Blocking of IL-6 signaling pathway prevents CD4\(^+\) T cell-mediated colitis in a Th17-independent manner

Daisuke Noguchi\(^1\), Daiko Wakita\(^1\), Masaki Tajima\(^1\), Shigeru Ashino\(^1\), Yoichiro Iwakura\(^2\), Yue Zhang\(^3\), Kenji Chamoto\(^1\), Hidemitsu Kitamura\(^1\) and Takashi Nishimura\(^1,3\)

\(^1\)Division of Immunoregulation Section of Disease Control, Institute for Genetic Medicine, Hokkaido University, Sapporo 060-0815, Japan
\(^2\)Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan
\(^3\)Division of ROYCE' Health Bioscience, Section of Disease Control, Institute for Genetic Medicine, Hokkaido University, Sapporo 060-0815, Japan

Keywords: colitis, IL-6, Th\(_1\), Th\(_17\)

Abstract

Naive CD4\(^+\) T cells rapidly proliferate to generate effector cells after encountering an antigen and small numbers survive as memory T cells in preparation for future immunological events. In the present work, adoptive transfer of naive CD4\(^+\) T cells into RAG2\(^{-/-}\) mice caused the generation of memory-type effector T cells including Th1, Th2, Th17 and regulatory T cells, and eventually induced T cell-dependent colitis. We found here that blocking of the IL-6R with a specific mAb remarkably inhibited the CD4\(^+\) T cell-mediated colitis in parallel with the inhibition of Th17 cell generation. However, the transfer of naive CD4\(^+\) T cells prepared from IL-17\(^{-/-}\) mice still induced severe colitis. At the effector phase, the mAb significantly inhibited IL-17 but not IFN-\(\gamma\) production. The blockade of IL-6 signaling enhanced the generation of IL-4- and IL-10-producing CD4\(^+\) T cells, and inhibited up-regulation of tumor necrosis factor -\(\alpha\) mRNA expression in the colon. These findings clearly demonstrated that IL-6 is a critical factor for the induction of colitis by expansion of naive CD4\(^+\) T cells in RAG2\(^{-/-}\) mice. Thus, the IL-6-mediated signaling pathway may be a significant therapeutic target in T cell-mediated autoimmune diseases.

Introduction

The immune system is spatiotemporally regulated by complicated and attractive immune networks. In particular, CD4\(^+\) T cell subsets, which develop from naive CD4\(^+\) T cells after interaction with pathogenic antigen-activated antigen-presenting cell (APC), play a crucial role in the maintenance of immune homeostasis through cytokine production. Until recently, the known universe of adaptive CD4\(^+\) T cell responses was considered to be regulated by cytokines produced by two distinct CD4\(^+\) T cell subsets, Th1 and Th2 cells (1–3). Th1-derived cytokines (IFN-\(\gamma\), IL-2, etc.) are critical for inducing cellular immunity, while Th2-derived cytokines (IL-4, IL-5, IL-13, etc.) play an important role in initiating humoral immunity. Many studies have indicated that an imbalance in Th1/Th2 immunity is a major cause of various immune diseases. In addition, it has been reported that down-modulation of Th1 or Th2 immunity by the third group of immunoregulatory CD4\(^+\) T cells, regulatory T (Treg) cells, resulted in the prevention of immune diseases (4–6). More recently, another CD4\(^+\) T cells subset, IL-17-producing Th\(_1\) (Th17) cells, was discovered (7, 8) as a more probable candidate effector T cell subtype that induces chronic autoimmune diseases (9–12). So far, it has been demonstrated that the development of Th17 cells was inhibited by cytokines derived from both Th1 and Th2 cells. Moreover, combinations of immunosuppressive cytokines such as transforming growth factor \(\beta\) and the pro-inflammatory cytokine IL-6 enhanced the differentiation of Th17 cells (13–15).

In general, pathogenic autoreactive T cells are deleted in the thymus by negative selection mechanisms. If any autoreactive T cells escape deletion and home into the peripheral lymphoid tissues, they are usually unresponsive to autoantigens due to tolerance. However, repeated chronic inflammation and infectious disease may sometimes activate autoreactive T cells and trigger autoimmune disease. The immune balance mediated by Th17/Treg cells rather than Th1/Th2 cells is a more suitable explanation of how autoimmune diseases are initiated during chronic inflammation at vulnerable sites (16–20). Indeed, recent works have revealed that Th17 cells were closely associated with the development of some experimental autoimmune diseases such...
as experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA). The depletion of IL-17 or T<sub>H17</sub> cells decreased the severity of the diseases (10, 11, 21–23).

Pro-inflammatory cytokine IL-6, which is an essential factor for T<sub>H17</sub> induction is reportedly involved in various immunological disorders including autoimmune diseases. Atreya et al. (24) reported that transfer of naive CD4<sup>+</sup> T cells into SCID mice caused severe colitis, which could be blocked by anti-IL-6 R mAb. They also indicated that administration of the anti-IL-6R mAb caused reduction of cytokine mRNA levels such as tumor necrosis factor (TNF) in the colon, which is a well-known important effector molecule in the induction of colitis. On the other hand, recent studies also demonstrated an important role for IL-6 in the suppression of Treg function and in the development of pathogenic T<sub>H17</sub> cells, which are also involved in colitis induced in some experimental models (10, 25).

In the present work, we found that in vivo injection of anti-IL-6R mAb remarkably inhibits T cell-mediated colitis caused by the homeostatic proliferation of naive CD4<sup>+</sup> T cells adoptively transferred into RAG2<sup>−/−</sup> mice. Interestingly, administration of the anti-IL-6R mAb also strongly blocked the development of T<sub>H17</sub> cells from the transferred naive CD4<sup>+</sup> T cells. Thus, we initially supposed that anti-IL-6R mAb prevented colitis via inhibition of T<sub>H17</sub> generation. However, unexpectedly, transfer of naive CD4<sup>+</sup> T cells from IL-17<sup>−/−</sup> mice also induced severe colitis in this model. From these results, we concluded that IL-6 is a crucial cytokine for inducing severe colitis in RAG2<sup>−/−</sup> mice by expansion of pathogenic cells of different phenotypes derived from transferred naive BALB/c CD4<sup>+</sup> T cells. The detailed mechanism for IL-6-mediated colitis is also discussed in this paper.

Materials and methods

Mice

Wild-type (WT) BALB/c mice were purchased from Charles River Japan (Kanagawa, Japan). BALB/c-background RAG2 knockout (RAG2<sup>−/−</sup>) mice were a gift from Central Institute for Experimental Animals (Kanagawa, Japan). BALB/c-background IL-17A knockout (IL-17<sup>−/−</sup>) mice (26) were backcrossed 8 times. All mice used in this study were initiated in specific pathogen-free (SPF) conditions according to the guidelines of the Institute for Genetic Medicine, Hokkaido University.

mAbs

Rat anti-mouse IL-6R monoclonal IgG1 antibody, MR16-1 (24), was kindly provided by Chugai Pharmaceutical Co. Ltd, Shizuoka, Japan. PE-Cy5-anti-CD4 (GK1.5), APC-anti-IFN-γ (XMG1.2), PE-anti-IL-10 (JESS16E3) and PE-anti-CD62L (MEL-14) mAb were purchased from eBioscience (San Diego, CA, USA). Anti-CD3ε (145-2C11), FITC-anti-CD45RB (16A), FITC-anti-IL-4 (11B11) and PE-anti-IL-17A (TC11-18H10.1) mAbs were purchased from BD PharMingen (San Diego, CA, USA).

Cell preparation

CD45RB<sup>high</sup>/CD62L<sup>−</sup> naive CD4<sup>+</sup> T cells were enriched by nylon passage of spleen cells and isolated by FACSria Cell Sorting System (BD PharMingen) with anti-CD4, anti-CD62L and anti-CD45RB mAbs. In all cases, the purity was >99% and the isolated CD4<sup>+</sup> T cells and un-separated cells were used for the following experiments.

Experimental colitis

CD45RB<sup>high</sup>/CD62L<sup>−</sup>, naive CD4<sup>+</sup> T cells (5 x 10<sup>5</sup>) from BALB/c mice were intravenously injected into syngeneic RAG2<sup>−/−</sup> mice. The body weights were monitored for 10 weeks after the cell transfer. Histological analysis and clinical scoring were carried out as in previous reports (27). Briefly, 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 leukocytic infiltration and epithelial hyperplasia and 3, severe, extensive leukocytic infiltration, loss of goblet cells and marked epithelial hyperplasia. Histological evaluation was conducted in a blinded fashion.

Administration of anti-IL-6R mAb

Mice were given anti-IL-6R mAb (MR16-1) by intra-peritoneal injection, 2 mg at the time of T cell transfer and 500 μg twice weekly thereafter.

Flow cytometry

Surface expression levels of CD45RB or CD62L on CD4<sup>+</sup> T cells and carboxyfluorescein diacetate succinimidyl diester (CFSE)-labeled CD4<sup>+</sup> T cells were characterized by FACS Calibur (BD Bioscience). The intracellular Foxp3 level was analyzed by Anti-Mouse/Rat Foxp3 Staining Kit (eBioscience) according to the manufacturer’s instructions. For intracellular cytokine staining, whole cells were first stained with anti-CD4 mAb, and then fixed with 4% PFA. After permeabilization, the fixed cells were stained with anti-IL-17A, anti-IFN-γ or anti-IL-4 mAbs. The intracellular Foxp3 and cytokine levels of CD4<sup>+</sup> cells were analyzed by FACSCalibur (BD Bioscience).

Cytokine production and ELISA

CD4<sup>+</sup> T cells (1 x 10<sup>5</sup>) from peripheral lymph node (pLN) and mesenteric lymph node (mLN) were cultured in 250 µl of RPMI medium (Sigma) containing 10% FCS (BD Bioscience) plus penicillin and streptomycin in 96-well flat-bottom plates, and stimulated with anti-CD3ε mAb immobilized on the plate for 48 h. IL-17A, IFN-γ, IL-4 and IL-10 levels in the supernatants were determined by the OptEIA mouse ELISA system (BD PharMingen).

In vitro suppression assay

CD25<sup>high</sup>/CD4<sup>+</sup> T cells were isolated by FACSria Cell Sorting System (BD PharMingen) from the mLN of normal BALB/c mice or naive CD4<sup>+</sup> T cell-transferred RAG2<sup>−/−</sup> mice that had been injected or not with anti-IL-6R mAb after the transfer. CD25<sup>high</sup>/CD4<sup>+</sup> T cells (5 x 10<sup>5</sup>) prepared from the mLN of normal BALB/c mice were stimulated with anti-CD3ε mAb (5 μg/ml) and with APC (1 x 10<sup>5</sup>) prepared from spleen cells, which had been depleted of T cells and treated with
mitomycin C, in the presence or absence of the CD25\(^{high}\)CD4\(^+\) T cells (5 × 10\(^5\)) for 72 h. \(^{3}H\)-thymidine (\(\text{[}^{3}H\text{]TdR}\)) was added to the culture media for the last 6 h of culture. The \(\text{[}^{3}H\text{]TdR}\) incorporation by the proliferating cells was counted using a Packard Matrix 9600 \(\beta\)-counter (Packard).

**Quantitative PCR**

Naive CD4\(^+\) T cells (5 × 10\(^5\)) were adoptively transferred into RAG2\(^{-/-}\) mice and after 10 weeks the large intestines were removed from the mice. Total RNA was extracted by Isogen RNA extraction kit (Nippon Gene, Tokyo, Japan), and cDNA was prepared from the total RNA using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Subsequently, cytokine mRNA expression levels were evaluated by TaqMan quantitative PCR on an ABI PRISM 7700 sequencer (Applied Biosystems). Each reaction was processed at 50°C for 2 min and at 95°C for 10 min followed by 45 cycles of amplification at 95°C for 15 s and at 60°C for 1 min. Sequences of the primer pairs and TaqMan probes used for these analyses were as follows: TNF-\(\alpha\) forward, 5'-GTT-CTC-CTC-TTC-AAG-GGA-CAA-GGC-TG-3'; TNF-\(\alpha\) reverse, 5'-TCC-TGG-TAT-GAT-AGA-GCA-AAT-CGG-3'; TNF-\(\alpha\) probe, 5'-TAC-GTG-CTC-ACC-ACC-ACC-GTC-ACC-A-3'; IL-1\(\beta\) forward, 5'-GGA-AGA-CCA-GCC-ACC-ACC-C-3'; IL-1\(\beta\) reverse, 5'-AGA-CAA-ACC-GCT-ATT-CCA-TCT-TC-3'; IL-1\(\beta\) probe, 5'-TGC-AGC-TGG-AGA-GTG-TGG-ATC-CCA-A-3'; IL-17A forward, 5'-GCT-CCA-GAA-GGC-CCT-CAG-ACC-A-3'; IL-17A reverse, 5'-CTT-TCC-CTC-CGC-ATT-GAC-A-3'; IL-17A probe, 5'-ACC-TCA-ACC-GTG-CCA-CGT-CAC-3'; \(\beta\)-actin forward, 5'-AGC-CAT-GTA-CTG-AGC-CAT-CCA-3'; \(\beta\)-actin reverse, 5'-TCT-CGG-GAG-TCC-ATC-ACA-ATG-3'; \(\beta\)-actin probe, 5'-TGT-CCC-TGT-ATG-CCT-GTC-GTA-CCA-3'. Samples were normalized to the housekeeping gene \(\beta\)-actin according to the \(\Delta\Delta C_t\) method: \(\Delta\Delta C_t = \Delta C_{t,\text{sample}} - \Delta C_{t,\text{reference}}\). Percentages against the WT control sample were calculated for each sample.

**Statistical analyses**

All experiments were repeated at least three times. Mean values and standard deviations or standard error of means (SEMs) were calculated for data from three independent experiments and shown in the figures. Significant differences in the results were determined by the Student's t-test. The \(P < 0.05\) was considered as significant in the present experiments.

**Results**

**Naive CD4\(^+\) T cells differentiate into cytokine-producing cells in RAG2\(^{-/-}\) mice**

We first investigated whether proliferation of naive CD4\(^+\) T cells generates diverse effector T cells in lymphopenic RAG2\(^{-/-}\) mice under SPF conditions. Purified CD45RB\(^{high}\)CD62L\(^{+}\) naive CD4\(^+\) T cells derived from WT BALB/c mice that initially produced negligible levels of cytokines after anti-CD3e stimulation (data not shown) were transferred into syngeneic RAG2\(^{-/-}\) mice. As shown in Fig. 1(A), the adoptively transferred naive CD4\(^+\) T cells proliferated under lymphopenic SPF conditions within 7 days of transfer. The T cell proliferation was significantly higher in the mLNs than in the pLN. We further examined the phenotypic characteristics of the CD4\(^+\) T cells within the pLN and mLN at day 14 after transfer. The transferred CD4\(^+\) T cells showed a CD45RB\(^{high}\)CD62L\(^{+}\) or so-called 'effector-memory' phenotype at day 14. The presence of effector-memory T cells was more pronounced in the mLN than in the pLN (Fig. 1B). The data were consistent with the results of proliferation (Fig. 1A). These results suggest that the mLN environment may provide a stronger activating stimulus on naive CD4\(^+\) T cells compared with that of the pLNs in mice.

We next examined the function of CD4\(^+\) T cells expanded under lymphopenic SPF conditions. Whole CD4\(^+\) T cells from the pLN or mLN were stimulated with anti-CD3e antibody and the intracellular levels of IL-17, IFN-\(\gamma\) and IL-4 were evaluated by flow cytometry. Moreover, Foxp3-positive cells were analyzed in the absence of anti-CD3e stimulation. As shown in Fig. 1(C and D), although there were few IL-4-producing CD4\(^+\) T cells present, the numbers of IFN-\(\gamma\)-producing cells increased both in the pLN and in the mLN. The ratio of IFN-\(\gamma\)-positive cells and the production levels were higher in the mLN than in the pLN (Fig. 1C and D). These results suggest that naive T cell expansion preferentially induced T\(_{h}1\) cells rather than T\(_{h}2\) cells.

Interestingly, we found here that IL-17-producing CD4\(^+\) T and T\(_{h}17\) cells were induced under the same conditions. Although the ratios of IL-17-positive cells in the pLN and mLN were similar, the cytokine production levels in cells of the mLN were significantly higher than those of cells in the pLN after anti-CD3e stimulation (Fig. 1C and D). Additionally, Foxp3\(^+\) Treg cells were also generated, and the ratio was significantly higher in the pLN than in the mLN. Because CD45RB\(^{high}\)CD62L\(^{+}\) naive CD4\(^+\) T cells used in the present experiments did not include 'naturally occurring' Foxp3\(^+\) CD25\(^+\) T cells (data not shown), the Foxp3\(^+\)CD25\(^+\) T cells in the pLN and mLN must have differentiated from naive CD4\(^+\) T cells in the present expansion process as described previously (28). The accumulated evidence strongly suggests that these cytokine-producing cells and Treg cells would be involved in the resulting disorders, especially in tissues near the mLN.

**Administration of anti-IL-6R mAb inhibits the generation of T\(_{h}17\) but does not affect the number of Foxp3\(^+\)CD25\(^+\) Treg cells in vivo**

Next, we investigated the effect of the IL-6 signaling pathway on the generation of cytokine-producing CD4\(^+\) T cells in vivo. Naive CD4\(^+\) T cells from WT BALB/c mice were transferred into syngeneic RAG2\(^{-/-}\) mice with mAb against IL-6R. Two or 5 weeks later, the cytokine production pattern in the expanded in vivo CD4\(^+\) T cells was examined by intracellular staining. As shown in Fig. 2(A), the ratio of IFN-\(\gamma\)-producing cells in mLN did not differ between anti-IL-6R mAb-treated and control mice at 2 and 5 weeks but significantly increased in the pLN after the injection. There was no enhancement or reduction of IL-4-producing cells by the administration of anti-IL-6R mAb (data not shown). Interestingly, the percentage of IL-17-producing CD4\(^+\) T cells was significantly reduced in anti-IL-6R mAb-treated mice compared with controls at both 2 and 5 weeks.
Fig. 1. Naive CD4+ T cells differentiate into cytokine-producing cells during homeostatic proliferation. (A) CD45RBhighCD62L+ and naive CD4+ T cells (5 × 10⁵) from BALB/c mice were pre-stained with CFSE (dashed lines) and intravenously injected into syngeneic RAG2−/− mice (n = 2 per group). The fluorescence intensity of the fresh and adoptively transferred CD4+ T cells (thin lines) in pLN and mLN was detected by FACSCalibur on day 7. Three independent experiments (n = 2 per group) were carried out and representative data are included in the figure. (B) Expression levels of CD62L and CD45RB on the transferred CD4+ T cells at day 14, and the percentages of WT BALB/c CD4+ T cells in pLN and mLN were analyzed by FACS with the fluorescent marker-conjugated mAbs. Three independent experiments (n = 2 per group) were carried out and the representative profiles are indicated in the figure. (C) Cytokine production levels of the transferred CD4+ T cells in pLN and mLN on day 14 were evaluated by intracellular staining with anti-IL-17A, IFN-γ, IL-4 and CD4 mAbs after anti-CD3e mAb stimulation for 6 h. Foxp3 expression levels of the freshly isolated cells were evaluated by intracellular staining analysis with anti-Foxp3 mAb and anti-CD4 mAb on day 14 after the transfer. Three independent experiments (n = 2 per group) were carried out and the representative FACS profiles are indicated in the figure. Percentages of the positive populations were calculated from four independent experiments and the means and standard deviations (SDs) are indicated in the figure. (D) CD4+ T cells in pLN and mLN of the adoptively transferred mice were stimulated with anti-CD3e mAb for 48 h in vitro. IL-17A, IFN-γ and IL-4 productions from the CD4+ T cells were measured by ELISA. The cytokine levels were evaluated from the representative data of three independent experiments and the means and SDs were indicated in the figure.
The ratios of Foxp3+CD25+ T cells were increased from 2 to 5 weeks. After 5 weeks, in both pLN and mLN, Foxp3+CD25+ T cells existed at 10–20% of the whole CD4+ T cells, likewise in the case of WT BALB/c (data not shown).

The percentages of Foxp3+CD25+ T cells generated in the mice were not significantly altered by the injection of anti-IL-6R mAb compared with control mice. The in vivo injection of anti-IL-6R mAb significantly suppressed production of IL-17 by cells from both the pLN and the mLN at 2 weeks (Fig. 2B). Then, we isolated CD25highCD4+ T cells from the mLN of the adoptively transferred mice, which included Foxp3+ Tregs (Fig. 2A), and evaluated the inhibitory effect on CD4+ T cell proliferation. The effect was not significantly altered by the injection of anti-IL-6R mAb (Fig. 2C). Taken together, results show that the blockade of IL-6 signaling with anti-receptor mAb caused modulation of the balance in cytokine-producing cells generated under lymphopenic condition, suggesting the possibility of consequences in the subsequent CD4+ T cell-mediated immunological events.

**Anti-IL-6R mAb inhibits the CD4+ T cell-mediated colitis in a Th17-independent manner**

In order to address the effect of anti-IL-6R mAb on the function of cytokine-producing CD4+ T cells, we kept monitoring...
adoptively transferred mice for 10 weeks. During the present experiments, the mice with transferred naive CD4+ T cells showed severe diarrhea and anal prolapsus as reported in previous studies (27). Histological analysis of the colon was carried out on the mice and compared with untreated mice at 10 weeks after the adoptive transfer of CD4+ T cells. As shown in Fig. 3(A), the wall of the mucosal epithelium was obviously thickened in the colon of mice transferred with WT-CD4+ T cells. We could observe the typical symptoms of colitis such as hemorrhagic necrosis, infiltration of lymphocytes, neoangiogenesis and depletion of goblet cells. Interestingly, the mice transferred with IL-17−/− mouse-derived naive CD4+ T cells also presented with colitis. Furthermore, both colons from mice treated with anti-IL-6R mAb showed significantly diminished colitis. We evaluated the clinical score for each mouse in detail as described in previous studies. As shown in Fig. 3(B), the score for anti-IL-6R antibody-injected mice was significantly low compared with that for control mice. The average values ± SEMs of the scores were as follows: WT-control, 1.88 ± 0.13; WT-anti-IL-6R antibody, 1.03 ± 0.16; IL-17−/−-control, 1.95 ± 0.16 and IL-17−/−-anti-IL-6R antibody, 1.28 ± 0.18. As shown in Fig. 3(C), there was no loss of weight for the mice receiving WT-CD4+ T cells plus anti-IL-6R antibody compared with untreated mice. Consistent with the clinical score, the mice transferred with IL-17−/−-CD4+ T cells showed reduced body weights similar to the mice treated with WT-CD4+ T cells. The loss of weight for the mice with IL-17−/−-CD4+ T cells was also canceled by the anti-IL-6 mAb injection. Taken together, administration of the anti-IL-6R mAb prevented the development of experimental colitis induced by transferred CD4+ T cells from both WT and IL-17−/− mice. These evidences indicate that IL-6 signaling could be associated with the pathogenesis of the colitis and that the IL-17 production from the adoptive transferred CD4+ T cells was not essential for the disorder in the present model.

Our results suggested that the effect of the IL-6 signaling pathway on the development of colitis was independent of the generation of Th17 cells, although blockade of the IL-6 signaling pathway with anti-IL-6R mAb significantly inhibited Th17 cell generation in this experiment.

**Blockade of IL-6 signaling pathway with anti-IL-6R mAb promotes the generation of IL-4- and IL-10-producing CD4+ T cells in vivo**

We further investigated the IL-6-dependent mechanism for inducing CD4+ T cell-mediated colitis. As shown in Figs 1 and 2, naive CD4+ T cells differentiated into various effector T cells after proliferation under lymphopenic conditions. Ten weeks after the transfer of naive CD4+ T cells, we examined the surface phenotypes of the cells within the pLN and the mLN. As shown in Fig. 4(A), the transferred CD4+ T cells showed CD45RBlowCD62L− memory phenotypes. The percentages of the cells isolated from pLN were higher than those isolated from mLN at the effector phase. The in vivo injection of anti-IL-6R mAb did not alter the ratio of CD45RBlowCD62L− populations in both pLN and mLN.
Fig. 4. Blockade of IL-6 signaling pathway with anti-IL-6R mAb generates IL-4- and IL-10-producing CD4+ T cells in vivo. (A) Naive CD4+ T cells (5 × 10^5) from WT or IL-17−/− BALB/c mice were intravenously injected into syngeneic RAG2−/− mice together with or without anti-IL-6R mAb. Every 3.5 days, anti-IL-6R mAb was again intra-peritoneally injected into the mAb-treated group. Expression levels of CD45RB and CD62L on the CD4+ T cells in the pLN and mLN were analyzed by FACS with the fluorescent marker-conjugated mAbs on day 70. Three independent experiments (n = 5 per group) were carried out and the representative profiles are indicated in the figure. (B) CD4+ T cells in the pLN and mLN of the adoptively transferred mice were stimulated with anti-CD3ε mAb for 48 h in vitro. IL-4, IFN-γ, IL-10 and IL-17A productions by the CD4+ T cells were measured by ELISA. The cytokine levels were evaluated from the representative data of three independent experiments and the means and standard deviations (SDs) are indicated in the figure. (C) IL-4 and IL-10 production levels of the cells isolated from mLN of the mice injected or not with anti-IL-6R mAb on day 70 were analyzed by intracellular FACS with anti-CD4 and either anti-IL-4 or anti-IL-10 antibodies after anti-CD3ε mAb stimulation for 6 h. Three independent experiments (n = 5 per group) were carried out and the representative FACS profiles are indicated in the figure. The percentages of IL-4- or IL-10-producing CD4+ T cells against the total CD4+ T cells were evaluated from the representative data of three independent experiments and the means and SDs are indicated in the figure.
The cells were collected from pLN and mLN 10 weeks after the injection and were stimulated with anti-CD3e antibody in vitro. As shown in Fig. 4(B), IL-17 production by WT-CD4+ T cells was significantly inhibited in both the pLN and the mLN by the administration of anti-IL-6R mAb. Recently, it was reported that IL-17 from non-T cells in the gut was clearly implicated in an inflammatory bowel disease model (29). In order to evaluate the possibility that the IL-17 was produced by host-derived cells and it caused the colitis, we checked serum IL-17 levels and mRNA expression levels of IL-17 in the colon from mice injected with IL-17-deficient CD4+ T cells. We could not confirm systemic IL-17 production in the sera (data not shown); however, there was a small amount of IL-17 mRNA expression even after the transfer of IL-17-deficient CD4+ T cells (Fig. 5). Since it has been reported that non-T populations such as NK cells and macrophages also produced IL-17 (29), the IL-17 mRNA expression level was possibly derived from non-T cells in RAG2-/- mice. But the IL-17 mRNA expression from non-T cells was much lower than that observed in colon tissues of RAG2-/- mice transferred with WT-CD4+ T cells. Therefore, we concluded that the present experimental colitis was not affected by non-T cell-derived IL-17. All together, these results suggested that T cell-derived IL-17 is not required for the development of intestinal inflammation in the present colitis model. There was no alteration in the IFN-γ production from WT- or IL-17-deficient CD4+ T cells induced by the anti-IL-6R mAb (Fig. 4B). Surprisingly, IL-4 and IL-10 productions from WT- or IL-17-deficient CD4+ T cells in mLN were enhanced by the administration of anti-IL-6R mAb (Fig. 4B). The enhanced IL-10 production from CD4+ T cells by anti-IL-6R mAb was similar to that in previous reports, in which IL-10 mRNA levels increased in the colon tissue of IL-6-/- mice with drug-induced colitis compared with WT mice (30, 31). In the present experiment, we confirmed that blockade of IL-6 signaling by the anti-IL-6R mAb resulted in the up-regulation of IL-4 and IL-10 production by the transferred CD4+ T cells (Fig. 4C). A recent study demonstrated that IL-6 was involved in the function of Treg cells and shifted the development of Treg cells toward T17 cells in vitro (25, 32). Here, the level of Foxp3+CD25+ T cells in mLN was not elevated by the anti-IL-6R mAb treatment (data not shown). Therefore, anti-IL-6R antibody plays a critical role in enhancing the function of IL-10-producing CD4+ T cells. Because it was reported that cure of T cell-mediated colitis required IL-10 production (33-35), up-regulation of IL-10 levels by the anti-IL-6R mAb treatment might have a greater importance in the subsequent inhibition of the colitis. Anti-IL-6R mAb also promoted IL-4-producing cells. We are now investigating the precise mechanism of this in vivo.

We further examined mRNA expression levels of TNF-α, which is thought to be a critical effector cytokine in colitis. As shown in Fig. 5, IL-1β and TNF-α mRNA levels were enhanced in colon of mice with CD4+ T cell-mediated colitis. The up-regulation of both mRNA levels was significantly reduced by the injection of anti-IL-6R mAb in this model. Lamina propria (LP) cells were collected from the colon tissues of mice with colitis in this model according to previous studies (36, 37). We also confirmed the production of inflammatory cytokines such as TNF-α, IL-1β, and IL-17A from LP cells of the colitis mice. The production of TNF-α as well as IL-17A from LP cells was not observed in the colitis-free mice (data not shown). Since the immune-suppressive cytokine IL-10 reduces production of various inflammatory cytokines (38-40), the IL-10-producing CD4+ T cells generated by the injection of anti-IL-6R mAb may be involved in the suppression of TNF-α and IL-1β production in the colon. These data suggested that IL-6 signal-mediated effector cytokine storm in the colon tissues was involved in the immunological disorders in the CD4+ T cell-mediated colitis model.

**Discussion**

Disruption of the balance and homeostasis of effector T cell populations causes serious immunological disorders including autoimmune diseases. In the present study, we found that adoptive transfer of naive CD4+ T cells but not CD8+ T cells from BALB/c mice into syngeneic RAG2-/- mice generates various cytokine-producing cells and that interestingly the IL-6 signaling cascade has a critical role in the balance of cytokine production from the expanded T cells.

IL-6 is a pleiotropic cytokine that regulates multiple biological functions such as development of the nervous and...
hematopoietic systems, acute-phase responses and inflammatory and immune responses (41). Numerous studies have reported on the multi-functionality of this cytokine in various immunological disorders including autoimmune diseases (42–44). Recent studies demonstrated that IL-6 had important roles in the function of Tregs (25) and in the development of T<sub>H</sub>17 cells (10, 13, 14, 45). So far, Foxp3-positive Treg cells were identified as a key population that prevents the development of autoimmune diseases. In addition to Tregs, recent studies have revealed that IL-17-producing CD<sup>4</sup><sup>+</sup> T cells, T<sub>H</sub>17 cells, were closely associated with the development of some experimental autoimmune diseases such as EAE and CIA (11, 12, 29, 31, 46, 47).

In the present study, we found that adoptive transfer of naive CD<sup>4</sup><sup>+</sup> T cells into RAG2<sup>−/−</sup> mice generated T<sub>H</sub>17 cells as well as Foxp3-positive cells in addition to IFN-γ-producing T<sub>H</sub>1 cells. Recently, Paul et al. demonstrated that spontaneous and homeostatic expansion occurred during proliferation of naive CD<sup>4</sup><sup>+</sup> T cells in lymphopenic mice after adoptive transfer (48). We also confirmed that rapid expansion of naive CD<sup>4</sup><sup>+</sup> T cells occurs in the mLN within 1 week of transfer (Fig. 1A) and eventually caused severe colitis (Fig. 3), involving the generation of various cytokine-producing effector memory CD<sup>4</sup><sup>+</sup> T cells (Fig. 4). In this T cell-mediated colitis model mouse, blockade of the IL-6 signaling pathway remarkably inhibited the generation of T<sub>H</sub>17 cells (Figs 2A, B and 4B). However, the adoptive transfer of naive CD<sup>4</sup><sup>+</sup> T cells from IL-17<sup>−/−</sup> mice also caused subsequent colitis in this model. In the present experiments, similar numbers of Foxp3-positive cells, which did not originally exist in the transferred naive T cells (data not shown), were generated in the lymphopenic mice injected or not with anti-IL-6R mAb (Fig. 2A). In addition, the in vivo injection of anti-IL-6R mAb did not alter their inhibitory function (Fig. 2C). Moreover, we also found that blocking of the IL-6R with the specific antibody remarkably inhibits T cell-mediated colitis caused by the expansion of naive CD<sup>4</sup><sup>+</sup> T cells under lymphopenic conditions (Fig. 3). These evidences strongly suggested that the target of the IL-6 signaling cascade in the present colitis model was regulated by other effector T cells. We showed here that blocking of IL-6 signaling results in the up-regulation of IL-10-producing CD<sup>4</sup><sup>+</sup> T cells in the present colitis model (Fig. 4C). Previous papers indicated that the IL-10 mRNA level was significantly enhanced in the colon tissues of drug-induced colitis models using IL-6KO mice, resulting in suppression of the colitis (30, 31). Since many investigators reported that cure of T cell-mediated colitis required IL-10 production (5, 33–35), up-regulation of IL-10 levels by the anti-IL-6R mAb treatment might have implications required IL-10 production (5, 33–35), up-regulation of IL-10 levels by the anti-IL-6R mAb treatment might have implications for the regulation of T cell-mediated inflammatory conditions (Fig. 3). These evidences strongly suggested that the target of the IL-6 signaling cascade in the present colitis model was regulated by other effector T cells.

We finally conclude here that the IL-6 signaling pathway involved in the expansion of naive CD<sup>4</sup><sup>+</sup> T cells is critical for the subsequent generation of pathogenic effector T cells in colitis. Based on these findings, we consider the IL-6 signaling cascade to be a significant therapeutic target in T cell-mediated autoimmune diseases.

Acknowledgements

We would like to thank Mr Yasushi Uchiyama and Chugai Pharmaceutical Co. Ltd, (Shizuoka, Japan) for their kind gift of anti-mouse IL-6R mAb, MR16-1. We would also like to thank Mark J. Micalef (Intervet Co. Ltd, Ibaraki, Japan) for a kind revision of the manuscript.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIA</td>
<td>collagen-induced arthritis</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>[&lt;sup&gt;3&lt;/sup&gt;H]TdR</td>
<td>&lt;sup&gt;3&lt;/sup&gt;H-thymidine</td>
</tr>
<tr>
<td>LP</td>
<td>lamina propria</td>
</tr>
<tr>
<td>mLN</td>
<td>mesenteric lymph node</td>
</tr>
<tr>
<td>pLN</td>
<td>peripheral lymph node</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SPF</td>
<td>specific pathogen free</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>

References
