

IL-6–dependent spontaneous proliferation is required for the induction of colitogenic IL-17–producing CD8⁺ T cells

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We propose a novel role for interleukin (IL) 6 in inducing rapid spontaneous proliferation (SP) of naive CD8⁺ T cells, which is a crucial step in the differentiation of colitogenic CD8⁺ T cells. Homeostasis of T cells is regulated by two distinct modes of cell proliferation: major histocompatibility complex/antigen–driven rapid SP and IL-7/IL-15–dependent slow homeostatic proliferation. Using our novel model of CD8⁺ T cell–dependent colitis, we found that SP of naive CD8⁺ T cells is essential for inducing pathogenic cytokine–producing effector T cells. The rapid SP was predominantly induced in mesenteric lymph nodes (LNs) but not in peripheral LNs under the influence of intestinal flora and IL-6. Indeed, this SP was markedly inhibited by treatment with anti-IL-6 receptor monoclonal antibody (IL-6R mAb) or antibiotic–induced flora depletion, but not by anti-IL-7R mAb and/or in IL-15–deficient conditions. Concomitantly with the inhibition of SP, anti-IL-6R mAb significantly inhibited the induction of CD8⁺ T cell–dependent autoimmune colitis. Notably, the transfer of naive CD8⁺ T cells derived from IL-17^{–/–} mice did not induce autoimmune colitis. Thus, we conclude that IL-6 signaling is crucial for SP under lymphopenic conditions, which subsequently caused severe IL-17–producing CD8⁺ T cell–mediated autoimmune colitis. We suggest that anti-IL-6R mAb may become a promising strategy for the therapy of colitis.

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Homeostasis of T cells can be defined as a mechanism of restoration of immune balance, and maintenance of immune status after T cell depletion or expansion after immunological responses. Homeostasis of T cells is regulated by two distinct modes of cell proliferation: MHC/antigen–driven rapid spontaneous proliferation (SP) and IL-7/IL-15–dependent slow homeostatic proliferation (HP) (1–3). The definition of SP in CD4⁺ T cell homeostasis was first proposed by Min et al. (1). In this report, we initially propose a crucial role for IL-6 in SP of naive CD8⁺ T cells for inducing flora-specific colitogenic IL-17–producing CD8⁺ T (Tc17) cells.

It has been reported that the disruption of the Th1/Th2 immune balance causes various

immune diseases (4–8). However, recent discoveries suggested that a new subset of IL-17–producing CD4⁺ T (Th17) cells, rather than Th1/Th2 cells, might contribute to autoimmune diseases (9–11). Indeed, Th17 cells have been demonstrated to be crucial for inducing experimental autoimmune encephalomyelitis, rheumatoid arthritis, and inflammatory bowel disease (IBD) or colitis (12–16).

Idiopathic IBDs, including Crohn's disease and ulcerative colitis, are autoimmune diseases with symptoms including abdominal pain, weight loss, fever, and rectal bleeding. Now, these diseases are thought to be a cytokine-driven inflammation—triggered by excess IL-12/IL-23 and IFN- γ /IL-17 production for Crohn's disease, and excess type 2 cytokine production, chiefly IL-13, for ulcerative colitis—that affects the large intestine (2, 17).

The online version of this article contains supplemental material.

Although studies have addressed the involvement of CD4⁺ T cells in animal models of IBD (18), there is little information about the possible contribution of CD8⁺ T cells to the pathogenesis. In this report, we established a novel Tc17 cell-dependent IBD model and initially demonstrated that IL-6-dependent SP of naive CD8⁺ T cells was essential for the expansion of colitogenic Tc17 cells. Thus, we propose that the control of SP of naive CD8⁺ T cells by anti-IL-6R mAb will become a novel strategy for developing the therapy for autoimmune diseases.

RESULTS AND DISCUSSION

Adoptive transfer of naive CD8⁺ T cells causes severe autoimmune colitis

Severe colitis was induced by a single adoptive transfer of CD44^{low}CD62L⁺ naive CD8⁺ T cells into syngeneic RAG2^{-/-} mice. Transfer of naive CD8⁺ T cells or CD4⁺ T cells caused severe weight loss and thickening of the large intestinal wall compared with untreated control mice within 6 wk (Fig. 1, A and B). Hematoxylin-eosin (HE) staining of colon tissue sections revealed large numbers of infiltrating cells, in addition to hemorrhagic necrosis, neoangiogenesis, and depletion of goblet cells, in the CD8⁺ T cell-transferred mice, which were also assigned histological scores (Fig. 1 C). In addition, elevation of quantitative markers for enteropathy such as serum KC, serum amyloid A (SAA), and ICAM-1 (Fig. 1 D and not depicted) (19, 20) suggested that some inflammatory responses would be induced in the colon. However, it still remained unclear whether the CD8⁺ T cell-induced pathogenesis was specific to the large intestine. To make this point clear, we examined the induction of pathogenic inflammatory responses in various organs. The pathogenic responses were observed only in the large intestine, and not in other organs such as the liver, kidney, lung, heart, and brain (unpublished data). These pathological events strongly indicated that naive CD8⁺ T cells could be a critical population for the induction of autoimmune colitis under lymphopenic conditions.

A kinetics study revealed that IFN- γ , IL-17, and TNF- α were produced by CD8⁺ T cells, and these cytokine-producing cells preferentially expanded at the mesenteric LNs (mLNs) rather than the peripheral LNs (pLNs) within 1 wk of cell transfer (Fig. 1 E and not depicted). Concomitantly with the increase of cytokine-producing CD8⁺ T cells in mLNs, CD8⁺ T cells were infiltrated into colon tissues accompanied with inflammatory CD11b⁺ cells (Fig. 1 F). Almost all of the CD11b⁺ cells coexpressed F4/80 macrophage markers (unpublished data). These findings suggest that transferred naive CD8⁺ T cells would initially be stimulated in the mLNs and then migrate into the peripheral tissues at subsequent pathogenic stages. Thus, it appeared that the rapid expansion of naive CD8⁺ T cells to generate cytokine-producing effector CD8⁺ T cells would be closely related with the pathogenesis of autoimmune colitis.

Kinetics of SP essential for inducing pathogenic effector memory CD8⁺ T cells in mLNs

Recent studies have demonstrated that adoptive transfer of naive T cells into immunodeficient mice resulted in two distinct

types of proliferation (1). HP is driven by low-affinity self-MHC/peptide ligands and homeostatic cytokines such as IL-7 and IL-15 (21, 22), whereas SP exhibits rapid and massive proliferation induced by antigen recognition via the TCR (23). After transfer of CFSE-labeled naive CD8⁺ T cells into RAG2^{-/-} mice, dilution of the cellular fluorescence intensity was analyzed as an index of the cell proliferation at early stages. From a kinetics study (Fig. 2 A), we found that SP of naive CD8⁺ T cells occurred preferentially in the mLNs rather than in the pLNs within 5 d after the adoptive transfer. This rapid SP was detected neither in pLNs or mLNs at day 3. SP was not detectable in pLNs even at 7 d after CD8⁺ T cell transfer. At day 7, the numbers of CD8⁺ T cells collected from the mLNs were much larger than those from the pLNs (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20071133/DC1>). CD8⁺ T cells expanded by SP in mLNs but not pLNs, and rapidly acquired more characteristics of a “memory-type” phenotype, such as CD44 markers and a cytokine-producing ability, in the early stages (Fig. 2 B and not depicted). Therefore, these cytokine-producing effector memory cells might be critical for the initiation of colitis. SP in mLNs was not detectable in the following two cases: (a) when CD8⁺ T cells derived from OT-1-TCR transgenic mice were transferred into RAG2^{-/-} mice (Fig. 2 C), and (b) when RAG2^{-/-} mice were treated with antibiotics to deplete intestinal flora (Fig. 2 D). Moreover, it was demonstrated that SP was different from HP of CD8⁺ T cells judging from the evidence that naive CD8⁺ T cells transferred into OT-1/RAG2^{-/-} mice exhibited SP in mLNs, whereas HP was completely blocked in both pLNs and mLNs by endogenously existing OT-1 T cells (Fig. 2 E). Thus, these data clarified that SP, which was triggered with antibiotic-sensitive flora in mLNs via TCR, was distinct from HP induced under the influence of IL-7 and/or IL-15. Alternatively, SP was not an HP-related end stage of T cell proliferation. A previous report had indicated that adoptive transfer of naive CD8⁺ T cells into lymphopenic hosts elicited no pathology, including colitis (24). This might be because of the strain differences between C57BL/6 and BALB/c mice, which have genetically different predispositions in controlling their susceptibility to immune diseases (25), including our established colitis model (unpublished data).

Critical role of IL-6 for SP while inducing pathogenic effector T cells

In contrast to HP (26), it remains unclear which factors are involved in SP of naive CD8⁺ T cells in addition to antigen stimulation. We found that mRNA expression levels of IL-6 and TGF- β were significantly higher in the mLNs compared with pLNs (Fig. 3 A). To evaluate the role for IL-6 and TGF- β , as well as the homeostatic cytokines IL-7 and IL-15, in SP and HP, we performed the following experiments: (a) CD8⁺ T cells were transferred into RAG2^{-/-} mice treated with anti-IL-7R mAb; (b) IL-15^{-/-} mouse-derived CD8⁺ T cells were transferred into IL-15/RAG2 double-knockout mice; (c) IL-15^{-/-} CD8⁺ T cells were transferred into IL-15/RAG2 double-knockout mice treated with anti-IL-7R mAb;

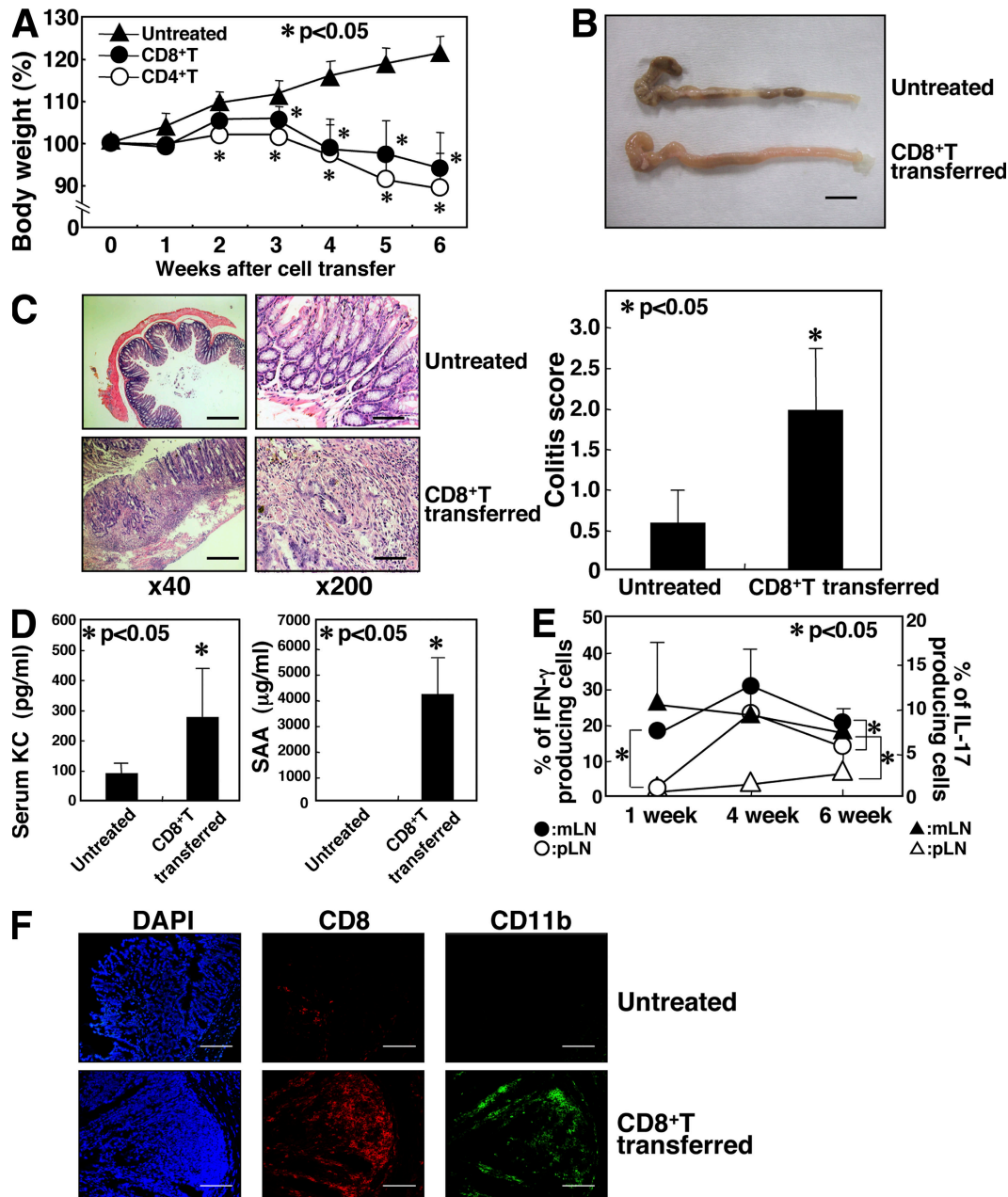


Figure 1. Adoptive transfer of naive CD8⁺ T cells into RAG2^{-/-} mice causes severe autoimmune colitis. 5×10^5 CD44^{low}CD62L⁺ naive CD8⁺ T cells from C57BL/6 mice were intravenously injected into RAG2^{-/-} mice. (A) Body weights of untreated ($n = 5$), CD8⁺ T cell-transferred ($n = 5$), or CD4⁺ T cell-transferred ($n = 5$) mice were monitored for 6 wk. Percentages of the resultant body weights against preinjection body weights were calculated every week. The means and SDs are indicated. (B) Colon tissues were obtained from control or CD8⁺ T cell-transferred mice after 9 wk. Morphology of the representative colon tissues is shown. Bar, 1 cm. (C) HE staining was performed on sections of the colon tissues. Histological pictures of the representative tissues are shown at two different magnifications. Means and SDs of the colitis score are indicated in the bar graph. Bars: (left) 1 mm; (right) 200 μm. (D) Serum KC and SAA levels of untreated or CD8⁺ T cell-transferred mice were measured by ELISA. The means and SDs are indicated. (E) Cytokine (IFN-γ and IL-17) production by CD8⁺ T cells from pLNs and mLNs of the adoptively transferred mice 1, 4, and 6 wk after cell transfer. IFN-γ-producing cells in mLNs or pLNs, and IL-17-producing cells in mLNs or pLNs are indicated. Means and SDs of the percentages of cytokine producing cells are shown. (F) Colon sections from untreated or CD8⁺ T cell-transferred mice were stained with DAPI, anti-CD8 mAb, and anti-CD11b mAb. Bars, 200 μm.

(d) CD8⁺ T cells were transferred into RAG2^{-/-} mice treated with anti-IL-6R mAb; and (e) CD8⁺ T cells were transferred into RAG2^{-/-} mice treated with anti-TGF-β mAb. Neither IL-7, IL-15, nor TGF-β contributed to SP, whereas

IL-7 and/or IL-15 were involved in HP (Fig. 3 B). It was also demonstrated that TGF-β had no effect on HP (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20071133/DC1>). Unexpectedly, however, SP was completely blocked by the

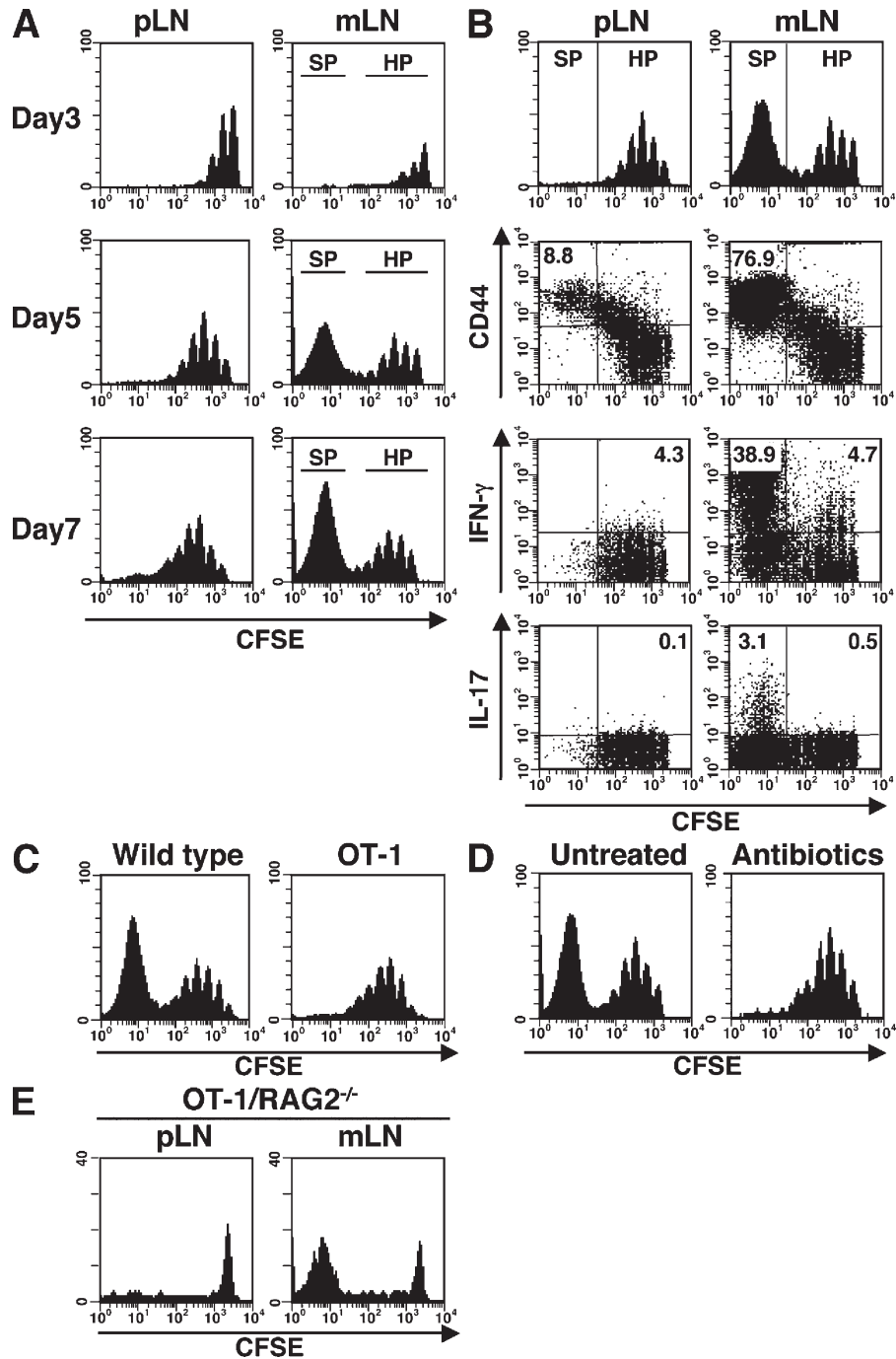


Figure 2. Kinetics of SP essential for inducing pathogenic effector memory T cells in mLNs. 5×10^5 cells naive CD8⁺ T cells from C57BL/6 mice were labeled with CFSE and intravenously injected into RAG2^{-/-} mice. (A) Proliferation of CD8⁺ T cells was monitored by flow cytometry 3, 5, and 7 d after the injection. The representative FACS profiles are indicated in the figure. (B) CD44 expression and cytokine production levels of CD8⁺ T cells from pLNs and mLNs in the adoptively transferred mice were examined by staining with mAbs against CD44, IFN- γ , or IL-17 at day 5. The representative FACS profiles are shown. Percentages are indicated. (C) CFSE-labeled CD8⁺ T cells from OT-1-TCR transgenic mice were intravenously injected into RAG2^{-/-} mice. Proliferation of the CD8⁺ T cells in mLNs was analyzed at day 7. The representative FACS profiles are indicated. (D) CFSE-labeled CD8⁺ T cells from C57BL/6 mice were intravenously injected into untreated and antibiotic-treated RAG2^{-/-} mice. Proliferation of the CD8⁺ T cells in the mLNs of the untreated and antibiotic-treated mice was analyzed by FACS. (E) CFSE-labeled CD8⁺ T cells from C57BL/6-background Ly5.1 mice were intravenously injected into RAG2^{-/-} or OT-1/RAG2^{-/-} mice. Proliferation of the CD8⁺ T cells in the pLNs or mLNs of OT-1/RAG2^{-/-} mice was analyzed by FACS.

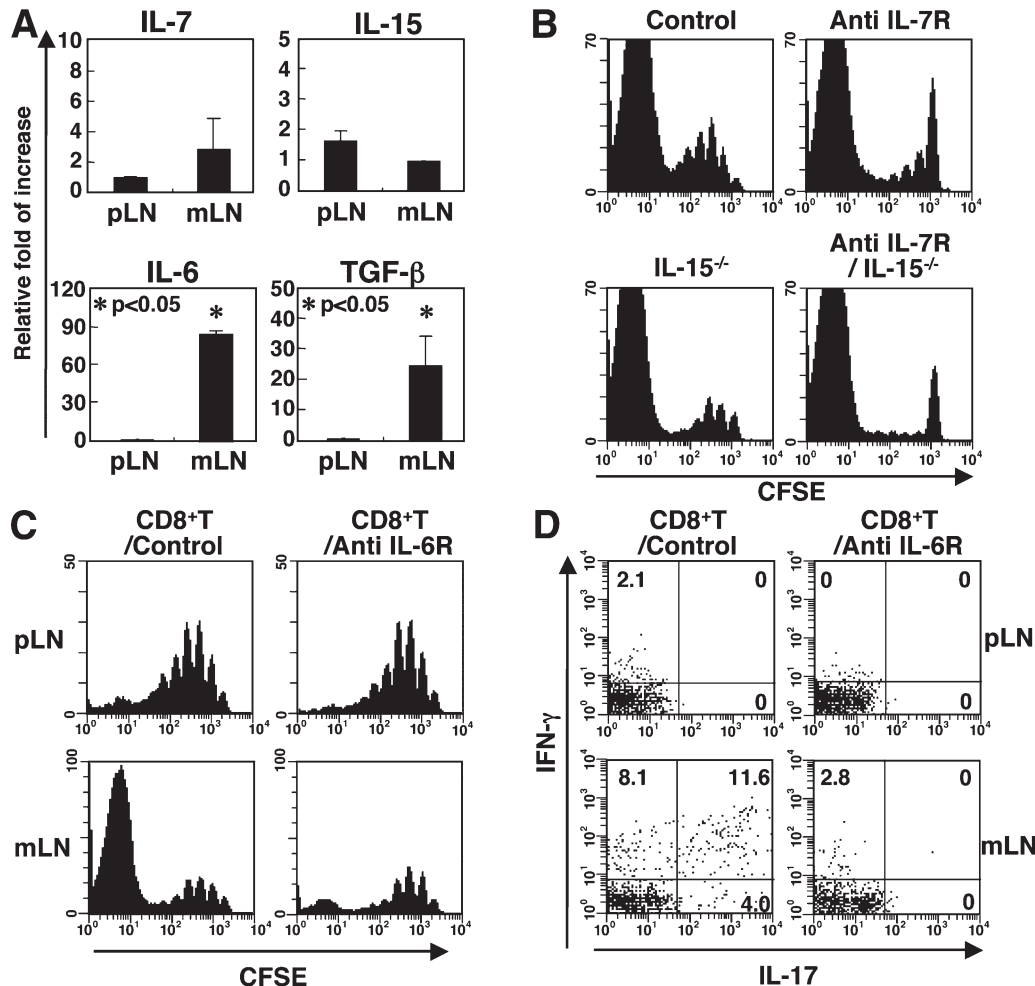


Figure 3. Critical role of IL-6 for SP while inducing pathogenic effector T cells. 5×10^5 naive CD8⁺ T cells from C57BL/6 mice were labeled with CFSE and intravenously injected into RAG2^{-/-} mice. (A) IL-7, IL-15, IL-6, and TGF-β mRNA expression levels in pLNs or mLNs of untreated RAG2^{-/-} mice were evaluated by real-time PCR. The means and SDs ($n = 4$ per group) are indicated. (B) SP in mLNs of RAG2^{-/-} mice treated with anti-IL-7R mAb and RAG2/IL-15 double-knockout mice were observed 7 d after the injection. The representative FACS profiles are indicated. (C) Proliferation of CD8⁺ T cells in the pLNs and mLNs of RAG2^{-/-} mice treated with anti-IL-6R mAb was observed, and the representative FACS profiles are indicated. (D) Cytokine (IFN-γ and IL-17) production by CD8⁺ T cells in the pLNs and mLNs of anti-IL-6R mAb-treated RAG2^{-/-} mice. The representative FACS profiles are shown. Percentages are indicated.

administration of anti-IL-6R mAb (Fig. 3 C). In contrast, HP was not affected by the anti-IL-6R mAb at all (Fig. 3 C). As shown in Fig. 3 D, both IFN-γ and IL-17A production were markedly inhibited by treatment with anti-IL-6R mAb (Fig. 3 D). From these findings, IL-6 signaling, but not IL-7, IL-15, or TGF-β, was considered to be critical for SP in the present model. It has been demonstrated that IL-6 was closely related to the survival of T cells (27, 28). However, there was no significant difference in CD8⁺ T cell apoptosis between control and anti-IL-6R mAb-treated mice (Fig. S3).

As described in Fig. 2, we clearly demonstrated that HP and SP of CD8⁺ T cells were totally distinct in T cell homeostasis, and that SP was not the end stage of HP. We also demonstrated that SP appeared to be triggered with antibiotic-sensitive flora antigen via TCR, consistent with a previous report (29). Therefore, at present, we consider that anti-IL-6R mAb does

not affect the apoptosis of HP-related T cells but instead inhibits the SP-related generation of flora-triggered pathogenic CD8⁺ T cells, which is accelerated in an IL-6-dependent manner. Indeed, IL-6 was demonstrated to promote TCR-mediated T cell proliferation directly (unpublished data).

Anti-IL-6R mAb inhibits CD8⁺ T cell-dependent autoimmune colitis

In vivo injection of anti-IL-6R mAb markedly suppressed the weight loss and abnormal thickening of the large intestine wall, which are associated with CD8⁺ T cell-induced colitis (Fig. 4 A). HE staining revealed that blockade of IL-6 signaling significantly suppressed cell infiltration into the large intestine (Fig. 4, B and C). Although the mRNA levels of various inflammatory cytokines, including TNF-α, IL-1β, IFN-γ, and IL-17, were up-regulated in the tissues from the colitis

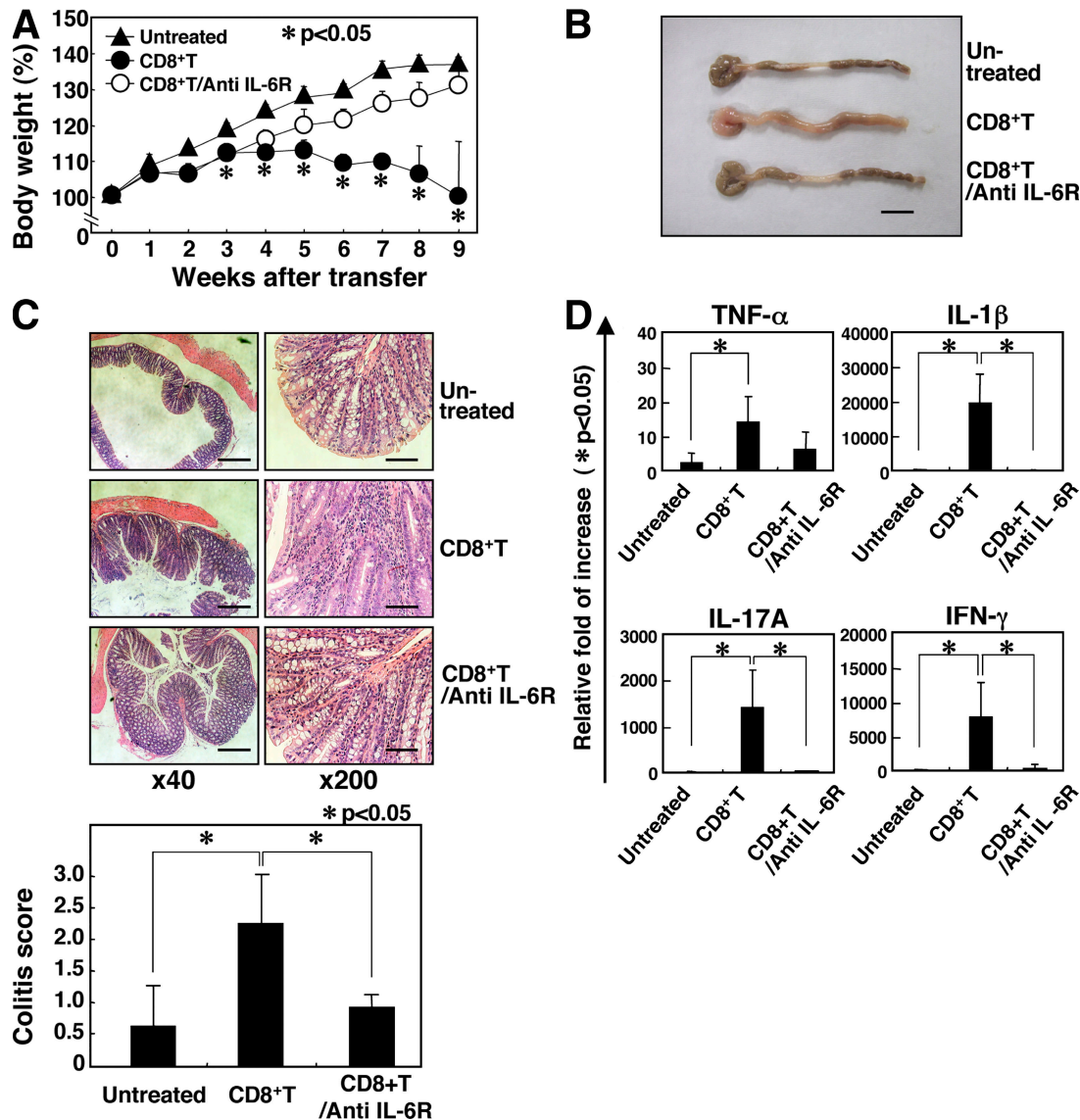


Figure 4. Anti-IL-6R mAb inhibits CD8⁺ T cell-dependent autoimmune colitis. 5×10^5 naive CD8⁺ T cells from C57BL/6 mice were intravenously injected into RAG2^{-/-} mice. (A) Body weight of untreated ($n = 4$), naive CD8⁺ T cell-transferred ($n = 3$), or naive CD8⁺ T cell-transferred/anti-IL-6R mAb-treated ($n = 3$) mice were monitored for 9 wk. Body weight percentages against the respective preinjection values were calculated every week. The means and SDs are indicated. (B) Colon tissues were obtained from untreated, control, or anti-IL-6R mAb-treated mice 9 wk after the cell transfer. Morphology of the representative colon tissues is shown. Bar, 1 cm. (C) HE staining was performed on the colon tissues from the untreated, control, or anti-IL-6R mAb-treated mice. The representative histological micrographs are shown at two different magnifications. Means and SDs of the colitis score are indicated in the bar graph. Bars: (left) 1 mm; (right) 200 μ m. (D) TNF- α , IL-1 β , IL-17A, and IFN- γ mRNA expression levels in the colon tissues from the untreated, control, and anti-IL-6R mAb-treated mice were determined by real-time PCR. The means and SDs are indicated.

mice, the expression levels in the anti-IL-6R mAb-treated mice were almost the same as those in the untreated normal mice (Fig. 4 D). This evidence demonstrated that IL-6 signaling was not only involved in the up-regulation of inflammatory cytokine levels in the colon tissues but was also related to the pathogenesis of CD8⁺ T cell-dependent colitis.

Requirement of Tc17 cells in autoimmune colitis

To define the role of IL-17 (11–14) on the pathogenesis of the CD8⁺ T cell-mediated autoimmune colitis, we performed

adoptive transfer of naive CD8⁺ T cells derived from IL-17^{-/-} mice. Deficiency of IL-17 in CD8⁺ T cells caused a remarkable suppression of pathology, including severe weight loss, thickening of the large intestinal wall, and colitogenic responses (Fig. 5, A–C). Thus, we propose that Tc17 cells would be critical effectors in the pathogenesis of autoimmune diseases. The generation of IFN- γ -producing CD8⁺ T cells by SP was observed in mLN even when IL-17^{-/-} CD8⁺ T cells were transferred into RAG2^{-/-} mice (Fig. 5 D), whereas the infiltration of CD8⁺ T cells into the colon tissues was greatly

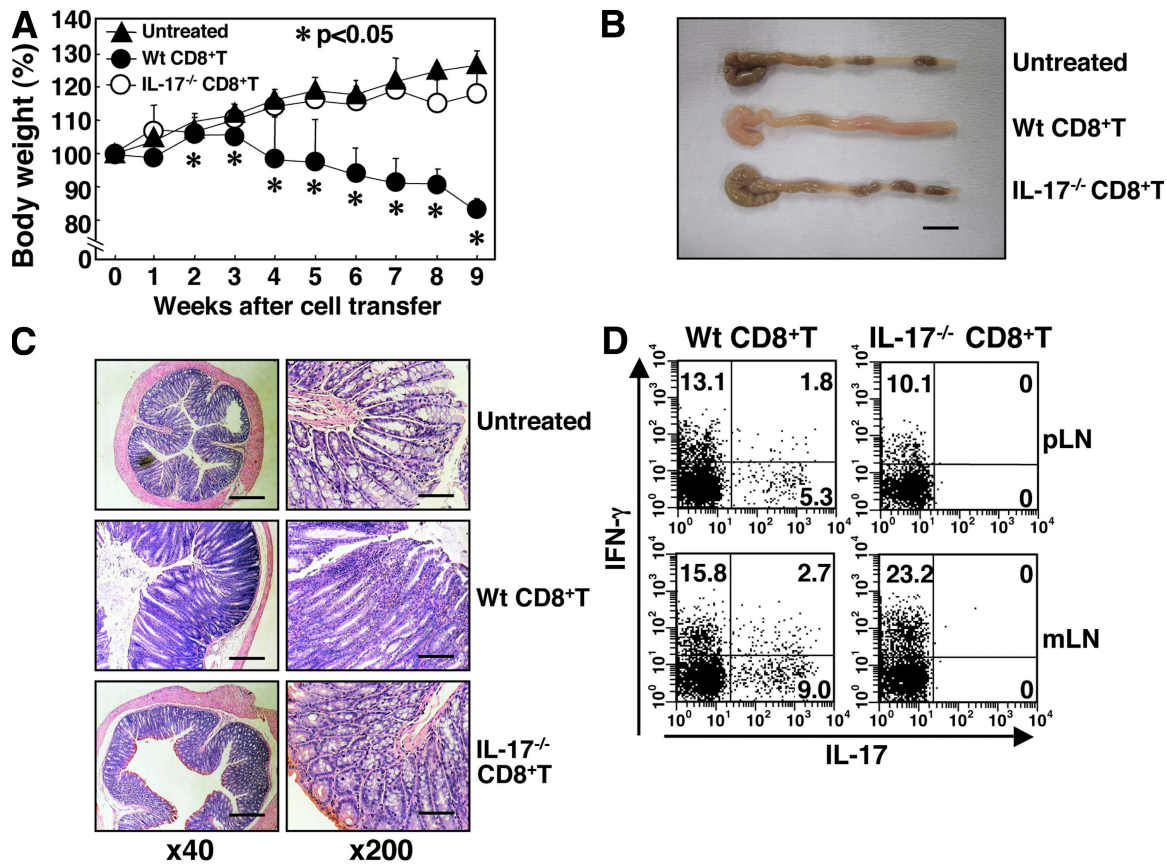


Figure 5. Requirement of Tc17 cells in autoimmune colitis. 5×10^5 naive CD8⁺ T cells from C57BL/6 WT and IL-17A^{-/-} mice were transferred into RAG2^{-/-} mice. (A) Body weights of untreated ($n = 4$), WT naive CD8⁺ T cell-transferred ($n = 4$), or IL-17A^{-/-} naive CD8⁺ T cell-transferred ($n = 4$) mice were monitored for 9 wk after the injection. Body weight percentages against the respective preinjection values were calculated every week. The means and SDs are indicated. (B) Colon tissues were obtained from the untreated, WT naive CD8⁺ T cell-transferred, or IL-17A^{-/-} naive CD8⁺ T cell-transferred mice. Morphology of the representative colon tissues is shown. Bar, 1 cm. (C) Colon sections from the untreated, WT naive CD8⁺ T cell-transferred, or IL-17A^{-/-} naive CD8⁺ T cell-transferred mice were stained with HE. The representative histological micrographs are shown at two different magnifications. Bars: (left) 1 mm; (right) 200 μm. (D) CD8⁺ T cells from the pLNs or mLNs of the adoptively transferred mice were intracellularly stained with anti-IFN-γ mAb and anti-IL-17 mAb. The representative FACS profiles are indicated. Percentages are shown.

inhibited by the IL-17-deficient condition (unpublished data). In addition, we have evidence that this colitis model was not induced when IFN-γ^{-/-} CD8⁺ T cells were transferred into RAG2^{-/-} mice, whereas SP of Tc17 cells was generated in mLNs (unpublished data). Therefore, IL-17 was required for the development of the CD8⁺ T cell-dependent colitis, but IL-17 might synergistically act with IFN-γ to induce the final colitogenic responses, including migration of effector CD8⁺ T cells and other inflammatory cells into the colon tissues. We are now investigating the critical role of both IL-17 and IFN-γ in colitis. As shown in Fig. 3 D, IL-17 and IFN-γ double-positive CD8⁺ T cells are rapidly proliferated in mLNs. Therefore, there is a good possibility that SP-induced IL-17 and IFN-γ double-producing Tc17 cells play a critical role in autoimmune colitis.

We have established a novel model of colitis by transfer of naive CD8⁺ T cells into syngeneic RAG2^{-/-} mice that mimicked the symptoms of IBD, such as weight loss and excess inflammatory cytokine production. So far, it has been

demonstrated that several cytokines, including IL-1β, TNF-α, IFN-γ, and IL-17, were involved in the induction of inflammation during colitis (30). However, little has been investigated about the physiological role of IL-17 production by CD8⁺ T cells in contrast to CD4⁺ T cells. Our results initially demonstrated a pivotal role of Tc17 cells in the pathogenesis of autoimmune colitis.

Adoptive transfer of naive CD8⁺ T cells underwent two distinct types of proliferation, SP and HP. We found that IL-6 signaling was critical for only SP, which was triggered by recognition of antibiotic-sensitive microbe antigens in the gut. The important role of antibiotic-sensitive flora-induced SP was demonstrated from the finding that intestinal flora depletion by antibiotics treatment caused the prevention of weight loss and colitis (unpublished data), in parallel with the blocking of SP, which is essential for pathogenic CD8⁺ T cell induction (Fig. 2 D). Moreover, IL-6-dependent SP was demonstrated to be critical for the differentiation of the final effector cells, Tc17 cells involved in autoimmune colitis.

This report initially proposes a novel mechanism for maintaining the homeostasis of CD8⁺ T cells, which is triggered under lymphopenic conditions in an IL-6-dependent manner. We designated this phenomenon as SP of CD8⁺ T cells, which also can be dissociated from IL-6-independent SP of CD4⁺ T cells (1, 18). Our results also indicate a novel application of anti-IL-6R mAb for the immunotherapy of Tc17 cell-mediated colitis, and propose that the IL-6-dependent SP could be a promising therapeutic target in autoimmune diseases such as IBD. We are now investigating the general role of SP in other CD8⁺ T cell-mediated immune diseases.

MATERIALS AND METHODS

Mice. WT C57BL/6 mice were purchased from Charles River Laboratories. C57BL/6-background RAG2^{-/-} mice were provided by M. Ito (Central Institute for Experimental Animals, Kanagawa, Japan). OT-1-TCR transgenic mice were provided by F.R. Carbone (University of Melbourne, Victoria, Australia). C57BL/6-background Ly5.1 mice were purchased from RIKEN Bioresource Center. C57BL/6-background IL-15^{-/-} mice were purchased from Taconic. OT-1/RAG2^{-/-} and IL-15^{-/-}/RAG2^{-/-} mice were bred in our facility. C57BL/6-background IL-17A^{-/-} mice were established as described previously (14). All mice used in the present studies were 5–8 wk old and maintained in specific pathogen-free conditions according to the guidelines for animal care of our institute. All mice were used in accordance with the guidance of an institutional committee at Hokkaido University.

Preparation and adoptive transfer of naive CD8⁺ T cells. Total lymphoid cells were recovered from the spleens and pLNs of the mouse strains indicated in the figures and resuspended in RPMI 1640 medium (Sigma-Aldrich) containing 10% FCS (BD Biosciences) plus penicillin and streptomycin (both from Meiji Seika). Erythrocytes were eliminated with 0.155 M NH₄Cl. The cells were passed through a nylon wool column for enrichment of T cells and were stained with FITC-CD44 mAb (IM7; eBioscience), PE-CD62L mAb (MEL-14; eBioscience), and PE-Cy7-CD8a mAb (53-6.7; BD Biosciences). CD44^{low}CD62L⁺ naive CD8⁺ T cells were isolated using a cell-sorting system (FACSARIA; BD Biosciences). The purity of the naive CD8⁺ T cells was consistently >98%. 5 × 10⁵ isolated naive CD8⁺ T cells per mouse were then intravenously injected into syngeneic RAG2^{-/-} mice.

Induction of experimental colitis. 5 × 10⁵ naive CD8⁺ T cells were intravenously injected into syngeneic RAG2^{-/-} mice. The body weights of the adoptively transferred mice were monitored for 6–9 wk. The colon tissues were recovered for histopathological analysis and HE staining at the time points indicated in the figures. Clinical scoring was performed as described previously (29). In brief, colitis was graded on a scale of 0–3, as follows: 0, minimal, indistinguishable from normal BALB/c mice; 1, mild; 2, moderate, low to intermediate degree of leukocyte infiltration and epithelial hyperplasia; and 3, severe, extensive leukocyte infiltration, loss of goblet cells, and marked epithelial hyperplasia. Histological evaluation was conducted in a blinded fashion.

Antibody treatment. Mice were intravenously injected with 1–2 mg anti-IL-6R mAb (MR16-1; a gift from Chugai Pharmaceutical Company Ltd., Shizuoka, Japan) at the time of T cell transfer, and were intraperitoneally injected with 500 μg of antibody 4 d after the transfer or every week up to the end of the colitis experiments. 500 μg anti-IL-7R mAb per mouse was intravenously injected before adoptive transfer. The mice were then intraperitoneally injected (500 μg per mouse) on days 2, 4, and 6 after T cell transfer.

Flow cytometric analysis. For analysis of cell-surface molecules, the cell samples indicated in the figures were stained with fluorescent dye-conjugated mAbs against the selected markers on ice. Cell proliferation was evaluated by monitoring the fluorescence intensity of the cells prestained with CFSE

(Invitrogen), according to the manufacturer's instructions. For detection of cytoplasmic cytokines, the indicated cells were stimulated with anti-CD3ε mAb (145-2C11; BD Biosciences) on 96-well flat-bottom plates for 6 h and treated with Brefeldin A for the final 2 h. The stimulated cells were stained with PE-Cy5-TCRβ mAb (H57-597; BD Biosciences) and fixed with 4% paraformaldehyde phosphate buffer solution (Wako). After treatment with permeabilizing solution (50 mmol/liter NaCl, 5 mmol/liter EDTA, 0.02% NaN₃, and 0.5% Triton X-100, pH 7.5), the cells were stained with PE-IL-17 mAb (TC11-18H10.1; BD Biosciences) and allophycocyanin-IFN-γ mAb (XMGI.2; eBioscience). Fluorescence signals from the cells were acquired by FACSCalibur and analyzed with CellQuest software (both from BD Biosciences). Data were collected with logarithmic amplification.

ELISA. Serum KC and SAA levels were determined by the mouse KC ELISA kit (R&D Systems) and the mouse SAA ELISA kit (Invitrogen), respectively. Assays were performed according to the manufacturers' instructions.

Immunohistochemical staining. Colons collected from mice were treated with optimum cutting temperature compound (Sakura Finetechnical Co.) and immediately frozen in liquid nitrogen. 4-μm sections of the colon tissues, fixed with acetone at 4°C for 10 min followed by blocking for 30 min, were incubated with anti-mouse CD8a mAb (53-6.7). After washing, the sections were further incubated with Alexa Fluor 546-conjugated goat anti-rat IgG (Invitrogen). The serial sections were incubated with biotinylated rat anti-mouse CD11b (M1/70; BD Biosciences), followed by detection with Alexa Fluor 488-conjugated streptavidin (Invitrogen). Each section was incubated with VECTASHIELD (Vector Laboratories) for 30 min, and the immunostaining images were analyzed with a laser scanning microscope (FluoView; Olympus).

Antibiotics treatment. Drinking water containing 1 g/liter ampicillin sodium (Meiji Seika), 1 g/liter neomycin sulfate (Nacalai Tesque), 500 mg/liter vancomycin (Nacalai Tesque), and 1 g/liter metronidazole (Nacalai Tesque) was provided for RAG2^{-/-} mice at 4 wk before cell transfer. 5 × 10⁵ naive CD8⁺ T cells stained with CFSE were intravenously injected into the antibiotic-treated mice, and the proliferation was determined by flow cytometry.

Real-time PCR. Total RNA was extracted from LNs or colon tissues of the mice indicated in the figures using the Isogen RNA extraction kit (Nippongene), according to the manufacturer's instructions. cDNA was prepared from the total RNA with RT (Invitrogen), oligo dT, and dNTP mixture (Promega). The indicated gene cDNAs were specifically amplified using a thermal cycler system (ABI PRISM 7700 Sequencer; Applied Biosystems) and using the corresponding primer pairs for mouse IL-7, IL-15, IL-6, TGF-β, IL-1β, TNF-α, IFN-γ, IL-17A, and β-actin. The sequences used were as follows: IL-7, (sense) 5'-GAGGTGGGTGTAGTCATGATGACT-3', (antisense) 5'-GGGTCTCTGGGAGTGATTATGG-3', and (probe) 5'-AGCCGGCTCTGCTGCAGTCC-3'; IL-15, (sense) 5'-GACCATGAAGAGGCAGTGCTT-3', (antisense) 5'-CAGCTCAGAGAGGTACGAAAGA-3', and (probe) 5'-CCACCTTGACACATGGCCCTCTGG-3'; IL-6, (sense) 5'-GAGGATACCACTCCCAACAGACC-3', (antisense) 5'-AAGTGCAATCATCGTTGTTTCATACA-3', and (probe) 5'-CAGAATTGCCATTGCACAACCTCTTTCTCA-3'; TGF-β, (sense) 5'-TGACGTCACTGGAGTTGTACGG-3', (antisense) 5'-GGTTTCATGTGATGGATGGTGC-3', and (probe) 5'-TTCAGCGCTCACTGCTCTTGTGACAG-3'; IL-1β, (sense) 5'-GAAAGACGGCACACCCACC-3', (antisense) 5'-AGACAAACCGCTTTTCCATCTTC-3', and (probe) 5'-TGCAGCTGGAGAGTGTGATCCCA-3'; TNF-α, (sense) 5'-GTTCTCTTCAAGGGACAAGGCTG-3', (antisense) 5'-TCCTGGTATGAGATAGCAAATCGG-3', and (probe) 5'-TACGTGCTCTCACCCACACCGTCA-3'; IFN-γ, (sense) 5'-GGATGCATTCATGAGTATTGC-3', (antisense) 5'-GCTTCCTGAGGCTGGATTTC-3', and (probe) 5'-TTTGAGGTCAACAACCCACAGGTCCA-3'; IL-17A, (sense) 5'-GCTCCAGAAGGCCCTCAGA-3', (antisense) 5'-CTTTCCCTCCGCATTGACA-3', (probe) 5'-ACCTCAACCGTTCCACGTCAC-3'; and β-actin, (sense) 5'-AGCCATGTACGTAGCATCCA-3', (antisense) 5'-TCTCCGGAGTCCATCACAATG-3', and

(probe) 5'-TGTCCTGTATGCCTCTGGTCGTACCA-3'. Samples were normalized to the housekeeping gene β -actin according to the $\Delta\Delta C_t$ method: $\Delta C_t = \Delta C_{t_{\text{sample}}} - \Delta C_{t_{\text{reference}}}$. Percentages against the WT control sample were calculated for each sample.

Statistics. All experiments were repeated at least three times. Mean values and SDs were calculated for data from three independent experiments and are shown in the figures. Statistical significance was calculated using the Student's *t* test. $P < 0.05$ was considered significant in the present experiments, as indicated with an asterisk.

Online supplemental material. Fig. S1 demonstrates that injection of anti-IL-6R mAb blocks proliferation of adoptively transferred naive CD8⁺ T cells in mLN. Fig. S2 shows that TGF- β signaling is not required for SP of CD8⁺ T cells. Fig. S3 demonstrates that anti-IL-6R mAb treatment does not induce apoptosis of naive CD8⁺ T cells. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20071133/DC1>.

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