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IL-6 Is Required for the Development of Th1 Cell-Mediated Murine Colitis

Mitsunari Yamamoto,* Kazuyuki Yoshizaki, † Tadamitsu Kishimoto, ‡ and Hiroaki Ito*‡

Proinflammatory cytokines have been demonstrated to play a crucial role in the pathogenesis of Crohn’s disease. Among those cytokines, strong expression of IL-6 has been repeatedly demonstrated. To examine the role for IL-6 in the pathogenesis of Crohn’s disease, we introduced anti-IL-6R mAb to a murine model of colitis. Colitis was induced in C.B-17 scid mice transferred with CD45RB<sup>high</sup> CD4<sup>+</sup> T cells from BALB/c mice. Anti-IL-6R mAb or rat IgG was administered weekly after T cell transfer. ICAM-1 and VCAM-1 expression were analyzed by immunohistochemistry. Colonic cytokine expression was determined by RT-PCR. Mice treated with mAb showed normal growth, whereas controls lost weight. The average colitis score was 0.64 for mAb-treated mice and 1.80 for controls. T cell expansion in treated mice was less remarkable than in the controls. Colonic ICAM-1 and VCAM-1 expression were markedly suppressed by mAb. IFN-γ, TNF-α, and IL-1β mRNA were reduced by the treatment. The results presented here show a crucial role for IL-6 in the pathogenesis of murine colitis and suggest a therapeutic potential of anti-IL-6R mAb for treatment of human Crohn’s disease. The Journal of Immunology, 2000, 164: 4878–4882.

Efforts have been made to elucidate the pathogenesis of Crohn’s disease (CD), but the mystery remains unsolved. The problem is that CD affects young adults, and there is no satisfactory therapy. Whatever may trigger the initial event, there is substantial evidence that the chronic intestinal inflammation may be shaped by Th1 cell activation and subsequent overproduction of proinflammatory cytokines such as IL-1β, TNF-α, and IL-6 (1). Various immunosuppressive therapies have been shown to benefit patients with CD. Corticosteroids are the most commonly used, but they only relieve the symptoms, without improvement of endoscopically visible lesions (2). Azathioprine and other immunosuppressive medications are also used, but their effectiveness in inducing remission remains controversial, and their utility is limited because of their side effects (3). Recently, anti-TNF-α mAb has been shown to be useful in patients with steroid-unresponsive CD (4). This might prove a great boon to patients, but its safety and efficacy in long-term treatment have not yet been established. It has been shown that IL-6 is present at very high levels in both serum and intestinal tissue from patients with CD (5). The soluble form of IL-6R (sIL-6R) is also elevated (6), and this is known to potentiate IL-6 activity by forming IL-6-sIL-6R complexes that bind the signaling-competent transmembrane receptor gp130 (7). IL-6 is known as a major modulator of inflammation (8). Therefore, IL-6 might play a crucial role in the pathogenesis of CD, and we considered that blocking IL-6 signaling should bring great benefits to patients with CD.

Congenic scid mice given CD45RB<sup>high</sup> CD4<sup>+</sup> T cells from normal mice develop Th1 cell-mediated colitis called wasting disease which resembles human CD (9). In this report, we utilized this model to examine the therapeutic potential of anti-IL-6R mAb (MR16-1), which blocks both the transmembrane and soluble forms of IL-6R.

Materials and Methods

Mice
BALB/c and C.B-17 scid mice were purchased from Charles River Breeding Laboratories (Shiga, Japan). Before use, the mice were maintained in the specific pathogen-free animal facility at the Institute of Experimental Animal Sciences, Osaka University Medical School. BALB/c mice were used at 7–9 wk, C.B-17 scid mice at 8–12 wk of age.

Anti-IL-6R mAb
Anti-IL-6R mAb, which is rat IgG1 mAb against murine IL-6R, was provided by Chugai Pharmaceutical Co. (Gotemba, Japan).

Purification of splenocyte subsets
All procedures were conducted under aseptic conditions. CD4<sup>+</sup> T cells were enriched from single-cell suspensions of BALB/c splenocytes in RPMI 1640 containing 2% FCS using an anti-L3T4 (anti-CD4) MACS magnetic separation system (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instruction. Briefly, splenocytes were incubated with anti-CD4 magnetic microbeads for 20 min at 40°C, washed, and then enriched by passage through magnetic flow columns. Enriched CD4<sup>+</sup> T cells (96–97% purity, confirmed by FACS) were then labeled with PE-conjugated anti-CD4 magnetic microbeads (Miltenyi) and sorted into CD4<sup>+</sup>- (bright staining 13–17%) fractions on a FACS Vantage (Becton Dickinson, Sunnyvale, CA).

Cell transfer and administration of anti-IL-6R mAb
Each C.B-17 scid mouse was injected i.p. with 100 μl of PBS containing 4 × 10<sup>5</sup> CD45RB<sup>high</sup> CD4<sup>+</sup> T cells from normal BALB/c mice. Mice were also given either rat IgG or anti-IL-6R mAb by i.p. injection (2 mg at the time of T cell transfer and 1 mg weekly up to 8 wk).

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Histological and immunohistological analyses

Colons were removed from recipient mice 8 wk after T cell reconstitution and were frozen in OCT compound at −80°C. For histological analyses, 6-μm sections were fixed in 10% Formalin and stained with hematoxylin and eosin. 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. For immunohistochemistry, staining of the sections was performed using an immunofluorescence staining method. Six-micrometer sections were incubated with PE-conjugated anti-ICAM-1 (BE29G1, rat IgG2a; Leico Technologies, St. Louis, MO) and FITC-conjugated anti-CD4. For staining of VCAM-1, anti-VCAM-1 (MK2, rat IgG1; Immunotech, Marseille, France) and FITC-conjugated anti-rat IgG (Vector Laboratories, Burlingame, CA) were used. Fluorescence micrographs were taken by using a LSM 310 microscope (Zeiss, Oberkochen, Germany).

RT-PCR for cytokine expression

The expression of IFN-γ, TNF-α, IL-1β, IL-4, IL-6, IL-10, and TGF-β were determined by RT of total RNA, followed by PCR. Total RNA was isolated from the colonic tissue by the acid guanidium thiocyanate-phenol chloroform extraction method. cDNAs were synthesized by incubating 1 μg of total RNA with 600 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD) in the presence of 0.5 U of oligo(dT) (Pharmacia, Piscataway, NJ), 3 mg of acetylated BSA (Life Technologies), and 40 U of RNase inhibitor (Promega, Madison, WI) in a volume of 30 ml for 1 h at 37°C and then for 5 min at 96°C to stop the reaction. PCR of the cDNA was performed in a final volume of 50 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% (v/v) gelatin, 0.2 mM of dNTP (Pharmacia), 1.25 µl of AmpliTaq DNA polymerase (Perkin-Elmer, Branchburg, NJ), and each primer at 0.5 μM, using the geneAmp 2400 PCR system (Perkin-Elmer). The amplification cycles were 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min. The PCR products were separated by electrophoresis on 1.5% agarose gel after 25–35 cycles for IFN-γ, TNF-α, IL-4, IL-6, IL-10, or 20–22 cycles for gylceraldehyde-3-phosphate dehydrogenase (G3PDH), and were visualized by ethidium bromide staining. Amplification of G3PDH served as a control for sample loading and integrity. The following primers were used: IFN-γ sense, 5′-GAA-AGC-CTA-GGA-AGT-CTG-AAT-AAC-TCC-3′; IFN-γ antisense, 5′-ATC-AGC-GAC-TCC-TTT-TTC-GCT-T-3′; TNF-α sense, 5′-ATG-AGC-ACA-GAA-AGC-ATG-ATC-CCC-3′; TNF-α antisense, 5′-CCA-AAG-TAG-ACC-TGG-ATT-TAT-AGA-3′; IL-1β sense, 5′-ATG-GCA-ACT-GTT-CTT-CGT-GAA-CTC-AAC-TCC-3′; IL-1β antisense, 5′-CAG-CAC-AGC-TAT-AGA-TTC-TTC-TCT-TF-3′; IL-4 sense, 5′-ATG-GCT-GTC-ACC-CCC-CAG-CTA-GT-3′; IL-4 antisense, 5′-GCT-CTT-TAG-GCT-TTC-CAG-GAA-GTC-3′; IL-6 sense, 5′-GAT-GCT-ACC-AA-AAT-CGT-GAT-ATA-ATC-3′; IL-6 antisense, 5′-ACC-CTG-GGT-TGA-AGG-GCA-3′; IL-10 sense, 5′-ACC-TAG-TGC-GTAAG-TGC-CCC-AGG-CA-3′; IL-10 antisense, 5′-CTA-TGC-AGT-GTA-GAG-TAG-TCT-CAA-A-3′; G3PDH sense, 5′-CGG-TTC-GTA-TGC-GTG-GAT-GGA-GTC-3′; and G3PDH antisense, 5′-GTT-ATT-ATG-GGG-GTC-GTG-GAT-GGA-A-3′. TGF-β was analyzed using quantitative competitive PCR mouse TGF-β (Maxim Biotech, South San Francisco, CA).

Isolation of lamina propria lymphocytes (LPLs) and analysis by FACS

LPLs were isolated from the colon as described previously (10) and incubated with PE-conjugated anti-ICAM-1, FITC-conjugated anti-CD4 (RM4-5, rat IgG2a; PharMingen), anti-Mac-1 (M1/70, rat IgG2b; PharMingen), and FITC-conjugated anti-rat IgG. Flow cytometric analysis was performed on a FACSscan (Becton Dickinson).

Statistical analysis

Data are expressed as means ± SEM. Results were analyzed using the t test. A p value of <0.05 was considered to be statistically significant.

Results

Anti-IL-6R mAb prevents wasting disease

To test whether blocking IL-6 signaling could prevent wasting disease, scid mice restored with CD45RBlow CD4+ T cells were treated with anti-IL-6R mAb, control rat IgG, or PBS alone. As shown in Fig. 1, the mice restored with CD45RBlow CD4+ T cells and given PBS alone started to lose body weight by 3 wk after T cell transfer (wasting disease), whereas mice restored with CD45RBlow CD4+ T cells and treated with anti-IL-6R mAb experienced weight gain similar to that seen in normal scid mice. The final average weight of the mice given control rat IgG was indistinguishable from that of the mice given PBS alone. Although it seemed that rat IgG had partial protection, it was not statistically significant. The average body weight at the end of observation was: normal scid; 110.5 ± 0.9%, anti-IL-6R mAb-treated mice; 108.9 ± 1.9%, mice given PBS alone; and 88.7 ± 3.0%, control rat IgG treatment; 93.3 ± 5.0% of their initial weight. We also examined the mice restored with both CD45RBlow and CD45RBlow CD4+ T cells which were protected from wasting disease. The change of body weight was very similar to the normal scid mice and the mice treated with anti-IL-6R mAb (data not shown).

FIGURE 1. Anti-IL-6R mAb prevents wasting disease. C.B-17 scid mice were reconstituted with 4 × 10⁶ CD45RBlow CD4+ T cells and treated weekly with anti-IL-6R mAb (●, n = 11), PBS (○, n = 16), or control rat IgG (△, n = 5) for up to 8 wk. Body weights of these mice were compared with normal unconstituted scid mice (□, n = 8). Data represent the average ± SEM.
Anti-IL-6R mAb treatment prevents the development of colitis

The mice were sacrificed 8 wk after T cell transfer, and the colons were removed for histological evaluation. In gross examination, colons of the mice restored with CD45RB<sup>high</sup> CD4<sup>+</sup> T cells and given PBS alone were obviously enlarged, and the walls were diffusely thickened, whereas colons from the mice treated with anti-IL-6R mAb were almost indistinguishable from normal unreconstituted scid mice. Further observation under the microscope clearly demonstrated the therapeutic effect of anti-IL-6R mAb on the development of colitis. Thirteen of 16 mice restored with CD45RB<sup>high</sup> CD4<sup>+</sup> T cells and given PBS alone showed moderate to severe colitis, with an average score of 2.3 ± 0.2 (Fig. 2), characterized by marked epithelial hyperplasia, extensive leukocyte infiltration, and goblet cell depletion. Inflammatory infiltrates, which consisted mainly of mononuclear cells, were observed in the lamina propria. The mice restored with CD45RB<sup>high</sup> CD4<sup>+</sup> T cells and given control rat IgG showed similar results with an average colitis score of 1.8 ± 0.6 (Fig. 3B). In contrast, treatment with anti-IL-6R mAb provided significant protection from colitis: 7 of 11 had mild colitis and 4 of 11 had minimal pathological changes, yielding an average colitis score of 0.6 ± 0.2 (Fig. 3C).

Anti-IL-6R mAb suppressed the expression of ICAM-1 and VCAM-1

Cell adhesion between vascular endothelial cells and leukocytes is an important process for inflammatory changes. We examined the expression of ICAM-1 and VCAM-1 in the colons by means of immunohistochemistry. In the mice restored with CD45RB<sup>high</sup> CD4<sup>+</sup> T cells and given control rat IgG, there was a massive accumulation of ICAM-1-positive cells in the lamina propria. Vascular endothelial cells were also positive for ICAM-1 (Fig. 4b). In contrast, the immunoreactivity for ICAM-1 was less intense and no vascular staining was seen in the mice restored with CD45RB<sup>high</sup> CD4<sup>+</sup> T cells and treated with anti-IL-6R mAb (Fig. 4c). VCAM-1 was positive in vascular endothelial cells in the mice given control rat IgG (Fig. 4e). Again, the expression of VCAM-1 was markedly suppressed in the mice treated with anti-IL-6R mAb (Fig. 4f). Analysis of isolated LPLs by FACS revealed that most of the

FIGURE 3. Prevention of colitis in mice treated with anti-IL-6R mAb. A, Normal unreconstituted scid mice. B, Severe colitis (grade 3) in mice reconstituted with 4 × 10<sup>5</sup> CD45RB<sup>high</sup> T cells and given control rat IgG. C, Prevention of colitis in mice restored with 4 × 10<sup>5</sup> CD45RB<sup>high</sup> T cells and treated with anti-IL-6R mAb. Colonic sections were stained with hematoxylin and eosin and photographed at ×40 magnification.

FIGURE 4. ICAM-1 and VCAM-1 expression was suppressed by anti-IL-6R mAb treatment. Frozen tissue sections of colons from T cell-restored mice given control rat IgG (b and e), mice treated with anti-IL-6R mAb (c and f), and normal scid mice (a and d) were stained by immunofluorescence for the expression of ICAM-1 (a–c, ×200 magnification) and VCAM-1 (c–e, ×200 magnification).
ICAM-1+ cells were Mac-1+ and CD4+ and were most likely macrophages (data not shown).

**Anti-IL-6R mAb reduced the expansion of CD4+ T cells in the recipient scid mice**

Increase of transferred T cells in the recipient scid mice was discussed in a previous report, with the scid mice that received CD45RBhigh CD4+ T cells having more CD4+ T cells than originally injected and the majority of CD4+ T cells being recovered from the spleens (9). To examine the effects of anti-IL-6R mAb on the expansion of T cells in the recipients, we compared colonic infiltration of CD4+ cells by immunohistochemistry and determined the number of CD4+ T cells in the spleen. Fig. 5 clearly demonstrates marked reduction of CD4+ cells in the colon of mAb-treated mice compared with those with colitis. As shown in Table I, the average number of splenic CD4+ T cells recovered from mice that received CD45RBhigh CD4+ T cells and given PBS alone was 2.05 ± 0.37 × 10^6 and that from mice given control rat IgG was 2.22 ± 0.54 × 10^6. In contrast, treatment with anti-IL-6R mAb significantly suppressed the expansion of CD4+ T cells. The cell number in mice given anti-IL-6R mAb was 0.83 ± 0.19 × 10^6, which was significantly lower than in the other groups. Therefore, anti-IL-6R mAb suppressed the expansion of both colonic and splenic CD4+ T cells.

**Colonic expression of IFN-γ and proinflammatory cytokines were also reduced by treatment with anti-IL-6R mAb**

Wasting disease is a Th1-mediated colitis, and Th1 cytokines, especially IFN-γ and TNF-α, have been demonstrated to play an important role in its pathogenesis (9). Therefore, by using RT-PCR, we compared cytokine expression in the colons of the mice that developed wasting disease with the mice successfully treated with anti-IL-6R mAb. As shown in Fig. 6, the mice into which CD45RBhigh CD4+ T cells were transferred and given control rat IgG, which developed wasting disease and colitis, showed strong expression of IFN-γ, TNF-α, IL-1β, and IL-6 mRNA, whereas the normal unreconstituted scid mice exhibited only slight expression of those cytokines. The mice restored with both CD45RBhigh and CD45RBlow CD4+ T cells, which were protected from the development of colitis, showed similar patterns of cytokine expression to normal scid mice. In the colons of the mice restored with CD45RBhigh and treated with anti-IL-6R mAb, mRNA for IFN-γ was almost undetectable, IL-1β was remarkably reduced, and the level of TNF-α expression was as low as in normal scid mice and mice cotransferred with CD45RBlow CD4+ T cells. As compared with the other cytokines, the amount of IL-6 mRNA in mAb-treated mice was considerable but slightly less than in colitic mice. Recently, colitis-suppressing potential of CD45RBhigh CD4+ T cells, T regulatory-1 cells, and their effector cytokines have been discussed (11, 12). Therefore, we examined IL-4, IL-10, and TGF-β. As shown in Fig. 6, IL-10 and TGF-β mRNA were detectable almost equally in all experimental groups, whereas IL-4 was undetectable in any mice.

**Discussion**

The data in this report provide evidence that IL-6 plays a crucial role for the pathogenesis of murine wasting disease, and hold out hope that anti-IL-6 strategy will be effective in the treatment of human CD.

Murine wasting disease is considered to be a Th1-mediated pathogenesis, as is human CD. Treatment of wasting disease by blocking Th1 cytokines was discussed in a previous report, with only two doses of anti-IFN-γ mAb given to mice soon after T cell transfer preventing the development of colitis for up to 12 wk, whereas continual neutralization of TNF with anti-TNF-α and -β mAb only reduced the incidence of severe disease and anti-TNF mAb only for the first 3–4 wk had no effect, which suggested that anti-TNF might act primarily to block TNF effector function (9). Th1 cytokine IFN-γ is the major physiologic activator of macrophages and activated macrophages produce proinflammatory cytokines, including IL-1, TNF-α, and IL-6. Furthermore, IL-6 is produced by various kinds of cells following IL-1 and TNF-α stimulation (13). IL-6 is considered to play a central role in the

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**Table I. Expansion of CD4+ T cells in C.B-17 scid mice**

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<thead>
<tr>
<th>Reconstitution and Treatment</th>
<th>Splenic CD4+ T Cells (×10⁶)</th>
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<tbody>
<tr>
<td>CD45RBhigh T cells + PBS</td>
<td>2.05 ± 0.37</td>
</tr>
<tr>
<td>CD45RBhigh T cells + rat IgG</td>
<td>2.22 ± 0.54</td>
</tr>
<tr>
<td>CD45RBhigh T cells + anti-IL-6R mAb</td>
<td>0.83 ± 0.19*</td>
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*CD4+ T cell number in the spleen was determined by FACS analysis 8–9 wk after T cell transfer. Data represent the means ± SEM for the group. The number of splenic CD4+ T cells recovered from anti-IL-6R mAb-treated mice was significantly lower than that of mice given PBS alone or control rat IgG (*, p < 0.02, t test).
regulation of inflammatory and immune reactions, and the requirement for IL-6 has been demonstrated for murine models of rheumatoid arthritis and multiple sclerosis (14, 15). CD shares characteristics with these diseases in terms of oligoclonal expansion, activation of CD4+ T cells, and participation of proinflammatory cytokines. Therefore, it is not surprising that anti-IL-6 mAb prevented the development of wasting disease and colitis. So far, we have not determined whether continual administration of anti-IL-6 mAb is indispensable or not. Treatment starting the following day after T cell transfer was equally effective (data not shown). Questions such as how many doses are required, and how much dosage is necessary remain to be answered.

Proinflammatory cytokines are known to up-regulate a spectrum of cell adhesion molecules, including ICAM-1, VCAM-1, selectins, and integrins. In the study of human inflammatory bowel disease, ICAM-1+ inflammatory infiltrates and venules were demonstrated to increase in parallel to the degree of inflammation, whereas VCAM-1 was not significantly enhanced in the inflamed mucosa (16, 17). It has been shown that IL-6 in the presence of sIL-6R augmented ICAM-1 expression of endothelial cells, but did not modify the expression of VCAM-1 (8), which may explain the difference between ICAM-1 and VCAM-1 expression in human CD. Stimulation with concomitant IL-6 and sIL-6R was required because endothelial cells express only the gp130 signal-transducing chain and not the subunit-specific IL-6R (8). Adhesion molecules in murine colitis appeared somewhat different from those in human CD. There was enhanced expression of both ICAM-1 and VCAM-1 in wasting disease mice, and treatment with anti-IL-6 mAb remarkably reduced the expression of both adhesion molecules. Reduction of expression was notable in small vessel walls as the mAb significantly reduced the expression of both adhesion molecules.

IL-6-6R mAb suppressed wasting disease and colitis through the induction of TGF-β.

In summary, the results show the requirement of IL-6 in the development of wasting disease and colitis. Experiments addressing the mechanism for the abrogation of colitis indicate the effects of IL-6 blockade on ICAM-1 and VCAM-1 expression, T cell proliferation, and IFN-γ and proinflammatory cytokine production. This study will provide a basis for the practical application of anti-IL-6 mAb to the treatment of human CD.

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References