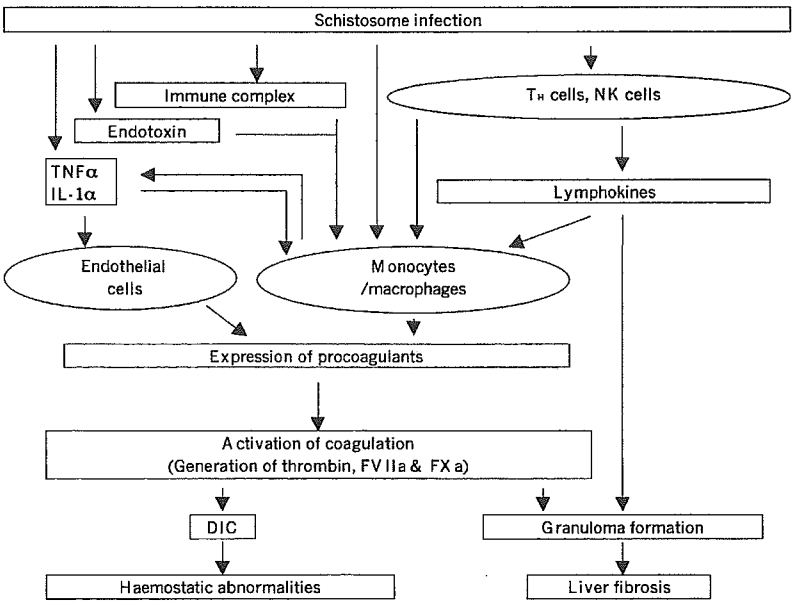

Fig. 1. The diagrammatic representation of a hypothetical mechanisms involved in the development of haemostatic abnormalities in hepatosplenic schistosomiasis.

Recent evidences suggest that serine proteases and their receptors might play a major role in the host defense mechanism at the interface between coagulation and inflammation [37]. Thrombin and some upstream proteases of the extrinsic coagulation cascade, such as FXa and FVIIa, are known to participate vascular permeability, cell migrations, endothelial functions and cellular events involved in inflammation by binding to protease activation receptors (PARs) or effector cell protease receptors (EPRs) on the surface of target cells [24,37–39]. Extravascular activation of coagulation proteases and fibrin deposition can occur in certain pathological situations that are associated with increased vascular permeability, inflammation and tissue fibrosis [40–44]. A number of nonvascular cells, including macrophages, fibroblasts and epithelial cells, express tissue factor [47,48] and are therefore capable of triggering the extrinsic coagulation cascade. Accordingly, it can be possible that the localized expression of procoagulants in the livers, spleens and intestines play a pivotal role in the activation of extrinsic coagulation cascade and in the pathogenesis of granuloma formation and tissue deterioration (inflammation and fibrosis) in *S. mansoni*-infected animals through the actions of activated serine proteases on mitogenesis [45,47], chemotaxis [46], cytokine expression [48,49] and cell functions of target cells (Fig. 1).

4. Inducing mechanism of procoagulants in schistosomiasis mansoni

The mechanism involved in procoagulant induction by schistosome infection remains obscure. The vascular endothelium participates in the control of haemostasis through mechanisms that involve the synthesis and release of coagulation and anticoagulation factors. When the endothelium is damaged, the balance between both factors is lost and lean toward procoagulant-dominant state [24,39]. Activation of coagulation proteases is one of the earliest events, and is usually initiated when tissue factor, expressed on the surface of vascular smooth muscle cells, activated endothelial cells and platelets, triggers the extrinsic coagulation cascade by binding to circulating FVII. In vitro

studies have shown that cultured endothelial cells can induce TF expression in response to many kinds of agents, such as pro-inflammatory cytokines [50–52], lymphokines [52], LPS [53], or PMA [53]. In the patients with advanced hepatosplenic schistosomiasis high levels of pro-inflammatory cytokines, IL-1α and TNFα, and LPS have been detected in the sera of these patients. It is, therefore, assumed that TF expression of endothelial cells by stimulation with these agents may participate in the activation of extrinsic coagulation cascade. However, no direct evidence indicating TF expression on the surface of vascular endothelium has been obtained in the patients with this endemic disease.

Tanabe and his colleagues have conducted a series of experiments to clarify the inducing mechanism of FX activating procoagulant, and have demonstrated that thioglycollate-induced peritoneal macrophages can produce high level of FX activating procoagulant in vitro when stimulated with splenic or hepatic lymphocytes that are prepared from *S. mansoni*-infected mice and sensitized/activated with SWAP antigen or T cell mitogens [55]. In this assay condition, optimal macrophage procoagulant production has been recorded when stimulated with splenic lymphocytes that are isolated from the infected mice and sensitized with SWAP antigen. Among lymphocyte sub-populations, $CD4^+$ helper T cells and NK cells, but not $CD8^+$ T cells and B cells, isolated from the spleens of *S. mansoni*-infected mice, have potently promoted the macrophage procoagulant expression. They have also demonstrated the augmentation of macrophage procoagulant activity by incubation with LPS, IFNγ and GM-CSF, but not with TNFα, IL-1α, IL-4, IL-6, and IL-12. A synergistic increase in macrophage procoagulant production has also been found in the incubation with any two agents among IFNγ, GM-CSF and LPS. Moreover, lymphocyte-mediated macrophage procoagulant production has been markedly inhibited by addition of anti-IFNγ monoclonal antibody.

These evidences indicate that macrophages can induce FX activating procoagulant through interaction with activated T helper cells or NK cells, lymphokines and/or LPS. Moreover, significantly higher activities of macrophage procoagulants have been induced by sensitized splenic lymphocytes with SWAP antigen as compared with those induced by LPS, pro-inflammatory cytokines or sensitized lymphocytes with LPS. This finding probably indicates that host immune mechanisms play a pivotal role in the induction of macrophage FX activating procoagulant in the tissues of *S. mansoni*-infected animals. Accordingly, it may be possible that host immune responses to schistosome eggs and adults participate in not only granuloma formation and tissue fibrosis, but also the development of haemostatic abnormalities in schistosomiasis mansoni (Fig. 1).

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Mucosal T Cells Expressing High Levels of IL-7 Receptor Are Potential Targets for Treatment of Chronic Colitis[1]

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The IL-7/IL-7R-dependent signaling pathway plays a crucial role in regulating the immune response in intestinal mucosa. Here we demonstrate the pivotal role of this pathway in the development and treatment of chronic colitis. T cells expressing high levels of IL-7R were substantially infiltrated in the chronic inflamed mucosa of TCR α-chain knockout mice and IL-7 transgenic mice. Transfer of mucosal T cells expressing high levels of IL-7R, but not T cells expressing low levels of IL-7R, from these mice into recombinase-activating gene-2$^{-/-}$ mice induced chronic colitis. Selective elimination of T cells expressing high levels of IL-7R by administrating small amounts of toxin-conjugated anti-IL-7R Ab completely ameliorated established, ongoing colitis. These findings provide evidence that therapeutic approaches targeting mucosal T cells expressing high levels of IL-7R are effective in the treatment of chronic intestinal inflammation and may be feasible for use in the therapy of human inflammatory bowel disease. *The Journal of Immunology,* **2003, 171: 1556–1563.**

Interleukin-7 is a nonredundant cytokine for the development of lymphocyte lineage cells (1). Abundant IL-7 expression has been demonstrated in the bone marrow stroma, thymus, spleen, and liver. However, a potential role for IL-7 in peripheral nonlymphoid tissues remained unclear. We have demonstrated that IL-7 is produced by intestinal epithelial cells and regulates mucosal lymphocytes (2). Following our study, other investigators demonstrated that IL-7 is crucial for the development of TCR-$\gamma\delta$ T cells and the formation of Peyer's patches in the intestinal mucosa of the mouse (3–6). TCR-$\gamma\delta$ intraepithelial lymphocytes (IELs)[3] are completely absent from IL-7R knockout mice, and their number is extremely decreased in IL-7 knockout mice. It has been demonstrated that IL-7 expression under intestinal fatty acid binding protein promoter in intestinal epithelial cells of IL-7 knockout mice was sufficient for the development of extrathymic TCR-$\gamma\delta$ IELs (7). The effect of IL-7 expression on the development of Peyer's patches further emphasized the critical role for IL-7 in the ontogeny of the mucosal immune system. Moreover, we have recently demonstrated the presence of a novel lymphoid tissue, designated cryptopatches, in murine intestinal mucosa, where clusters of IL-7R$^+$c-Kit$^+$ lympho-hemopoietic progenitor develop in an IL-7-dependent fashion (8, 9). All these findings indicated that intestinal epithelial cell-derived IL-7 plays a crucial role in the organization of mucosal lymphoid tissues and in the regulation of the normal immune response in the intestinal mucosa.

However, the role of IL-7/IL-7R-dependent signals during inflammation and in human intestinal disease is poorly understood. We tried to clarify the mechanism by which locally produced IL-7 regulates mucosal lymphocytes and the role of mucosal IL-7/IL-7R-dependent signals in chronic intestinal inflammation. We have demonstrated that mucosal IL-7/IL-7R-mediated immune responses are dysregulated at the chronic inflammation sites in human ulcerative colitis (our unpublished observation). We have also demonstrated that IL-7 transgenic (Tg) mice developed chronic colitis (10, 11). IL-7 Tg mice frequently showed rectal prolapse and remittent intestinal bleeding at 8–12 wk of age. Histopathological examination of the colonic tissues revealed the development of chronic colitis that mimicked histopathological characteristics of ulcerative colitis in humans. Of note, IL-7 protein accumulation was significantly decreased in the epithelial cells of the inflamed region of chronic colitis, both in human ulcerative colitis and in the colitis region of IL-7 Tg mice. IL-7R$^+$ cells were significantly infiltrated in the lamina propria at the colitis lesions. These findings in human ulcerative colitis and IL-7 Tg mice indicated that chronic inflammation of the colonic mucosa may be mediated by dysregulation or down-regulation of epithelial cell-derived IL-7 and by infiltration of IL-7R$^+$ T cells in both human and rodents.

Here we demonstrate the essential role of the mucosal IL-7R-dependent pathway in the development of chronic intestinal inflammation. We provide evidence that infiltration of highly IL-7R α-chain-expressing T cells is a common characteristic of chronic colitis, and new therapeutic approaches targeting mucosal T cells expressing high levels of IL-7R were successful in the treatment of chronic intestinal inflammation in mouse models without deletion

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[3] Abbreviations used in this paper: IEL, intraepithelial lymphocyte; DSS, dextran sulfate sodium; LPL, lamina propria lymphocyte; RAG, recombinase-activating gene; Tg, transgenic.

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of cells with low or intermediate expression of IL-7R. We have shown that the mucosal IL-7R-dependent signaling pathway in the colonic mucosa was dysregulated in human ulcerative colitis. Therefore, our results indicated the potential of targeting mucosal T cells expressing high levels of IL-7R for the therapy of human inflammatory bowel disease.

Materials and Methods

Mice

TCR α-chain knockout (TCR$\alpha^{-/-}$) mice with a background of C57BL/6 were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 recombinase-activating gene-2 (RAG-2)$^{-/-}$ mice were provided by Central Laboratories for Experimental Animals (Kawasaki, Japan). BALB/c and C.B.17-SCID mice were purchased from Japan Clea (Tokyo, Japan). IL-7 Tg mice carrying murine IL-7 cDNA under the control of the SRα promoter were established as previously described (10). In some experiments wild-type littermates were used as controls. Mice were maintained at the animal care facility of Tokyo Medical and Dental University. The review board of the university approved our experimental animal studies.

Induction of experimentally induced colitis

For chemically induced colitis, we used dextran sulfate sodium (DSS)-induced, oxazolone-induced, and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-immune colitis models (12). These models developed acute or short term colitis. CD4$^+$CD45RB^{high} T cell transfer model using C.B.17-SCID mice (13) was also used as a chronic colitis model.

Histological and immunohistochemical analyses

Colonic tissues were embedded at −80°C. Six-micrometer sections were placed on glass slides and stained with H&E. The severity of colitis was graded by histological findings. The disease score (0, normal; 1, mild; 2, moderate; 3, severe colitis) in stained sections were determined according to the degree of inflammation as previously described (14). For the staining of IL-7R$^+$ cells, sections were incubated with 10 μg/ml of anti-IL-7R mAb (A7R34, provided by Dr. T. Sudo, Toray Industries, Tokyo, Japan). Isotype-matched control Abs were used as controls. Staining of the sections was performed using the avidin-biotin complex method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Staining was then visualized using diaminobenzidine solution, and the slides were then counterstained with hematoxylin.

Preparation of colonic lamina propria lymphocytes (LPLs)

For the isolation of LPLs from colon, nonadherent mesenteric tissues were removed, and the entire colon was opened longitudinally, washed, and cut into pieces. The dissected mucosa was incubated with Ca^{2+}, Mg^{2+}-free HBSS containing 1 mM DTT (Life Technologies, Gaithersburg, MD) for 30 min at 37°C with gentle stirring, and this step was repeated. The residing tissue fragments were washed and incubated with collagenase A (Roche, Mannheim, Germany) for 2 h at 37°C. The supernatants were collected and washed, and the lymphocyte fraction was isolated on discontinuous Percoll gradients of 75 and 40%.

Flow cytometry

The profile of LPLs of the colon was analyzed by flow cytometry. To detect the expression of a variety of molecules on the cell surface, isolated cells were preincubated with a FcγR-blocking mAb (CD16/32; 2.4G2; BD PharMingen, San Diego, CA) for 20 min, followed by incubation with FITC-, PE-, or biotin-labeled specific Abs for 30 min on ice. The mAbs used were anti-CD4 mAb (anti-L3T4, RM4-5; BD PharMingen), TCR-β mAb (H57-597; BD PharMingen), and anti-IL-7R mAb. Biotinylated Abs were detected with PE-streptavidin (BD PharMingen). Standard two-color flow cytometric analysis was performed using FACSCalibur (BD Biosciences, Mountain View, CA) with CellQuest software. Staining with control irrelevant isotype-matched mAbs assessed background fluorescence. Dead cells were eliminated from analysis by propidium iodide staining.

Cytokine-specific ELISA

To measure cytokine production, isolated LPLs were cultured in medium supplemented with 1 μg/ml of soluble anti-CD28 mAb (37.51; BD PharMingen) in 96-well plates precoated with 10 μg/ml of anti-CD3 mAb (145-2C11; BD PharMingen) in PBS. Culture supernatants were collected, and the cytokine concentrations of IL-2, IL-4, IL-10 and IFN-γ were determined by specific ELISA (Endogen, Woburn, MA).

Cell transfer experiments

CD4$^+$ T cells were separated from colonic LPLs from colitis-free TCR$\alpha^{-/-}$ mice (4 wk of age), TCR$\alpha^{-/-}$ mice with chronic colitis (20 wk of age), and IL-7 Tg mice (60 wk of age) by MACS (Miltenyi Biotech, Bergisch Gladbach, Germany). We then sorted IL-7R$^+$ T cells using FACSVantage (BD Biosciences). The purity of IL-7R$^+$ T cells was confirmed by flow cytometry and was >97%. The purified IL-7R$^+$ T cells were i.p. transferred into RAG-2$^{-/-}$ mice (8–10 wk of age). Mice were sacrificed 4–6 wk after cell transfer for analysis. In some experiments purified CD4$^+$IL-7R$^+$ T cells from colitic mice were further separated into cells expressing high and low levels of IL-7R. In the histogram of IL-7R expression determined by flow cytometry, the top 30% of the cells were separated as IL-7R^{high} cells and the bottom 30% of the cells were separated as IL-7R^{low}. We then transferred 5×10^5 of either IL-7R^{high} or IL-7R^{low} mucosal T cells into RAG-2$^{-/-}$ mice.

Administration of saporin-conjugated anti-IL-7R mAb

We conjugated the plant toxin saporin to our anti-IL-7R mAb (A7R34) as a custom service at Advance Targeting System (Carlsbad, CA). We then treated chronic colitis in TCR$\alpha^{-/-}$ mice from 20–24 wk of age by i.p. injection of this toxin-conjugated anti-IL-7R mAb at a dose of 10 μg, once a week for 6 wk. As a control, the same amount of a mixture of free anti-IL-7R mAb (10 μg) and saporin (unconjugated) was injected. All mice were sacrificed on the day after the last treatment, and colitis lesions were evaluated.

Statistical analysis

The results were expressed as the mean ± SD. For statistical analysis, we used the program StatView for Macintosh (Abacus Concepts, Berkley, CA) and MS Office (Excel; Microsoft, Redmond, WA) and analyzed the data with Student's *t* test.

Results

Infiltration of T cells expressing high levels of IL-7R in the colonic mucosa of TCR$\alpha^{-/-}$ mice with chronic colitis

We assessed whether the mucosal IL-7R-dependent immune response is dysregulated in the development of acute and chronic intestinal inflammation. As acute or short term colitis models, we used chemically induced colitis models, including DSS-induced, oxazolone-induced, and TNBS-immune colitis models. As chronic or long term colitis models, CD4$^+$CD45RB^{high} T cell transfer into the SCID mice model and TCR$\alpha^{-/-}$ mice (14) were examined. No changes in IL-7/IL-7R-mediated immune responses were observed in the inflamed colonic mucosa of chemically induced acute colitis models (data not shown). TNBS-treated mice developed short term colitis, but IL-7R$^+$ T cells did not infiltrate the lamina propria of the inflamed mucosa. This was also observed in oxazolone-treated mice and the DSS-induced mouse colitis model. In contrast, IL-7R$^+$ T cells were remarkably infiltrated in lamina propria of chronically inflamed mucosa of CD4$^+$CD45RB^{high} T cell-transferred SCID mice. These results are consistent with the findings in IL-7 Tg mice and in human ulcerative colitis. The results suggested that mucosal IL-7R-dependent immune responses were involved in chronic intestinal inflammation, but not in acute inflammation.

To prove this possibility, we then focused on TCR$\alpha^{-/-}$ mice. Our TCR$\alpha^{-/-}$ mice spontaneously developed chronic colitis at 8–16 wk of age (14). We assessed the expression of IL-7R on infiltrated mucosal T cells before and after the development of colitis. In TCR$\alpha^{-/-}$ mice, previous reports showed that CD4$^+$TCRβ^{dim} T cells mediate chronic colitis (15, 16). Flow cytometric analysis of isolated LPLs demonstrated that CD4$^+$ TCRβ^{dim} T cells were demonstrable in the colonic mucosa of both colitis-free TCR$\alpha^{-/-}$ mice and mice with chronic colitis. However, IL-7R$^+$TCRβ^{dim} T cells were remarkably increased in colonic LPLs after the development of colitis (Fig. 1*A*). In the colonic mucosa of colitis-free TCR$\alpha^{-/-}$ mice, TCRβ^{dim} LPLs were demonstrable, but only half of these cells expressed IL-7R. In contrast, almost all TCRβ^{dim} cells in the colonic mucosa of TCR$\alpha^{-/-}$

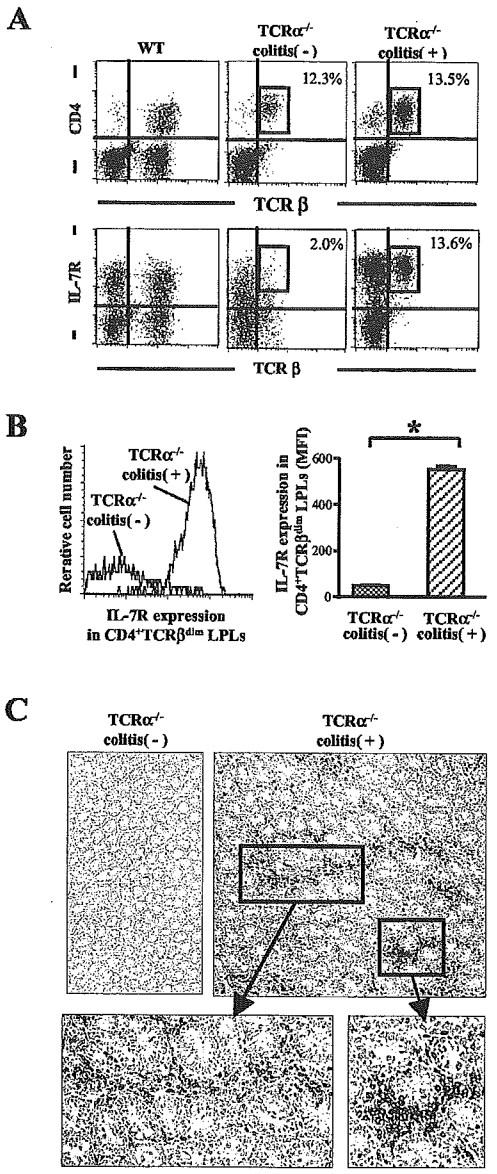

FIGURE 1. Infiltration of LPLs expressing IL-7R at a high level in the colonic mucosa of TCR$\alpha^{-/-}$ mice with chronic colitis. *A*, Flow cytometric analysis of isolated LPLs demonstrated that CD4$^+$TCRβ^{dim} T cells were demonstrable in the colonic mucosa of both colitis-free TCR$\alpha^{-/-}$ mice (n = 18) and TCR$\alpha^{-/-}$ mice with chronic colitis (n = 28), but not in that of wild-type littermates (WT; n = 32). However, IL-7R$^+$TCRβ^{dim} T cells were remarkably increased in colonic LPLs after the development of colitis. In the colonic mucosa of colitis-free TCR$\alpha^{-/-}$ mice, TCRβ^{dim} LPLs were demonstrable, but only half these cells expressed IL-7R. In contrast, almost all TCRβ^{dim} T cells in the colonic mucosa of TCR$\alpha^{-/-}$ mice with chronic colitis expressed IL-7R. *B*, The degree of IL-7R expression in CD4$^+$TCRβ^{dim} LPLs in the colonic mucosa of TCR$\alpha^{-/-}$ mice with chronic colitis (n = 28) was significantly (*, $p < 0.001$) higher than that in the colonic mucosa of colitis-free TCR$\alpha^{-/-}$ mice (n = 18; mean fluorescence intensity (MFI), 553 ± 21 and 41 ± 5, respectively). *C*, There were only a few IL-7R$^+$ cells in the colonic mucosa of colitis-free TCR$\alpha^{-/-}$ mice (n = 10) or wild-type mice (n = 21). In contrast, cells expressing IL-7R at a high level detected by immunohistochemistry were predominantly infiltrated in the lamina propria at chronic colitis lesions in TCR$\alpha^{-/-}$ mice (n = 20). These data are representative of four separate series of experiments.

mice with chronic colitis expressed IL-7R. Moreover, the degree of IL-7R expression in CD4$^+$TCRβ^{dim} LPLs of the colonic mucosa of TCR$\alpha^{-/-}$ mice with chronic colitis was significantly ($p <$ 0.001) higher compared with that in the colonic mucosa of colitis-free TCR$\alpha^{-/-}$ mice (Fig. 1*B*). The degrees of IL-7R expression determined by the mean fluorescence intensity in flow cytometric histogram were 553 ± 21 and 41 ± 5, respectively. To confirm that infiltrated CD4$^+$ T cells expressed IL-7R at high level in the chronic inflamed colonic mucosa, we then performed immunohistochemistry. There were only a few IL-7R$^+$ cells in the colonic mucosa of colitis-free TCR$\alpha^{-/-}$ mice or wild-type mice. In contrast, T cells expressing intense staining signals of IL-7R were predominantly infiltrated in the lamina propria at chronic colitis lesions in TCR$\alpha^{-/-}$ mice (Fig. 1*C*). Most of these infiltrated cells expressed CD4. In addition, IL-7R transcript was up-regulated in chronic colitis lesions, as determined by RT-PCR (data not shown). These results further reinforce the concept that IL-7R-mediated immune responses are dysregulated in chronic intestinal inflammation. The expansion of LPLs expressing high levels of IL-7R in the colonic mucosa was a characteristic feature of the chronic colitis lesion.

Transfer of IL-7R^{high} mucosal T cells induced chronic colitis in immunodeficient mice

To prove the hypothesis that T cells expressing high levels of IL-7R in the lamina propria of the colonic mucosa mediated the development of chronic intestinal inflammation, we first performed transfer experiments of mucosal T cells expressing IL-7R into immunodeficient mice. IL-7R$^+$CD4$^+$ T cells were isolated from the colonic mucosa of TCR$\alpha^{-/-}$ mice that developed chronic colitis or wild-type mice by sorting and then were transferred into syngeneic RAG-2$^{-/-}$ mice (Fig. 2*A*). Phenotypic analysis of IL-7R$^+$CD4$^+$ LPLs from TCR$\alpha^{-/-}$ mice with chronic colitis revealed that these isolated cells consisted of 80% TCRβ^{dim} and 20% $\gamma\delta$, 95% CD45RB^{low} and 5% CD45RB^{high}, and CD25$^-$ cells (Fig. 2*B*). Cytokine production of isolated IL-7R$^+$CD4$^+$ LPLs in TCR$\alpha^{-/-}$ mice with chronic colitis and that in wild-type mice after stimulation with anti-CD3 mAb and anti-CD28 mAb were quite different. Isolated IL-7R$^+$CD4$^+$ LPLs from TCR$\alpha^{-/-}$ mice with chronic colitis produced significantly higher amounts of IL-2 ($p < 0.001$), IFN-γ ($p < 0.05$), and IL-4 ($p < 0.01$) compared with those from wild-type mice (Fig. 2*C*). IL-10 production was decreased in isolated IL-7R$^+$CD4$^+$ LPLs from TCR$\alpha^{-/-}$ mice with chronic colitis ($p < 0.05$ compared with that from wild-type mice).

All recipient mice transferred 5×10^5 IL-7R$^+$CD4$^+$ LPLs from TCR$\alpha^{-/-}$ mice with chronic colitis developed severe colitis within 4–6 wk (Fig. 2*D*). Colonic inflammation occurred more rapidly and was more severe in the recipient mice compared with the original TCR$\alpha^{-/-}$ mice. Histopathological examination of the colonic tissues revealed that inflammatory cell infiltration and goblet cell depletion were prominent throughout the colon. Crypt abscesses, Paneth cell metaplasia, and infiltration of eosinophils were also observed in the inflammatory lesions. These features resembled the histopathological characteristics of the colitic lesion of TCR$\alpha^{-/-}$ mice and our IL-7 Tg mice (10, 14–16). In contrast, transfer of IL-7R$^+$CD4$^+$ LPLs from wild-type mice into RAG-2$^{-/-}$ mice did not produce colitis in the mice during the observation period. In addition, IL-7R$^-$CD4$^+$ LPLs from both mice never induced colitis. In the chronic colitis lesion of RAG-2$^{-/-}$ mice transferred IL-7R$^+$CD4$^+$ LPLs from TCR$\alpha^{-/-}$ mice with chronic colitis, IL-7R$^+$ T cells were remarkably infiltrated in the lamina propria (Fig. 2*D*). Flow cytometric analysis revealed that these infiltrated LPLs mainly consisted of TCRβ^{dim}, CD4$^+$, CD4$^+$CD45RB^{low}, and CD25$^-$ cells (Fig. 2*E*).

To eliminate the possibility that not mucosal T cells expressing high levels of IL-7R, but merely CD4$^+$TCRβ^{dim} T cells mediated

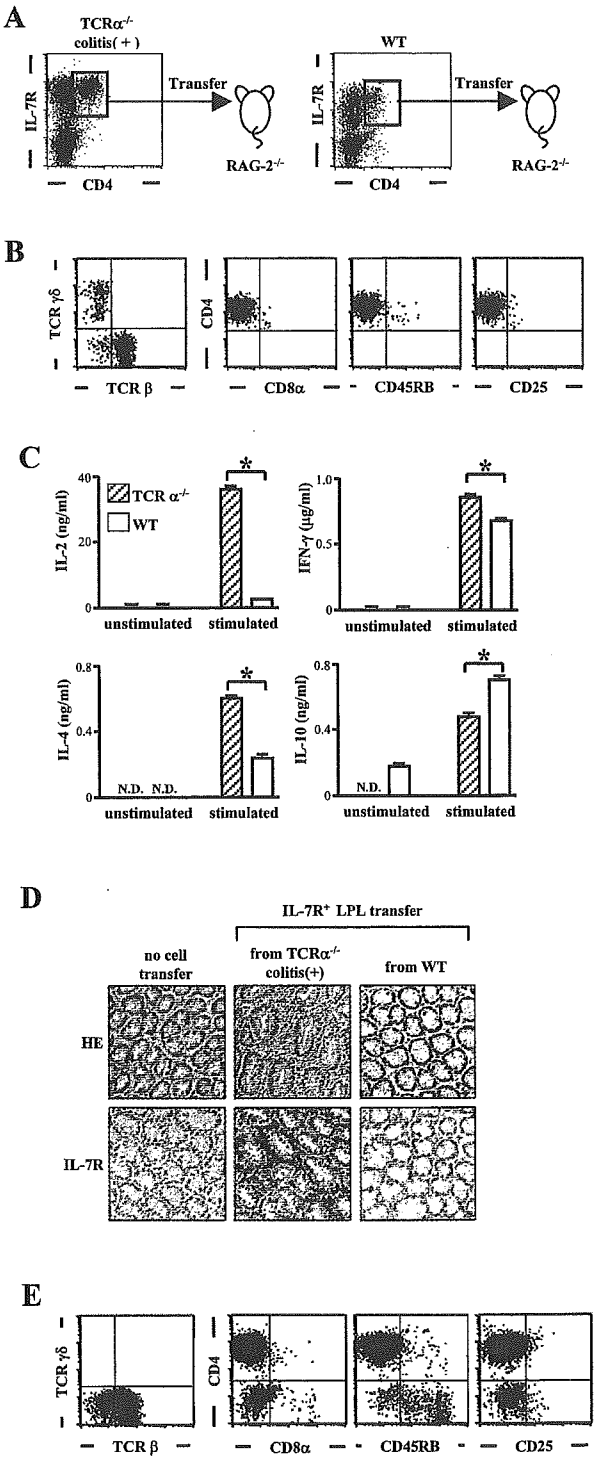

FIGURE 2. Transfer of IL-7R$^+$CD4$^+$ LPLs from TCR$\alpha^{-/-}$ mice into RAG-2$^{-/-}$ mice induced chronic colitis. *A*, IL-7R$^+$CD4$^+$ T cells were isolated from the colonic mucosa of both TCR$\alpha^{-/-}$ mice with chronic colitis and wild-type mice by sorting and then were transferred into syngeneic RAG-2$^{-/-}$ mice. *B*, Phenotypic analysis of IL-7R$^+$CD4$^+$ LPLs from TCR$\alpha^{-/-}$ mice with chronic colitis (n = 10) revealed that these isolated cells consisted of 80% TCRβ^{dim} and 20% $\gamma\delta$, 95% CD45RB^{low} and 5% CD45RB^{high}, and CD25$^-$ cells. *C*, Cytokine production of isolated IL-7R$^+$CD4$^+$ LPLs from TCR$\alpha^{-/-}$ mice with chronic colitis (n = 8) and wild-type mice (n = 10) after stimulation with anti-CD3 mAb and anti-CD28 mAb presented remarkable differences. Isolated IL-7R$^+$CD4$^+$ LPLs from TCR$\alpha^{-/-}$ mice with chronic colitis produced significantly higher amounts of IL-2 (*, $p < 0.001$), IFN-γ (*, $p < 0.05$), and IL-4 (*, $p < 0.01$) compared with those from wild-type mice. IL-10 production was decreased in isolated IL-7R$^+$CD4$^+$ LPLs from TCR$\alpha^{-/-}$ mice with chronic colitis (*, $p < 0.05$ compared with that from wild-type mice). *D*, All the recipient RAG-2$^{-/-}$ mice that were transferred 5 $\times$ 10^5 IL-7R$^+$CD4$^+$ LPLs/body from TCR$\alpha^{-/-}$ mice with chronic colitis developed severe colitis at 4–6 wk after transfer (n = 30). Colonic inflammation occurred at earlier periods and more severely in these mice than in the original TCR$\alpha^{-/-}$ mice. Histopathological examination of the colonic tissues revealed that inflammatory cell infiltration and goblet cell depletion were most prominent throughout the colon. In contrast, transfer of the same numbers of IL-7R$^+$CD4$^+$ LPLs from wild-type mice into RAG-2$^{-/-}$ mice did not produce colitis during the observation period (n = 10). In chronic colitis lesion of RAG-2$^{-/-}$ mice transferred CD4$^+$IL-7R$^+$ LPLs from TCR$\alpha^{-/-}$ mice with chronic colitis, IL-7R$^+$ T cells were remarkably infiltrated in the lamina propria. In the colonic mucosa of RAG-2$^{-/-}$ mice that were transferred IL-7R$^+$ LPLs from wild-type mice, IL-7R$^+$ T cells were not infiltrated in the lamina propria. These data are representative of four separate series of experiments. *E*, Flow cytometric analysis revealed that infiltrated IL-7R$^+$ LPLs mainly consisted of TCRβ^{dim}, CD4$^+$, CD4$^+$CD45RB^{low}, and CD25$^-$ cells in the chronic colitis lesion of RAG-2$^{-/-}$ mice that were transferred IL-7R$^+$CD4$^+$ LPLs from TCR$\alpha^{-/-}$ mice with chronic colitis (n = 8).

chronic inflammation, we performed another set of transfer experiments using purified and sorted IL-7R$^+$CD4$^+$ T cells from the mucosa of TCR$\alpha^{-/-}$ mice with chronic colitis. Purified IL-7R$^+$CD4$^+$ LPLs from TCR$\alpha^{-/-}$ mice with chronic colitis were further separated into cells expressing IL-7R at high and low levels. In the flow cytometric histogram of IL-7R expression, the highest 30% of IL-7R-expressing T cells were separated as IL-7R^{high} cells, and the lowest 30% of IL-7R expressing T cells were separated as IL-7R^{low} cells (Fig. 3*A*). We transferred 5 $\times$ 10^5 cells/body of those sorted cells into RAG-2$^{-/-}$ mice. All recipient RAG-2$^{-/-}$ mice that were transferred IL-7R^{high} LPLs developed severe colitis within 4–6 wk after transfer (Fig. 3*B*). In sharp contrast, none of mice that were transferred IL-7R^{low} LPLs developed colitis during the observation period. Assessment of the severity of colitis examined by histological scores showed a significant ($p < 0.001$) difference between mice transferred IL-7R^{high} LPLs and those given IL-7R^{low} LPLs (Fig. 3*C*). These results supported the concept that IL-7R^{high} T cells, not merely CD4$^+$TCRβ^{dim} T cells, in lamina propria of colonic mucosa induced chronic colitis.

To further eliminate the possibility that not IL-7R^{high} mucosal T cell, but TCRβ^{dim} T cells mediated chronic inflammation, we performed another transfer experiment using IL-7R$^+$ T cells from IL-7 Tg mice. In IL-7 Tg mice, purified IL-7R$^+$ T cells from colitic lesions of IL-7 Tg mice contained no TCRβ^{dim} T cells (Fig. 4*A*). We also sorted IL-7R^{high} and IL-7R^{low} CD4$^+$ T cells from the colonic mucosa of IL-7 Tg mice that developed chronic colitis and transferred these sorted cells as well as whole IL-7R$^+$ T cells into RAG-2$^{-/-}$ mice (Fig. 4*B*). All recipient mice that were transferred IL-7R^{high} mucosal T cells or whole IL-7R$^+$ T cells from IL-7 Tg mice developed severe colitis within 4 wk (Fig. 4*C*). In contrast, transfer of IL-7R^{low} T cells did not induce inflammation. Similar to the transfer experiments with IL-7R^{high} mucosal T cells from TCR$\alpha^{-/-}$ mice, colonic inflammation occurred more rapidly and was more severe in the recipient mice compared with the colitis in original IL-7 Tg mice. Histopathological examination of the colonic tissues of those transferred mice revealed that inflammatory cell infiltration and goblet cell depletion were most prominent throughout the colon. Crypt abscesses, Paneth cell metaplasia, and infiltration of eosinophils were also observed in the colitis lesions. Assessment of the severity of colitis by histological scores showed a significant ($p < 0.001$) difference between mice transferred IL-7R^{high} LPLs and those given IL-7R^{low} from the colitic lesion of IL-7 Tg mice (Fig. 4*D*). All these results indicated that mucosal T cells expressing high levels of IL-7R mediated the development of

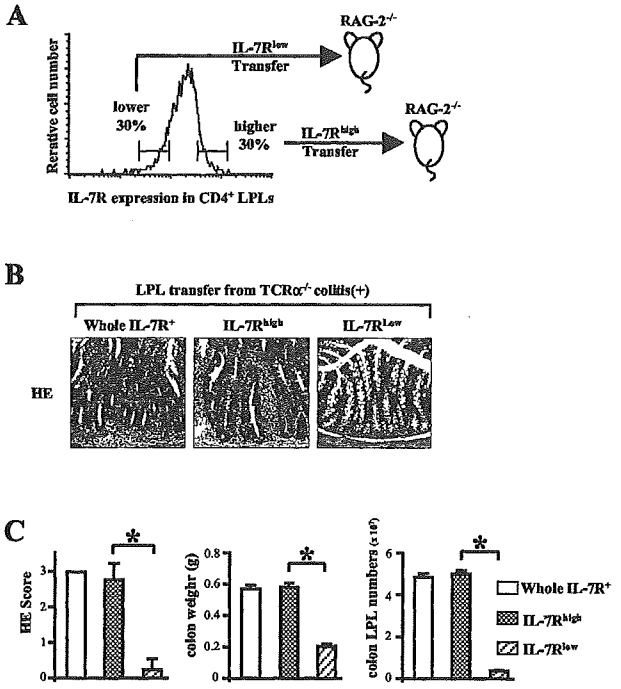

FIGURE 3. Transfer of LPLs expressing IL-7R at a high level from TCR$\alpha^{-/-}$ mice into RAG-2$^{-/-}$ mice induced chronic colitis. *A*, Purified IL-7R$^+$CD4$^+$ LPLs from TCR$\alpha^{-/-}$ mice with chronic colitis were further separated into cells expressing IL-7R at high and low levels. In the flow cytometric histogram of IL-7R expression, the top 30% of IL-7R-expressing T cells were separated as IL-7R^{high} cells, and the bottom 30% of IL-7R expressing T cells were separated as IL-7R^{low} cells. We transferred 5×10^5 cells/body of those sorted cells into RAG-2$^{-/-}$ mice. *B*, All recipient RAG-2$^{-/-}$ mice that were transferred IL-7R^{high} LPLs ($n = 12$) developed severe colitis within 4–6 wk. In sharp contrast, none of mice that were transferred IL-7R^{low} LPLs from same mice ($n = 9$) developed colitis during the observation period. *C*, Assessment of the severity of the colitis by histological scores showed a significant (*, $p < 0.001$) difference between the recipient mice transferred IL-7R^{high} LPLs and those given IL-7R^{low}. These data are representative of three separate series of experiments.

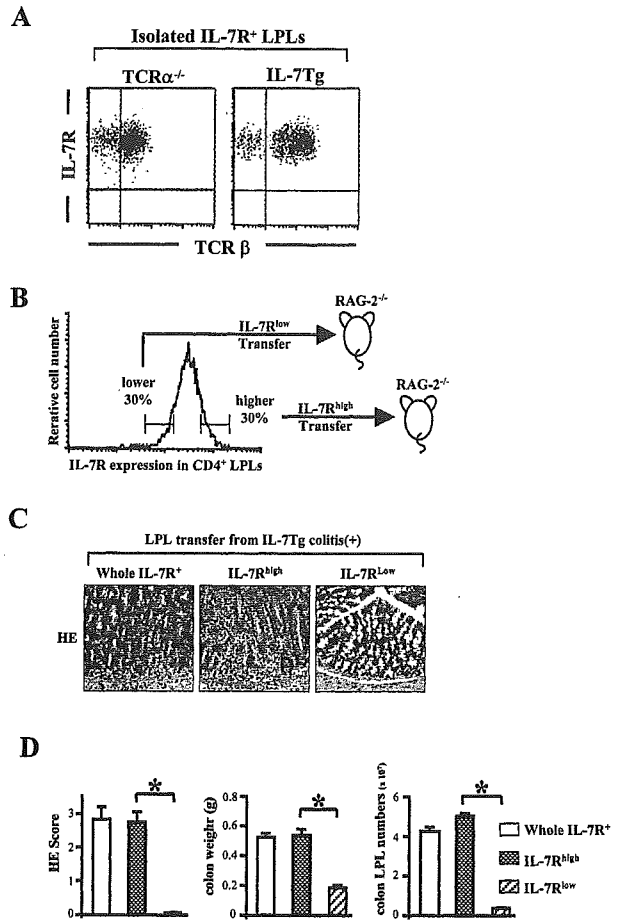

FIGURE 4. Transfer of sorted IL-7R^{high} LPLs from IL-7 Tg mice into RAG-2$^{-/-}$ mice induced chronic colitis. *A*, Purified CD4$^+$ LPLs from the colonic mucosa of IL-7 Tg mice with chronic colitis contained no TCRβ^{dim} T cells. *B*, We sorted IL-7R^{high} and IL-7R^{low} CD4$^+$ T cells from the colonic mucosa of IL-7 Tg mice with chronic colitis as described in Fig. 3 and transferred these sorted as well as unfractionated IL-7R$^+$ T cells into RAG-2$^{-/-}$ mice. *C*, All recipient mice that were transferred IL-7R^{high} mucosal T cells ($n = 12$) as well as unfractionated IL-7R$^+$ T cells from IL-7 Tg mice ($n = 8$) developed severe colitis within 4 wk after cell transfer. In contrast, transfer of IL-7R^{low} T cells did not induce inflammation ($n = 8$). Similar to TCR$\alpha^{-/-}$ IL-7R^{high} mucosal T cell transfer experiments, colonic inflammation occurred more rapidly and was more severe in the recipient mice compared with that in the original IL-7 Tg mice. Histopathological examination of the colonic tissues revealed that inflammatory cell infiltration and goblet cell depletion were prominent throughout the colon. *D*, Assessment of the severity of colitis by histological scores showed a significant (*, $p < 0.001$) difference between the recipient mice that were transferred either IL-7R^{high} LPLs or IL-7R^{low} and the colitic IL-7 Tg mice. These data are representative of three separate series of experiments.

chronic intestinal inflammation. Therefore, therapeutic approaches targeting IL-7R-mediated immune responses are thought to be feasible.

Successful treatment of established, ongoing chronic colitis in TCR$\alpha^{-/-}$ mice by selective elimination of LPLs expressing high levels of IL-7R using saporin-conjugated anti-IL-7R Ab

To correct the dysregulation of mucosal IL-7/IL-7R-mediated immune responses, we attempted to control mucosal T cells expressing high levels of IL-7R. On the basis of previous findings, we then tried to eliminate mucosal T cells expressing high levels of IL-7R by toxin-based destruction of IL-7R-expressing cells. A plant toxin, saporin, was conjugated to our anti-IL-7R mAb (17). In preliminary experiments we confirmed that a low concentration (10 μg/ml) of saporin-conjugated anti-IL-7R mAb inhibited the proliferation of IL-7-dependent cell line DW34 cells expressing IL-7R at a high level, but not of LPLs and spleen cells from wild-type mice. Using 10 μg/ml of this toxin-conjugated anti-IL-7R mAb, we found that this agent did not inhibit the in vitro proliferation of CD4$^+$ spleen cells that expressed IL-7R at low and intermediate levels from wild-type mice, but did inhibit that of IL-7R^{high} T cells from chronically inflamed mucosa of TCR$\alpha^{-/-}$ mice (data not shown). In preliminary experiments treatment of wild-type mice by i.p. injection of small amounts (10 μg/body) of saporin-conjugated anti-IL-7R mAb once a week for 6 wk did not cause any change in the total cell number and phenotypic change in spleen cells or LPLs (data not shown). Depletion of IL-7R$^+$ or CD4$^+$ cells was not observed even after six treatments with 10 μg/body of saporin-conjugated anti-IL-7R mAb. We then assessed the therapeutic effect of this saporin-conjugated anti-IL-7R mAb in the established, ongoing colitis of TCR$\alpha^{-/-}$ mice. Since all untreated TCR$\alpha^{-/-}$ mice developed colitis within 16 wk of age in our series, we started the treatment of established colitis in these mice at 20 wk of age. We treated chronic colitis in TCR$\alpha^{-/-}$ mice by i.p. injection of small amounts (10 μg/body) of saporin-conjugated anti-IL-7R mAb, once a week for 6 wk. Selective elimination of IL-7R^{high} LPLs by the administration of small amounts of

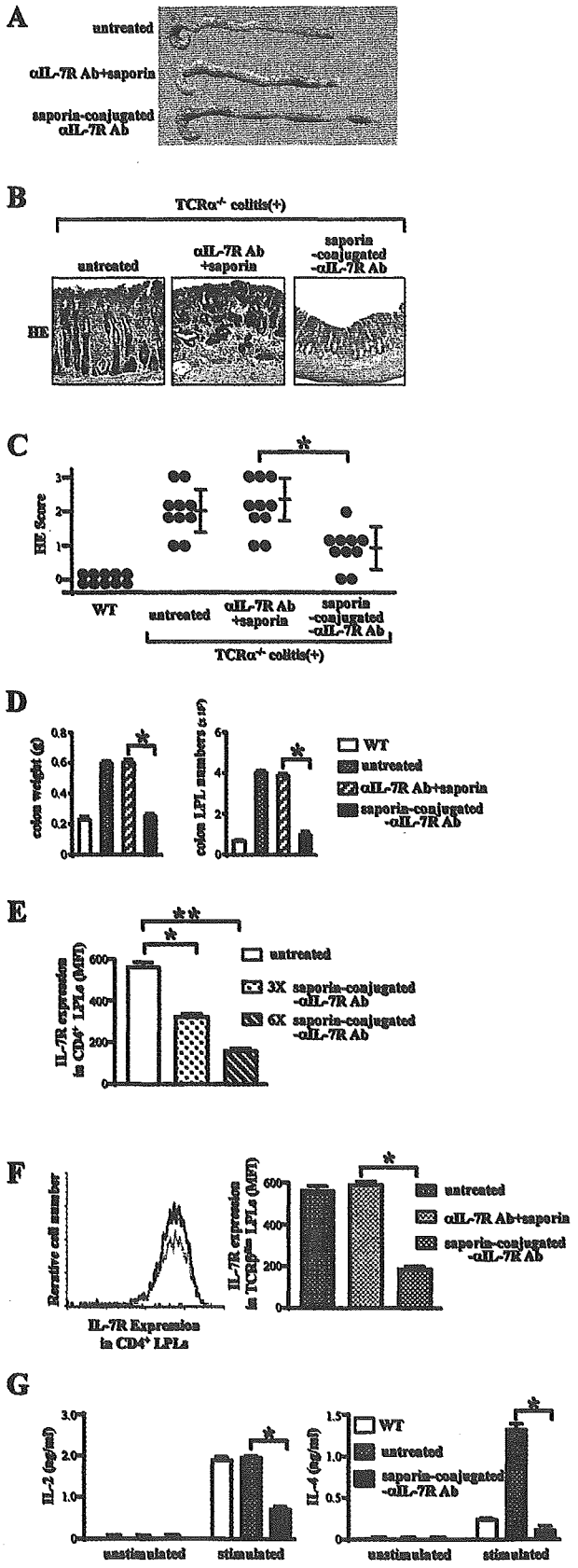

FIGURE 5. Successful treatment of established, ongoing chronic colitis in TCR$\alpha^{-/-}$ mice by the selective elimination of LPLs expressing IL-7R at a high level using saporin-conjugated anti-IL-7R Ab. All untreated TCR$\alpha^{-/-}$ mice developed colitis within 16 wk of age in our series; therefore, we started the treatment of this established, ongoing colitis in these mice at 20 wk of age. We treated chronic colitis in the TCR$\alpha^{-/-}$ mice by i.p. injection of small amounts (10 μg/body) of saporin-conjugated anti-IL-7R mAb, once a week for 6 wk. *A*, Gross inspection of the colon in TCR$\alpha^{-/-}$ mice revealed a complete reduction in the inflammatory activity after treatment with saporin-conjugated anti-IL-7R mAb (n = 16), comparable to the colitis observed in wild-type mice (n = 20). In contrast, TCR$\alpha^{-/-}$ mice treated with a mixture of free anti-IL-7R mAb and saporin (not conjugated) using the same protocol (n = 14) developed severe colitis, comparable to that in untreated TCR$\alpha^{-/-}$ mice. *B*, Histological analysis of saporin-conjugated anti-IL-7R mAb-treated mice showed the dramatic decrease in colonic inflammation, comparable to the histology of the colonic mucosa in wild-type mice. In contrast, TCR$\alpha^{-/-}$ mice treated with a mixture of free anti-IL-7R mAb and saporin developed severe colitis. *C*, The histological score was significantly (*, $p < 0.01$) decreased after saporin-conjugated anti-IL-7R mAb treatment (n = 10) compared with that after treatment with a mixture of free anti-IL-7R mAb and saporin (n = 10). *D*, The colonic wet weight and isolated cell number of colonic LPLs were significantly (*, $p < 0.001$) decreased in TCR$\alpha^{-/-}$ mice after saporin-conjugated anti-IL-7R mAb treatment (n = 16) compared with those after treatment with a mixture of free anti-IL-7R mAb and saporin (n = 14). The decrease reached the level in wild-type mice. *E*, Flow cytometric analysis of isolated LPLs revealed that the degree of IL-7R expression on CD4$^+$ LPLs from the colonic mucosa of TCR$\alpha^{-/-}$ mice with chronic colitis was gradually and significantly (*, $p < 0.01$; **, $p < 0.001$) decreased after saporin-conjugated anti-IL-7R mAb treatment. The decrease in IL-7R expression was more prominent after six treatments (6X; n = 16) than that after three treatments (3X; n = 5). *F*, Saporin-conjugated anti-IL-7R mAb treatment induced a significant (*, $p < 0.001$) decrease in IL-7R expression on CD4$^+$ LPLs in the colonic mucosa of TCR$\alpha^{-/-}$ mice with chronic colitis (n = 16) compared with that after treatment with a mixture of free anti-IL-7R mAb and saporin (n = 14). The degree of IL-7R expression in CD4$^+$ LPLs after treatment with a mixture of free anti-IL-7R mAb and saporin was comparable to that in untreated TCR$\alpha^{-/-}$ mice (n = 20). *G*, Saporin-conjugated anti-IL-7R mAb treatment induced a significant (*, $p < 0.001$) reduction in IL-2 production by CD4$^+$ mucosal T cells after stimulation with anti-CD3 and anti-CD28 mAbs. IL-4 production increased in CD4$^+$ LPLs from untreated TCR$\alpha^{-/-}$ mice after the same stimulation. This increase was significantly (*, $p < 0.001$) reduced in CD4$^+$ LPLs from saporin-conjugated anti-IL-7R mAb-treated TCR$\alpha^{-/-}$ mice, and production was below the level in wild-type mice. These data are representative of five separate series of experiments.

saporin-conjugated anti-IL-7R mAb completely ameliorated established colitis in TCR$\alpha^{-/-}$ mice. Gross inspection of the colon in TCR$\alpha^{-/-}$ mice revealed complete reduction of inflammatory activity after treatment with saporin-conjugated anti-IL-7R mAb, comparable with that in wild-type mice. In contrast, TCR$\alpha^{-/-}$ mice treated with a mixture of free anti-IL-7R mAb and saporin (not conjugated) using the same protocol developed severe colitis, comparable with that in untreated TCR$\alpha^{-/-}$ mice (Fig. 5*A*). Histological analysis of saporin-conjugated anti-IL-7R mAb-treated mice showed the dramatic decrease in colonic inflammation, comparable with the histology of the colonic mucosa in wild-type mice. In contrast, TCR$\alpha^{-/-}$ mice treated with a mixture of free anti-IL-7R mAb and saporin developed severe colitis (Fig. 5*B*). The histological score assessing the severity of inflammation was significantly ($p < 0.01$) decreased after saporin-conjugated anti-IL-7R mAb treatment compared with that after treatment with a mixture of free anti-IL-7R mAb and saporin (Fig. 5*C*). Colonic wet weight and isolated total cell number of colonic LPLs were significantly ($p < 0.001$) decreased in TCR$\alpha^{-/-}$ mice after saporin-conjugated anti-IL-7R mAb treatment compared with those after treatment with a mixture of free anti-IL-7R mAb and saporin (Fig. 5*D*). The decrease in colonic weight and total LPL number reached the level in wild-type mice. Flow cytometric analysis of isolated LPLs revealed that the degree of IL-7R expression on CD4$^+$ LPLs from the colonic mucosa of TCR$\alpha^{-/-}$ mice with chronic colitis was gradually and significantly ($p < 0.01$) decreased after saporin-conjugated anti-IL-7R mAb treatment (Fig. 5*E*). The decrease in

IL-7R expression was more prominent after six treatments than after three treatments. Subsequently, saporin-conjugated anti-IL-7R mAb treatment induced a significant ($p < 0.001$) decrease in IL-7R expression on $CD4^+$ LPLs in the colonic mucosa of $TCR\alpha^{-/-}$ mice with chronic colitis compared with that after treatment with a mixture of free anti-IL-7R mAb and saporin (Fig. 5*F*). The degree of IL-7R expression in $CD4^+$ LPLs after treatment with a mixture of free anti-IL-7R mAb and saporin was comparable to that in untreated $TCR\alpha^{-/-}$ mice. Saporin-conjugated anti-IL-7R mAb treatment induced a significant ($p < 0.001$) reduction in IL-2 production by $CD4^+$ LPLs after stimulation with anti-CD3 and anti-CD28 mAbs (Fig. 5*G*). IL-4 production was increased in $CD4^+$ LPLs from untreated $TCR\alpha^{-/-}$ mice after stimulation, and this increase was significantly ($p < 0.001$) reduced to the level in wild-type mice in saporin-conjugated anti-IL-7R mAb-treated $TCR\alpha^{-/-}$ mice. These results indicate that successful treatment of established, ongoing chronic colitis was achieved by the selective elimination of LPLs expressing IL-7R at a high level without deletion of cells expressing with low or intermediate levels of IL-7R.

Discussion

A potential role for IL-7/IL-7R-mediated immune responses in the intestinal inflammation was unclear. We have demonstrated that IL-7 Tg mice developed chronic colitis that mimicked histopathological characteristics of human ulcerative colitis. In the colonic mucosa of IL-7 Tg mice with chronic colitis, a decrease in IL-7 protein accumulation in the epithelial cells and marked infiltration of $IL\text{-}7R^+$ T cells in the lamina propria were demonstrable (10, 11). We also showed the decrease in IL-7 protein accumulation in the epithelial cells and infiltration of $IL\text{-}7R^+$ T cells in the lamina propria at the chronic inflammation sites of patients with ulcerative colitis (our unpublished observations). These findings suggest that dysregulation of the mucosal IL-7/IL-7R system is a common phenomenon in chronic inflammation sites of the colonic mucosa. In the present study we confirmed this concept in various chronic colitis mice models. Interestingly, dysregulation of the mucosal IL-7/IL-7R system is not apparent in the acute colitis mouse model. This was consistent with our previous findings. In fact, IL-7 Tg mice developed acute colitis with infiltrating neutrophils and T cells at 1–3 wk of age. In the acute colitis stage, IL-7 protein expression was significantly increased in the inflamed colonic mucosa. This contrasted with the decreased IL-7 expression in the chronic colitis stage, but was consistent with the findings that IL-7 expression was increased in colonic mucosa of patients with acute *Salmonella* enterocolitis and in severely inflamed mucosa in ulcerative colitis at acute exacerbation (our unpublished observations). The reason for substantial proliferation of mucosal $IL\text{-}7R^+$ T cells in chronic colitis, although IL-7 expression in the epithelial cells is decreased, remains unclear. Recent reports indicated that the serum concentration of IL-7 is strongly related to $CD4^+$ T cell lymphopenia, and IL-7 is produced by dendritic-like cells within peripheral lymphoid tissues in HIV disease (18). We are currently investigating the extraintestinal source of IL-7 in murine colitis models.

The most important finding of the present study was that attempts could be feasible in the treatment of chronic intestinal inflammation by the regulation of a mucosal IL-7R-dependent signaling pathway. Increasing evidence showed that chronic colitis in murine models has been successfully prevented by the administration of various mAbs or cytokines and by the establishment of double-knockout mice (19–21). However, few attempts resulted in adequate treatment of the established, ongoing colitis. We prevented chronic colitis in $TCR\alpha^{-/-}$ mice by establishment of $TCR\alpha^{-/-} \times IL\text{-}7R^{-/-}$ double-knockout mice (our unpublished observation). Moreover, we successfully treated established, ongoing colitis in $TCR\alpha^{-/-}$ mice with Ab-based therapy targeting the IL-7R-dependent signaling pathway. We treated chronic colitis in $TCR\alpha^{-/-}$ mice by infusion of free and toxin-conjugated anti-IL-7R mAb. Blockade of the IL-7R-dependent signaling pathway by anti-IL-7R mAb partially abrogated established colitis (our unpublished observation). Importantly, selective elimination of $IL\text{-}7R^{high}$ T cells by the administration of small amounts of saporin-conjugated anti-IL-7R mAb completely ameliorated ongoing colitis in $TCR\alpha^{-/-}$ mice. This saporin-conjugated anti-IL-7R mAb did not inhibit the in vitro proliferation of $CD4^+IL\text{-}7R^+$ spleen cells from normal mice, but did inhibit that of $IL\text{-}7R^{high}$ T cells from chronically inflamed mucosa of $TCR\alpha^{-/-}$ mice. This observation indicated that small amounts of saporin-conjugated anti-IL-7R mAb inhibited the proliferation or induced cell death of T cells expressing IL-7R at a high level that infiltrated in the chronic inflamed mucosa, but did not have an effect on cells expressing IL-7R at low or intermediate levels. This is explained by the fact that the amount of saporin binding to our anti-IL-7R mAb was extremely low. These results strongly confirmed that chronic inflammation in the colonic mucosa is mediated by the dysregulation of the mucosal IL-7/IL-7R signaling pathway. Treatment of wild-type mice with the same amount of saporin-conjugated anti-IL-7R mAb did not cause any change in the total cell number or a phenotypic change in spleen cells or LPLs. Depletion of $IL\text{-}7R^+$ or $CD4^+$ cells was not observed even after six treatments with 10 μg/body of saporin-conjugated anti-IL-7R mAb. Therefore, a therapy regulating LPLs expressing IL-7R at a high level is feasible in the treatment of chronic colitis without the deletion of cells expressing IL-7R at low or intermediate levels.

The mechanism by which the elimination of $IL\text{-}7R^{high}$ T cells leads to the amelioration of ongoing colitis should be defined. Our study showed that $IL\text{-}7R^{high}$ LPLs infiltrated in the lamina propria of colonic mucosa were activated and produced Th1- and Th2-type cytokines. Those activated $IL\text{-}7R^{high}$ mucosal T cells eventually produce inflammatory and proinflammatory cytokines that trigger a nonspecific inflammatory cascade. Therefore, it is not surprising that elimination of LPLs expressing IL-7R at a high level leads to the inhibition of ongoing colitis in chronic colitis mice.

Several clinical applications of IL-7 have been proposed, and many have been tested in mice (1, 22, 23). The major areas in which IL-7 appears to hold some clinical promise are antitumor activity, enhancement of lymphopoiesis, promotion of stem cell engraftment, and enhanced antimicrobial activity. However, only a few clinical applications have been conducted targeting IL-7R-bearing cells. Only a single trial was proposed for the therapy for hematologic malignancies by toxin-based destruction of IL-7R-bearing cells. Sweeney et al. (24) have constructed a recombinant fusion protein, DAB389 IL-7, composed of the catalytic and transmembrane domains of diphtheria toxin, fused to IL-7. They demonstrated that DAB389 IL-7 has a selective cytotoxic effect only on cells bearing the IL-7R, and that entry into target cells was mediated through the receptor. These results indicated that DAB389 IL-7 may be a novel reagent that possesses potential as a therapeutic agent against IL-7R-bearing cell-mediated disorders. They have also constructed an IL-2 version of the diphtheria toxin-based fusion toxin, DAB-IL-2, and applied this to the treatment of cutaneous T cell lymphoma (25). Preliminary studies using DAB-IL-2 for the treatment of severe rheumatoid arthritis and severe methotrexate-resistant psoriasis have also been reported (26, 27). Therefore, DAB389 IL-7 may be promising in the treatment of disorders other than hematological malignancies. All previous attempts were conducted to eliminate every IL-7R-bearing cell. The

present study was the first attempt to eliminate only T cells expressing IL-7R at a high level by toxin-based destruction of cells for the treatment of nonmalignant disorders, and we are currently investigating whether DAB389 IL-7 is effective in the treatment of chronic colitis.

This study provides a basis for practical application of therapy targeting T cells expressing IL-7R at a high level for the treatment of chronic intestinal inflammation in human inflammatory bowel disease. Human inflammatory bowel disease is thought to result from an inappropriate activation of the mucosal immune system driven by luminal flora (28). The activation of key immune cell populations is eventually accompanied by the production of a wide variety of nonspecific mediators of inflammation, including various other inflammatory and proinflammatory cytokines, chemokines, and growth factors. We suggest that T cells expressing IL-7R at a high level are one such key immune cell population. Therefore, therapeutic approaches targeting mucosal T cells expressing IL-7R at a high level may be feasible for the therapy of human inflammatory bowel disease.

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ANTIBODY ISOTYPE RESPONSES TO PARAMYOSIN, A VACCINE CANDIDATE FOR SCHISTOSOMIASIS, AND THEIR CORRELATIONS WITH RESISTANCE AND FIBROSIS IN PATIENTS WITH *SCHISTOSOMA JAPONICUM* IN LEYTE, THE PHILIPPINES

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Abstract. We examined whether antibody isotype responses to paramyosin (PM), a vaccine candidate for schistosomiasis, are associated with age-dependent resistance and pathology in liver fibrosis using human sera collected from139 individuals infected with *Schistosoma japonicum* in Leyte, the Philippines. Here, we report that IgA and IgG3 responses to PM

showed a positive correlation with age and that the epitopes responsible were localized predominantly within the N-terminal half of PM. In addition, the IgG3 response to PM was associated with serum level of procollagen-III-peptide (P-III-P), an indicator of progression of liver fibrosis. These results imply that IgG3 against PM may not only provoke age-dependent resistance to *S. japonicum* infection but also enhance granuloma formation. In contrast, levels of IgE to PM and to multiple PM fragments showed a negative correlation with P-III-P level. Thus, in contrast to IgG3, increases in PM-specific IgE may contribute to suppression of liver pathogenesis in schistosomiasis.

INTRODUCTION

A number of epidemiological studies have suggested the occurrence of age-dependent, acquired resistance to reinfection with *Schistosoma mansoni*,[1] *S. haematobium*,[2] and *S. japonicum*.[3,4] Age-dependent resistance is correlated with specific antibody isotype responses to the schistosome antigens, especially IgE responses to adult worm antigens (AWA).[5-8] In addition, IgA specific to parasite antigens was shown to be associated with resistance.[9,10] Thus, IgE and IgA may play a role in mediating protective immunity. On the other hand, IgM, IgG2, and IgG4 have been suggested to block killing by antibody-dependent cellular cytotoxicity (ADCC) of the parasites, acting as a "blocking antibody".[6,11] Nevertheless, the responses of various isotypes are controversial in their ability to provoke an immune effector mechanism.

Paramyosin (PM) is an invertebrate myofibrillar protein and is one of six candidate vaccines against schistosomiasis.[12] Vaccination with recombinant PM induced a significant reduction in worm recovery after challenge infection with *S. japonicum* in mice, pigs, and water buffaloes as experimental animal models.[13,14] Immunohistochemical and immunoelectron microscopic analyses indicated that PM is localized on the surface of cercaria, schistosomula, and adult *S. japonicum*, as well as in the muscle layers, suggesting that the surface PM could evoke ADCC.[15,16] Indeed, passive transfer of PM-specific monoclonal IgE in mice at an early stage of challenge infection resulted in reduction of worm burden.[17]

In humans, antibody isotype responses against *S. japonicum* PM have been reported. A study in the Philippines showed that IgA titers to AWA are correlated with age and the major target of IgA was PM, suggesting a role of anti-PM IgA in acquired immunity.[9] In contrast, antibody responses to PM were not correlated with susceptibility in another study in

China.[18] These discrepancies may have been due to geographical differences of both human and parasite populations and differences in the PM epitopes recognized by the specific antibody isotypes, some of which would be protective with others acting as blocking antibodies.

The major etiology of schistosomiasis is periportal fibrosis, which is a consequence of prolonged granuloma formation surrounding the deposited parasite eggs in the liver. From the practical view of vaccine development, schistosome vaccines are required not only to reduce worm burden but also to improve liver fibrosis. With regard to the roles of isotype responses to parasite antigens in fibrosis, analyses of IgE-deficient mice infected with either *S. japonicum* or *S. mansoni* indicated that IgE induces granuloma formation.[19,20] In addition, increased levels of IgG4 to parasite egg antigens in schistosomiasis mansoni patients with liver fibrosis have been demonstrated.[21] Interestingly, PM has been suggested to be involved in granuloma formation in mice infected with *S. mansoni*.[22,23] Thus, it is important to examine the role of isotype responses to PM in liver fibrosis for schistosome vaccine development.

The present study was performed to determine whether isotype responses against PM are involved in age-dependent resistance and liver fibrosis in human *S. japonicum* infection. We demonstrate that IgG3 and IgA against PM were correlated positively with aging, while the epitopes recognized varied among isotypes. In addition, we observed a positive correlation between IgG3 responses to PM and serum level of procollagen-III-peptide (P-III-P), an indicator of progression of liver fibrosis. Surprisingly, IgE specific to PM showed negative correlation negatively with P-III-P level, suggesting the involvement of IgE-PM interactions in liver fibrosis. The possibility of using PM as a schistosome vaccine is also discussed.

MATERIALS AND METHODS

Study design and evaluation of liver fibrosis. The study was carried out in villages on Leyte, the Philippines, in which schistosomiasis japonica is endemic. In this area, mass screening by stool examination followed by treatment with praziquantel against *S. japonicum* infection was conducted from 1981 to 1999, as part of the National Schistosomiasis Control Program of the Philippines. In July and August 1999, outpatients from Schistosomiasis Research Hospital, who were diagnosed as having *S. japonicum* infection by stool examination, were enrolled in the present study. The purpose and protocols of the study were explained to and written consent obtained from all the patients. All enrolled patients underwent serological and ultrasonographic (US) examinations. Patients positive for hepatitis B surface antigen on radioimmunoassay (RIA; cut off index > 2.0) and/or anti-HCV antibody (second generation) and alcoholics with bright liver on ultrasonography (US; alcohol consumption > 80 ml/d for 5 yrs or more) were excluded from the study.

A total of 139 patients were selected for further analyses. The degree of liver fibrosis was estimated by US and classified into four stages (Type 0: normal pattern; Type 1: linear pattern; Type 2: tubular pattern; Type 3: Network pattern) as described.[24,25] Serum levels of procollagen-III-peptide (P-III-P), type-IV collagen (Type-IV), and total bile acids (TBA) were measured in only 133 of the 139 blood specimens, the other six specimens having been lost during analyses. Eight control sera were collected from healthy adult volunteers who lived in Japan and were free from *S. japonicum* infection.

Schistosome antigens and recombinant paramyosins. The soluble adult worm antigens (AWA) were extracted from adult worms of the Yamanashi strain of *S. japonicum* by repeated freezing and thawing.[17] After centrifugation at 10,000 *g* for 30 min at 4°C, the supernatant

was recovered and cryopreserved at –80°C until use. Full-length *S. japonicum* PM and six truncated forms were designated as PM (1--866 amino acids), PM1 (1--164 amino acids), PM2 (157--302 amino acids), PM3 (297--451 amino acids), PM4 (447--602 amino acids), PM5 (597--742 amino acids), and PM6 (734--866 amino acids). The PM cDNAs were amplified by PCR using the *S. japonicum* PM cDNA[16] as a template and the following primers: PM, 5'-CGGGATCCCATATGATGAATCACGATACAG-3' and 5'-GCGGATCCTACATCATACTTGTTGC-3'; PM1, 5'-CGGGATCCCATATGATGAATCACGATACAG-3' and 5'-CGGGATCCCCGGGTACCGAGCTCGACTTTTGATTCAGCTGATTG-3': PM2, 5'-CGGGATCCATATGGTCGACGAATTCGCTAAGCAATCAGCTGAATC-3' and 5'-CGGGATCCCTCGAGAAGCTTGAATTCCTCTGTTTTACTC-3'; PM3, 5'-CGGGATCCGAGTAAAACAGAGGAATTC-3' and 5'-CGGGATCCCAGCTTCTAATTGAGACCA-3'; PM4, 5'-CGGGATCCGTCTCAATTAGAAGCTGAA-3' and 5'-CGGGATCCCAACTTCATTTGCCAGCTG-3'. The amplified cDNAs were digested with *Nde*I/*Bam*HI (PM, PM1, and PM2) or *Bam*HI (PM3 and PM4) and subcloned into the expression vector, pET14b. cDNA for PM5 was derived by *Pvu*II/*Eco*RI digestion of the PM cDNA, end-filled, and subcloned into the *Eco*RV site of the pT7Blue-T vector (Novagen Inc., Madison, WI). The *Nde*I/*Bam*HI fragment carrying the PM5 cDNA was subcloned into pET14b. The cDNA of PM6 was derived by *Pst*I/*Bam*HI digestion of the PM cDNA, end-filled, and subcloned into the end-filled *Xho*I site of pET14b. Transformation of bacteria, induction of expression, and purification of recombinant PMs with an N-terminal His6-tag were carried out as described.[13] PM was found to contain many degraded forms and was purified further using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electro-elution. Finally, the recombinant PMs were stored in a

solution of 10 mM sodium phosphate (pH 7.2), 1 M NaCl, and 4 M urea at –80°C until use.

Measurement of antibody titer specific to the schistosome antigens in human sera. Enzyme-linked immunosorbent assay (ELISA) was carried out using SWA, the full-length PM, and a series of recombinant PMs. Briefly, 96-well microtiter plates were coated with 5 μg/ml of SWAP or 1 μg/ml of PMs. After washing out the unbound antigens 3 times with PBS containing 0.05% Tween 20 (PBST), the plates were blocked with blocking solution containing 0.5% bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, MO) in PBST for 30 min at room temperature. The plates were further washed 3 times with PBST. The human sera were diluted 1:100 with blocking solution for detection of IgG, IgG1, IgG2, and IgG3, and to 1:50 for IgG4, IgE, and IgA, and then incubated overnight at 4°C. The plates were washed 5 times with PBST and incubated with HRP-conjugated anti-human IgG1, IgG2, IgG3, IgG4, IgA (anti-IgG: EY Laboratories, Inc., San Mateo, CA; IgG1, IgG2, IgG3, and IgG4: Southern Biotechnology Associates Inc., Birmingham, AL; IgA: ICN Biomedicals, Costa Mesa, CA), or biotinylated anti-human IgE (Vector Laboratories, Inc., Burlingame, CA) at 1:1000 for 1 hr at room temperature. The plates were then washed 5 times with PBST. For detection of IgE, the plates were further treated with a VECTASTAIN® Elite ABC standard kit under the conditions recommended by the manufacturer (Vector Laboratories). The assays were developed with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and the optical density was measured at 405 nm using a microplate reader (Model MTP-22, Corona Electrics Co., Ltd., Ibaraki, Japan) with reference at 492 nm.

Statistical analysis. StatView™ version 4.0 (Abacus Concepts Inc., Berkeley, CA) was used for all data analyses. Optical densities of serum concentrations of P-III-P and Type-IV and the antibody titers were log transformed before analyses. We used Student's *t*-test to evaluate differences between log-transformed means and Pearson's correlation coefficient to quantify associations between age, ultrasonographic evaluation, and log transformed data for P-III-P,