MLN, PB, and BM, as compared with the paired Ly5.1$^+$MyD88$^{+/+}$CD4$^+$ T cells (Fig. 4*B*). Furthermore, the ratio of IFN-γ-expressing cells within total MyD88$^{-/-}$ LP CD4$^+$ T cells was significantly lower compared with that in total MyD88$^{+/+}$ LP CD4$^+$ T cells (Fig. 4*C*). Consistent with the lower expression of IFN-γ in MyD88$^{-/-}$ LP CD4$^+$ T cells, expression of the activation marker CD69 on MyD88$^{-/-}$ LP or SP CD4$^+$ cells was significantly lower than on MyD88$^{+/+}$ LP or SP CD4$^+$ T cells, respectively (Fig. 4*D*).

RAG-2$^{-/-}$ mice transferred with MyD88$^{-/-}$ colitogenic LP CD4$^+$ donor cells develop milder colitis

To next assess the role of MyD88-dependent pathway in persistent colitis, we next examined colitogenic LP CD4$^+$ T cell-mediated colitis model (17), which lacks the impact of naive T cell priming, activation, and differentiation phase required in the former CD4$^+$CD45RB^{high} T cell-transferred colitis model. We first confirmed that RAG-2$^{-/-}$ mice transferred with MyD88$^{-/-}$CD4$^+$CD45RB^{high} T cells do develop colitis to a similar extent to mice transferred with MyD88$^{+/+}$CD4$^+$ CD45RB^{high} T cells at the late stage of 10 wk after transfer as confirmed by the weight curve (Fig. 5*A*), albeit the ongoing disease activity index (Fig. 5*B*) and histological assessment (Fig. 5, *C* and *D*) delayed onset and kinetics. Consistent with these findings, the recovered cell number was equivalent between mice transferred with MyD88$^{+/+}$ or MyD88$^{-/-}$ CD4$^+$CD45RB^{high} T cells (Fig. 5*E*). Furthermore, the expression of activation (CD69)/differentiation (IL-7Rα, CD44, and CD62L) on LP CD4$^+$ T cells showed no difference between two groups of mice (Fig. 5*F*), indicating that MyD88 deficiency solely contributes to the delayed kinetics of the development of colitis.

We thus isolated the LP CD4$^+$ T cells from colitic recipient mice transferred with either MyD88$^{+/+}$ or MyD88$^{-/-}$ CD4$^+$CD45RB^{high} T cells at 10 wk after transfer, to use for the subsequent memory T cell transfer. We transferred the isolated colitic LP CD4$^+$ T cells into new RAG-2$^{-/-}$ mice to focus on the persistence of colitogenic CD4$^+$ memory T cells (Fig. 6*A*). Similar with the results using CD4$^+$CD45RB^{high} T cell-mediated colitis model in Fig. 2, the recipient mice transferred with colitic MyD88$^{-/-}$ LP CD4$^+$ T cells showed milder wasting disease (Fig. 6*B*) with milder clinical signs of colitis at 4 wk after retransfer, as compared with mice transferred with colitic MyD88$^{+/+}$ LP CD4$^+$ T cells (Fig. 6*C*). Histological examination also revealed that mice transferred with MyD88$^{-/-}$ LP CD4$^+$ T cells developed milder colitis at 4 wk after retransfer as compared with mice transferred with MyD88$^{+/+}$ LP CD4$^+$ T cells (Fig. 6*D*). The difference was statistically confirmed by histological scoring of colon sections, which showed as follows: mice transferred with MyD88$^{+/+}$ LP CD4$^+$ T cells, 17.8 ± 0.86 and mice transferred with MyD88$^{-/-}$ LP CD4$^+$ T cells, 9.4 ± 1.86 ($p < 0.01$) (Fig. 6*E*). Furthermore, a significantly lower number of CD4$^+$ T cells was recovered from SP, LP, and MLN of mice transferred with MyD88$^{-/-}$ donor cells as compared with mice transferred with MyD88$^{+/+}$ donor cells (Fig. 6*F*). As shown in Fig. 6*G*, LP CD4$^+$ T cells from mice transferred with MyD88$^{-/-}$ LP donor cells produced significantly less IFN-γ and IL-17 as compared with those from mice transferred with MyD88$^{+/+}$ LP donor cells.

To further assess the expansive activity of colitic LP CD4$^+$ memory T cells, we again performed in vivo competition experiments. The same number (2.0×10^5 cells/mouse) of colitic Ly5.1$^+$MyD88$^{+/+}$ and Ly5.2$^+$MyD88$^{-/-}$ LP donor cells obtained from colitic mice transferred with Ly5.1$^+$MyD88$^{+/+}$ or Ly5.2$^+$MyD88$^{-/-}$ CD4$^+$CD45RB^{high} T cells at 10 wk after transfer was coinjected i.p. into identical RAG-2$^{-/-}$ mice (Fig. 7*A*). Six wk after cotransfer, a significantly lower proportion of Ly5.2$^+$ MyD88$^{-/-}$CD4$^+$ T cells was recovered from the inflamed LP, SP, and MLN, as compared with the paired Ly5.1$^+$MyD88$^{+/+}$CD4$^+$ T cells (Fig. 7*B*). Furthermore, the ratio of IFN-γ-expressing CD4$^+$ T cells within total MyD88$^{-/-}$ LP CD4$^+$ T cells was significantly decreased as compared with that within total MyD88$^{+/+}$ LP CD4$^+$ T cells (Fig. 7*C*).

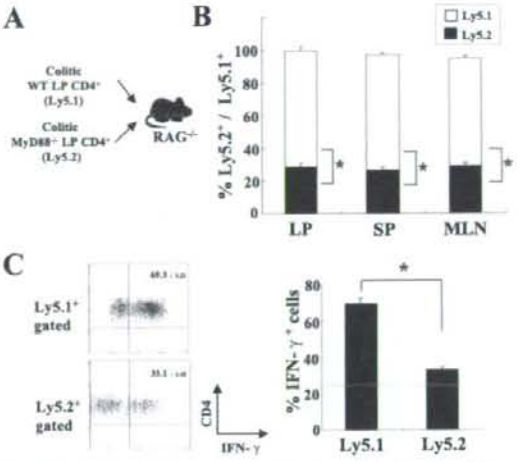

FIGURE 7. Expansion activity of colitic MyD88$^{+/+}$ LP donor cells predominates over that of MyD88$^{-/-}$ donor cells in an in vivo competition assay. *A*, The same number (2.0×10^5 cells/mouse) of colitic LP MyD88$^{+/+}$ (WT) (Ly5.1$^+$) and MyD88$^{-/-}$ (Ly5.2$^+$) CD4$^+$ T cells mice was injected i.p. into RAG-2$^{-/-}$ mice ($n = 6$). *B*, Six weeks after transfer, LP, SP, and MLN CD4$^+$ T cells were isolated from mice, and the ratio of Ly5.1$^+$ and Ly5.2$^+$ CD4$^+$ cells was determined by flow cytometry. *, $p < 0.01$. *C*, The frequencies of IFN-γ-producing cells per the total Ly5.1$^+$ or Ly5.2$^+$ cells were analyzed in the indicated subpopulations by flow cytometry. Data are represented as mean ± SEM of three independent experiments. *, $p < 0.01$.

MyD88 signaling contributes to the lymphopenia-driven rapid proliferation of colitogenic CD4$^+$ T cells

To finally examine the effect of MyD88 signaling on the lymphopenia-driven rapid proliferation (18) of the colitogenic CD4$^+$ memory T cells, we used the in vivo CFSE dilution method to examine cells undergoing proliferation after a short period from transfer. First, the LP CD4$^+$ T cells obtained from colitic RAG-2$^{-/-}$ mice transferred with either MyD88$^{+/+}$ or MyD88$^{-/-}$ CD4$^+$CD45RB^{high} T cells at 10 wk after transfer were labeled with CFSE and adoptively cotransferred into new RAG-2$^{-/-}$ mice. Cell divisions were determined 10 days after cotransfer by assessing the CFSE dilution (Fig. 8*A*). As depicted in Fig. 8*B*, the markedly delayed division pattern of CD4$^+$ T cells from mice transferred with MyD88$^{-/-}$ donor cells was observed as compared with that in mice transferred with MyD88$^{+/+}$ donor cells. This difference was statistically confirmed by comparing the CFSE$^+$ cells between Ly5.1$^+$ and Ly5.2$^+$ cells (Fig. 8*C*), indicating that the MyD88-dependent signaling pathway in T cells promotes the rapid proliferation of colitogenic CD4$^+$ memory T cells in a lymphopenic condition.

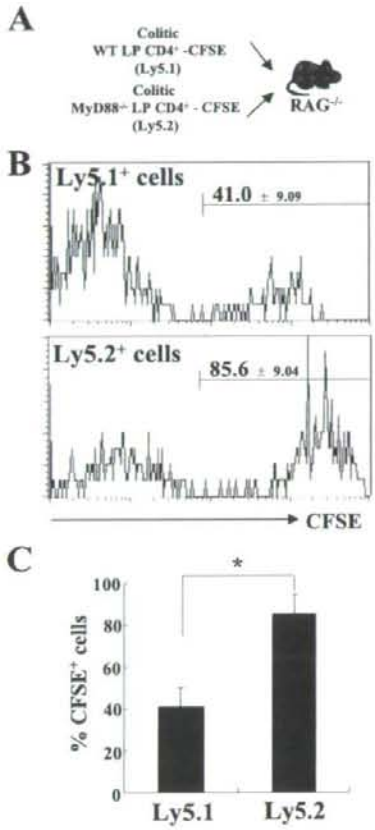

FIGURE 8. MyD88 pathway contributes to the lymphopenia-driven rapid proliferation of colitogenic $CD4^+$ T cells. *A*, The same number (2.0×10^6 cells/mouse) of CFSE-labeled colitic LP MyD88$^{+/+}$ (WT) ($Ly5.1^+$) and MyD88$^{-/-}$ ($Ly5.2^+$) $CD4^+$ T cells were coinjected i.p. into new RAG-2$^{-/-}$ mice ($n = 6$). *B*, The donor cells in the host spleen were analyzed 10 days after transfer by staining CD4, Ly5.1, and Ly5.2. Histograms show CFSE profiles of the two donor cell types in the host spleen. Data are representative of five independent experiments. *C*, Percentages of positive CFSE staining per total $Ly5.1^+$ or $Ly5.2^+$ cells were analyzed in the indicated subpopulations by flow cytometry. Data are represented as mean ± SEM of three independent experiments. *, $p < 0.05$.

Discussion

In the present study, we demonstrated that the MyD88-dependent signaling pathway in T cells directly modulates the proliferation and survival of TLRs/MyD88-expressing colitogenic $CD4^+$ T cells during the development and persistence of colitis. So far, it has been believed that T cell activation and expansion is induced and maintained by TCR signaling through the interaction with Ag-loading DCs that are primarily activated by PAMP/TLR-induced maturation (21). However, this study provides a new pathway by which the MyD88-dependent signaling pathway within $CD4^+$ T cells may directly play a pivotal role in the acquired immune components of chronic colitis by enhancing PAMP-specific immune responses collaborating with Ag-specific TCR signaling and homeostatic cytokines, such as IL-7 and IL-15 (18, 22–24).

How do commensal bacteria-derived PAMPs contribute to the maintenance of colitogenic $CD4^+$ T cells during the perpetuation of colitis? In other words, from where do colitogenic $CD4^+$ T cells receive proliferative and/or survival signals to sustain chronic colitis? First, it is well-known that commensal bacteria are essentially required for the development and the persistence of colitis, because 1) almost all models of T cell-mediated colitis do not develop colitis under the germfree condition (4–6), and 2) several groups elegantly demonstrated the requirement of specific Ags for the development and persistence of colitis by showing that colitis is induced and sustained by administration of OVA peptide-expressing *Escherichia coli* into OVA-specific TCR-transgenic mice in an Ag-specific manner (25, 26). These results indicated that TCR signaling through Ags, especially Ags derived from commensals, are needed for the development and persistence of colitis. Second, in addition to Ags derived from commensal bacteria, we here showed that the MyD88-dependent signaling pathway directly bolsters up the proliferation and survival of colitogenic $CD4^+$ T cells. However, it is of note that RAG-2$^{-/-}$ mice transferred with MyD88$^{-/-}$ $CD4^+CD45RB^{high}$ T cells did develop colitis with $CD4^+$ T cell infiltration in the inflamed mucosa albeit the onset was delayed as compared with the control, indicating that the direct MyD88-dependent signaling pathway in colitogenic $CD4^+$ T cells may act as a costimulator to tune the essential TCR signaling for the maintenance of these cells. However, at the molecular level, it still remains unknown how the identical $CD4^+$ T cells coordinate TCR and TLR signaling initiated from the commensal bacteria for activation, proliferation, and survival. Further studies will be required to address this important issue.

So far, most studies regarding TLRs have focused on cells of the innate immune system, such as DCs, macrophages, and epithelial cells, and now it is recognized that members of TLRs play an essential role in the innate immune recognition allowing the detection of commensal bacteria, followed by the second activation of T cells (9–11). However, recent works showed that conventional TCR $\alpha\beta^+CD4^+$ T cells also express TLRs (12), suggesting that PAMPs may directly modulate the function of $CD4^+$ T cells. Importantly, Gelman et al. (27) recently reported that TLR signaling in primary $CD4^+$ T cells directly enhances proliferation through MyD88 and PI3K-dependent pathway, in response to a T cell-dependent Ag. Thus, the present study may add the identification of the role of TLR signaling in the activation/function of the pathogenic memory $CD4^+$ T cells. Although we showed that the MyD88-dependent signaling pathway positively reinforces the proliferation and survival of colitogenic $CD4^+$ T cells in colitic mice, it has been previously reported that TLR-4 is predominantly expressed on regulatory $CD4^+CD25^+$ T cells rather than $CD4^+CD45RB^{high}$ naive cells, and TLR-4-specific signaling by LPS increases the regulatory $CD4^+CD25^+$ T cell activity, resulting in suppression of inflammatory responses in vivo (16). We slightly, but substantially, detected TLR-4 mRNA as well as other TLRs in colitic LP $CD4^+$ T cells, thus it is interesting to know how the stimulatory and inhibitory TLR-signaling pathway in T cells orchestrates the complicated immune responses in chronic colitis.

Such characteristics of TLR/MyD88-expressing colitogenic $CD4^+$ T cells raise another important question of whether the colitogenic $CD4^+CD44^{high}CD62L^-$ IL-7Rα^{high} T cells (Fig. 2) can be defined as effector-memory T cells rather than just effector T cells under the persistent presence of commensal Ags and/or self Ags, because it is accepted that memory T cells are generated after Ag clearance for the first time, but not under persistent presence of Ags, shown in models of chronic viral infections to $CD8^+$ T cells, using lymphocytic choriomeningitis virus or influenza A virus infections (28). Because the candidate Ags for colitogenic $CD4^+$ T cells are thought to be derived

from the intestinal bacterial Ags that are never eliminated from the body, it is doubtful whether colitogenic CD4$^+$ memory T cells can be generated under such a situation. However, as recent studies have suggested that persistent presence of Ags is rather required for the long-term maintenance of CD4$^+$ memory T cells, it is possible that the nature of CD4$^+$ memory T cells is quite different from that of CD8$^+$ memory T cells (29, 30). Thus, the present results may support another idea that the persistent presence of both commensal bacteria-derived PAMPs and specific Ags is required for the maintenance of long-term colitogenic CD4$^+$ memory T cells, and the subsequent progressive, disabling disease course.

It is also possible that nonpathogenic commensals stimulate TLR signaling of colitogenic CD4$^+$ memory T cells to sustain the disease without providing specific Ags for such cells. In other words, it should be verified whether specific Ags or PAMPs from the commensal bacteria are essential for the priming or memory phase. Consistently, the current study also provides an explanation of why a common recurrence of IBD is observed during complication of microbial infection, such as acute *Salmonella enterocolitis*, which may possibly supply large amounts of "bystander" PAMPs (31).

Finally, an important point should also be discussed: whether the present experimental design solely assesses the role of direct TLR signaling in various stages of CD4$^+$ T cells during the development of chronic colitis, because MyD88 is also involved in signaling downstream of endogenous cytokines, IL-1 and IL-18, in addition to TLR signaling (13, 14). Further studies will be required to address this issue by assessing which TLR is the most important for the stimulation of colitogenic CD4$^+$ T cells, followed by in vivo experiment using the corresponding TLR^{null} mice.

In summary, we here demonstrate that the MyD88-dependent pathway that mediates downstream signals of TLRs is crucially involved in the proliferative and survival responses of colitogenic CD4$^+$ T cells, which is required for the perpetuation of chronic colitis. Thus, in addition to the specific commensal Ags, homeostatic cytokines, and costimulatory molecules, therapeutic approaches targeting PAMPs may be feasible in the treatment of IBD.

Disclosures

The authors have no financial conflict of interest.

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Systemic, but Not Intestinal, IL-7 Is Essential for the Persistence of Chronic Colitis[1]

Takayuki Tomita, Takanori Kanai,[2] Yasuhiro Nemoto, Teruji Totsuka, Ryuichi Okamoto, Kiichiro Tsuchiya, Naoya Sakamoto, and Mamoru Watanabe

We previously demonstrated that IL-7 is produced by intestinal goblet cells and is essential for the persistence of colitis. It is well known, however, that goblet cells are decreased or depleted in the chronically inflamed mucosa of animal colitis models or human inflammatory bowel diseases. Thus, in this study, we assess whether intestinal IL-7 is surely required for the persistence of colitis using a RAG-1/2$^{-/-}$ colitis model induced by the adoptive transfer of CD4$^+$CD45RB^{high} T cells in combination with parabiosis system. Surprisingly, both IL-7$^{-/-}$ × RAG-1$^{-/-}$ and IL-7$^{+/+}$ × RAG-1$^{-/-}$ host mice developed colitis 4 wk after parabiosis to a similar extent of colitic IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice that were previously transferred with CD4$^+$CD45RB^{high} T cells. Of note, although the number of CD4$^+$ T cells recovered from the spleen or the bone marrow of IL-7$^{-/-}$ × RAG-1$^{-/-}$ host mice was significantly decreased compared with that of IL-7$^{+/+}$ × RAG-1$^{-/-}$ host mice, an equivalent number of CD4$^+$ T cells was recovered from the lamina propria of both mice, indicating that the expansion of CD4$^+$ T cells in the spleen or in the bone marrow is dependent on IL-7, but not in the lamina propria. Development of colitis was never observed in parabionts between IL-7$^{+/+}$ × RAG-1$^{-/-}$ host and noncolitic IL-7$^{-/-}$ × RAG-1$^{-/-}$ donor mice that were transferred with CD4$^+$CD45RB^{high} T cells. Collectively, systemic, but not intestinal, IL-7 is essential for the persistence of colitis, suggesting that therapeutic approaches targeting the systemic IL-7/IL-7R signaling pathway may be feasible in the treatment of inflammatory bowel diseases. ***The Journal of Immunology*****, 2008, 180: 383–390.**

Inflammatory bowel disease (IBD)[3] are caused by chronic inflammatory responses in the gut wall, commonly take persistent courses, but in some patients relapse after remissions (1–6). Because the recurrent disease usually mimics the primary disease episode, it is possible that the disease is caused by the repeated activation and expansion of colitogenic effector CD4$^+$ T cells arising from common long-lived colitogenic memory CD4$^+$ T cells, which latently reside in their target tissues or in some reservoirs. Nevertheless, the nature of the colitogenic memory CD4$^+$ T cells over time is not fully understood.

IL-7 is secreted by stromal cells in the bone marrow (BM) and thymus, and epithelial cells including the intestine (7–10). Recent findings revealed that IL-7 is an important cytokine supporting the survival of resting naive and memory CD4$^+$ T cells, but not effector CD4$^+$ T cells (9–16). We have previously demonstrated that, 1) IL-7 is constitutively produced by intestinal goblet epithelial cells (8), 2) IL-7 transgenic (Tg) mice, in which IL-7 overexpression was driven by SRα promotor, developed chronic colitis that mimicked histopathological characteristics of human IBD (17), 3) mucosal CD4$^+$IL-7Rα^{high} T cells in CD4$^+$CD45RB^{high} T cell-transferred colitic mice are colitogenic (18), and 4) IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice transferred with colitogenic lamina propria (LP) CD4$^+$ T cells isolated from colitic CD4$^+$CD45RB^{high} T cell-transferred mice did not develop colitis (19).

Somewhat at odds, however, we also found that production of intestinal IL-7 was dramatically decreased in the inflamed mucosa of colitic IL-7 Tg mice in accordance with depletion of goblet cells (17). Because our IL-7 Tg mice were established by expressing IL-7 under regulation of the ubiquitous SRα promoter, it was possible that intestinal IL-7 is indeed decreased at the site of mucosal inflammation due to depletion of goblet cells, which is a feature often seen in the inflamed mucosa of human IBD, but systemic IL-7 of other tissue origin, such as BM (20) and thymus (21), is rather critical for the maintenance of colitogenic memory CD4$^+$ T cells. Based on these complex backgrounds, in this study, we assess the distinct requirement of intestinal or systemic IL-7 in the development and persistence of colitis using a RAG-1/2$^{-/-}$ colitis model (22, 23) induced by adoptive transfer of CD4$^+$CD45RB^{high} T cells in combination with parabiosis system.

Materials and Methods

Animals

C57BL/6-Ly5.2 mice were purchased from Japan CLEA. C57BL/6-Ly5.1 mice and C57BL/6-Ly5.2-RAG-2-deficient (RAG-2$^{-/-}$) mice were obtained from Taconic Farms and Central Laboratories for Experimental Animals. C57BL/6-Ly5.2-background RAG-1$^{+/-}$ and IL-7$^{+/-}$ mice were provided from Dr. Rosa Zamoyska (National Institute for Medical Research, London, U.K.) (24). These mice were intercrossed to generate IL-7$^{-/-}$ × RAG-1$^{-/-}$ and IL-7$^{+/+}$ × RAG-1$^{-/-}$ littermate mice in the Animal Care Facility of Tokyo Medical and Dental University (TMDU). Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of TMDU. Donors and recipients were used at 6–12 wk of

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

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[2] Address correspondence and reprint requests to Dr. Takanori Kanai, Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. E-mail address: taka.gast@tmd.ac.jp

[3] Abbreviations used in this paper: IBD, inflammatory bowel disease; BM, bone marrow; LP, lamina propria; SP, spleen; Tg, transgenic; IEL, intraepithelial cell; HPF, high power field; DAPI, 4′, 6′-diamidino-2-phenylindole; LN, lymph node.

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age. All experiments were approved by the regional animal study committees and were done according to institutional guidelines and Home Office regulations.

Parabiosis experimental design

To assess the specific requirement of mucosal or systemic IL-7 in the development of colitis, we performed adoptive transfer experiment in combination with a parabiosis system using IL-7$^{+/+}$ × RAG-1$^{-/-}$ and IL-7$^{-/-}$ × RAG-1$^{-/-}$ littermate recipients (Fig. 1*A*). For adoptive transfer, CD4$^+$ T cells were first isolated from SP cells of C57BL/6-Ly5.2 mice using the anti-CD4 (L3T4)-MACS system (Miltenyi Biotec) according to the manufacturer's instruction. Enriched CD4$^+$ T cells (96–97% pure, as estimated by FACSCalibur (BD Biosciences)) were then labeled with PE-conjugated anti-mouse CD4 (RM4–5; BD Pharmingen) and FITC-conjugated anti-mouse (16A; BD Pharmingen). CD4$^+$CD45RB^{high} cells were purified using a FACSAria (BD Biosciences). This population was >98.0% pure on reanalysis. IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice (n = 18) and IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice (n = 6) were then injected i.p. with 3 × 10^5 splenic CD4$^+$CD45RB^{high} T cells from normal C57BL/6-Ly5.2 mice. After 6 wk post transfer, IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice, but not IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice, transferred with CD4$^+$CD45RB^{high} T cells developed a wasting disease and colitis as previously reported (19).

We then conducted parabiosis surgery according to institutional guidelines and Home Office regulations. In brief, sex-matched mice were anesthetized before surgery, and incisions were made in the skin on the opposing flanks of the donor and recipient animals. Surgical sutures were used to bring the body walls of the two mice into direct physical contact. The outer skin was then attached with surgical staples. For this parabiosis experiment, we divided colitic IL-7$^{+/+}$ × RAG-1$^{-/-}$ (n = 18) mice that were previously transferred with CD4$^+$CD45RB^{high} T cells into three groups: Group 1, colitic IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice joined with normal C57BL/6-Ly5.1 mice (n = 6); Group 2, colitic IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice joined with new IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice (n = 6); Group 3, colitic IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice joined with new IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice (n = 6). As Group 4, noncolitic IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice previously transferred with CD4$^+$CD45RB^{high} T cells were joined with new IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice (n = 6). All mice were observed for clinical signs, such as hunched posture, piloerection, diarrhea, and blood in the stool. At autopsy, mice were assessed for a clinical score (25) that is the sum of three 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) (25).

Histological examination

Tissue samples were fixed in PBS containing 10% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with H&E. Three tissue samples from the proximal, middle, and distal parts of the colon were prepared. The sections were analyzed without prior knowledge of each mouse. 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 follows: mucosa damage, 0; normal, 1; 3–10 intraepithelial cells (IEL)/high power field (HPF) and focal damage, 2; >10 IEL/HPF and rare crypt abscesses, 3; >10 IEL/HPF, multiple crypt abscesses and erosion/ulceration, submucosa damage, 0; normal or widely scattered leukocytes, 1; focal aggregates of leukocytes, 2; diffuse leukocyte infiltration with expansion of submucosa, 3; diffuse leukocyte infiltration, muscularis damage, 0; normal or widely scattered leukocytes, 1; widely scattered leukocyte aggregates between muscle layers, 2; leukocyte infiltration with focal effacement of the muscularis, 3; extensive leukocyte infiltration with transmural effacement of the muscularis.

Tissue preparations

Single cell suspensions were prepared from SP, LP, and BM as previously described (18). To isolate LP CD4$^+$ T cells, the entire length of the 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 and then treated with 3.0 mg/ml collagenase (Roche) and 0.01% DNase (Worthington Biochemical) for 2 h. The cells were pelleted two times through a 40% isotonic Percoll solution, and then 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. BM cells were obtained by flushing two femurs with cold RPMI 1640. For in vitro assay, only live cells were counted by using trypan blue staining method, and confirmed that the viability of cells was almost the same (>96% live) among the sample groups.

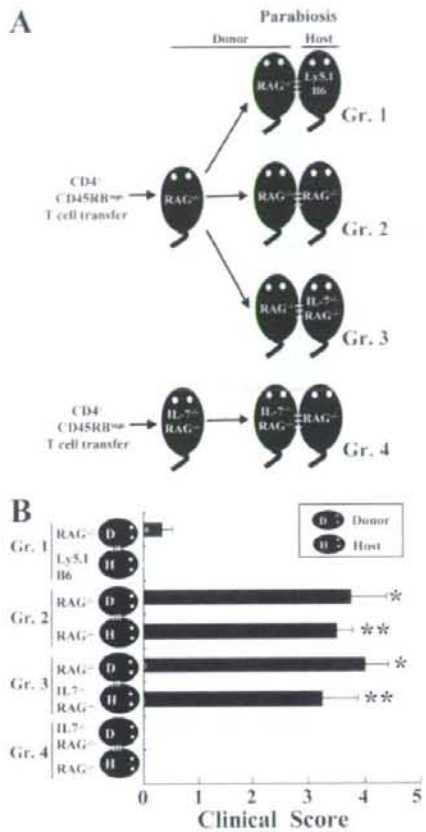

FIGURE 1. Host IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice in parabionts with diseased IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice show a wasting disease and clinical signs of colitis. *A*, Parabiosis experimental design. For an adoptive transfer, splenic CD4$^+$CD45RB^{high} T cells were isolated from C57BL/6-Ly5.2 mice, and then transferred into female IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice (n = 18) and IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice (n = 6). Six wk after transfer, IL-7$^{+/+}$ × RAG-1$^{-/-}$, but not IL-7$^{-/-}$ × RAG-1$^{-/-}$, mice transferred with CD4$^+$CD45RB^{high} T cells developed a wasting disease and colitis. As parabiosis pairs, Group 1 parabionts were joined between colitic donor IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice and normal host C57BL/6-Ly5.1 mice (n = 6 pairs). Group 2 parabionts were joined between colitic donor IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice and new host IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice (n = 6 pairs). Group 3 parabionts were joined between colitic donor IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice and new host IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice (n = 6 pairs). Group 4 parabionts were joined between noncolitic donor IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice and new host IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice (n = 6 pairs). Jointed animals were maintained for 4 wk after surgery. Gr. 1, Group 1; Gr. 2, Group 2; Gr. 3, Group 3; and Gr. 4, Group 4. *B*, Clinical scores were determined at 4 wk after surgery as described in *Materials and Methods*. Data are indicated as mean ± SEM of six mice in each group. *, $p < 0.01$, vs Group 1 donor mice. **, $p < 0.01$, vs Group 1 host mice.

Reverse transcription polymerase chain reaction

Total RNA was isolated by using Isogen reagent (Nippon Gene). Aliquots of 5 μg total RNA were used for complementary DNA synthesis in a reaction volume of 20 μl using random primers. One microliter of reverse transcription product was amplified with 0.25 U of rTaq DNA polymerase

(Toyoba) in a 50 μl reaction. Sense and antisense primers and the cycle numbers for the amplification of each gene were as follows: sense IL-7, 5′-GCCTGTCACATCATCTGAGTGCC-3′ and antisense IL-7, 5′-CAG GAGGCATCCAGGAACTTCTG-3′ for IL-7 (35 cycles); and sense G3PDH, 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ and antisense G3PDH, 5′-CATGTAGGCCATGAGGTCCACCAC-3′ for G3PDH (30 cycles). The amplification for each gene was logarithmic under these conditions. PCR products were separated on 1.8% agarose gels, stained with ethidium bromide, and visualized with a Lumi-Imager F1 (Roche).

Immunohistochemistry

We used consecutive cryostat colon sections in all studies. Immunohistochemistry using purified mAb against mouse CD4 (RM4–5; BD Pharmingen) or biotin-conjugated polyclonal IL-7 Ab (BAF407; R&D Systems) was performed. In brief, O.C.T. compound-embedded tissue samples were cut into serial sections 6-μm thick, placed on coated slides, and fixed with 4% paraformaldehyde phosphate buffer solution for 10 min. Slides were then incubated with the primary Ab at 4°C overnight, followed by staining with AlexaFluor 488 goat anti-rat IgG for CD4 detection or AlexaFluor 488 streptavidin (Molecular Probes) for IL-7 detection at room temperature for 60 min. All slides were counterstained with 4′, 6′ -diamidino-2-phenylindole (DAPI; Vector Laboratories) and observed under a confocal microscopy (LSM510 Carl Zeiss).

Cytokine ELISA

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

Flow cytometry

To detect the surface expression of a variety of molecules, isolated SP, BM, 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-, PerCP-, allophycocyanin-labeled Abs for 30 min on ice. The following mAbs were obtained from BD Pharmingen: anti-CD4 mAb (RM4–5), anti-CD45RB mAb (16A), anti-CD45.1 (Ly5.1; A20), and anti-CD45.2 (Ly5.2; 104). Standard four-color flow cytometric analyses were obtained using the FACSCalibur and analyzed by CellQuest software. Background fluorescence was assessed by staining with control irrelevant isotype-matched mAbs.

Statistical analysis

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

Results

IL-7$^{-/-}$ × RAG-1$^{-/-}$ host mice joined with colitic IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice develop a wasting disease

We have previously demonstrated that IL-7 is essential for the development and the persistence of colitis as a survival factor for colitogenic $CD4^+$ memory T cells (19). Furthermore, we have found that IL-7 Tg mice, in which IL-7 was systemically overproduced, develop colitis spontaneously, but production of intestinal IL-7 was conversely decreased in the inflamed mucosa because of depletion of the goblet cells. Based on such paradoxical findings, in this study, we assess whether intestinal or systemic IL-7 is essential for the perpetuation of colitis, by adoptive transfer experiment in combination with parabiosis system using IL-7$^{+/+}$ × RAG-1$^{-/-}$ and IL-7$^{-/-}$ × RAG-1$^{-/-}$ littermate recipients (Fig. 1*A*). To this end, we first induced chronic colitis by adoptive transfer of splenic $CD4^+CD45RB^{high}$ T cells from normal C57BL/6-Ly5.2 mice into IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice (Fig. 1*A*). Consistent with our previous report (19), the transferred IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice manifested progressive weight loss from 3 wk after transfer and clinical symptoms of colitis 6 wk after transfer (data not shown). In contrast, the $CD4^+CD45RB^{high}$ T cell-transferred IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice showed no clinical signs of colitis and weight loss (data not shown) (19), indicating that IL-7 is essential for the development of colitis.

At 6 wk after transfer, we next generated four groups of parabionts (Fig. 1*A*). In parabionts between colitic IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice that has been previously transferred with $Ly5.2^+CD4^+CD45RB^{high}$ T cells and normal C57BL/6-Ly5.1 host mice (Group 1) (Fig. 1*A*), clinical symptoms, such as diarrhea, anorectal prolapse, and hunched posture, gradually decreased over time in IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice as compared with the mice at the time of surgery, and completely disappeared at 4 wk after surgery by assessing the clinical score (Fig. 1*B*). C57BL/6-Ly5.1 host mice were consistently healthy during the observed period (Fig. 1*B*). In parabionts between colitic IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice and new IL-7$^{+/+}$ × RAG-1$^{-/-}$ host mice (Group 2) (Fig. 1*A*), all the IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice were consistently diseased (Fig. 1*B*), and clinical symptoms of colitis gradually increased in new IL-7$^{+/+}$ × RAG-1$^{-/-}$ host mice, which reached to the equal level of the paired IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice at 4 wk after surgery (Fig. 1*B*). In parabionts between colitic IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice and new IL-7$^{-/-}$ × RAG-1$^{-/-}$ host mice (Group 3) (Fig. 1*A*), IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice remained diseased to a similar level of IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice in Group 2 (Fig. 1*B*), and notably, IL-7$^{-/-}$ × RAG-1$^{-/-}$ host mice, albeit with the absence of intestinal IL-7, were gradually sick and clinical symptoms of colitis reached to the equal level of paired IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice and the IL-7$^{+/+}$ × RAG-1$^{-/-}$ host mice in Group 2 at 4 wk after surgery (Fig. 1*B*). In sharp contrast, in parabionts between the nondiseased IL-7$^{-/-}$ × RAG-1$^{-/-}$ donor mice that were transferred with $CD4^+CD45RB^{high}$ T cells and new IL-7$^{+/+}$ × RAG-1$^{-/-}$ host mice (Group 4) (Fig. 1*A*), both IL-7$^{-/-}$ × RAG-1$^{-/-}$ donor and IL-7$^{+/+}$ × RAG-1$^{-/-}$ host mice were consistently healthy during the observed period (Fig. 1*B*), indicating that $CD4^+CD45RB^{high}$ T cell-transferred IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice never retained colitogenic $CD4^+$ T cells.

IL-7$^{-/-}$ × RAG-1$^{-/-}$ host mice parabiosed with colitic IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice develop Th1-mediated colitis

Four wk after surgery, the colons from parabionts between IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice and C57BL/6-Ly5.1 host mice in Group 1 and parabionts between IL-7$^{-/-}$ × RAG-1$^{-/-}$ donor mice and IL-7$^{+/+}$ × RAG-1$^{-/-}$ host mice in Group 4 were macroscopically normal (data not shown). In contrast, the colon from all mice in Groups 2 and 3, regardless of IL-7$^{+/+}$ × RAG-1$^{-/-}$ or IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice and as donors or hosts, were equally enlarged and had a greatly thickened wall (data not shown). In addition, the enlargement of spleen was also present in donors and hosts of Groups 2 and 3 mice (data not shown). Histological examination showed that in colons from Group 1, donor IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice, which initially had clinical symptoms of colitis, exhibited no pathological change 4 wk after surgery, and were indistinguishable from the colons of C57BL/6-Ly5.1 host mice (Fig. 2*A*, *left*). In turn, we could not detect any pathological finding in Group 4 parabionts between IL-7$^{-/-}$ × RAG-1$^{-/-}$ donor mice and IL-7$^{+/+}$ × RAG-1$^{-/-}$ host mice. In contrast, all the donor and host mice in Groups 2 and 3 parabionts showed prominent epithelial hyperplasia with glandular elongation and a massive infiltration of mononuclear cells (Fig. 2*A*, *left*). This difference was also confirmed by histological scoring of colon sections (Fig. 2*B*), showing that the host mice in parabionts in Groups 2 and 3 developed colitis comparable to the paired diseased donor mice that had sustained colitis, while all the donor and host mice in Groups

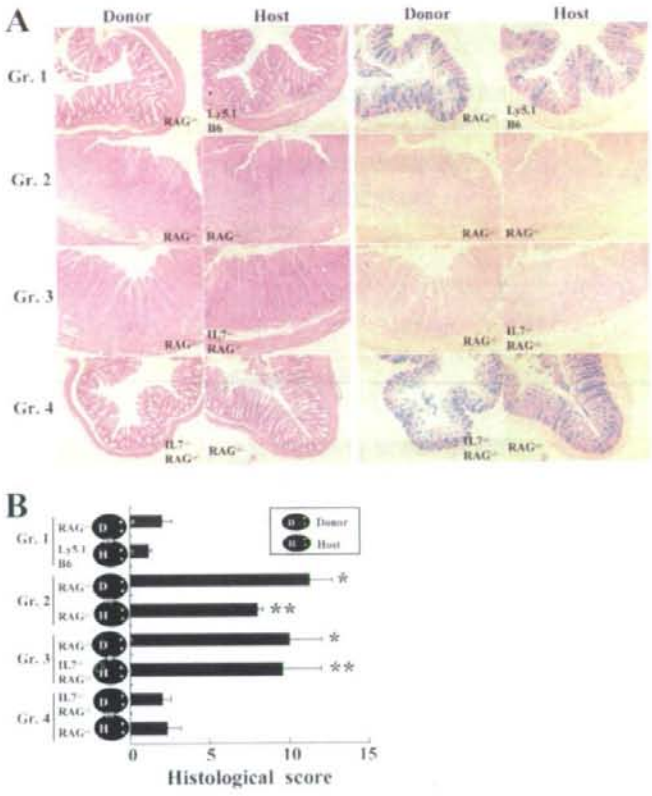

FIGURE 2. IL-7$^{-/-}$ × RAG-1$^{-/-}$ host mice in parabionts with diseased IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice develop colitis. *A*, Histological examination by H&E staining (*left*) and Alcian blue staining (*right*) of the colon from each group at 4 wk after surgery. Representative of four separate samples in each group. Original magnification, ×100. *B*, Histological scoring of the colon from Groups 1–4 at 4 wk after surgery. Data are indicated as the mean ± SEM of six mice in each group. *, $p < 0.01$, vs Group 1 donors. **, $p < 0.01$, vs Group 1 hosts. Gr., Group.

1 and 4 did not develop colitis. Furthermore, acid mucin production examined by Alcian blue staining revealed a marked decrease of mucin-producing goblet cells in all colitic mice in Groups 2 and 3 in contrast to mice in Groups 1 and 4 (Fig. 2*A*, *right*).

To clarify that newly developed colitis in host mice of Groups 2 and 3 was surely mediated by the infiltration of immigrant CD4$^+$ T cells from donor mice, but not by innate immune cells such as granulocytes and macrophages, we next assessed colonic infiltration of CD4$^+$ T cells by immunohistochemistry. Fig. 3 clearly demonstrated marked infiltration of CD4$^+$ T cells in the colon of host mice as well as in donor mice in parabionts of Groups 2 and 3. In contrast, only a small population of CD4$^+$ T cells was found in the host and donor mice in Groups 1 and 4 (Fig. 3). Especially, although the IL-7$^{+/+}$ × RAG-1$^{-/-}$ host mice in Group 1 had severe wasting disease with symptoms of colitis before surgery, there were only a few infiltrated CD4$^+$ T cells observed in colonic LP, indicating that the previous colitis was suppressed and cured by certain immigrant suppressor cells derived from normal host mice.

We next examined the cytokine production by LP CD4$^+$ T cells from each mouse in Groups 1–4. As shown in Fig. 4, LP CD4$^+$ T cells from donor and host mice in Groups 2 and 3 produced significantly higher amounts of IFN-γ and TNF-α as compared with those from mice in Groups 1 and 4, indicating that colitic LP CD4$^+$ T cells in IL-7$^{-/-}$ × RAG-1$^{-/-}$ host mice or IL-7$^{+/+}$ × RAG-1$^{-/-}$ host mice of Groups 2 and 3 have functions of Th1-mediated immune responses. Importantly, the elevated production of these cytokines in Groups 2 and 3 was dependent on the presence of colitis, but not on the expression of IL-7 in the colon.

Expansion of CD4$^+$ T cells is dependent on IL-7 in the SP or BM but is independent of IL-7 in the LP

We have previously reported that BM retaining colitogenic CD4$^+$ T cells in colitic mice might play a critical role as a reservoir for persisting colitis (18). Furthermore, BM is physiologically a major source of IL-7, contributing to the development of B cells (24). To further investigate the role of intestinal and/or systemic IL-7 in consecutive immunopathology of the parabiosis model, we next compared the composition of CD4$^+$ T cells in the LP, BM, and SP of donor and host mice in each parabiont using flow cytometry at 4 wk after surgery. The recovered cell numbers of CD3$^+$CD4$^+$ T cells from the donor and host LP in Groups 2 and 3 were significantly higher as compared with those of the paired donor and host colitic mice in Groups 1 and 4 parabionts, respectively (Fig. 5*A*). Furthermore, the recovered cell numbers of CD3$^+$CD4$^+$ T cells in the donor and host BM (Fig. 5*B*) and SP (Fig. 5*C*) in Groups 2 and 3 were significantly higher as compared with those of the paired donor and host colitic mice in Group 4, but not in Group 1, parabionts, respectively. In contrast, IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice that were previously transferred with CD4$^+$CD45RB^{high} T cells and C57BL/6-Ly5.1 host mice in Group 1 sustained a normal number of cells in the BM and SP (Fig. 5, data not shown). Most importantly, although the number of CD3$^+$CD4$^+$ T cells recovered from the SP or BM of the IL-7$^{-/-}$ × RAG-1$^{-/-}$ host mice in Group 3 was significantly decreased compared with that of the

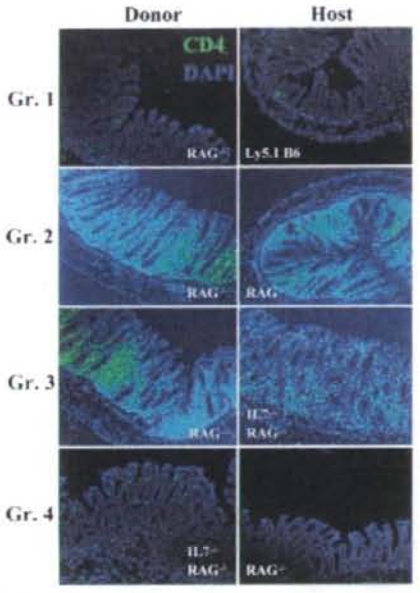

FIGURE 3. IL-7$^{-/-}$ × RAG-1$^{-/-}$ host mice in parabionts developed colitis with the marked infiltration of immigrant CD4$^+$ T cells from donor mice. CD4 immunostaining and DAPI counterstaining of the colon from Groups 1–4 at 4 wk after surgery. Frozen sections were fixed with 4% paraformaldehyde phosphate buffer solution and stained with anti-mouse CD4 mAb, followed by AlexaFluor 488 goat anti-rat IgG as secondary Ab and DAPI counterstaing. A large number of CD4$^+$ T cells were infiltrated in the colonic mucosa of IL-7$^{-/-}$ × RAG-1$^{-/-}$ host mice (Group 3) as well as in that of IL-7$^{+/+}$ × RAG-1$^{-/-}$ host mice (Group 2). Representative of four separate samples in each group. Original magnification: ×100. Gr., Group.

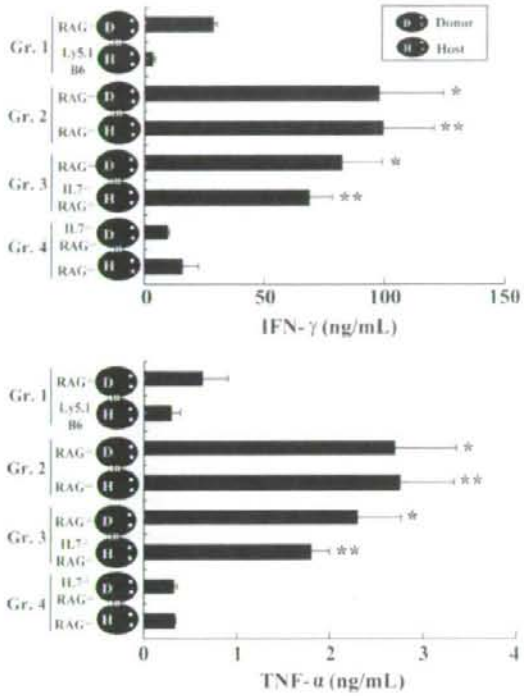

FIGURE 4. IL-7$^{-/-}$ × RAG-1$^{-/-}$ host mice in parabionts develop Th1-mediated colitis. LP CD4$^+$ T cells were prepared from colons at 4 wk after surgery and stimulated with anti-CD3 and anti-CD28 mAbs for 48 h. Concentrations of IFN-γ, and TNF-α in culture supernatants were measured by ELISA. Data are indicated as the mean ± SEM of six mice in each group. *, $p < 0.01$, vs Group 1 donors. **, $p < 0.01$, vs Group 1 hosts. Gr., Group.

IL-7$^{+/+}$ × RAG-1$^{-/-}$ host mice in Group 2, an equivalent number of CD4$^+$ T cells was recovered from the LP of both host mice in Groups 2 and 3, indicating that the expansion of CD4$^+$ T cells in the SP and BM is dependent on IL-7, but is independent in the LP.

Further analysis of Group 1 mice using a four-colored CD3/CD4/Ly5.1/Ly5.2 FACS staining revealed that >95% of total CD4$^+$ T cells were derived from Ly5.1$^+$ cells and most resident Ly5.2$^+$CD4$^+$ T cells decreased to only 5–10% of total CD4$^+$ T cells in SP and BM in Group 1 IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice (Fig. 5). Interestingly, although the absolute number of LP CD4$^+$ T cells was significantly decreased in Group 1 IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice as compared with those of IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice in Groups 2 and 3 colitic parabionts, ~50% of total LP CD4$^+$ T cells remained to be Ly5.2$^+$, suggesting that 1) colitogenic LP Ly5.2$^+$CD3$^+$CD4$^+$ T cells were resistant to the suppression by Ly5.1-derived cells as compared with Ly5.2$^+$CD3$^+$CD4$^+$ T cells in other sites and/or 2) they remained in the intestine, and in other words could not exit, and redistribute outside the intestine. Furthermore, small but substantial percentages (1–5%) of total CD4$^+$ T cells in each tissue of host C57BL/6-Ly5.1 mice were donor-derived Ly5.2$^+$ cells, indicating that two-way recirculation of CD4$^+$ T cells from the donor to the host and vice versa had been established and most of Ly5.2$^+$ colitogenic CD4$^+$ T cells in both donor and host mice had undergone the contraction under a certain suppressive mechanism including suppression by CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells derived from host C57BL/6 mice.

IL-7 is not detected in host IL-7$^{-/-}$ × RAG-1$^{-/-}$ host mice after parabiosis

Studies showing engraftment of BM-derived cells to various non-hemopoietic tissues including epithelial cells after BM transplantation are now on topic (26, 27), and we have previously demonstrated that human BM cells have a potential to repopulate the gastrointestinal epithelia by detecting Y-chromosomes in female cases that have undergone BM transplantation using male donor cells (28). It was thus needed to assess whether this was the case with our parabiosis setting, and if so, it was interesting to know whether IL-7 was produced by engrafted colonic epithelial cells derived from the BM of IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice in IL-7$^{-/-}$ × RAG-1$^{-/-}$ host mice after surgery in Group 3. As shown in Fig. 6*A*, immunohistochemistry revealed that IL-7 is detected in uninflamed colonic epithelia of both IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor and C57BL/6 host mice in Group 1 and IL-7$^{+/+}$ × RAG-1$^{-/-}$ host, but not in IL-7$^{-/-}$ × RAG-1$^{-/-}$ donor, mice in Group 4. Consistent with previous findings (17), IL-7 expression was detectable, but markedly decreased in inflamed colonic epithelia in Groups 2 and 3 of IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice along with the decreased goblet cells, in both host and donor mice (Fig. 2*A*, *right*). In contrast, IL-7 was not detected in the inflamed colonic epithelia of Group 3 IL-7$^{-/-}$ × RAG-1$^{-/-}$ host mice (Fig. 6*A*). Consistent with these results, further RT-PCR analysis for IL-7 mRNA expression showed that IL-7 mRNA was not detected in

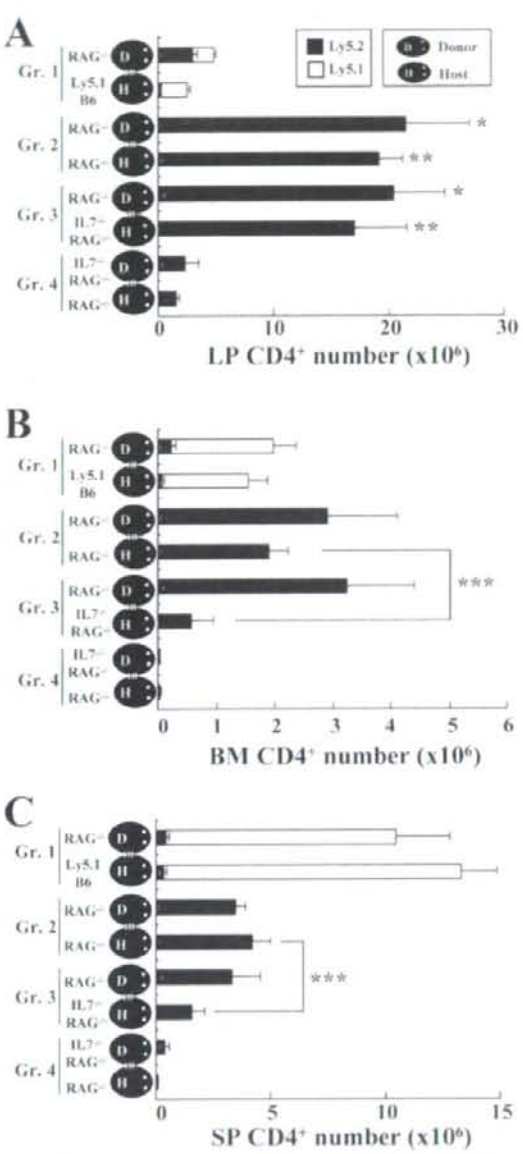

FIGURE 5. Expansion of BM and SP, but not of LP, $CD4^+$ T cells in IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice in parabionts is dependent on IL-7. LP (*A*), BM (*B*), and SP (*C*) $CD4^+$ T cells were isolated from each mouse of Groups 1–4 at 4 wk after surgery, and the number of $CD4^+$ cells were determined by flow cytometry. Data are indicated as the mean ± SEM of six mice in each group. *, $p < 0.01$, vs Group 1 donors. **, $p < 0.01$, vs Group 1 hosts. ***, $p < 0.01$, vs Group 2 hosts. For cells in Group 1 parabionts, cells were stained with anti-CD45.1 mAb and anti-CD45.2 mAb to discriminate between donor or host origin. Gr., Group.

colitic IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice in Group 3, and was markedly decreased in colitic IL-$7^{+/+}$ × RAG-$1^{-/-}$ donor and host mice in Groups 2 and 3 in clear contrast to that of control C57BL/6 mice (Fig. 6*B*).

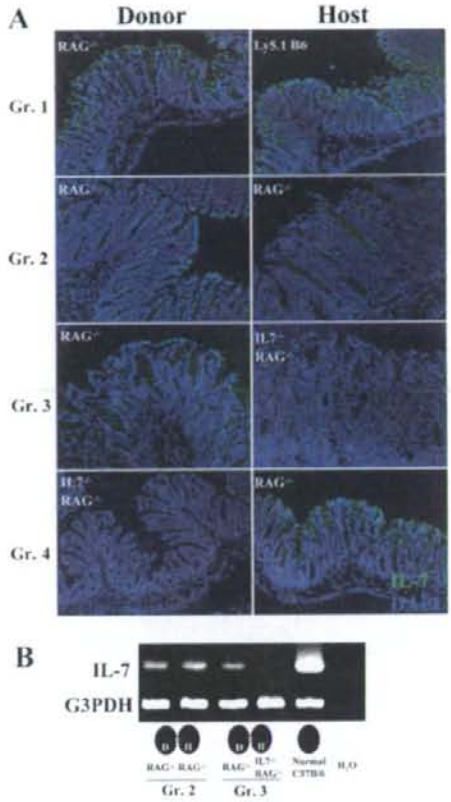

FIGURE 6. IL-7 is not detected in host IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice in parabionts with diseased IL-$7^{+/+}$ × RAG-$1^{-/-}$ donor mice. *A*, Frozen sections of colon from each mouse in Groups 1–4 at 4 wk after surgery were stained with polyclonal anti-IL-7 Abs. Representative of five separate samples in each group. Original magnification: ×100. *B*, Expression of IL-7 mRNA in the whole colon was determined by RT-PCR. Representative of five separate samples in each group. Gr., Group.

Discussion

In this study, we demonstrated that intestinal IL-7 is not essential for the development and perpetuation of colitis by showing that IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice parabiosed with colitic IL-$7^{+/+}$ × RAG-$1^{-/-}$ donor mice develop a wasting disease and severe colitis. Because we previously demonstrated that IL-7 is needed to develop and sustain colitis by showing a lack of colitis development in IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice transferred with $CD4^+CD45RB^{high}$ T cells or colitogenic LP $CD4^+$ T cells (19), in this study, we suggest that IL-7 production from tissues other than the intestine, such as BM, is sufficient, or rather may be essential to develop and sustain the chronic colitis.

Before starting this study, we confronted a paradox between two facts. The first fact is that IL-7-producing goblet cells are easily decreased or depleted in patients with severe ulcerative colitis (29), colitic IL-7 Tg mice (17) and in the present model of colitis (Fig. 2*A*, *right*) resulting in the decreased IL-7 production in the intestine, and the second fact is that IL-7 appeared to be indispensable for the development and persistence of chronic colitis by adoptive transfer experiment using IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice (19). Based on these backgrounds, we hypothesized that intestinal IL-7 is

indeed important to establish GALT, such as Payer's patches and cryptopatches, and also to maintain IELs (30), but not needed to develop and sustain colitis, since many Ags, such as intestinal bacterial Ags, may be sufficient to stimulate colitogenic $CD4^+$ T cells in the intestinal LP without stimuli from IL-7. To prove it, we performed a combinational experiment using adoptive transfer and parabiosis systems in the present study. Although the parabiosis system seems to be somewhat artificial and problematic on some level as two mice, host and donor, are forced to have a surgical stress and behavioral limitation (Groups 3 and 4), mice laboring colitogenic $CD4^+$ T cells are surgically joined, resulting in prompt development of anastomoses of blood vessels within a few days. Even in the present setting, it is noteworthy that IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice joined with colitic IL-$7^{+/+}$ × RAG-$1^{-/-}$ donor mice developed a wasting disease and colitis to the similar level of colitic IL-$7^{+/+}$ × RAG-$1^{-/-}$ donor mice over time.

In this parabiosis system, however, it was also possible that certain stem cells that are committed to differentiate into IL-7-producing mesenchymal cells or epithelial cells homed to the intestine, and might have been involved in the development and persistence of colitis in IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice joined with colitic IL-$7^{+/+}$ × RAG-$1^{-/-}$ donor mice (Group 3). To rule out this possibility, we also demonstrated that IL-7 expression was not detected in the colon of the IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice both at the protein and mRNA levels (Fig. 6). Consistent with the present result, another group demonstrated that restoring intestinal IL-7 expression to IL-$7^{-/-}$ mice did not result in the development of colitis (31). Collectively, the current results clearly indicate that intestinal IL-7 is not essential, but systemic IL-7 from extraintestinal sites is essential, for the development and sustainment of colitis.

It is also very important to know why IL-7 is decreased in the inflamed mucosa of colitis in terms of pathogenesis of chronic colitis. In other words, it is possible that the lack or decrease of IL-7 production in inflamed mucosa of colitis is pathologically needed to maintain chronic colitis. Consistent with this hypothesis, we previously demonstrated that although IL-7 promoted proliferation of human LP IL-7Rα-expressing $CD4^+$ T cells, double stimuli by IL-7 and anti-CD3 mAb conversely suppressed it (21). In addition, Fluur and colleagues (32) very recently reported that IL-7 induces Fas-mediated T cell apoptosis by inducing Fas expression on $CD4^+$ T cells. Thus, it appears that intestinal IL-7 physiologically plays a key role in the elimination of pathological LP $CD4^+$ T cells activated by intestinal bacteria. Further studies will be needed to address this issue.

Interestingly, the recovered cell number of LP $CD4^+$ T cells was equivalent between host and donor mice both in Group 2 and 3, although it was likely that total production of IL-7 in Group 3 parabionts between one IL-$7^{+/+}$ mouse and one IL-$7^{-/-}$ mouse was approximately half compared with that in Group 2 parabionts between two IL-$7^{+/+}$ mice. Because it seems that the production of IL-7 is maintained at a constant rate and is uninfluenced by extrinsic stimuli (33, 34), this result indicates that factors other than IL-7, such as stimulation by commensal bacteria might control the homeostasis of cell number in the LP, but not in the BM and SP. Further studies will be needed to address this issue.

BM is a major source of IL-7 in the body (26). In contrast to the LP, it is noteworthy that the number of $CD4^+$ T cells recovered from the BM and SP of the colitic IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice (Group 3) was significantly decreased compared with that of the IL-$7^{+/+}$ × RAG-$1^{-/-}$ host mice. Regarding this result, we recently demonstrated that $CD4^+$ effector-memory-like T (T_{EM}-like) cells reside in the BM of colitic SCID and RAG-1/2$^{-/-}$ mice induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells (20). Importantly, these resident BM $CD4^+$ T_{EM}-like cells are closely attached to IL-7-producing stromal cells in the colitic BM. Most importantly, the accumulation of BM $CD4^+$ T_{EM}-like cells was significantly decreased in IL-7-deficient recipients reconstituted with the colitogenic LP $CD4^+$ T_{EM}-like cells. Together with the present study, these findings suggest that the BM $CD4^+$ T_{EM}-like cells residing in mice with chronic colitis play a critical role as a reservoir for lifelong persisting colitis in an IL-7-dependent manner. However, it is still possible that IL-7 produced by sites other than intestine or BM, such as skin, liver, eye, lymph nodes (LN), and SP, also contribute to the development and perpetuation of colitis. In this regard, we very recently demonstrated that splenectomized LN-null lymphotoxin $\alpha^{-/-}$ × RAG-$2^{-/-}$ mice transferred with colitogenic LP $CD4^+$ T cells develop colitis (35), suggesting that IL-7 production at least by LN and SP does not appear to be essential. To further clarify the role of IL-7 produced by BM mesenchymal cells in the pathogenesis of chronic colitis, BM chimera of IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice, which are lethally irradiated and transplanted with the BM cells from IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice, may be quite beneficial. Interestingly, however, it is also well known that extraintestinal complications of IBD patients such as skin, liver, and mucocutaneous manifestations (36) appears to be closely associated with sites of local IL-7 production by keratinocytes, hepatocytes, and uvea cells. Although no inflammation was not observed at least in liver and skin in the present model of colitis (data not shown), further studies will be needed to address this issue.

Clinicopathologically, IBD is characterized by chronic intestinal inflammation. Surgery does not cure IBD, especially Crohn's disease, as relapse is a rule after remission, suggesting that IBD is not a circumscribed disease, but rather a systemic disease mediated by colitogenic memory $CD4^+$ T cells distributing throughout the body via the bloodstream, which may hide in their reservoir, such as BM. Consistent with this hypothesis, recent findings showing usefulness of leukocytoapheresis, which removes peripheral blood cells for the treatment of refractory IBD patients (37, 38), suggests that recirculation of colitogenic memory $CD4^+$ T cells from the gut to some reservoir and vice versa, may play a role in the perpetuation of chronic colitis. Furthermore, we have recently demonstrated that FTY720 that has an ability to inhibit circulation of lymphocytes prevents the development of SCID/RAG-1/2$^{-/-}$ colitis induced by adoptive transfer of LP colitogenic $CD4^+$ T_{EM}-like cells (39). Together with the current results, it would be possible that the circulation of colitogenic $CD4^+$ T_{EM}-like cells is quite active in IBD, making them continue to circulate in the blood and migrate to IL-7-producing reservoir from the IL-7-depleted LP.

In summary, in this study, we demonstrated that systemic IL-7, but not intestinal IL-7, is essential for the development and perpetuation of colitis, suggesting that therapeutic approaches targeting systemic IL-7 using the biologics against IL-7 may be feasible in the treatment of IBD.

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Disclosures

The authors have no financial conflict of interest.

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Adiponectin stimulates IL-8 production by rheumatoid synovial fibroblasts

Kanako Kitahara [a,b], Natsuko Kusunoki [a], Terutaka Kakiuchi [b], Toru Suguro [c], Shinichi Kawai [a,*]

[a] Division of Rheumatology, Department of Internal Medicine, Toho University School of Medicine, 6-11-1 Omori-Nishi, Ota-ku, Tokyo 143-8541, Japan
[b] Department of Immunology, Toho University School of Medicine, Tokyo, Japan
[c] Department of Orthopaedic Surgery, Toho University School of Medicine, Tokyo, Japan

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ABSTRACT

The adipokines are linked not only to metabolic regulation, but also to immune responses. Adiponectin, but not leptin or resistin induced interleukin-8 production from rheumatoid synovial fibroblasts (RSF). The culture supernatant of RSF treated with adiponectin induced chemotaxis, although adiponectin itself had no such effect. Addition of antibody against adiponectin, and inhibition of adiponectin receptor gene decreased adiponectin-induced IL-8 production. Nuclear translocation of nuclear factor-kappa B was increased by adiponectin. The induction of interleukin-8 was inhibited by mitogen-activated protein kinase inhibitors. These findings suggest that adiponectin contributes to the pathogenesis of rheumatoid arthritis.

Rheumatoid arthritis (RA) is a systemic, chronic, inflammatory disease that affects multiple joints. An important recent development in our understanding of RA is that patients with RA experience an acceleration of atherosclerosis that cannot be solely explained by the increased prevalence of traditional cardiovascular risk factors [1].

Adiponectin is an adipokine that is mainly secreted from white adipose tissue. It is believed to be beneficial because plasma levels of adiponectin are reduced in metabolic syndrome [2]. It has been reported that adiponectin exerts an anti-atherosclerotic effect [3,4]. Interestingly, recent studies have reported increased levels of adiponectin in RA patients [5,6], findings which appear paradoxical in light of the higher prevalence of atherosclerosis in RA. The existence of this paradox raises the possibility that adiponectin plays a novel role in the pathogenesis of RA. Moreover, the effect that adipokines have not only on the metabolic system, but also on inflammation, has recently become of clinical interest, and the role of adiponectin remains controversial [2,7,8].

RA is characterized by proliferative synovitis in multiple joints. The infiltration and activation of leukocytes results in progressive destruction of joint. Chemokines are pivotal in the recruitment of leukocytes and angiogenesis. In RA, IL-8 is an important chemokine, and high levels of IL-8 have been detected in RA patients [9,10]. In the present study, we examined the direct effect of adiponectin on IL-8 production by RSF.

Materials and methods

Reagents. Reagents used in this study were purchased from the following sources; recombinant human full-length adiponectin from Biovendor (Brno, Czech Republic); recombinant human leptin from Sigma (Missouri, USA); recombinant human resistin from Axxora (CA, USA); human recombinant IL-8 from Chemicon (USA); Goat anti-human AdipoR1 and AdipoR2 antibodies from Santa Cruz Biotechnology (CA, USA); SB203580 from Sigma; SP600125 and PD98059 from Calbiochem (Darmstadt, Germany). Stock solutions were dissolved in phosphate-buffered saline (PBS) or dimethyl sulfoxide (DMSO) when appropriate. The final concentration of DMSO in experiments was always <0.1% and control wells contained an equivalent concentration of the vehicle.

Cell culture. Rheumatoid synovial cells were prepared as described previously [11]. The synovial tissues were obtained from patients who fulfilled the 1987 revised criteria for RA [12] or patients who fulfilled the criteria for osteoarthritis [13]. Research protocol has approved by the ethics committee of Toho University (approval number: 16002, 19021). Synovial cells were cultured in RPMI 1640 with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) under the standard conditions.

Evaluation of cytokine secretion. RSF were cultured in 96-well plates for 24 h. The cells were then incubated with various concentrations of adipokines, human recombinant IL-1β (BD biosciences), or PBS in RPMI containing 1% FBS. Cytokine concentrations in the media were measured by enzyme-linked immunosorbent assay (ELISA) (IL-8, R&D systems, Minneapolis, USA; IL-1 β, IL-6, and TNF-α, BioSource International Inc., CA, USA).

* Corresponding author. Fax: +81 3 5753 8513.
E-mail address: skawai@med.toho-u.ac.jp (S. Kawai).

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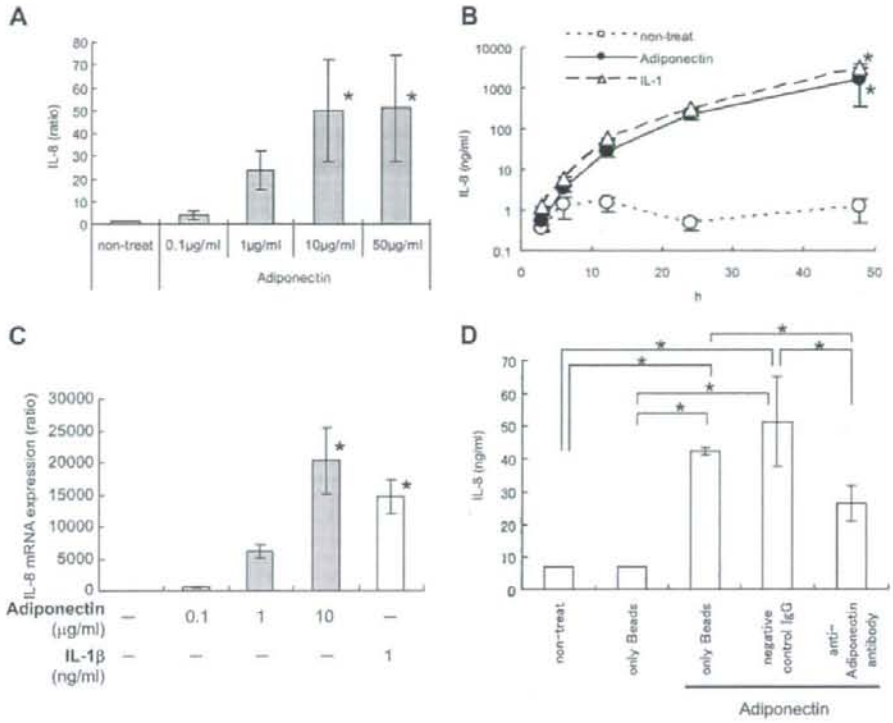

Fig. 1. IL-8 induction by adiponectin in RSF. (A) Cells were treated with each dose of adiponectin for 24 h and levels of IL-8 in culture supernatant were measured by ELISA. The IL-8 level of control (non-treat) was 0.11–10.03 ng/ml. $^{*}P < 0.05$ vs. non-treat. (B) Cells were treated with 10 μg/ml of adiponectin, 1 ng/ml of IL-1 (positive control), or PBS for 3, 6, 12, 24, or 48 h and levels of IL-8 were measured by ELISA. $^{*}P < 0.05$ vs. non-treat. (C) Expression of IL-8 mRNA as determined by real-time PCR. $^{*}P < 0.05$ vs. non-treat. (D) (From right) Adiponectin was incubated with anti-adiponectin antibody, negative control IgG, or PBS and with sepharose beads at 4 °C overnight. PBS incubated with/without sepharose beads were used as negative controls. Then supernatant was collected and added into cultured RSF. IL-8 concentration was measured by ELISA. $^{*}P < 0.05$.

PCR. Total RNA was isolated from the cells using the RNeasy Mini Kit (Qiagen), and reverse-transcribed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). One microgram of each reverse-transcription product was used as a template. The primers of IL-8 (GenBank accession no. NM_000584.2) [14], AdipoR1 (GenBank accession no. NM_015999)/AdipoR2 (GenBank accession no. NM_024551) [15] and GAPDH (GenBank accession no. NM_002046) were purchased from Sigma. For real-time PCR, probes and primer pairs for IL-8 and β-actin were purchased from Applied Biosystems. The results were normalized for β-actin as an endogenous control.

Western blot. The cells were lysed in Triton lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, and a protease inhibitor cocktail (Pierce Biotechnology). After electrophoresis of cell lysates, the SDS–PAGE separated proteins were electroblotted onto Immobilon-P poly (vinylidene difluoride) membranes with a semidry blotter (Atto, Tokyo, Japan). The membranes were probed with an appropriate primary antibody and a HRP-conjugated secondary antibody. Protein bands were detected with an enhanced chemiluminescence Western blot analysis system.

Inhibition experiments of adiponectin with anti-adiponectin antibody. Adiponectin was incubated with mouse anti-adiponectin monoclonal antibody (Chemicon), mouse monoclonal IgG1 negative control (Chemicon), or PBS and with Protein-G sepharose beads (GE Healthcare Bio-Sciences) at 4 °C overnight. Then supernatant was collected and added into RSF cultured in 96-well plates (2×10^4 cells/well).

RNA interference and transfection. Small-interfering RNAs (*RNAi*) were designed and synthesized by Invitrogen. The sequences of the *RNAi* were as follows: AdipoR1: AAUAGACGGUGUGAAAGAGCCAG GA and UCCUGGCUCUUUCACACCGUCUAUU; AdipoR2: AGCAUG AUGGGCUUGUAAGAGAGGG and CCCUCUCUUACAAGCCCAUCAUG CU. The cultured RSF were transfected with 120 nM of *RNAi* by Lipofectamine RNAiMAX (Invitrogen). Cells transfected with nonfunctional *RNAi* (Invitrogen) were used as negative controls. Seventy-two hours after the transfection, cells were recultured for analysis by ELISA or PCR.

Chemotaxis assay. Polymorphonuclear cells (PMN) were prepared using Polymorphprep (Axis-Shield, Norway) and suspended in RPMI containing 1% FBS. For measurement of PMN migration, a 96-well chemotaxis chamber (Neuro Probe, USA) was used, as previously described [16]. The migrated cells were assessed by Cell Counting Kit (Dojindo Molecular Technologies, Kumamoto, Japan). The ratio of directed migration to random migration (D/R ratio) was calculated: D/R = total fluorescence of wells containing chemoattractant/total fluorescence of wells containing vehicle.

Nuclear extracts and NF-κB assay. Nuclear protein extraction from cultured RSF was performed with the nuclear extract kit (Active Motif, Tokyo, Japan). Nuclear extract was obtained from RSF treated with adiponectin (10 μg/ml), IL-1 β (1 ng/ml), or PBS for 3 h. Nuclear translocation of active NF-κB was assessed by ELISA (Active Motif, Tokyo, Japan).

Statistical analysis. All experiments were repeated as least three times using RSF obtained from three different patients. Differences

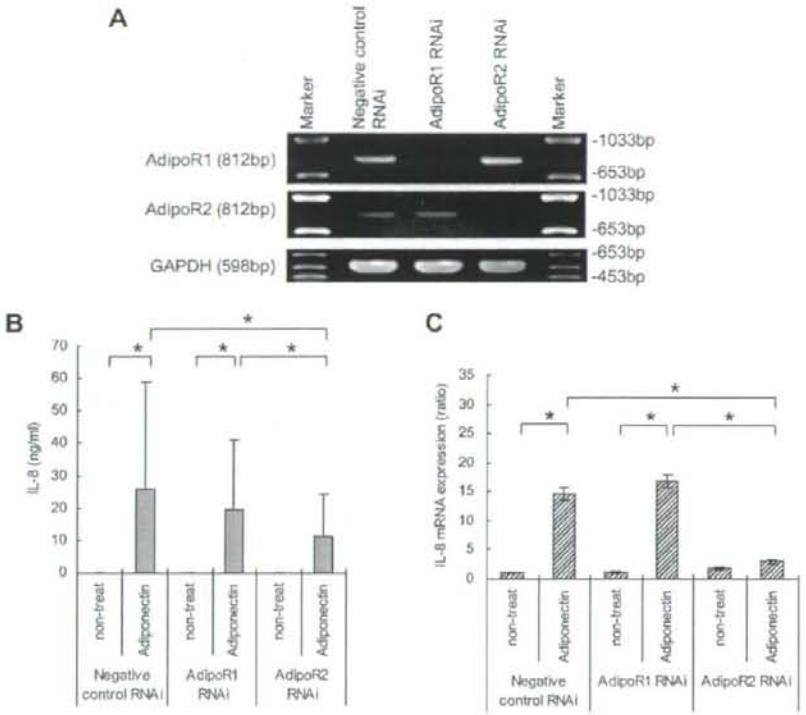

Fig. 2. Specific inhibition of adiponectin receptors by RNAi. (A) RSF were transfected with AdipoR1, AdipoR2, or Negative control RNAi as described (Methods), and mRNA levels of AdipoR1 or AdipoR2 was analyzed by RT-PCR. (B, C) After the transfection of RNAi, RSF were treated with 10 μg/ml of adiponectin or PBS for 24 h, and levels of IL-8 were measured by ELISA (B) and by real-time PCR (C). $^{*}P < 0.05$.

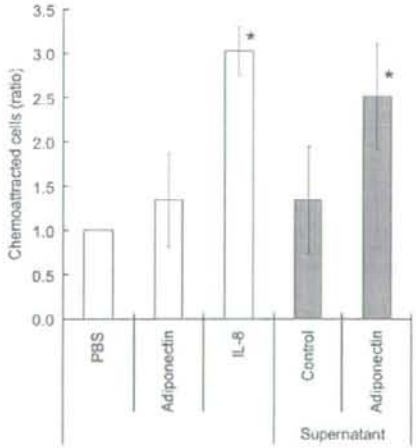

Fig. 3. PMN migration induced by supernatant of RSF treated with adiponectin. RSF were treated with 10 μg/ml of adiponectin or PBS; culture supernatants were collected after 24 h. Either 29 μl of vehicle, adiponectin (10 μg/ml), cell culture supernatants, or IL-8 dilutions (100 ng/ml) (positive control) was added to the bottom chamber, and 25 μl of PMN suspension was placed onto a filter. After incubation for 1 h, the *D/R* ratio of cells that migrated into the bottom chamber was assessed as described (Methods). $^{*}P < 0.05$ vs. vehicle.

between groups were assessed by Tukey's test. The statistical analysis was performed using the SPSS 12.0 (SPSS Inc., Chicago, IL, USA).

Results

Expression of AdipoR1 and AdipoR2 on RSF

To date, two specific adiponectin receptors—AdipoR1 and AdipoR2—have been cloned [17]. We performed RT-PCR and western blot to investigate whether the adiponectin receptors were present on RSF. Both mRNA and protein expression of these adiponectin receptors were detected in all RSF used in this study (data not shown).

Adiponectin-induced cytokine production by RSF

After stimulation of RSF with adiponectin, leptin, or resistin, IL-8 levels in the culture supernatants were determined. A dose-dependent increase in IL-8 was detected in the culture supernatants of RSF treated with adiponectin, which suggests that adiponectin stimulated IL-8 production by RSF (Fig. 1A). A physiological concentration (10 μg/ml) of serum adiponectin induced the highest IL-8 production. However, physiological concentrations of leptin and resistin did not stimulate IL-8 production (data not shown). The IL-8 induction by adiponectin increased in a time-dependent manner (Fig. 1B). To confirm the IL-8 expression, real-time PCR were performed. Expression of IL-8 mRNA was dose-dependently increased by adiponectin (Fig. 1C). In addition to IL-

8 production, IL-6 production by adiponectin from RSF was observed (data not shown), as previously reported by others [18,19]. In contrast, neither of the cytokines IL-1β and TNF-α could be detected by ELISA.

IL-8 induction by adiponectin was inhibited by presence of anti-adiponectin antibody (Fig. 1D). To confirm IL-8 production by adiponectin, specific inhibition of adiponectin receptors expression was accomplished with RNAi (Fig. 2A). Treatment with AdipoR2 RNAi blocked the IL-8 production (Fig. 2B) and mRNA expression (Fig. 2C) by RSF, meanwhile AdipoR1 RNAi treatment had few effects.

We also examined the effect of adiponectin on IL-8 production in synovial fibroblasts obtained from patients with osteoarthritis. Adiponectin even concentration at 20 μg/ml weakly induced IL-8 production in synovial fibroblasts of osteoarthritis patients, which were less than one tenth when compared to the maximal responses of those in RSF (data not shown).

Effect of adiponectin on chemotaxis of human polymorphonuclear cells

We evaluated the biological effect of supernatant of RSF that were treated with adiponectin on chemotaxis of polymorphonuclear cells obtained from healthy human subjects. Adiponectin alone had no effect on chemotaxis; however, the culture supernatant of RSF treated with adiponectin induced significant chemotaxis of human polymorphonuclear cells *in vitro* (Fig. 3).

Intracellular signaling

As shown in Fig. 4A and B, nuclear translocation of p50 and p65 NF-κB was increased by adiponectin. A signal decrease was noted only in the presence of wild-type competitor oligonucleotides, which confirms that the assay specifically measures binding of p50 and p65 to their target sites. Other NF-κB family members, namely p52, RelB, and c-Rel, were not activated by adiponectin (data not shown).

The effects of SB203580, SP600125, and PD98059, which are MAPK inhibitors for p38, JNK, and ERK, respectively, were evaluated to investigate signal pathways (Fig. 5A–C). Production of IL-8 by adiponectin was significantly inhibited by the addition of SB203580 or SP600125. Although the effect of PD98059 was not significant, there was a tendency toward inhibition. These results suggest that adiponectin induces IL-8 from RSF via NF-κB and MAPK pathways.

Discussion

In the present study, we demonstrated that adiponectin, but not leptin or resistin stimulated IL-8 production by RSF that express specific receptors for adiponectin. Addition of antibody against adiponectin, and inhibition of adiponectin receptor gene decreased adiponectin-induced IL-8 production. Supernatant obtained from RSF treated with adiponectin significantly induced chemotaxis of PMN; adiponectin alone had no such effect. These results support the hypothesis that adiponectin has a local proinflammatory role in RA. The core IL-8 promoter contains an NF-κB site and previous studies have highlighted the contribution of the MAPK pathways to IL-8 gene expression [20]. We noted that adiponectin induced nuclear translocation of activated p50 and p65 NF-κB and that MAPK inhibitors reduced adiponectin-induced IL-8 production by RSF.

Previous reports have shown that adiponectin exerts an anti-atherosclerotic effect on endothelial cells by inhibiting the expression of TNF-induced IL-8 [3] and adhesion molecules [4]. Adiponectin has also been found to inhibit endothelial NF-κB signaling [21], which may contribute to inhibition of monocyte adhesion to endothelial cells. These findings do not accord with our results, possibly because of differences in the cells and culture conditions used in the experiments. In contrast, Ehling et al. [18] showed that adiponectin induced IL-6 and matrix metalloproteinase-1 production by RSF, suggesting that adiponectin has the potential to promote arthritis, a findings confirmed by our data. Tang et al. [19] also showed that adiponectin induced IL-6 production by RSF. Although their reports indicated a significance of AdipoR1 in adiponectin-induced IL-6 production, our results suggested that AdipoR2 might be a major receptor involved in adiponectin-induced IL-8 production. Indeed, levels of adiponectin in synovial fluid and sera have been shown to be significantly

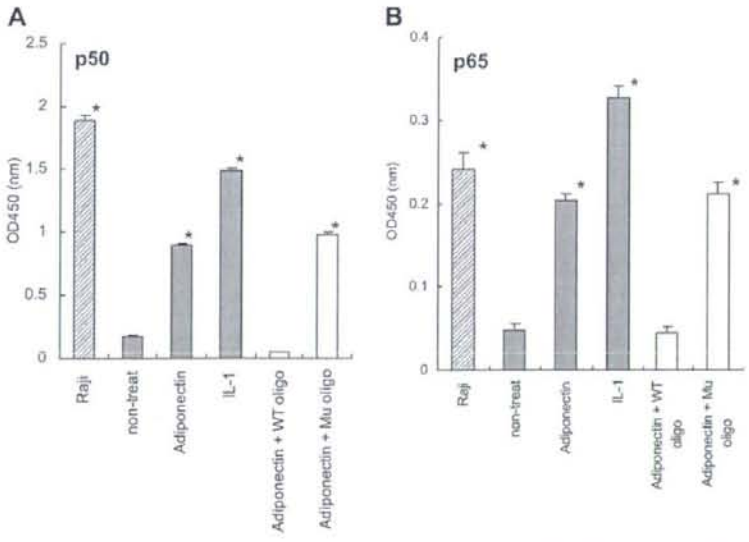

Fig. 4. Nuclear translocation of the activated NF-κB. Nuclear translocation of the activated (A) p50 and (B) p65 NF-κB were assessed by ELISA. RSF were treated with adiponectin, IL-1, or PBS for 3 h, and nuclear extracts were obtained. Raji nuclear extracts were used as positive controls. $^{*}P < 0.05$ vs. non-treat. WT oligo, wild-type oligonucleotides; Mu oligo, mutated oligonucleotides.

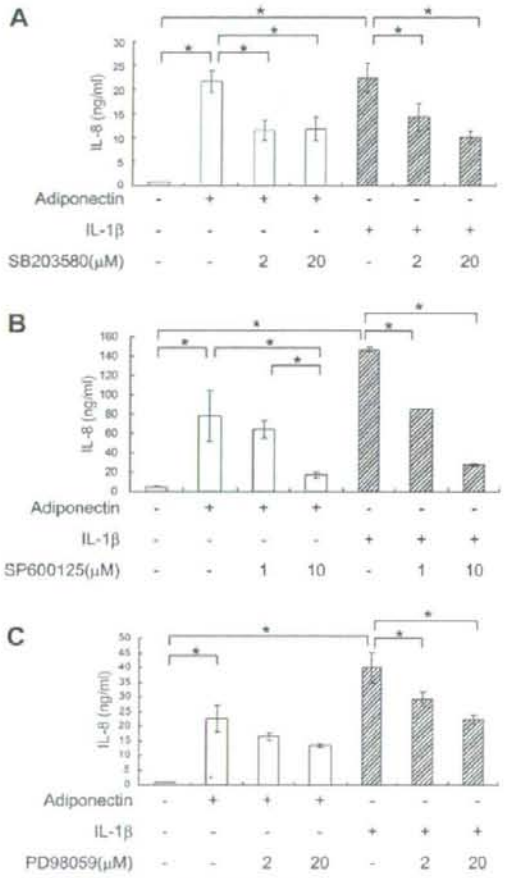

Fig. 5. Effect of MAPK inhibitors on adiponectin-stimulated IL-8 production. Cells were treated with (A) SB203580, (B) SP600125, or (C) PD98059 for 1 h prior to the treatment with 10 μg/ml of adiponectin, 1 ng/ml of IL-1, or PBS. IL-8 levels after 24 h were measured by ELISA. $^{*}P < 0.05$.

elevated in patients with RA [5,6,22], which indicates that adiponectin might have a role in the pathogenesis of RA.

Anti-TNF-α treatment has been reported to be associated with increase in adiponectin levels [23–25], however, this might merely reflect the fact that TNF-α negatively regulates adiponectin transcription [26]. In the present study, we examined the direct effect of adiponectin on RSF and found that adiponectin stimulated IL-8 production by RSF. This result supports the hypothesis that adiponectin alone has a proinflammatory effect on RSF. Some recent studies also have found that adiponectin promotes inflammation by inducing production of several proinflammatory cytokines [27,28].

In summary, we have shown that adiponectin significantly increases IL-8 production by RSF and supernatant of RSF treated with adiponectin induced PMN chemotaxis, suggesting that adiponectin might play a proinflammatory role in RA.

Acknowledgments

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REVIEW ARTICLE

Pro-apoptotic effect of nonsteroidal anti-inflammatory drugs on synovial fibroblasts

Natsuko Kusunoki · Ryuta Yamazaki · Shinichi Kawai

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Abstract Rheumatoid arthritis (RA) is a systemic inflammatory disease that mainly affects the articular synovial tissues. Although the etiology of RA has not yet been elucidated, physical and biochemical inhibition of synovial hyperplasia, which is the origin of articular destruction, may be an effective treatment for RA. Nonsteroidal anti-inflammatory drugs (NSAIDs) have long been used for the treatment of RA. The mechanism of action of NSAIDs generally involves the inhibition of cyclooxygenase (COX) at sites of inflammation. Thus, NSAIDs were not generally considered to have a so-called anti-rheumatic effect, including inhibition of progressive joint destruction and induction of remission. However, certain conventional NSAIDs and celecoxib, a selective COX-2 inhibitor, have been reported to inhibit synovial hyperplasia by inducing the apoptosis of human synovial fibroblasts. Therefore, it has been suggested that such NSAIDs may not only have an anti-inflammatory effect but also an anti-rheumatic effect. In this review, we summarize findings about the pro-apoptotic effect, in other words, anti-proliferative effect of NSAIDs on synovial fibroblasts from patients with RA.

Keywords Apoptosis · NSAIDs · Celecoxib · Synovial fibroblasts

N. Kusunoki · S. Kawai (✉)
Division of Rheumatology, Department of Internal Medicine, Toho University School of Medicine, 6-11-1 Omori-Nishi, Ota-ku, Tokyo 143-8541, Japan
e-mail: skawai@med.toho-u.ac.jp

R. Yamazaki
Yakult Central Institute for Microbiological Research, Tokyo, Japan

Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disease that mainly affects the articular synovial tissues. It is thought that an autoimmune response to the synovium is induced in RA patients when genetic factors are combined with various environmental ones, resulting in the occurrence of chronic inflammation. However, the etiology of RA has not yet been elucidated.

Guidelines for the management of RA by American College of Rheumatology have been published [1] and were also prepared in 2004 by a research group of the Japanese Ministry of Health, Labour and Welfare [2]. These guidelines cover a wide variety of drugs, including the new biological preparations, as well as glucocorticoids, disease-modifying anti-rheumatic drugs (DMARDs), and nonsteroidal anti-inflammatory drugs (NSAIDs) that have long been used for the treatment of rheumatic diseases [3]. Although the role of NSAIDs in the treatment of RA has been decreasing, these are still convenient drugs to employ for their anti-inflammatory and analgesic effects.

The mechanism of action of NSAIDs generally involves the inhibition of cyclooxygenase (COX) at sites of inflammation. As a result, these drugs exhibit a therapeutic effect by inhibiting the production of inflammatory mediators known as prostaglandins (PGs), including PGE_2 and PGI_2. In the treatment of RA, NSAIDs are used as symptomatic therapy with analgesic and anti-inflammatory effects mediated via the inhibition of COX. However, other mechanisms of action of NSAIDs except for COX inhibition have also been discussed [3]. For instance, certain NSAIDs have been reported to inhibit inflammation by suppression of nuclear factor (NF)-κB due to IκB-kinase β inhibition in mononuclear cells [4]. In this review, we summarize data about the

pro-apoptotic effect of NSAIDs on synovial fibroblasts of patients with RA.

Synovial proliferation and apoptosis

Healthy synovial tissue is essential for normal joint function. In RA, hyperplasia of the synovium and formation of granulation tissue (pannus) occur together with the infiltration of inflammatory cells, such as T cells and macrophages (Fig. 1). Activated pannus may eventually cause the destruction of bone and cartilage via the release of various mediators, including inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α [5]. Synovial hyperplasia may be due to an increase in the proliferation of cells composing the pannus. This has been suggested by detection of the increased expression of various markers of proliferation and growth factors, including platelet-derived growth factor, basic fibroblast growth factor, and transforming growth factor-β [6]. Another reason for the formation of pannus might be a decrease of apoptosis [7]. Physical and biochemical inhibition of synovial hyperplasia, which is the initial factor leading to articular destruction, may be effective treatments for RA [8].

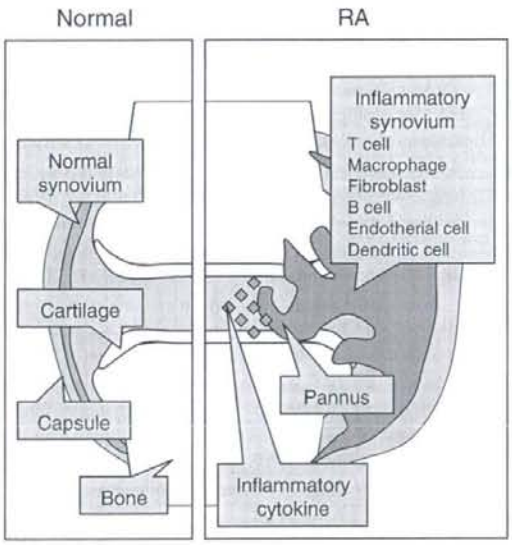

Fig. 1 Synovial proliferation in rheumatoid arthritis (RA). In articular joint of RA, hyperplasia of the synovium and formation of granulation tissue (pannus), which were composed of inflammatory cells, such as T cells, macrophages, and B cells, secrete inflammatory mediators to articular cavity

Several molecules, which induce apoptosis in vitro have been reported as preventing agents in in vivo experimental models of RA. Peroxisome proliferator-activated receptor γ (PPARγ) is an intranuclear transcription factor that promotes differentiation of adipocytes [9]. It also inhibits production of proinflammatory cytokines by macrophage [10]. On the other hand, Kawahito et al. [11] reported that articular destruction is significantly inhibited by administration of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), an endogenous ligand of PPARγ, by induction of apoptosis on synovial fibroblasts in experimental arthritis of rats. Administration of an anti-Fas antibody that also induces apoptosis inhibits arthritis in mice [12, 13]. Stimulation of RA synovial fibroblasts by macrophage inhibitory factor (MIF) reduces apoptosis, while MIF knock-out mice have less severe arthritis due to increased apoptosis in the synovium [14]. Although these findings suggest the possibility of achieving an anti-rheumatic effect by inhibiting hyperplasia of the synovial tissues, there have been no clinical studies of pro-apoptotic agents such as PPARγ ligands and anti-Fas antibodies targeting this endpoint.

Pro-apoptotic action of NSAIDs on synovial fibroblasts

Conventional NSAIDs

First, we found that some of the conventional NSAIDs (inhibitors both of COX-1 and COX-2), indomethacin, diclofenac, oxaprozin, and zaltoprofen, all inhibited the proliferation of RA synovial fibroblasts by the induction of apoptosis, which was confirmed by detection of DNA fragmentation [15] (Fig. 2). On the other hand, ketoprofen, acetaminophen, and NS-398, a selective COX-2 inhibitor, did not induce apoptosis of RA synovial fibroblasts. Since PPARγ ligands such as 15d-PGJ_2 and troglitazone have a pro-apoptotic effect on synovial fibroblasts [11], we hypothesized that the mechanism of these pro-apoptotic NSAIDs was mediated by PPARγ activation.

Some conventional NSAIDs, such as ibuprofen, indomethacin, flufenamic acid, and fenoprofen, are known to cause the transcriptional activation of PPARγ in C3H10T1/2 mouse fibroblast cells [16]. In addition, activation of PPARγ was obtained by ibuprofen, indomethacin, and fenoprofen in human monocytes [10]. Kawahito et al. [11] reported that 15d-PGJ_2, an endogenous ligand of PPARγ, ameliorate experimental arthritis of rats. Therefore, we investigated the effect of NSAIDs on PPARγ activity in RA synovial fibroblasts, one of the target cells in RA (Fig. 3). PPARγ activation was measured by luciferase reporter gene assay. Indomethacin, diclofenac, oxaprozin, and zaltoprofen induced PPARγ activation, while ketoprofen, acetaminophen, and NS-398,

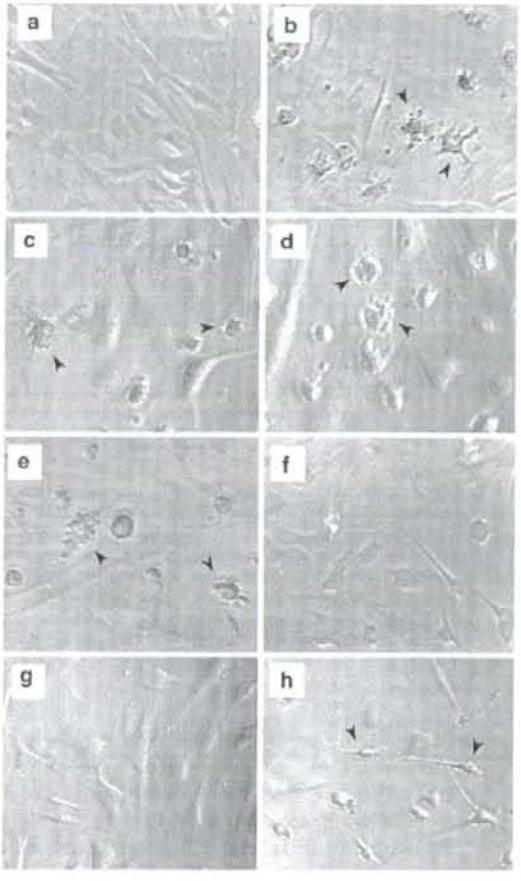

Fig. 2 Morphology of NSAID-treated rheumatoid synovial fibroblasts. Rheumatoid synovial cells were untreated (**a**) or treated with 300 μM indometacin (**b**), 100 μM diclofenac (**c**), 300 μM oxaprozin (**d**), 300 μM zaltoprofen (**e**), 300 μM ketoprofen (**f**), 300 μM acetaminophen (**g**), or 300 μM NS-398 (**h**) for 24 h. Cell morphology was observed with a light microscope. *Arrows* indicate representative morphological changes of synovial fibroblasts (×60). Reprinted from Yamazaki et al. [15], with kind permission from American Society for Pharmacology and Experimental Therapeutics

which do not induce apoptosis of RA synovial fibroblasts, did not promote PPARγ activation. Furthermore, the ability of NSAIDs and PPARγ ligands to stimulate the activation of PPARγ correlated with their ability to decrease cell viability and ability to induce DNA fragmentation in synovial fibroblasts.

Then we studied sodium salicylate and aspirin, which are the historical NSAIDs, to assess their apoptosis-inducing effect on RA synovial fibroblasts [17]. At relatively higher concentrations comparable to those that cause COX inhibition, sodium salicylate and aspirin induced

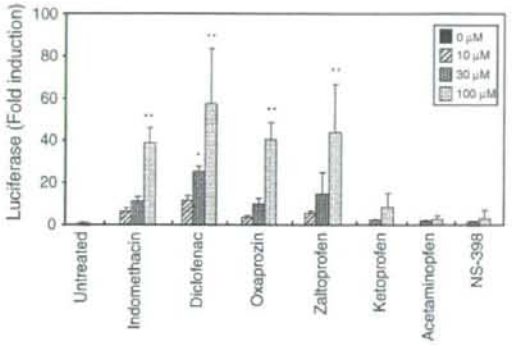

Fig. 3 Effect of NSAIDs on activation of peroxisome proliferator-activated receptor γ (PPARγ) in rheumatoid synovial cells. Rheumatoid synovial cells were cotransfected with a PPAR response element-driven luciferase reporter plasmid, PPARγ expression plasmid, and internal control plasmid. The transfected cells were treated with NSAIDs for 18 h. The fold-induction of luciferase activity is relative to untreated control cells. Data are the mean ± SD for triplicate cultures. Results are representative of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.001$ versus untreated control cells. Reprinted from Yamazaki et al. [15], with kind permission from American Society for Pharmacology and Experimental Therapeutics

apoptosis of these cells. It was also suggested that these drugs induced apoptosis via a mechanism independent of COX inhibition. Sodium salicylate and aspirin are known as potent inhibitors of the transcription factor NF-κB [18], and it has been shown that inhibition of the NF-κB pathway by pyrrolidinedithiocarbamate or N-acetylcysteine is linked to the induction of apoptosis in a variety of cells [19–21]. However, our additional study revealed that these inhibitors of NF-κB did not cause apoptosis of RA synovial fibroblasts [17]. Moreover, salicylates did not promote the activation of PPARγ in our experiments, so the mechanism of their pro-apoptotic effects on RA synovial fibroblasts is still unknown.

Celecoxib

COX-2 is an isozyme of COX that is markedly induced by inflammatory stimuli and is considered to be closely related to the process of inflammation. Celecoxib, which selectively inhibits COX-2, was developed by investigating the 3D structure of COX-2. In addition to inhibition of COX-2, celecoxib has been reported to inhibit the proliferation of various cancer cells, mainly by inducing apoptosis [22]. We investigated the effect of selective COX-2 inhibitors on apoptosis in RA synovial fibroblasts [23] (Fig. 4). Among six selective COX-2 inhibitors (celecoxib, etodolac, meloxicam, nimesulide, NS-398, and rofecoxib), only celecoxib induced the apoptosis of RA synovial fibroblasts, whereas the other COX-2 inhibitors did not. This indicated