+ T cells. *Nat. Immunol.* 10: 149–157.

32. Cella, M., A. Fuchs, W. Vermi, F. Facchetti, K. Otero, J. K. Lennerz, J. M. Doherty, J. C. Mills, and M. Colonna. 2009. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature* 457: 722–725.

33. Zenewicz, L. A., G. D. Yancopoulos, D. M. Valenzuela, A. J. Murphy, S. Stevens, and R. A. Flavell. 2008. Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. *Immunity* 29: 947–957.

34. Chiang, E. Y., G. A. Kolumam, X. Yu, M. Francesco, S. Ivelja, I. Peng, P. Gribling, J. Shu, W. P. Lee, C. J. Refino, et al. 2009. Targeted depletion of lymphotoxin-alpha-expressing TH1 and TH17 cells inhibits autoimmune disease. *Nat. Med.* 15: 766–773.

European Journal of
Immunology

IL-2 is positively involved in the development of colitogenic CD4⁺ IL-7Rα^{high} memory T cells in chronic colitis

Kaori Kameyama*[1], Yasuhiro Nemoto*[1], Takanori Kanai[2],
Tamako Shinohara[1], Ryuichi Okamoto[1], Kiichiro Tsuchiya[1],
Tetsuya Nakamura[1], Naoya Sakamoto[1], Teruji Totsuka[1],
Toshifumi Hibi[2] and Mamoru Watanabe[1]

[1] Department of Gastroenterology and Hepatology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan
[2] Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan

IL-2 and IL-7 share a common γ-chain receptor and are critical for T-cell homeostasis. We aimed to clarify the reciprocal roles of IL-2 and IL-7 in the development and persistence of chronic colitis. We performed a series of adoptive transfers of IL-2$^{-/-}$ CD4⁺CD45RB^{high} T cells into RAG-2$^{-/-}$ mice and assessed the role of IL-2 in the induction of IL-7Rα on colitogenic CD4⁺ T cells and the development of chronic colitis. RAG-2$^{-/-}$ mice transferred with WT but not with IL-2$^{-/-}$ CD4⁺CD45RB^{high} T cells developed Th1/Th17-mediated colitis. Consistently, re-expression of IL-7Rα was severely impaired on IL-2$^{-/-}$ but not on WT CD4⁺ T cells from the transferred mice. To exclude a contribution of the preclinical autoimmunity of IL-2$^{-/-}$mice, WT Ly5.1⁺ or IL-2$^{-/-}$ Ly5.2⁺ CD4⁺CD45RB^{high} T cells from GFP mice previously transplanted with the same number of WT and IL-2$^{-/-}$ BM cells were transferred into RAG-2$^{-/-}$ mice. RAG-2$^{-/-}$ mice transferred with IL-2$^{-/-}$-derived CD4⁺ CD45RB^{high} T cells did not develop colitis, but their splenic CD4⁺ T cells changed from effector-memory to central-memory type. These results show that IL-2 is critically involved in the establishment and maintenance of IL-7-dependent colitogenic memory CD4⁺IL-7Rα^{high} T cells.

Key words: Animal models · CD4⁺ T cell · Cytokines · Memory cell · Mucosal immunity

Introduction

The inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis, are caused by chronic inflammatory responses in the gut wall [1–3]. Although the aetiology of IBD is uncertain, there is much evidence suggesting that the pathogenesis of IBD involves dysregulated recognition of intestinal bacterial antigens, resulting in the generation of colitogenic CD4⁺ effector and memory T cells [4–10]. However, how colitogenic CD4⁺ T cells are generated and maintained in patients with IBD remains unknown.

During T-cell priming and maintenance of colitogenic CD4⁺ effector and memory T cells, cytokines may provide critical signals. IL-2 is produced by activated T cells early after antigenic stimulation and is essential for proliferation of T cells in the effector phase, at least in vitro [11, 12]. More recently, it has been shown that exposure to IL-2 in the effector phase is required for successful long-term survival of CD4⁺ T cells and their

Correspondence: Dr. Takanori Kanai
e-mail: takagast@sc.itc.keio.ac.jp

*These authors contributed equally to this work.

differentiation into memory T cells [13]. Interestingly, IL-2 does not seem to be needed for T-cell proliferation *in vivo* because IL-2$^{-/-}$ and IL-2R$\alpha^{-/-}$ mice develop autoimmune diseases, including chronic colitis, and extensive proliferation of T cells [14, 15]. Thus, the development of such diseases *in vivo* is explained entirely by the lack of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells, which are dependent on IL-2 for their development and maintenance [16–18].

In contrast to IL-2, IL-7 is produced not by lymphocytes but by stromal cells in the BM and thymus and by epithelial cells [19–21]. This cytokine is important for supporting the survival of naïve and memory CD4$^+$ T cells, but not that of effector CD4$^+$ T cells [21–23]. Our previous studies on the pathogenesis of IBD demonstrated that: (i) IL-7 is constitutively produced by intestinal goblet epithelial cells [20], (ii) IL-7 transgenic mice developed chronic colitis [24], (iii) mucosal CD4$^+$IL-7Rα^{high} T cells in CD4$^+$CD45RB^{high} T-cell-transferred colitic mice are colitogenic [25, 26], and (iv) IL-7 is essential for the persistence of colitis because IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice transferred with colitogenic CD4$^+$ T cells did not develop colitis [27].

IL-2 and IL-7 share a common γ-chain receptor and are critical cytokines for T-cell homeostasis [21, 22]. To clarify the reciprocal roles of IL-2 and IL-7 in the development and persistence of chronic colitis, we performed a series of adoptive transfers of WT or IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells into RAG-2$^{-/-}$ mice.

Results

IL-2$^{-/-}$ mice sustain substantial numbers of naïve CD4$^+$ T cells that have reduced levels of IL-7Rα

IL-2$^{-/-}$ mice develop spontaneous autoimmune syndrome from 4–6 wk of age and develop IBD at 10–12 wk of age [14]. We first analysed the phenotypic characteristics of CD4$^+$ T cells of young (3.5-wk-old) IL-2$^{-/-}$ and WT mice. The proportions of CD4$^+$CD44^{high} or CD45RB^{low} memory T cells and CD4$^+$CD44^{low} or CD45RB^{high} naïve T cells in the spleen (SP) of IL-2$^{-/-}$ mice were reciprocally higher or lower, respectively, than those of the paired WT mice (Fig. 1A and B). However, it is noteworthy that naïve T cells were detected in the SP of young IL-2$^{-/-}$ mice at this stage, indicating that continuous generation of naïve T cells occurs in these mice. As IL-2 is essential for the development and maintenance of CD4$^+$CD25$^+$Foxp3$^+$ Treg [17], expression of CD25 and Foxp3 on/in SP CD4$^+$ T cells in IL-2$^{-/-}$ mice was markedly impaired compared with that in WT mice (Fig. 1A and B). In contrast, SP CD4$^+$CD45RB^{high} populations from IL-2$^{-/-}$ and WT mice did not contain Treg (Fig. 1B). Interestingly, the positive frequency of IL-7Rα expression on SP CD4$^+$ T cells in IL-2$^{-/-}$ mice significantly reduced, which is in sharp contrast to that in WT mice (Fig. 1A and C). This suggests that SP CD4$^+$ T cells in IL-2$^{-/-}$ mice include a substantial number of effector T cells at a young age despite the absence of clinical manifestations or impaired naïve or memory T cells. Surprisingly, we found that the positive frequency of IL-7Rα expression on the SP CD4$^+$

CD45RB^{high} naïve T cells of IL-2$^{-/-}$ mice was also significantly reduced compared with that of WT mice (Fig. 1B and D), suggesting that IL-2 is involved in the development and maintenance of CD4$^+$ naïve T cells (Fig. 1B). This was also the case with the SP CD4$^+$CD45RB^{low} T cells of IL-2$^{-/-}$ mice, which may be explained by an increased number of effector T cells and/or impaired development of memory T cells.

RAG-2$^{-/-}$ mice transferred with IL-2$^{-/-}$ CD4$^+$ CD45 Rb^{high} T cells do not develop colitis

Since the positive frequency of IL-7Rα expression on IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T donor cells reduced (Fig. 1), the possibility remained that the impaired naïve CD4$^+$ T cells themselves were critically involved in the development of spontaneous colitis in IL-2$^{-/-}$ mice. To first assess the effect of IL-2 deficiency on the initial developmental process of colitis, CD4$^+$CD45RB^{high} T cells from young WT or non-colitic IL-2$^{-/-}$ mice were injected intraperitoneally into RAG-2$^{-/-}$mice (Fig. 2A). As a negative control, RAG-2$^{-/-}$ mice were transferred with WT CD4$^+$ CD45RB^{high} T cells and WT CD4$^+$CD25$^+$ Treg (Fig. 2A). Consistent with previous report [27], the RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells manifested weight loss from 5 wk after transfer (Fig. 2B). Clinical symptoms of colitis as shown by clinical scores were apparent 7 wk after transfer (Fig. 2D). In contrast, RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells and Treg showed no weight loss or clinical symptoms of colitis (Fig. 2B and D). However, RAG-2$^{-/-}$ mice transferred with IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells did not show wasting (Fig. 2B) or clinical symptoms of colitis throughout the observation period of 7 wk after transfer (Fig. 2D). Overall, the assessment of colitis according to clinical score showed a clear difference between RAG-2$^{-/-}$ mice transferred with IL-2$^{-/-}$ and those transferred with WT CD4$^+$CD45RB^{high} T cells (Fig. 2D). At 7 wk after transfer, the colon of RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells, but not with IL-2$^{-/-}$ CD4$^+$ CD45RB^{high} T cells or a combination of WT CD4$^+$CD45RB^{high} and Treg, was enlarged and had a greatly thickened wall (Fig. 2C). Enlargement of the SP was also present in RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells but not in other groups (Fig. 2C).

Histological examination showed a massive infiltration of mononuclear cells in the lamina propria (LP) of the colon of RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells (Fig. 2E). In contrast, the inflammation was mostly abrogated and only a few mononuclear cells were observed in the LP of the colon of RAG-2$^{-/-}$ mice transferred with IL-2$^{-/-}$ CD4$^+$ CD45RB^{high} T cells as well as in RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells and Treg (Fig. 2E). This difference was confirmed by histological scoring of colon sections (Fig. 2F). Further quantitative evaluation of LP CD4$^+$ T-cell infiltration was done using flow cytometry (Fig. 2G). The number of LP CD4$^+$ cells recovered from the colon of RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells far exceeded the number originally
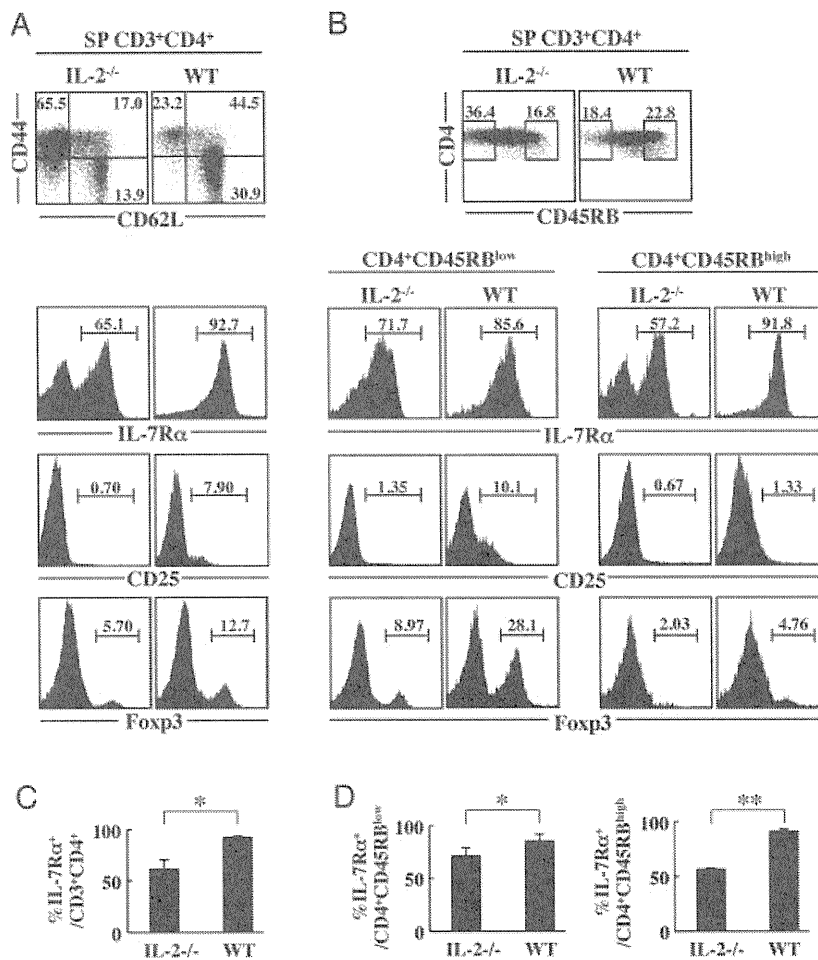
Figure 1. Phenotypic characteristics of IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells. (A) Identification and characterization of SP CD4$^+$ T cells in young IL-2$^{-/-}$ and WT mice. FACS analysis shows the expression of CD44 and CD62L, IL-7Rα, CD25, or Foxp3 on/in SP CD4$^+$ T cells. (B) Characterization of SP CD4$^+$CD45RB^{high} and CD45RB^{low} T cells in young IL-2$^{-/-}$ and WT mice. (C) The percentage of IL-7Rα$^+$ cells in SP CD3$^+$CD4$^+$ T cells was determined using a FACSCalibur. (D) The percentage of IL-7Rα$^+$ cells in the CD4$^+$CD45RB^{low} or CD45RB^{high} T-cell populations was determined using a FACSCalibur. Data are representative of six mice per group. (A and B) or show mean+SEM (C and D; n = 6 per group). *p = 0.011 (Mann-Whitney U) and **p = 0.011 (Student's t).

injected, indicating extensive T-cell proliferation and survival in the inflamed colon, which was mostly abrogated in RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells and Treg or RAG-2$^{-/-}$ mice transferred with IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells (Fig. 2G).

As shown in Fig. 1B, however, there were two populations of IL-2$^{-/-}$CD4$^+$CD45RB^{high} T cells, IL-7Rα^{high} and IL-7Rα^{low}. Thus, it is possible that IL-7Rα^{low} cells suppress IL-7Rα^{high} cells when they are transferred into RAG-2$^{-/-}$ mice as IL-2$^{-/-}$CD4$^+$ CD45RB^{high} T cells. To rule out this possibility, we performed another in vivo experiment. CD4$^+$CD45RB^{high} T cells from the SP of 4- to 5-wk-old IL-2$^{-/-}$ mice were divided into two populations, IL-7Rα^{high} and IL-7Rα^{low} by cell sorting, and each population was separately transferred into RAG-2$^{-/-}$ hosts. As a positive control, a same number of WT CD4$^+$CD45RB^{high} T cells were again transferred into RAG-2$^{-/-}$ mice (Fig. 3A). Mice transferred with IL-7Rα^{high} or IL-7Rα^{low} did not develop clinical or histological aspects of colitis, whereas mice transferred with WT CD4$^+$

CD45RB^{high} T cells did develop severe colitis (Figs. 3B–D). However, a proportion of IL-7Rα^{high} IL-2$^{-/-}$CD4$^+$CD45RB^{high} T cells converted to IL-7Rα^{low} and vice versa (data not shown) after they were transferred to RAG-2$^{-/-}$ mice, which shows that the expression of IL-7Rα on IL-2$^{-/-}$CD4$^+$CD45RB^{high} T cells is flexible, rather than fixed on naïve cells.

To determine the effect of these transfers on Th1/Th17 development, we next measured IFN-γ and IL-17 production by anti-CD3/CD28-stimulated CD4$^+$ LP T cells. As shown in Fig. 4A, the production of IFN-γ and IL-17 by anti-CD3/CD28-stimulated CD4$^+$ LP T cells from RAG-2$^{-/-}$ mice transferred with IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells or from RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells and Treg was significantly lower than that from RAG-2$^{-/-}$ mice transferred with WT CD4$^+$ CD45RB^{high} T cells. To assess the cell surface markers on isolated SP and LP cells in each group, we then performed flow cytometric analysis. As shown in Fig. 4B, the transferred IL-2$^{-/-}$ SP and LP CD4$^+$ T cells may differentiate into CD44^{high} memory T cells in

the absence of colitis, as was the case with the paired $CD4^+$ T cells in colitic RAG-$2^{-/-}$ mice transferred with WT $CD4^+$ CD45RB^{high} T cells and non-colitic RAG-$2^{-/-}$ mice transferred with WT $CD4^+$CD45RB^{high} T cells and WT Treg. It is noteworthy that the percentages of the central memory type of $CD44^{high}CD62L^+$ T cells (T_{CM}) in the SP (Fig. 4B and C, left) and
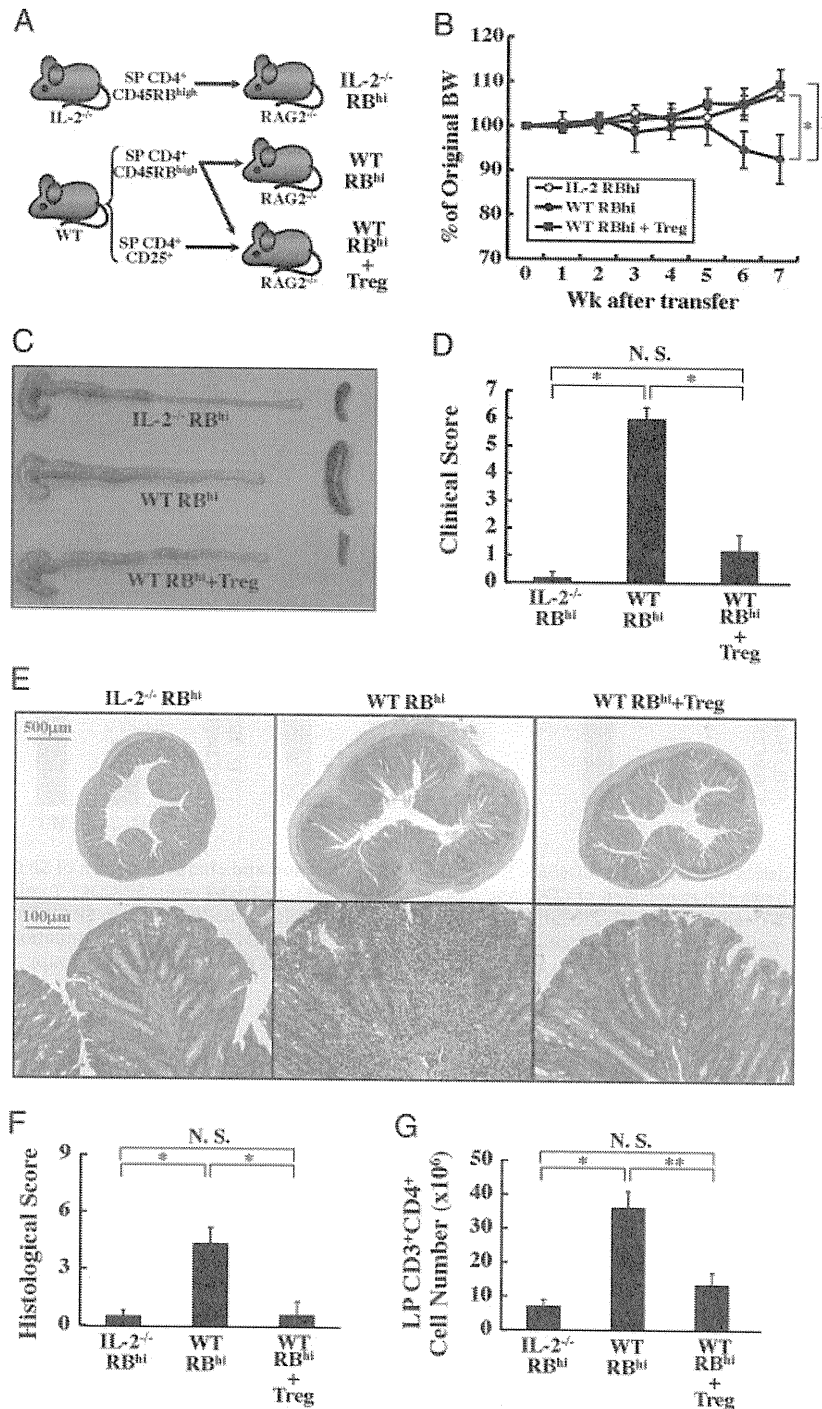

Figure 2. RAG-$2^{-/-}$ mice transferred with IL-$2^{-/-}$ $CD4^+$CD45RB^{high} T cells do not develop colitis. We performed two independent experiments. (A) Experimental design. RAG-$2^{-/-}$ mice were transferred with SP WT or IL-$2^{-/-}$ $CD4^+$CD45RB^{high} T cells (3×10^5 cells *per* mouse) or a mixture of WT $CD4^+$ CD45RB^{high} T cells (3×10^5) and WT $CD4^+$CD25$^+$ Treg (1×10^5). (B) The change in body weight over time is expressed as a percentage of the original weight. $^*p = 0.049$ (Welch's t), and $^{**}p = 0.049$ (Student's t). (C) Gross appearance of the colon and spleen. (D) Clinical scores. Data are expressed as the mean $\pm$ SEM of six mice *per* group. $^*p = 0.014$ (Mann-Whitney U.) (E) Histological results for the colons of each group. Original magnification, $\times 40$ (upper) and $\times 200$ (lower). (F) Histological scoring. $^*p = 0.014$ (Mann-Whitney U). (G) The number of $CD4^+CD3^+$ cells in colonic LP for each group. Cell number was determined using FACS. $^*p = 0.0049$ (Welch's t), and $^{**}p = 0.014$ (Mann-Whitney U). Data are expressed as the mean $\pm$ SEM of eight mice *per* group.
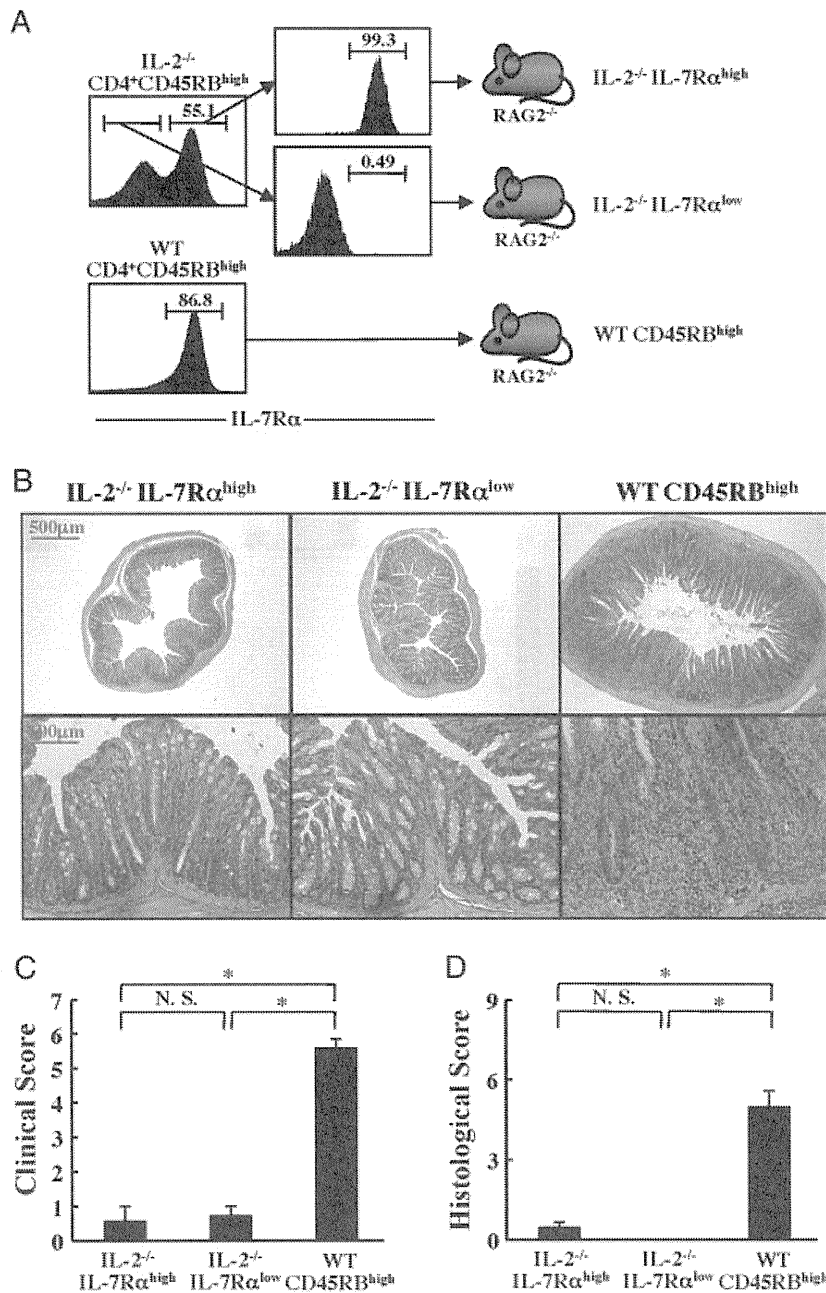
Figure 3. Either RAG-2$^{-/-}$ mice transferred with IL-2$^{-/-}$ CD4$^+$CD45RB^{high}IL-7Rα^{high} T cells or those transferred with IL-2$^{-/-}$ CD4$^+$CD45RB^{high} IL-7Rα^{low} T cells do not develop colitis. We performed one independent experiment. (A) Experimental design. RAG-2$^{-/-}$ mice were transferred with IL-7Rα^{high}CD4$^+$CD45RB^{high} T cells (3 × 10^5) or IL-7Rα^{low}CD4$^+$CD45RB^{high} T cells (3 × 10^5) from the spleen of IL-2$^{-/-}$ mice. As a positive control, we transferred the same number of WT CD4$^+$CD45RB^{high} T cells into RAG-2$^{-/-}$ mice. (B) Histological results for the colons of each group. Original magnification, × 40 (upper) and × 200 (lower). (C) Clinical scores. (D) Histological scoring. Data are expressed as the mean ± SEM of six (C) and five (D) mice per group. N.S., not significant. *p = 0.014 (Mann-Whitney U). N.S., not significant.

MLN (data not shown) IL-2$^{-/-}$ CD4$^+$ T cells were significantly higher than those in the SP and MLN of colitic RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells or non-colitic RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells and WT Treg. In contrast, LP CD4$^+$ T cells in all groups exhibited characteristics of effector-memory type of CD44^{high}CD62L$^-$ (T$_{EM}$)

cells, which is consistent with the non-lymphoid nature of the LP (Fig. 4B and C, right) [28, 29]. Furthermore, the positive frequency of IL-7Rα expression on SP and LP IL-2$^{-/-}$ CD4$^+$ T cells also significantly reduced, as compared with the paired cells from colitic RAG-2$^{-/-}$ mice transferred with WT CD4$^+$ CD45RB^{high} T cells or non-colitic RAG-2$^{-/-}$ mice transferred with
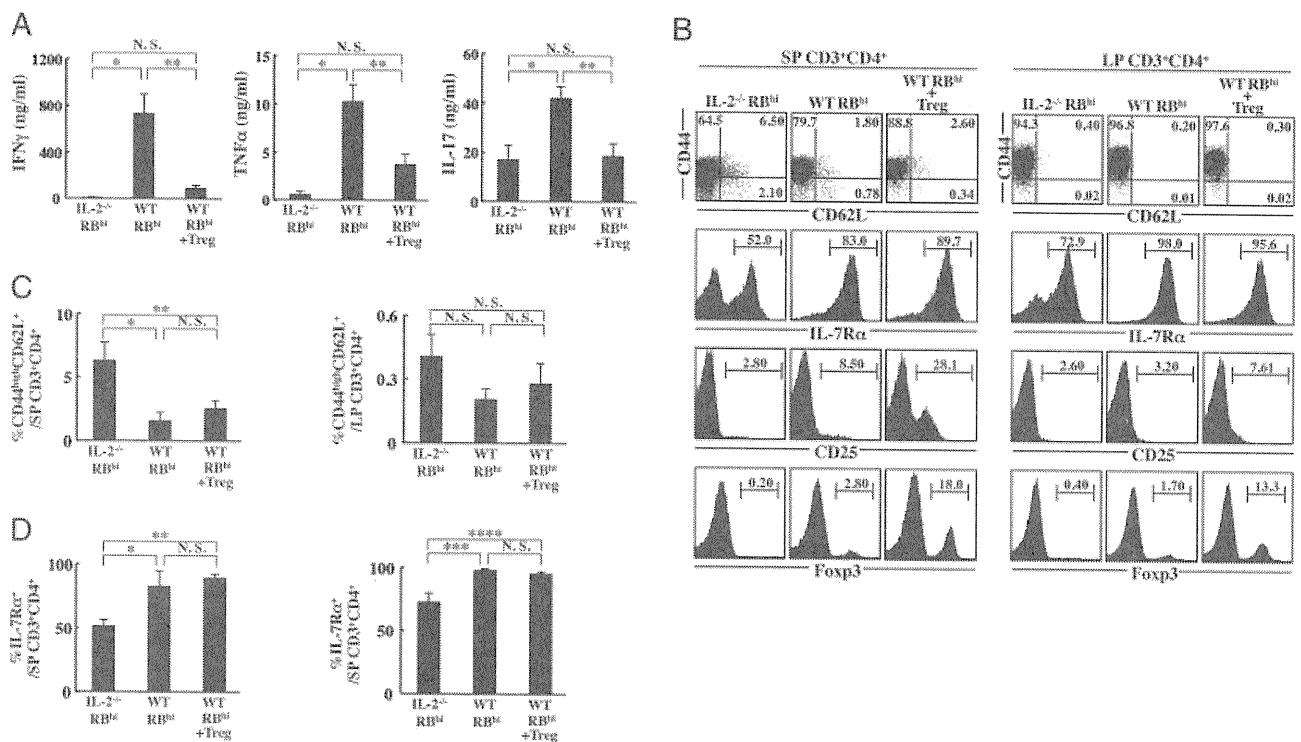
Figure 4. IL-7Rα expression of memory CD4$^+$ T cells in IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T-cell-transferred RAG-2$^{-/-}$ mice was impaired. We performed two independent experiments. (A) Cytokine production by LP CD4$^+$ T cells. LP CD4$^+$ T cells were stimulated with plate-coated anti-CD3 mAb and soluble anti-CD28 mAb for 48 h. Cytokine concentration in the supernatants were measured using ELISA. Data are expressed as the mean ± SEM of eight mice per group. *p = 0.021 (Mann-Whitney U), and **p = 0.014 (Mann-Whitney U). (B) Expression of various cell surface markers on SP and LP CD3$^+$CD4$^+$ T cells was determined by FACS. Representative results from eight mice per group for CD44, CD62L, IL-7Rα, CD25, and Foxp3 are shown. (C) The percentage of T$_{CM}$ cells was determined using FACS. Data are expressed as the mean ± SEM of eight mice. *p = 0.0001 (Student's t), **p = 0.047 (Student's t), and N.S., not significant. (D) The percentage of CD3$^+$CD4$^+$ T cells positive for IL-7Rα$^+$ is shown. Data are expressed as the mean ± SEM of eight mice per group. *p = 0.0001 (Student's t). **p = 0.0009 (Mann-Whitney U), ***p = 0.001 (Mann-Whitney U), ****p = 0.017 (Mann-Whitney U), and N.S., not significant.

WT CD4$^+$CD45RB^{high} T cells and WT Treg (Fig. 4B and D). In addition, CD4$^+$CD25$^+$Foxp3$^+$ Treg did not emerge in the SP or LP of RAG-2$^{-/-}$ mice transferred with IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells, which is in contrast to RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells, which contained a substantial number of inducible Treg (Fig. 4B), albeit fewer than RAG-2$^{-/-}$ mice transferred with WT CD4$^+$CD45RB^{high} T cells and WT Treg, which contained a mixture of naturally occurring and inducible Treg (Fig. 4B).

Paracrine IL-2 from WT CD4$^+$ T cells enables IL-2$^{-/-}$ LP CD4$^+$ T cells to induce mild colitis in an IL-7-dependent manner

To further assess the role of IL-2 signalling in the expansion of CD4$^+$ donor cells, we performed *in vivo* competition experiments. First, the same number of CD4$^+$CD45RB^{high} donor cells from Ly5.1$^+$ WT and Ly5.2$^+$ IL-2$^{-/-}$ mice were co-injected intraperitoneally into RAG-2$^{-/-}$ mice (Fig. 5A). As expected, recipient mice developed severe colitis 6 wk after co-transfer (data not shown), and a significantly lower proportion of Ly5.2$^+$ IL-2$^{-/-}$ CD4$^+$ T cells were observed in the inflamed LP and SP compared

with the paired Ly5.1$^+$ WT CD4$^+$ T cells (Fig. 5B). Furthermore, the positive frequency of IL-7Rα expression on IL-2$^{-/-}$ LP CD4$^+$ cells was markedly reduced, as compared with that on WT LP CD4$^+$ cells (Fig. 5C).

We next addressed the question of whether LP IL-2$^{-/-}$ CD4$^+$ T cells sustained in colitic RAG-2$^{-/-}$ mice transferred with a mixture of WT and IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells (Fig. 5A) have the potential to induce colitis when transferred to new RAG-1$^{-/-}$ mice, because it was considered possible that a small but substantial number of IL-2$^{-/-}$ LP CD4$^+$ T cells in those mice (Fig. 5) would gain colitogenicity by using paracrine IL-2 from surrounding WT LP CD4$^+$ T cells. If they were colitogenic, it would also be necessary to examine whether IL-7 is also needed for the development of colitis by those cells to assess whether they are effector or memory T cells (Fig. 5A), as is the case with colitic WT LP CD4$^+$ T cells as previously demonstrated by our group [27]. To this end, we isolated LP WT (Ly5.1$^+$) and IL-2$^{-/-}$ (Ly5.2$^+$) CD4$^+$ T cells from colitic RAG-2$^{-/-}$ mice previously transferred with the same number of WT and IL-2$^{-/-}$ CD4$^+$ CD45RB^{high} T cells and then separately retransferred them into new IL-7$^{+/+}$ × RAG-1$^{-/-}$ or IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice (Fig. 5A). As expected, IL-7$^{+/+}$ × RAG-1$^{-/-}$ recipients transferred with WT CD4$^+$ T cells (WT→IL-7$^{+/+}$) developed severe colitis 4–6 wk
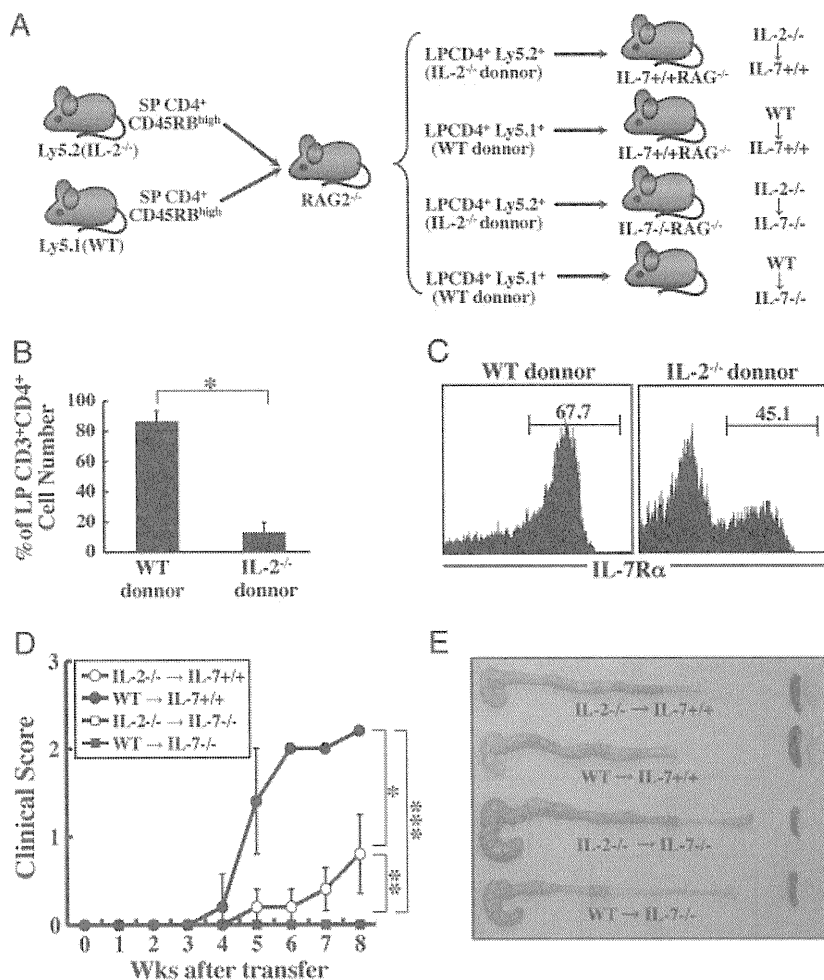
Figure 5. IL-7 is essential for the development of colitis in mice transferred with IL-2$^{-/-}$ LP CD4$^+$ T cells obtained from colitic mice previously transferred with a mixture of WT and IL-2$^{-/-}$ CD4$^+$CD45RB^{high} T cells. We performed one independent experiment. (A) Experimental design. RAG-2$^{-/-}$ mice were co-injected i.p. with a mixture of WT (Ly5.1$^+$) and IL-2$^{-/-}$ (Ly5.2$^+$) CD4$^+$CD45RB^{high} T cells. Eight weeks after transfer, WT and IL-2$^{-/-}$ LP CD4$^+$ T cells were isolated and separately retransferred into IL-7$^{+/+}$ × RAG-1$^{-/-}$ or IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice. (B) Numbers of WT (Ly5.1$^+$) and IL-2$^{-/-}$ (Ly5.2$^+$) LP CD3$^+$CD4$^+$ T cells recovered from colitic RAG-2$^{-/-}$ donor mice. Data are expressed as the mean ± SEM of five mice per group. $^*p = 0.049$ (Mann-Whitney U). (C) Expression of IL-7Rα on SP WT and IL-2$^{-/-}$ CD4$^+$ T cells of colitic RAG-2$^{-/-}$ donor mice was determined using FACS. (D) Clinical score after retransfer into new IL-7$^{+/+}$ × RAG-1$^{-/-}$ or IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice. Data are expressed as the mean ± SEM of five mice per group. $^*p = 0.025$, $^{**}p = 0.034$, $^{***}p = 0.034$ (Mann-Whitney U). (E) Gross appearance of the colon, SP, and MLN 8 wk after retransfer.

after transfer, which was characterized by significant weight loss, diarrhoea, higher total clinical scores (Fig. 5D), and thickening of the colonic wall with inflammation (Fig. 5E). In contrast, IL-7$^{-/-}$ × RAG-1$^{-/-}$ recipients transferred with WT CD4$^+$ T cells (WT→IL-7$^{-/-}$) appeared healthy, exhibited no signs of colitis until 8 wk after transfer (Fig. 5D), and exhibited no apparent thickening of the colonic wall (Fig. 5E). As expected, IL-7$^{-/-}$ × RAG-1$^{-/-}$ recipients transferred with IL-2$^{-/-}$ CD4$^+$ T cells (IL-2$^{-/-}$→IL-7$^{-/-}$) did not develop colitis. However, IL-7$^{+/+}$ × RAG-1$^{-/-}$ recipients transferred with IL-2$^{-/-}$ CD4$^+$ T cells (IL-2$^{-/-}$→IL-7$^{+/+}$) exhibited clinical signs of colitis and a thickened colonic wall (Fig. 5E) 8 wk after transfer, albeit less severely than WT→IL-7$^{+/+}$ mice (Fig. 5E).

Histological examinations revealed no evident pathological changes in the colons of WT→IL-7$^{-/-}$ or IL-2$^{-/-}$→IL-7$^{-/-}$ mice

in contrast to colitic WT→IL-7$^{+/+}$ or IL-2$^{-/-}$→IL-7$^{+/+}$ mice, which showed prominent epithelial hyperplasia with massive infiltration of mononuclear cells (Fig. 6A). This was confirmed by assessing each histological score (Fig. 6B). Furthermore, the score of IL-2$^{-/-}$→IL-7$^{+/+}$ mice was significantly less than that of WT→IL-7$^{+/+}$ mice (Fig. 6B). Consistent with this, the average number of LP CD4$^+$ T cells recovered from colitic IL-2$^{-/-}$→ IL-7$^{+/+}$ mice was significantly less than that of WT→IL-7$^{+/+}$ mice (Fig. 6C). The number of LP CD4$^+$ cells in IL-2$^{-/-}$→IL-7$^{-/-}$ or WT→IL-7$^{-/-}$ mice was almost zero (Fig. 6C). Furthermore, the positive frequency of IL-7Rα expression on SP CD4$^+$ T cells obtained from IL-2$^{-/-}$ CD4$^+$ T-cell-transferred mice was markedly reduced in both IL-7$^{+/+}$ and IL-7$^{-/-}$ recipients, whereas that from WT CD4$^+$ T-cell-transferred mice was not impaired in IL-7$^{+/+}$ or IL-7$^{-/-}$ recipients (Fig. 6D).