was increased in the peripheral blood mononuclear cells of patients with RA compared with healthy control subjects (7). It is therefore necessary to determine if autoimmune arthritis is a Th1- or a Th17-associated disorder.

The lineage commitment of each Th cell subset from naive CD4+ T cells is dependent on the expression of specific transcription factors induced under the particular cytokine environment. Differentiation of Th1 cells is dependent on the expression of the transcription factor T-bet, which is induced by IFNγ/STAT-1 signaling pathways and directly activates the production of IFNγ (8,9). Similarly, Th17 cell differentiation in mice is dependent on the transcription factor retinoic acid receptor–related orphan nuclear receptor γt (RORγt) induced by transforming growth factor β (TGFβ) and IL-6 (10). Previous studies showed that these transcription factors negatively regulate the differentiation of other T cell subsets by direct co-interaction and/or indirect effects of cytokines produced from each T cell subset (11,12). How the predominant differentiation of CD4+ T cells affects the development of autoimmune arthritis remains unclear, however.

In the present study, CIA was induced in C57BL/6 (B6) mice and T-bet–transgenic (Tg) mice under the control of the CD2 promoter. The results showed that CIA was significantly suppressed in T-bet–Tg mice as compared with B6 mice. IL-17 production was not detected in type II collagen (CII)–reactive T cells from T-bet–Tg mice, and a significant reduction in IL-17 production by CII-reactive CD4+ T cells from T-bet–Tg mice was observed even when they were cocultured with splenic dendritic cells (DCs) from B6 mice. IFNγ production was also reduced in T-bet–Tg mice as compared with B6 mice, and levels of IFNγ in CII-reactive CD4+ T cells from T-bet–Tg mice were not different from those in B6 mice. Inhibition of Th17 cell differentiation and predominant differentiation of Th1 cells under an in vitro condition favoring Th17 cell differentiation was observed in T-bet–Tg mice, and surprisingly, this inhibition was also observed in T-bet–Tg/IFNγ$^{-/-}$ mice. These results indicate suppression of Th17 cell differentiation by overexpression of T-bet, but not IFNγ. Our findings support the notion that the suppression of autoimmune arthritis in T-bet–Tg mice might be due to the direct inhibition of Th17 cell differentiation by T-bet overexpression in T cells.

MATERIALS AND METHODS

Mice. CD2 T-bet–Tg mice (12) were prepared by backcrossing mice on a C57BL/6 background. IFNγ$^{-/-}$ mice were obtained from The Jackson Laboratory. Littermates of T-bet–Tg mice were used as controls in all experiments. All mice were maintained under specific pathogen–free conditions, and the experiments were conducted in accordance with the institutional ethics guidelines.

Induction of CIA and assessment of arthritis. Native chicken CII (Sigma-Aldrich) was dissolved in 0.01*M* acetic acid and emulsified in Freund's complete adjuvant (CFA). CFA was prepared by mixing 5 mg of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco) and 1 ml of Freund's incomplete adjuvant (Sigma-Aldrich). Mice ages 8–10 weeks were injected intradermally at the base of the tail with 200 μg of CII in CFA on days 0 and 21. Arthritis was evaluated visually, and changes in each paw were scored on a scale of 0–3, where 0 = normal, 1 = slight swelling and/or erythema, 2 = pronounced swelling, and 3 = ankylosis. The scores in the 4 limbs were then summed (maximum score 12).

Histopathologic scoring. For histologic assessment, mice were killed on day 42 after the first immunization, and both rear limbs were removed. After fixation and decalcification, joint sections were cut and stained with hematoxylin and eosin. Histologic features of arthritis were quantified by 2 independent observers (YK and IM) who were blinded with regard to the study group, and a histologic score was assigned to each joint based on the degree of inflammation and erosion, as described previously (13). The severity of inflammation was scored on a scale of 0–5, where 0 = normal, 1 = minimal inflammatory infiltration, 2 = mild infiltration with no soft tissue edema or synovial lining cell hyperplasia, 3 = moderate infiltration with surrounding soft tissue edema and some synovial lining cell hyperplasia, 4 = marked infiltration, edema, and synovial lining cell hyperplasia, and 5 = severe infiltration with extended soft tissue edema and marked synovial lining cell hyperplasia. The severity of bone erosion was also scored on a scale of 0–5, where 0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, and 5 = severe erosion with full-thickness defects in the cortical bone.

Analysis of cytokine profiles and cytokine and transcriptional factor gene expression. Inguinal and popliteal lymph nodes were harvested from each mouse on day 10 after the first immunization with CII. Single-cell suspensions were prepared, and lymph node cells (2×10^5/well on a 96-well round-bottomed plate) were cultured for 72 hours in RPMI 1640 medium (Sigma-Aldrich) containing 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 50 μ*M* 2-mercaptoethanol in the presence of 100 μg/ml of denatured chicken CII. The supernatants were analyzed for IFNγ, IL-4, IL-10, and IL-17 by enzyme-linked immunosorbent assay (ELISA) using specific Quantikine ELISA kits (R&D Systems).

Lymphocytes harvested on day 10 after immunization were used to obtain complementary DNA (cDNA) by reverse transcription, using a commercially available kit. A TaqMan Assay-on-Demand gene expression product was used for real-time polymerase chain reaction (PCR; Applied Biosystems). The expression levels of *Ifng*, *Il17a*, *Tbx21*, *Rorc*, *Il12a*, and *Il23a* were normalized relative to the expression of *gapdh*. Analyses were performed with an ABI Prism 7500 apparatus (Applied Biosystems).

Criss-cross coculture with CD4+ T cells and CD11c+ splenic dendritic cells. Ten days after the first CII immunization, CD4+ cells in draining lymph nodes were isolated by

positive selection, using a magnetic-activated cell sorter (MACS) system with anti-CD4 monoclonal antibody (mAb; Miltenyi Biotec). After treatment with mitomycin C, CD11c+ cells were isolated from the spleen by positive selection, using a MACS system with anti-CD11c mAb (Miltenyi Biotec). Criss-cross coculture for 72 hours was performed with 1×10^5 CD4+ cells and 2×10^4 CD11c+ cells in 100 μg/ml of denatured CII-containing medium. Cytokine production and transcription factor expression were then analyzed.

Measurement of collagen-specific immunoglobulin titers. Serum was collected from the mice on day 56 after the first immunization. A total of 10 μg/ml of CII in phosphate buffered saline (PBS) was coated overnight at 4°C onto 96-well plates (Nunc MaxiSorp; Nalge Nunc). After washes with washing buffer (0.05% Tween 20 in PBS), the blocking solution, including 1% bovine serum albumin in PBS, was applied for 1 hour. After washing, 100 μl of diluted serum was added, and the plates were incubated for 1 hour at room temperature. After further washing, horseradish peroxidase–conjugated anti-mouse IgG, IgG1, IgG2a, or IgG2b (1:5,000 dilution) in blocking solution was added, and the plates were incubated for 1 hour at room temperature. After washing, tetramethylbenzidine was added, and the optical density was read at 450 nm using a microplate reader.

Purification of CD4+ cells and in vitro T cell cultures. CD4+ cells (1×10^6/well) were cultured in medium with 1 μg/ml of soluble anti-CD3ε mAb (eBioscience), 1 μg/ml of soluble anti-CD28 mAb (BioLegend), 10 μg/ml of anti-IFNγ mAb (BioLegend), and 10 μg/ml of anti–IL-4 mAb (BioLegend) for a neutral condition. For Th17 cell differentiation, CD4+ cells (1×10^6/well) were cultured in medium with 1 μg/ml of soluble anti-CD3ε mAb, 1 μg/ml of soluble anti-CD28 mAb, 3 ng/ml of human TGFβ (R&D Systems), 20 ng/ml of mouse IL-6 (eBioscience), 10 μg/ml of anti-IFNγ mAb, and 10 μg/ml of anti–IL-4 mAb. On day 4, cells were restimulated for 4 hours with 50 ng/ml of phorbol myristate acetate and 500 ng/ml of ionomycin and used in the experiments.

Surface and intracellular staining and fluorescence-activated cell sorter (FACS) analysis. GolgiStop (BD PharMingen) was added during the last 6 hours of each culture. Cells were stained extracellularly, fixed, and permeabilized with Cytofix/Cytoperm solution (BD PharMingen). Then, intracellular cytokine staining was performed according to the manufacturer's protocol, using fluorescein isothiocyanate (FITC)–conjugated anti-IFNγ (BD PharMingen) and phycoerythrin (PE)–conjugated anti–IL-17 (BD PharMingen) or FITC-conjugated anti–IL-17 (BioLegend). A Treg cell staining kit (eBioscience) was used to stain T-bet, RORγt, and FoxP3 in cultured cells according to the manufacturer's protocol, using PE-conjugated anti–T-bet (eBioscience), allophycocyanin-conjugated anti-RORγt (eBioscience), and PE-conjugated anti-FoxP3 (eBioscience). Samples were analyzed with a FACSCalibur flow cytometer (Becton Dickinson), and data were analyzed with FlowJo software (Tree Star).

Statistical analysis. Data are expressed as the mean ± SEM or the mean ± SD. Differences between groups were examined for statistical significance using Student's *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Construction of the T-bet transgene and tissue distribution of transcription factors and cytokine production in naive mice. To generate transgenic mouse lines that express high levels of T-bet specifically in T cells, mouse T-bet cDNA was inserted into a VA vector containing a human CD2 transgene cassette (14). To confirm the expression of the transgene, reverse transcription–PCR (RT-PCR) was performed to monitor the expression of *Tbx21* (coding for T-bet) in organs from the T-bet–Tg mice. *Tbx21* messenger RNA (mRNA) expression was detected in the lymphatic system and in nonlymphatic organs in T-bet–Tg mice, and the expression levels were higher than those in B6 mice (data available upon request from the author). Analysis by semiquantitative RT-PCR and quantitative PCR (data not shown) revealed that the expression levels of other transcription factors (*Gata3*, *Rorc*, and *Foxp3*) in T-bet–Tg mice were not different from those in B6 mice. As previously reported by Ishizaki et al (14), high production of IFNγ was observed even when CD4+ T cells isolated from the spleen of T-bet–Tg mice were cultured under neutral conditions (data available upon request from the author).

Failure to induce CIA and low CII-specific IgG production in T-bet–Tg mice. To assess whether T cell–specific T-bet expression affects the development of arthritis, we induced CIA in T-bet–Tg mice and in wild-type B6 mice. The incidence and severity of arthritis in T-bet–Tg mice were markedly suppressed compared with those in B6 mice (Figure 1A). Surprisingly, the majority of T-bet–Tg mice were essentially free of arthritis, and even when arthritis was present, it was of the mild type. Consistent with these findings, histologic analyses of the joints obtained from each mouse 42 days after immunization revealed that joint inflammation and destruction were significantly suppressed in T-bet–Tg mice compared with B6 mice (Figures 1B and C). These results indicated that enforced expression of T-bet in T cells suppressed the development of CIA.

Because the levels of CII-specific IgG correlate well with the development of arthritis (15), we examined CII-specific IgG production in T-bet–Tg mice. CII-specific IgG, IgG1, IgG2a, and IgG2b levels were significantly lower in T-bet–Tg mice than in B6 mice, as determined by ELISA (Figure 1D). Thus, enforced expression of T-bet in T cells suppresses the development of CIA and CII-specific IgG production.

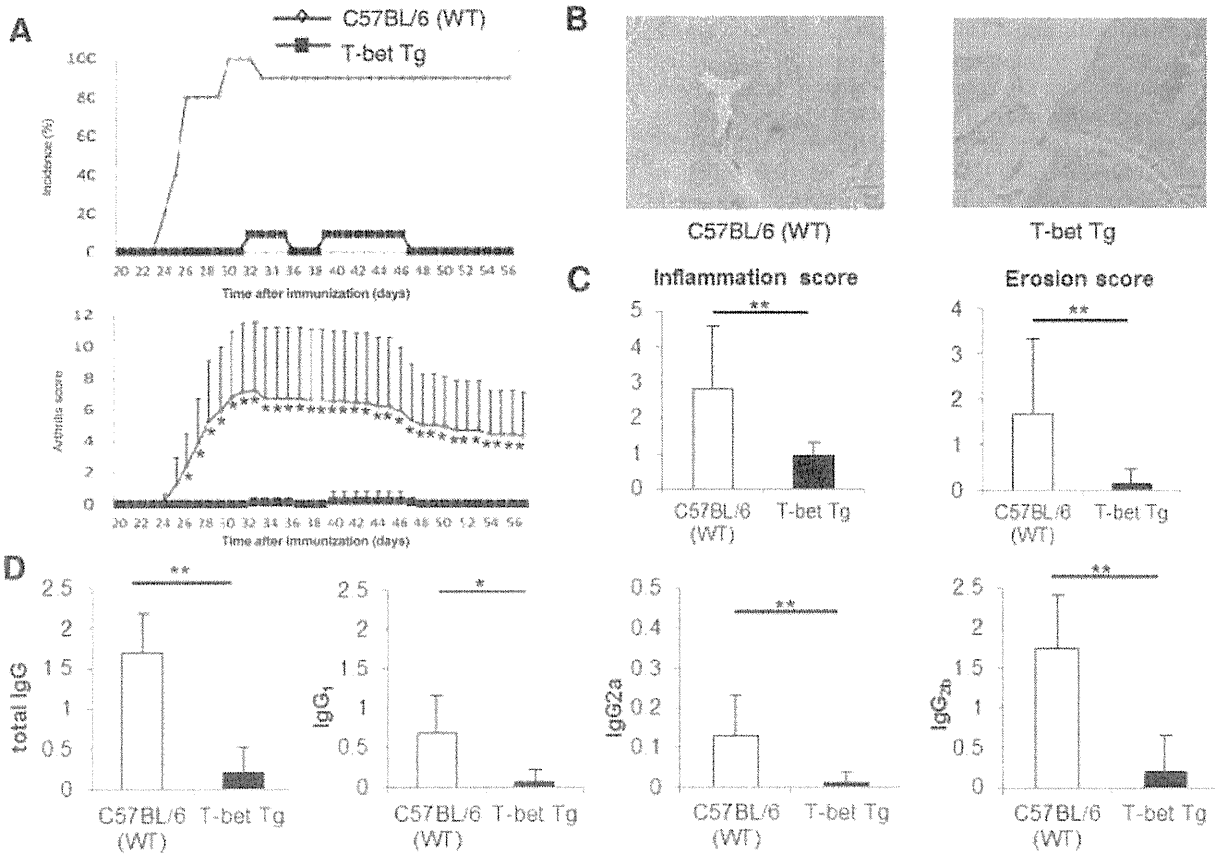

Figure 1. Significant suppression of collagen-induced arthritis (CIA) and type II collagen (CII)–specific IgG production in T-bet–transgenic (Tg) mice. On days 0 and 21, mice were immunized intradermally at several sites at the base of the tail with chicken CII emulsified with Freund's complete adjuvant. **A**, Incidence and severity of CIA. The arthritis score was determined as described in Materials and Methods. Data were obtained from 2 independent experiments involving 10 C57BL/6 (wild-type [WT]) mice and 11 T-bet–Tg mice. **B**, Hematoxylin and eosin–stained sections of the hind paws of mice obtained 6 weeks after the first immunization. Original magnification × 40. **C**, Inflammation and bone erosion scores in 7 C57BL/6 mice and 5 T-bet–Tg mice 6 weeks after the first immunization. Scores were determined as described in Materials and Methods. **D**, Serum levels of CII-specific IgG, IgG1, IgG2a, and IgG2b levels in 10 C57BL/6 mice and 11 T-bet–Tg mice 8 weeks after the first immunization, as measured by enzyme-linked immunosorbent assay. Values in **A**, **C**, and **D** are the mean ± SD. * = $P < 0.05$; ** = $P < 0.01$ by Student's *t*-test.

Suppression of CII-reactive IL-17 production and IL-17 mRNA expression in T-bet–Tg mice. Because enforced T-bet expression in T cells suppressed the development CIA, we examined antigen-specific cytokine production and transcription factor expression in mice with CIA. CD4+ T cells harvested from draining lymph nodes were stimulated with CII in vitro, and then various cytokine levels in the supernatants were measured by ELISA. IL-17 production by CII-reactive T cells was significantly reduced in T-bet–Tg mice as compared with B6 mice (Figure 2A). IFNγ production by CII-reactive T cells also tended to be decreased in T-bet–Tg mice.

We analyzed CII-reactive cytokine and transcription factor mRNA expression levels by real-time PCR (Figure 2B). Similar to the ELISA results, *Il17a* expression tended to be lower in T-bet–Tg mice than in B6 mice. No difference in *Ifng* expression was observed between B6 and T-bet–Tg mice (Figure 2B). *Tbx21* expression tended to be higher in T-bet–Tg mice, whereas *Rorc* expression was lower in T-bet–Tg mice than in B6 mice ($P < 0.05$). The level of expression of *Il12a* (coding for IL-12p35) was also higher in T-bet–Tg mice than in B6 mice ($P < 0.05$). However, there was no difference in the expression levels of *Il23a* (coding for IL-23p19) between B6 mice and T-bet–Tg mice. These

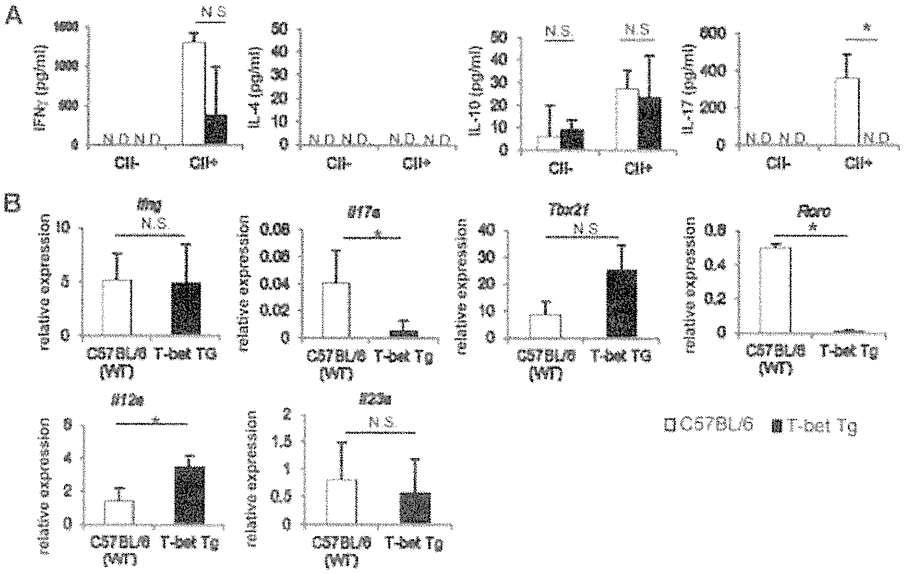

Figure 2. No production of interleukin-17 (IL-17) and low production of interferon-γ (IFNγ) in type II collagen (CII)–reactive CD4+ T cells. **A,** Ten days after the first CII immunization, lymphocytes derived from the draining lymph nodes of C57BL/6 (wild-type [WT]) mice and T-bet–transgenic (Tg) mice were cultured for 72 hours in the presence or absence of 100 μg/ml of denatured CII. Levels of IL-17, IFNγ, IL-4, and IL-10 in the supernatants were measured by enzyme-linked immunosorbent assay. **B,** After culture of lymphocytes with CII, cDNA was obtained, and levels of *Ifng*, *Il17a*, *Tbx21*, *Rorc*, *Il12a*, and *Il23a* expression were analyzed by real-time polymerase chain reaction. Values are the mean ± SD of 3 mice. * = $P < 0.05$ by Student's *t*-test. ND = not detected; NS = not significant.

results suggest that overexpression of T-bet on CD4+ T cells suppressed the expression of RORγt and IL-17.

No reduction of RORγt expression on CII-reactive CD4+ T cells in T-bet–Tg mice. CD4+ T cells from T-bet–Tg and B6 mice were cultured in vitro with CII, and analyses of T-bet and RORγt expression on CD4+ T cells were carried out by the intracellular staining method. T-bet expression on CII-reactive CD4+ T cells was significantly higher in T-bet–Tg mice than in B6 mice (Figure 3A). Surprisingly, the majority of T-bet+ CII-reactive T cells expressed RORγt in both the B6 mice and the T-bet–Tg mice (Figure 3A). Although there was no significant difference in the mean fluorescence intensity of RORγt between B6 mice and T-bet–Tg mice, the number of RORγt+ cells tended to be lower in T-bet–Tg mice (data available upon request from the author).

Moreover, in the case of CD4+ T cells examined under conditions favoring Th17 differentiation, RORγt expression on CD4+ T cells from T-bet–Tg mice was lower than that on cells from B6 mice (Figure 3B). Interestingly, most of the RORγt+ cells also expressed T-bet in the T-bet–Tg mice, and the proportion of IL-17–producing RORγt+ CD4+ T cells was lower in the T-bet–Tg mice than in the B6 mice. These findings support the notion that overexpression of T-bet not only suppresses RORγt expression on CD4+ T cells but also inhibits the production of IL-17 from RORγt+ T cells.

To investigate whether the suppression of arthritis and low antigen-specific cytokine production observed in T-bet–Tg mice was related to Treg cells, the next experiment analyzed FoxP3 expression on CD4+ T cells harvested from draining lymph nodes 10 days after immunization. There was no significant difference in the percentage of FoxP3+ cells among the CD4+ T cells between B6 mice and T-bet–Tg mice (data available upon request from the author). Thus, Treg cells do not seem to be involved in the suppression of CIA in T-bet–Tg mice.

Decreased numbers of T cells in the lymph nodes, spleen, and thymus of T-bet–Tg mice. To evaluate the low cytokine response and the low population of CII-reactive RORγt+CD4+ T cells in T-bet–Tg mice with CIA, we analyzed the lymphocyte subsets in the draining lymph nodes and spleen after immunization. The percentage and absolute number of CD3+ T cells were lower in both the draining lymph nodes and the

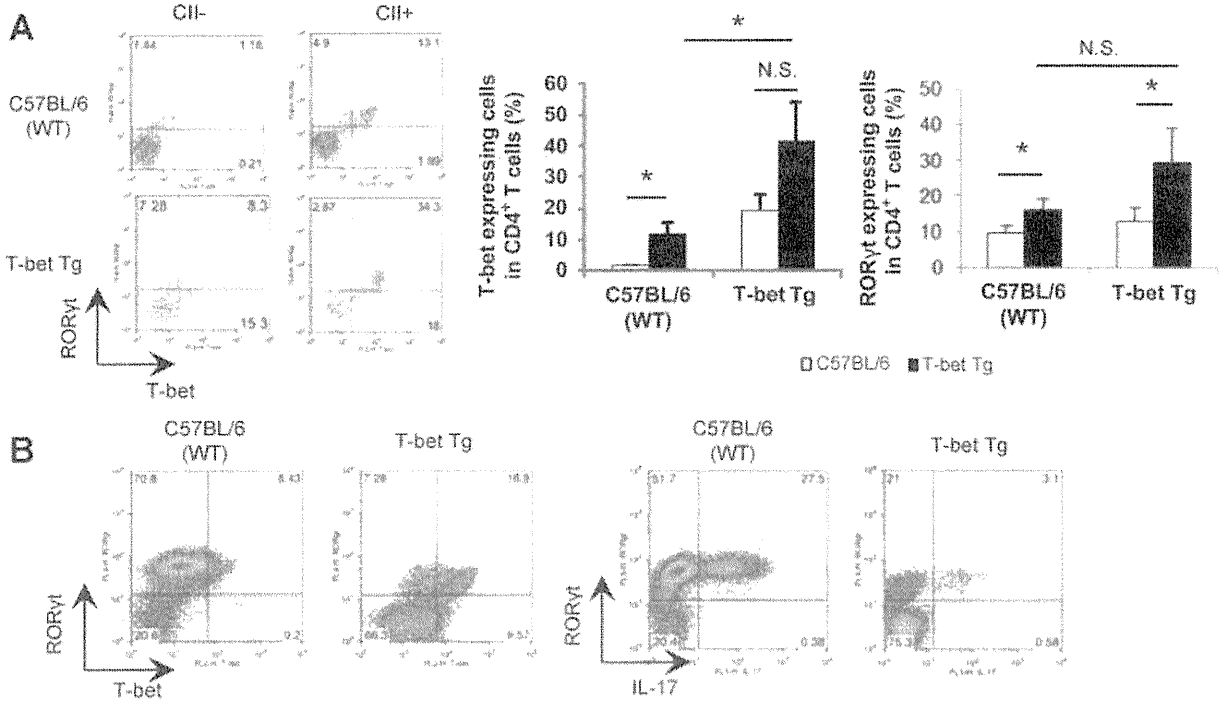

Figure 3. Suppression of Th17 cell differentiation by enforced expression of T-bet in T cells despite expression of retinoic acid receptor–related orphan nuclear receptor γt (RORγt). **A**, Ten days after the first type II collagen (CII) immunization, lymphocytes derived from the draining lymph nodes of C57BL/6 (wild-type [WT]) and T-bet–transgenic (Tg) mice were cultured for 72 hours in the presence or absence of 100 μg/ml of denatured CII. Levels of T-bet and RORγt expression on CD4+ T cells were analyzed by intracellular staining. Numbers in each compartment of the histograms are the percentage of transcription factor–expressing cells gated on CD4+ T cells. Values in the bar graphs are the mean ± SD of 3 mice per group. * = $P < 0.05$ by Student's *t*-test. NS = not significant. **B**, CD4+ T cells were isolated from the spleen of C57BL/6 and T-bet–Tg mice by magnetic-activated cell sorting and were then cultured for 96 hours with soluble anti-CD3 antibody, soluble anti-CD28 antibody, interleukin-6 (IL-6), and transforming growth factor β. Cytokine production and transcription factor expression on CD4+ T cells were analyzed by intracellular staining. Representative histograms from flow cytometric analysis of T-bet and RORγt expression with IL-17 production are shown. Numbers in each compartment are the percentage of positive cells gated on CD4+ T cells.

spleen of T-bet–Tg mice as compared with B6 mice (Figures 4A and B). The absolute number of CD4+ and CD8+ T cells also tended to be lower in T-bet–Tg mice (Figure 4B). Moreover, analysis of the thymus showed a significantly low number of total thymocytes in T-bet–Tg mice and the presence of an abnormal proportion of T precursor cells, such as a low number of double-positive T cells and CD4 single-positive T cells in T-bet–Tg mice (Figure 4C). These results suggest abnormal T cell development in the thymus of T-bet–Tg mice.

Inhibition of IL-17 production by CII-reactive CD4+ T cells in T-bet–Tg mice. To clarify whether T-bet overexpression on CD4+ T cells directly affects cytokine production, we performed criss-cross experiments using CD4+ T cells from B6 and T-bet–Tg mice, as well as DCs from B6 and T-bet–Tg mice in CII–containing medium, and measured IL-17 and IFNγ levels in the supernatants by ELISA. IL-17 production was detected in CII-reactive CD4+ T cells from B6 mice and in DCs from T-bet–Tg mice. Interestingly, IL-17 production was significantly reduced, even when CD4+ T cells from T-bet–Tg mice were cocultured with DCs from B6 mice (Figure 5A). These observations suggest that T-bet overexpression on CD4+ T cells is responsible for the inhibition of CII-reactive IL-17 production. No difference in IFNγ production was noted among the experimental conditions (Figure 5A), suggesting that reduced IFNγ production by CII-reactive CD4+ T cells from T-bet–Tg mice (Figure 2) was probably related to the reduced numbers of CD4+ T cells in draining lymph nodes. Moreover, intracellular staining revealed that RORγt expression was suppressed and T-bet expression was increased, even when CD4+ T cells from T-bet–Tg

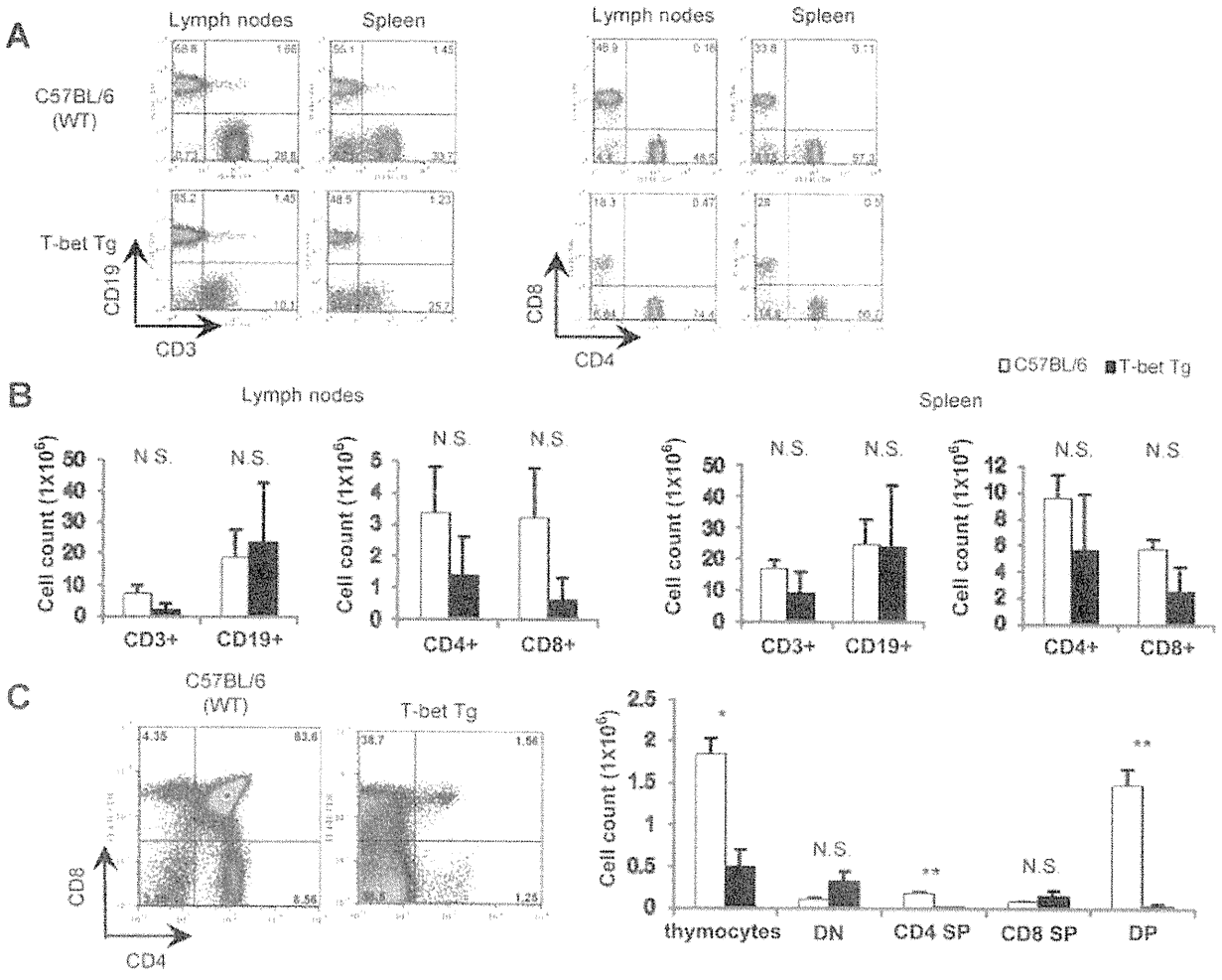

Figure 4. Decreased number of CD3+ T cells in spleen and lymph nodes and abnormal development of T precursor cells in the thymus in T-bet–transgenic (Tg) mice. **A**, Ten days after first immunization, the proportion of lymphocytes in draining lymph nodes and spleen were analyzed by fluorescence-activated cell sorting (FACS), and the absolute numbers of cells were calculated. Numbers in each compartment are the percentage of the parent population. **B**, The absolute numbers of CD3+, CD19+, CD4+, and CD8+ T cells in the lymph nodes and spleen of C57BL/6 (wild-type [WT]) and T-bet–Tg mice were determined. Values are the mean ± SD of 3 mice per group. NS = not significant. **C**, The proportion of T precursor cells in the thymus of nonimmunized mice was analyzed by FACS, and the absolute numbers of thymocytes, double-negative (DN) T cells, CD4 and CD8 single-positive (SP) T cells, and double-positive (DP) T cells were determined. Values in the bar graphs are the mean ± SD of 3 mice per group. * = $P < 0.05$; ** = $P < 0.01$ by Student's *t*-test.

mice were cocultured with DCs from B6 mice (Figure 5B). These results indicate that T-bet overexpression on CD4+ T cells suppressed CII-reactive IL-17 production by inhibition of the expression of RORγt.

Overexpression of T-bet directly suppresses Th17 cell differentiation via IFNγ-independent mechanisms. To clarify whether IFNγ production influences Th17 cell differentiation, we generated T-bet–Tg/IFNγ$^{-/-}$ mice. CD4+ T cells were isolated from the spleen of T-bet–Tg, T-bet–Tg/IFNγ$^{-/-}$, and B6 mice and were then cultured for Th17 cell differentiation. FACS analysis demonstrated that the proportion of IL-17–producing CD4+ T cells was lower in T-bet–Tg mice than in B6 mice, whereas the proportion of IFNγ-producing CD4+ T cells was higher in T-bet–Tg mice. Similarly, the proportion of IL-17–producing CD4+ T cells was also lower in T-bet–Tg/IFNγ$^{-/-}$ mice, although no IFNγ-producing CD4+ T cells were detected in

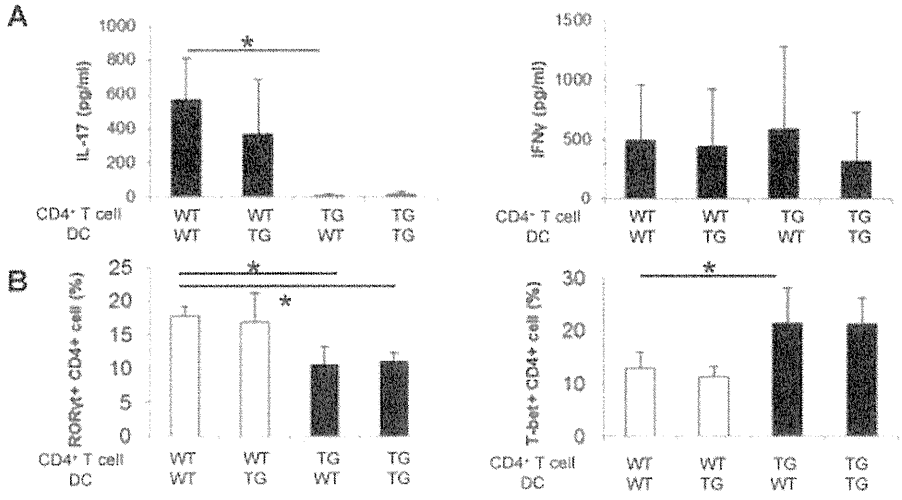

Figure 5. Impaired antigen-specific Th17 cell responses in T-bet–transgenic (Tg) mice with collagen-induced arthritis (CIA). Ten days after the first type II collagen (CII) immunization, CD4+ cells were isolated from draining lymph nodes of C57BL/6 (wild-type [WT]) mice and T-bet–Tg (TG) mice by positive selection using magnetic-activated cell sorting (MACS) with anti-CD4 monoclonal antibody (mAb). After treatment with mitomycin C, CD11c+ cells were isolated from the spleen by positive selection using a MACS system with anti-CD11c mAb. Criss-cross coculture for 72 hours was performed with 1×10^5 CD4+ cells and 2×10^4 CD11c+ cells in 100 μg/ml of denatured CII–containing medium. **A,** Levels of interleukin-17 (IL-17) and interferon-γ (IFNγ) in culture supernatants were measured by enzyme-linked immunosorbent assay. **B,** Expression of retinoic acid receptor–related orphan nuclear receptor γt (RORγt) and T-bet expression on CD4+ T cells were analyzed by intracellular staining. Representative data from flow cytometric analysis of the percentage of RORγt+ or T-bet+ cells in the CD4+ T cell subset are shown. Values are the mean ± SD of 3 mice per group. * = $P < 0.05$ by Student's *t*-test. DC = dendritic cells.

T-bet–Tg/IFN$\gamma^{-/-}$ mice (Figure 6). These results strongly support the view that inhibition of Th17 cell differentiation in T-bet–Tg mice cannot be due to overproduction of IFNγ, indicating that overexpression of T-bet directly suppresses Th17 cell differentiation in T-bet–Tg mice.

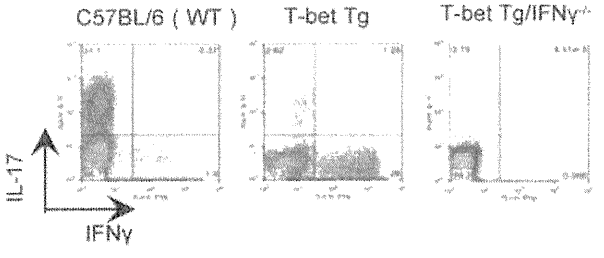

Figure 6. Suppressed expression of interleukin-17 (IL-17) by T-bet overexpression independently of interferon-γ (IFNγ) in T-bet–transgenic (Tg) mice. CD4+ T cells were isolated from the spleen of C57BL/6 (wild-type [WT]), T-bet–Tg, and T-bet–Tg/IFN$\gamma^{-/-}$ mice by magnetic-activated cell sorting and then cultured for 96 hours with soluble anti-CD3 monoclonal antibody (mAb), soluble anti-CD28 mAb, IL-6, and transforming growth factor β. IFNγ and IL-17 production by CD4+ cells was analyzed by intracellular cytokine staining. Numbers in each compartment are the percentage of cells secreting cytokines.

DISCUSSION

Recent studies showed that IL-17 plays a crucial role in the development of CIA (3) and other types of experimental arthritis (2). In contrast, it has been reported that IFNγ can suppress IL-17 production in vitro (16) and has antiinflammatory effects on the development of experimental arthritis (4,5). T-bet is a transcription factor known to induce the differentiation of naive CD4+ T cells to Th1 cells (8). Although the absence of T-bet can result in severe IL-17–mediated experimental autoimmune myocarditis via dysregulation of IFNγ (17), several studies have shown that T-bet is essential for the development of several models of autoimmunity, such as experimental autoimmune encephalitis (18,19), colitis (20), and diabetes mellitus (21). Nevertheless, the effect of T-bet expression on Th17 cell differentiation and function during arthritis remains unclear.

T-bet–Tg mice overexpress T-bet and mainly produce IFNγ in their T cells (14). Previous studies in T-bet–Tg mice suggested that overexpression of T-bet and a predominant Th1 response affect the pathogenesis of various diseases (14,22,23). To examine whether T-bet overexpression on T cells affects the regulation of

autoimmune arthritis, we induced CIA in T-bet–Tg mice and found marked suppression of CIA in T-bet–Tg mice.

To determine the reason for the low incidence of CIA in T-bet–Tg mice, we measured CII-reactive cytokine production and expression in vitro. IL-17 production from CII-reactive CD4+ T cells and *Il17a* expression were reduced in T-bet–Tg mice as compared with B6 mice. Although a predominant Th1 cell response was reported by Ishizuka et al (14), CII-specific IFNγ production was reduced in T-bet–Tg mice, and no significant difference was observed in *Ifng* expression between B6 mice and T-bet–Tg mice. Furthermore, *Il12a* expression was significantly higher in T-bet–Tg mice than in B6 mice, suggesting that overexpression of T-bet on T cells seems to affect innate immune cells, because the main producers of IL-12 are DCs and macrophages, not CD4+ T cells.

In criss-cross coculture experiments with CD4+ T cells and splenic DCs from B6 mice and T-bet–Tg mice, CII-reactive IL-17 production was also reduced even when CD4+ T cells from T-bet–Tg mice were cocultured with DCs from B6 mice, although there was no significant difference in IL-17 production by CD4+ T cells from B6 mice cocultured with DCs from either B6 mice or T-bet–Tg mice. In contrast, no difference in IFNγ production was observed under all coculture conditions examined. Moreover, suppression of RORγt expression and high expression of T-bet on CD4+ T cells were observed even when CD4+ T cells from T-bet–Tg mice were cocultured with DCs from B6 mice. These findings indicate that T-bet overexpression on CD4+ T cells might suppress CII-reactive IL-17 production resulting from suppression of RORγt expression in an IFNγ-independent manner, and that overexpression of T-bet has no direct effect on DC function.

CII-specific IgG levels correlate well with the development of arthritis (15). We observed significant suppression of CII-specific IgG production in the T-bet–Tg mice as compared with the B6 mice. A previous study showed that IL-17 is required for anti-CII antibody production (3). Therefore, the suppression of anti-CII antibody formation might be due to lower CII-reactive IL-17 production in T-bet–Tg mice.

To evaluate the low cytokine response to CII in T-bet–Tg mice, we analyzed lymphocytes obtained after immunization from draining lymph nodes and spleen. The percentage and absolute number of T cells tended to be lower in both the draining lymph nodes and spleen of T-bet–Tg mice compared with B6 mice. Moreover, significantly lower numbers of total thymocytes and an abnormal proportion of T precursor cells were observed in T-bet–Tg mice. The latter phenomenon could be due to T-bet transgene expression on double-negative thymic cells in T-bet–Tg mice. Because previous observations showed that T-bet interferes with GATA-3 function (11) and that GATA-3 was required for the development of early thymic T cells (24), one of the reasons for abnormal T cell development in the thymus might be the dysfunction of GATA-3 by overexpression of T-bet. These results suggest that overexpression of T-bet in thymic T cells affects T cell development, is responsible for the low number of T cells in spleen and lymph nodes, and is related to the low cytokine production against CII in T-bet–Tg mice.

To assess the effect of T-bet on CD4+ T cell differentiation in T-bet–Tg mice, we performed in vitro induction of Th17 cells. Analysis of T-bet–Tg mice showed a reduction in IL-17–producing CD4+ T cells and an increase in IFNγ-producing CD4+ T cells in spite of the condition favoring Th17 differentiation, which indicates suppression of Th17 cell differentiation and predominance of Th1 cell differentiation in vitro in T-bet–Tg mice. These results did not contradict the previous findings that the phenotype of polarized Th1 cells was not affected by Th cell–polarizing conditions (25). It is possible that suppression of CII-reactive IL-17 production in T-bet–Tg mice was not associated with IFNγ. For this reason, we generated T-bet–Tg/IFNγ$^{-/-}$ mice and performed in vitro induction of Th17 cells in these mice. Surprisingly, in T-bet–Tg/IFNγ$^{-/-}$ mice, the levels of IL-17–producing CD4+ T cells were also markedly reduced under Th17 cell differentiation–favoring conditions, indicating an IFNγ-independent suppressive pathway against Th17 cell differentiation. Although previous studies showed that suppression of Th17 cell differentiation was mediated through IFNγ signal transduction (16), our findings allow us to propose a new hypothesis: Th17 cell differentiation is regulated by a pathway that is distinct from the IFNγ signaling pathway. Therefore, we suggest that T-bet expression either directly or indirectly suppresses Th17 cell differentiation via an IFNγ-independent mechanism.

Tbx21 expression was significantly higher in T-bet–Tg mice as compared with B6 mice, and FACS analysis of CII-reactive CD4+ T cells revealed a significantly higher percentage of T-bet+ cells among the CD4+ T cell subset in T-bet–Tg mice. While there was no significant difference in the percentage of RORγt+ cells among the CD4+ T cell subset in T-bet–Tg mice as compared with B6 mice, *Rorc* expression was down-regulated on CII-reactive CD4+ T cells in T-bet–Tg mice. In the case of CD4+ T cells under

conditions favoring Th17 cell differentiation, RORγt expression on CD4+ T cells from T-bet–Tg mice was lower than that on cells from B6 mice. Interestingly, most of the RORγt+ cells also expressed T-bet in T-bet–Tg mice, and the proportion of IL-17–producing RORγt+ T cells in the CD4+ cell subset was lower in T-bet–Tg mice than in B6 mice. These findings support the notion that overexpression of T-bet not only suppresses RORγt expression on CD4+ T cells, but also inhibits the production of IL-17 from RORγt+ T cells.

Previous studies showed that RORγt expression is positively regulated by several transcription factors, such as runt-related transcription factor 1 (RUNX-1), interferon regulatory factor 4, and STAT-3 (26–28). Lazarevic et al (29) recently reported that T-bet prevented RUNX-1–mediated activation of the gene encoding RORγt, followed by the suppression of Th17 cell differentiation. In addition to direct promotion of RORγt expression, RUNX-1 also acts as a coactivator, together with RORγt, and induces the expression of *Il17a* and *Il17f* (26); therefore, T-bet inhibits IL-17 production by RORγt+ cells induced by RUNX-1 (29). Although further studies will be required to identify the effect of T-bet overexpression on the function of RUNX-1, it might be associated with the suppression of Th17 cell differentiation that was observed in the T-bet–Tg mice.

In conclusion, our results demonstrated that overexpression of T-bet in T cells suppressed the development of autoimmune arthritis. The regulatory mechanism of CIA might involve dysfunction of CII-reactive Th17 cell differentiation by overexpression of T-bet via IFNγ-independent pathways. These findings should enhance our understanding of the pathogenesis of autoimmune arthritis and help in the development of new therapies for RA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Sumida had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Therapeutic Targeting of the Interleukin-6 Receptor

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interleukin-6, humanized anti-interleukin-6 receptor antibody, tocilizumab, autoimmune, inflammation

Abstract

Interleukin (IL)-6 is a typical cytokine featuring redundancy and pleiotropic activity. It contributes to host defense against pathogens, but dysregulation of IL-6 production plays a significant pathological role in various autoimmune and inflammatory diseases. Because IL-6 blockade was expected to constitute a novel strategy for the treatment of such diseases, tocilizumab, a humanized anti-IL-6 receptor antibody (anti-IL-6RAb), was developed. Clinical trials have demonstrated the efficacy of anti-IL-6RAb for patients with rheumatoid arthritis, Castleman's disease, and juvenile idiopathic arthritis, resulting in approval of this innovative biologic for the treatment of these diseases, and it can be expected to become a novel drug for various other autoimmune and inflammatory diseases. In murine models of autoimmune diseases, anti-IL-6RAb induces Treg and inhibits Th17 and/or Th1 differentiation, indicating that anti-IL-6RAb may be able to repair Th17/Treg imbalance in human diseases as well.

INTRODUCTION

IL-6: interleukin-6
AA: amyloid A

Human interleukin-6 (IL-6) is a secreted 21-kDa glycoprotein with a four-helix bundle structure containing 184 amino acids (1). IL-6 has a wide variety of functions because it acts not only on B cells but also on T cells, hepatocytes, hematopoietic progenitor cells, and fibroblasts (**Figure 1**) (2, 3). In liver, IL-6 strongly induces a broad spectrum of acute-phase proteins such as C-reactive protein (CRP), serum amyloid A (SAA), haptoglobin, antichymotrypsin, fibrinogen, and hepcidin, whereas it reduces albumin, fibronectin, transferrin, and cytochrome P450 (CYP) (4, 5). Changes in the levels of these proteins are commonly observed in acute and chronic inflammatory diseases, and prolonged inflammation leads to a pathological state. For example, high levels of hepcidin block iron transporter ferroportin 1 on macrophages, hepatocytes, and gut epithelial cells, leading to hypoferremia and anemia of inflammation (6), whereas a high level of SAA over long periods results in amyloid A (AA) amyloidosis (7). In lymphocytes, IL-6 induces B cell differentiation into immunoglobulin-producing cells. $CD4^+$ T helper cells show distinct effector functions after their differentiation, and a subset of IL-17-producing T helper cells (Th17) plays a crucial role in the induction of autoimmune tissue injury (8). Combined with transforming growth factor β (TGF-β), IL-6 is essential for Th17 differentiation from naive $CD4^+$ T cells, whereas IL-6 inhibits the generation of regulatory T cells (Treg) induced by TGF-β (8, 9). IL-6 also acts on $CD8^+$ T cells to induce cytotoxic T cells (10). In addition to the effects of IL-6 on acquired immunity,

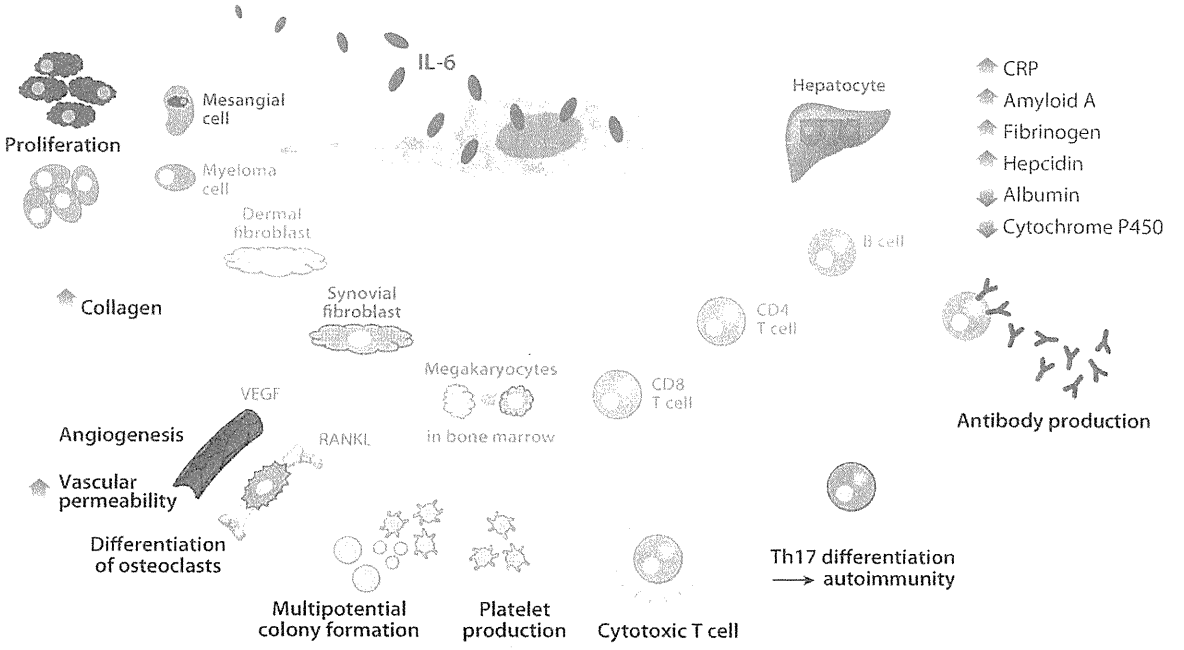

Figure 1

Interleukin-6 (IL-6), a multifunctional cytokine. IL-6 induces cell differentiation and specific gene expression. It induces production of acute-phase proteins such as CRP, amyloid A, fibrinogen, and hepcidin, whereas it reduces synthesis of albumin and cytochrome P450 in hepatocytes. IL-6 promotes immunoglobulin synthesis in activated B cells as well as Th17 and cytotoxic T cell differentiation from naïve T cells. In bone marrow, IL-6 induces maturation of megakaryocytes to platelets and activation of hematopoietic stem cells. IL-6 also acts on synovial fibroblast cells to produce RANKL and VEGF, which promote differentiation of osteoclasts and angiogenesis, respectively. Furthermore, IL-6 stimulates dermal fibroblasts to produce collagen and the growth of cells such as myeloma/plasmacytoma cells and mesangial cells. Abbreviations: CRP, C-reactive protein; RANKL, receptor activator of NF-κB ligand; VEGF, vascular endothelial growth factor.

IL-6 performs an important function as an in vivo SOS signal in the early phase of infections or injuries. In infectious inflammation, IL-6 is produced by monocytes and macrophages after the stimulation of Toll-like receptors (TLRs) with distinct pathogen-associated molecular patterns (PAMPs) of microbes via the myeloid differentiation factor 88 (MyD88)-dependent pathway (11). In noninfectious inflammation, such as burn or traumatic injury, damage-associated molecular patterns (DAMPs) from damaged or dying cells stimulate TLRs to produce IL-6 (12).

RA: rheumatoid arthritis

Anti-IL-6RAb: anti-interleukin-6 receptor antibody

The pathological significance of IL-6 for various diseases has been the subject of numerous reports. The relationship was first demonstrated in a case of cardiac myxoma. The culture fluid obtained from the myxoma tissues of a patient who presented with fever, arthritis with positivity for antinuclear factor, increased CRP level, and hypergammaglobulinemia contained a large quantity of IL-6 (13), which suggested that IL-6 may contribute to chronic inflammation and autoimmunity. Subsequent studies have shown that dysregulation of IL-6 production is implicated in the pathogenesis of Castleman's disease (14), rheumatoid arthritis (RA) (15), and various other autoimmune, inflammatory, and malignant diseases (2, 3, 16–18). In patients with RA, high levels of the IL-6/soluble IL-6 receptor (sIL-6R) complex in synovial fluids induce osteoclast-like cell formation, which is responsible for joint destruction (19). Moreover, IL-6 production in bone marrow stromal cells generates the receptor activator of NF-κB ligand (RANKL), which is an essential factor for the differentiation and activation of osteoclasts and bone resorption (20). Enhanced angiogenesis and vascular permeability of synovial tissue are pathological features of RA resulting from the excess production of vascular endothelial growth factor (VEGF), which is induced by IL-6 in synovial fibroblasts (21). The promotional activities of IL-6 may also contribute to autoimmune skin diseases such as psoriasis owing to the proliferation of keratinocytes or systemic sclerosis owing to the collagen production in dermal fibroblasts (22, 23).

The IL-6 receptor (IL-6R) system consists of two chains: IL-6R, which is the 80-kDa IL-6-binding subunit that has a short cytoplasmic domain (24), and gp130, which is the 130-kDa transmembrane glycoprotein. The latter transduces the IL-6 signal into cells (**Figure 2**) (25). The broad range of expression of gp130 on various cells suggests that IL-6 has pleiotropic effects because naturally occurring sIL-6R is present in human serum. Furthermore, even in cells lacking transmembrane IL-6R, the IL-6/sIL-6R complex can transduce the IL-6 signal on gp130-expressing cells (26). After binding of IL-6 to IL-6R, the resultant IL-6/IL-6R complex associates with gp130, and the activated IL-6 receptor complex is formed as a hexameric structure that includes two molecules each of IL-6, IL-6R, and gp130 (27, 28). The IL-6 signal is transduced into cells via gp130-JAK-STAT3 (signal transducer and activator of transcription 3) and gp130-JAK-SHP-2 (SH2-domain containing protein tyrosine phosphatase-2) pathways (**Figure 2**). IL-6R is a cognate binding receptor for IL-6, whereas gp130 is shared by cytokines of the IL-6 family including leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, IL-11, cardiotrophin 1, cardiotrophin-like cytokine, and IL-27 (29–31). These cytokines often show overlapping functions with those of IL-6 via the common signal transducer gp130.

HUMANIZED ANTI-INTERLEUKIN-6 RECEPTOR ANTIBODY (TOCILIZUMAB)

Because of the biological activities of IL-6 and its pathological role in diseases, IL-6 blockade was expected to constitute a novel treatment strategy for inflammatory and autoimmune diseases (17, 18, 32). In response to these expectations, anti-IL-6R antibody (anti-IL-6RAb) (chemical name: tocilizumab) was developed, which is a humanized anti-IL-6R monoclonal antibody of the IgG1 class that was generated by grafting the complementarity-determining regions of a mouse antihuman IL-6R antibody onto human IgG1. Anti-IL-6RAb blocks IL-6-mediated signal

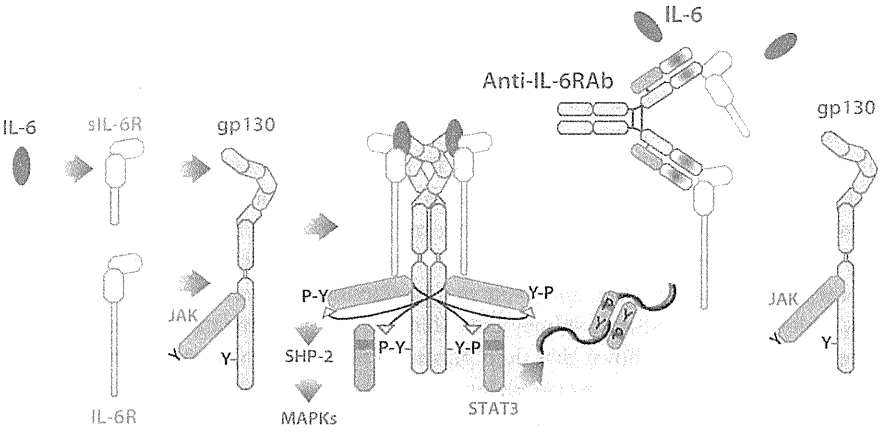

Figure 2

Anti-interleukin-6 receptor antibody (anti-IL-6RAb) blocks IL-6 binding to transmembrane IL-6R and soluble IL-6 receptor (sIL-6R). After binding of IL-6 to interleukin-6 receptor (IL-6R), the resultant IL-6/IL-6R complex associates with gp130 and induces the homodimerization of gp130, which triggers activation of Janus kinase family tyrosine kinases (JAKs) and tyrosine phosphorylation of gp130. The phosphorylated tyrosine motif of gp130 then recruits the signal transducer and activator of transcription 3 (STAT3) via the SH2-domain. Next, activated STAT3 translocates into the nucleus and regulates transcription for various sets of genes. Tyrosine-phosphorylated gp130 also recruits the SH2-domain containing protein tyrosine phosphatase-2 (SHP-2) and induces the association of SHP-2 with adaptor molecule growth factor receptor-bound protein 2 (Grb2), followed by activation of the son of Sevenless (SOS)/Ras–Raf–mitogen-activated protein kinase kinase (MEK)–MAP kinase (MAPK) pathway. Tocilizumab, a humanized anti-IL-6RAb, binds to transmembrane IL-6R and sIL-6R and competitively blocks binding of IL-6 to IL-6R or sIL-6R, leading to the inhibition of IL-6R-mediated signaling.

transduction by inhibiting IL-6 binding to membrane-bound IL-6R and sIL-6R (**Figure 2**). For RA, the recommended anti-IL-6RAb posology in Japan and the European Union is 8 mg kg^{-1} once every 4 weeks. In clinical terms, if free anti-IL-6RAb concentration is maintained at more than 1 μg ml^{-1}, CRP remains negative (33). The concentration of CRP is therefore a hallmark for determining whether IL-6 activity is completely blocked in vivo, although physicians should address the fact that CRP does not function as an acute inflammatory marker during anti-IL-6RAb treatment. Anti-IL-6RAb exposure for RA patients is not affected by concomitantly administered medications such as methotrexate, corticosteroids, and nonsteroidal anti-inflammatory drugs (34), but it may reverse IL-6-induced suppression of CYP activity and thus lead to reduced exposure to certain CYP3A4 substrates (35).

ANTI-INTERLEUKIN-6 RECEPTOR ANTIBODY FOR AUTOIMMUNE DISEASES

Rheumatoid Arthritis

RA is a chronic, progressive inflammatory disease of the joints and surrounding tissues characterized by intense pain, irreversible joint destruction, and systemic complications (36). The biological activities of IL-6 as described above and its elevation in serum as well as synovial fluids in patients with RA indicate that IL-6 is one of the key cytokines involved in the development of RA.

Seven Phase III clinical trials of anti-IL-6RAb demonstrated its efficacy either as monotherapy or in combination with disease-modifying antirheumatic drugs for adult patients with moderate to severe RA (37–43). A Cochrane database systematic review concluded that of patients taking

concomitant methotrexate, compared with placebo, anti-IL-6RAb-treated patients were four times more likely to achieve American College of Rheumatology (ACR) 50% improvement (38.8% versus 9.6%) and 11 times more likely to achieve Disease Activity Score remission (30.5% versus 2.7%) (44). Moreover, the SAMURAI (37) and LITHE studies (43) proved that radiological damage of joints was significantly inhibited by the treatment. As a result, anti-IL-6RAb has now been approved for the treatment of RA in more than 90 countries worldwide. The outstanding results obtained with biologics such as anti-IL-6RAb in the treatment of RA led to a change in the treatment objective from protection against joint destruction to prolongation of life expectancy with normal activities of daily living.

SLE: systemic lupus erythematosus

The safety and tolerability profiles of anti-IL-6RAb monotherapy for Japanese RA patients obtained from six initial trials and five long-term extensions have been reported (45). For these studies, 601 patients with moderate to severe RA and with a total exposure of 2,188 patient-years were enrolled. The medial treatment duration was 3.8 years. The incidence of adverse events (AEs), including abnormal laboratory test results, was calculated as 465.1 per 100 patient-years, with infections being the most common serious AEs (6.22 per 100 patient-years). Abnormalities in the laboratory test results, such as increases in lipid and liver function parameters, were common, but most were mild. Of the patients treated for more than 5 years, 59.7% met the Disease Activity Score 28 remission criteria at 5 years, which demonstrates the excellent tolerability and high efficacy of anti-IL-6RAb. A systemic literature review to assess the risk of AEs for RA patients treated with anti-IL-6RAb reported that pooled odds ratios indicated statistical significance for an increased risk of AEs for patients treated with 8 mg kg^{-1} of the antibody plus methotrexate compared with controls (odds ratio = 1.53; 95% confidence interval = 1.26–1.86), as well as a heightened risk of infection (odds ratio = 1.30; 95% confidence interval = 1.07–1.58) (46). However, no increases in the incidence of malignancy or hepatitis were detected. Anti-TNF (anti–tumor necrosis factor) inhibitors significantly increased the frequency of reactivation of tuberculosis (47), whereas anti-IL-6RAb did not produce an increase. In fact, Okada et al. (48) examined the effects of IL-6 and TNFα blockade on the development of tuberculosis infection in mice and observed that there was less tuberculosis infection for anti-IL-6RAb than for anti-TNFα antibody. In addition, we showed that interferon (IFN)-γ synthesis by means of a QuantiFERON®-TB test was suppressed by the addition of anti-TNF inhibitors but not by the addition of anti-IL-6RAb (49).

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease of unknown etiology that affects mainly young women (50). The elevation of serum as well as local IL-6 levels of SLE patients indicates that IL-6 plays a pathological role in SLE (51–53). Moreover, mice with epidermal loss of JunB were recently reported to develop SLE phenotype linked to increased epidermal IL-6 secretion, and facial skin biopsies of human SLE patients revealed low JunB protein expression and high IL-6 and activated STAT3 levels within lupus lesions (54). In murine SLE models, IL-6 blockade by means of anti-IL-6RAb or anti-IL-6Ab prevents the onset and progression of the disease (55, 56). An open-label Phase I dosage-escalation study was performed with 16 patients that had mild-to-moderate disease activity (57). The patients were assigned to receive different doses of anti-IL-6RAb (2 mg kg^{-1} for 4 patients, 4 mg kg^{-1} for 6 patients, and 8 mg kg^{-1} for 6 patients) given intravenously every 2 weeks for 12 weeks. Disease activity showed significant improvement in 8 of the 15 evaluable patients, whereas arthritis improved in all 7 patients who had arthritis at baseline. Levels of anti-double-stranded DNA antibodies decreased by a median of 47%. There were no significant changes in total lymphocytes or in overall T or B lymphocyte counts, whereas the frequency of $CD38^{high}CD19^{low}IgD^{-}$ plasma cells, which was higher for SLE patients than

for normal controls (mean 5.3% versus 1.2%), was significantly reduced to 3.1% at 6 weeks. This result indicates that anti-IL-6RAb also represents a promising therapeutic biologic for SLE.

SSc: systemic sclerosis
PM: polymyositis
TA: Takayasu arteritis
GCA: giant cell arteritis
CD: Crohn's disease

Systemic Sclerosis

Systemic sclerosis (SSc) is a connective tissue disease characterized by fibrosis of the skin and internal organs as well as pronounced alterations in the microvasculature (58). IL-6 expression is reportedly high in serum of SSc patients, and its elevation correlates with the skin score (59). Furthermore, in vitro studies have demonstrated that IL-6 may contribute to fibrosis by inducing collagen production (23) and α-smooth muscle actin expression by dermal fibroblasts, which leads to their differentiation into myofibroblasts (60). The clinical effect of anti-IL-6RAb was examined in two SSc patients who had been resistant to conventional treatment regimens. Both patients showed softening of the skin with respective reductions of 52% and 23% in the modified Rodnan total skin score (61). Histological examination showed thinning of the collagen fiber bundles in the dermis. These improvements suggest that anti-IL-6RAb appears to be a promising biologic for the treatment of SSc.

Polymyositis

Polymyositis (PM) is an inflammatory myopathy characterized by the clinical features of progressive symmetrical muscle weakness and mononuclear inflammatory cell infiltrates in muscle tissue (62). PM appears to be another suitable target disease for IL-6R blockade for two reasons. First, excessive expression of IL-6 has been found in the sera and infiltrating mononuclear cells in the muscles of PM patients (63, 64). Second, in models of experimental myositis induced by myosin or C protein, IL-6 blockade by either gene knockout (KO) or anti-IL-6RAb administration showed a preventive or therapeutic effect on myositis (65, 66). We administered anti-IL-6RAb to two PM patients who had been refractory to corticosteroids and immunosuppressive drugs. Creatine phosphokinase levels of both patients normalized, and magnetic resonance images showed the disappearance of high-intensity zones in the thigh muscles (67). These findings suggest that anti-IL-6RAb may also be effective as a novel drug for refractory PM.

Takayasu Arteritis and Giant Cell Arteritis

Takayasu arteritis (TA) and giant cell arteritis (GCA), which involve both large and medium-sized arteries, are examples of vasculitis syndrome. The pathogenesis of TA and GCA remains unclear, but IL-6 is clearly involved in their development (68, 69). Anti-IL-6RAb treatment for a 20-year-old woman with refractory active TA improved the clinical manifestations and abnormal laboratory findings (70), and the antibody treatment induced a rapid remission in 5 patients with GCA and 2 patients with TA (71), which strongly suggests that IL-6 inhibition may become a treatment option for both TA and GCA.

Crohn's Disease

Crohn's disease (CD) is a chronic inflammatory bowel disease of unknown etiology, but IL-6 has been demonstrated to play a significant role in its development (72). Elevated levels of IL-6 have been detected in the blood and in the cultures of colonic mucosal specimens from CD patients (73, 74). In a colitis mouse model generated by transfer of $CD45RB^{high}CD4^{+}$ T cells into SCID mice, anti-IL-6RAb prevented the occurrence of signs and symptoms of colitis (75). A pilot randomized trial of anti-IL-6RAb for 36 patients with active CD demonstrated that 80% of the patients given 8 mg kg^{-1} every 2 weeks showed a clinical response compared with only 31% of placebo-injected patients, indicating that anti-IL-6RAb may also serve as a promising drug for CD (76).

Colitis-associated cancer and intestinal perforation are the most serious complications of inflammatory bowel diseases. IL-6 and STAT3 reportedly play an important role in the survival of intestinal epithelial cells and development of colitis-associated cancer (77), so anti-IL-6RAb might be able to suppress colorectal cancer development. In worldwide clinical trials conducted by Roche, 26 cases of gastrointestinal (GI) perforation were found among RA patients treated with anti-IL-6RAb, and most cases appeared to be complications of diverticulitis (78). The rate for lower GI perforation was 1.9 per 1,000 patient-years, which was substantially lower than the 3.9 per 1,000 patient-years for RA patients exposed to corticosteroids. Although no GI perforation was observed in 23 CD patients treated with anti-IL-6RAb, clinical evaluation to assess whether the treatment leads to a decrease or increase in the incidence of GI perforation in CD patients is essential.

MS: multiple sclerosis

NMO: neuromyelitis optica

Relapsing Polychondritis

Relapsing polychondritis is a rare disease characterized by recurrent inflammation and cartilage destruction (79). Autoimmune reactions to antigens present in cartilage such as type II collagen and matrilin and excess generation of proinflammatory cytokines and chemokines are thought to evoke the disease symptoms (80). The involvement of laryngotracheal cartilage causes severe airway destruction and requires vigorous treatments with corticosteroids and immunosuppressive drugs. Two patients with relapsing polychondritis who had been refractory to conventional regimens were treated with anti-IL-6RAb. The treatment ameliorated clinical symptoms related to upper and lower airways, so that the prednisolone dose could be reduced (81). In one patient, airway narrowing of the bronchus was improved by one year of treatment, whereas in the other patient, gallium citrate uptake in the involved cartilages disappeared 21 months after treatment. Because of the rare occurrence of the disease, there is an urgent need for reports on clinical experience with anti-IL-6RAb in the treatment of relapsing polychondritis.

Acquired Hemophilia A

Acquired hemophilia A is a rare bleeding disorder characterized by the presence of autoantibodies that inhibit coagulation factor VIII (FVIII) activity (82). The etiology remains unknown, but failure of Treg activation may play a crucial role in FVIII inhibitor synthesis (83). Anti-IL-6RAb was administered to a patient with acquired hemophilia A who was refractory to corticosteroids and complicated by diabetes mellitus and glaucoma. The treatment increased the activity of FVIII in a rapid and sustained manner, so that the prednisolone dose could be reduced (84).

Multiple Sclerosis and Neuromyelitis Optica

Multiple sclerosis (MS) is a heterogeneous and complex autoimmune disease characterized by inflammation, demyelination, and axon degeneration in the central nervous system (CNS) (85). The disease may result from a primary defect in the immune system that targets components of the myelin sheath, resulting in secondary effects on neurons. Recent investigations have revealed that autoreactive Th17 and B cells may function as amplifiers and effectors in MS (86). IL-6 or IL-6 transcripts were elevated in the CNS of MS patients and in a murine experimental autoimmune encephalitis (EAE) model of MS (87–89), implicating IL-6 in the development of MS. Moreover, IL-6 blockade by anti-IL-6RAb impeded the development of EAE through the inhibition of antigen-specific Th17 and Th1 (90).

Neuromyelitis optica (NMO) is a chronic inflammatory CNS disorder that predominantly affects the spinal cord and optic nerves (91). Several studies have reported a marked increase of IL-6 in cerebrospinal fluid of patients with NMO (92, 93). Moreover, Chihara et al. (94) recently reported that the population of plasmablasts showing the $CD19^{int}CD27^{high}CD38^{high}CD180^{-}$

JIA: juvenile idiopathic arthritis

phenotype was selectively increased in the peripheral blood of NMO patients and that antiaquaporin 4 (AQP4) antibodies were produced mainly by the plasmablasts. IL-6 enhanced the survival of plasmablasts as well as their AQP4 antibody secretion, whereas anti-IL-6RAb lessened their survival. These findings suggest that IL-6 blockade could be a novel therapeutic strategy for autoimmune neurological diseases such as MS and NMO.

ANTI-INTERLEUKIN-6 RECEPTOR ANTIBODY FOR CHRONIC INFLAMMATORY DISEASES

Castleman's Disease

Castleman's disease is a lymphoproliferative disease characterized by benign hyperplastic lymph nodes, follicular hyperplasia, and capillary proliferation accompanied by vascular hyperplasia. Dysregulated IL-6 expression generated by transgenic mice produced a syndrome resembling Castleman's disease (95). IL-6 was highly expressed in hyperplastic lymph nodes of patients with Castleman's disease, and surgical removal of the solitary involved lymph node led to clinical improvement and reduced serum IL-6 concentration (14). This suggests that the generation of IL-6 by hyperplastic lymph nodes is the key element responsible for the various clinical symptoms. In a study by Soulier et al. (96), all HIV-positive and 50% of HIV-negative cases of multicentric Castleman's disease were infected with Kaposi sarcoma (KS)-associated herpes virus (KSHV; also known as human herpesvirus 8). KSHV encodes viral IL-6, which directly binds to and stimulates gp130 in the absence of IL-6R (97). Thus, both viral IL-6 and human IL-6 contribute to the pathogenesis of KSHV-infected Castleman's disease. The first evidence of the beneficial effect of IL-6 blockade was observed in a patient with Castleman's disease treated with a mouse anti-IL-6Ab (98). Subsequently, two open-label clinical trials of anti-IL-6RAb for Castleman's disease showed its marked ameliorative effect in clinical symptoms and laboratory findings (99, 100), leading to approval of anti-IL-6RAb as an orphan drug for Castleman's disease in Japan in 2005.

Systemic Juvenile Idiopathic Arthritis and Adult-Onset Still's Disease

Systemic juvenile idiopathic arthritis (JIA) is a subtype of chronic childhood arthritis that leads to joint destruction, functional disability, and growth impairment, accompanied by systemic inflammation (101). IL-6 is markedly elevated in blood and synovial fluid of JIA patients, and their IL-6 level correlates with disease activity (102). A Phase II dose-escalating trial starting with 2–8 mg kg^{-1} of anti-IL-6RAb at 2-week intervals was performed for 11 children with active systemic JIA who were refractory to corticosteroids. Overall improvement in arthritis and systemic features assessed on ACR Pedi 30%, 50%, and 70% improvement scales was seen in 90.9%, 90.9%, and 63.6% of the subjects, respectively (103). A randomized, double-blind, placebo-controlled, withdrawal Phase III trial for 56 patients with systemic JIA showed ACR Pedi 30%, 50%, and 70% responses in 91%, 86%, and 68% of the patients, respectively (104). On the basis of its outstanding efficacy for JIA, anti-IL-6RAb was approved as the first biologic drug for the treatment of systemic JIA in Japan. In a recent global Phase III trial (TENDER), 112 patients with severe systemic JIA who were refractory to conventional treatments, including TNF and IL-1 inhibitors, were randomized to receive placebo or anti-IL-6RAb (8 or 12 mg kg^{-1}, depending on body weight, every 2 weeks). Significantly higher responses for ACR Pedi 50%, 70%, and 90% scales were also noted in the anti-IL-6RAb-treated group at week 12 (85%, 71%, and 37% for the anti-IL-6RAb group versus 11%, 8%, and 5% for the placebo group) (105). Long-term treatment with anti-IL-6RAb has the potential to reserve growth retardation observed in systemic JIA patients because IL-6 inhibits growth hormone signaling (106).

Adult-onset Still's disease (AOSD) is a chronic inflammatory disease characterized by four cardinal symptoms: spiking fever, evanescent maculopapular rash, arthritis, and leukocytosis (107). Pathologically, it resembles systemic JIA and is considered to be an adult-onset type of systemic JIA. Several case and pilot studies have reported that anti-IL-6RAb treatment improved clinical symptoms and signs of AOSD patients who had been refractory to conventional treatments (108–111). The clinical efficacy of anti-IL-6RAb suggests that it may become a first-line biologic for the treatment of systemic JIA and AOSD.

AOSD: adult-onset Still's disease

PMR: polymyalgia rheumatica

RS3PE: remitting seronegative, symmetrical synovitis with pitting edema

Amyloid A Amyloidosis

AA amyloidosis is a serious complication of chronic inflammatory diseases in which amyloid fibril deposition causes progressive deterioration in various organs (7). SAA, an acute-phase protein produced in the liver, is an AA fibril precursor protein, and a sustained high concentration of SAA correlates with a progression of renal amyloid diseases (112). Chronic suppression of SAA levels leads to a regression or stabilization of the amyloid load (113). Because the activation of the SAA gene depends primarily on IL-6 (114, 115), anti-IL-6RAb administration causes a marked reduction of serum concentrations of SAA (40, 99, 100, 116, 117). Three case studies of AA amyloidosis complicated by RA reported on the ameliorative clinical effect of anti-IL-6RAb on GI symptoms due to intestinal amyloidosis (118–120). Surprisingly, AA fibril deposits disappeared in two cases after only three injections of anti-IL-6RAb (118, 120). This suggests that anti-IL-6RAb may be suitable as a first-line drug for patients with chronic inflammatory disease and AA amyloidosis (32).

Polymyalgia Rheumatica and Remitting Seronegative, Symmetrical Synovitis with Pitting Edema

Polymyalgia rheumatica (PMR) is a chronic inflammatory disorder that affects the elderly. PMR is characterized by aching and morning stiffness in the shoulders, neck, and pelvic girdle (68). Although the pathogenesis remains unknown, IL-6 has been identified as the only cytokine present at a consistently high level in patients with the active form of the disease and is recognized as the most sensitive indicator of disease activity (68, 121). Low doses (15–20 mg per day) of corticosteroids are effective, but 65% of patients with PMR experienced at least one steroid-related adverse event in a study by Gabriel et al. (122). Steroid-sparing or alternative drugs thus need to be developed. We reported a significantly beneficial effect of anti-IL-6RAb on a patient with long-standing steroid-refractory PMR complicated by diabetes, osteoporosis, and hypertension (123). After five injections of the antibody, symptoms such as pain and morning stiffness improved and the patient went into remission, so that the prednisolone dose could be reduced. In addition, anti-IL-6RAb treatment produced disease-free status in four patients with PMR complicated by GCA (71). Interestingly, two patients with PMR who were not treated with corticosteroids went into remission.

Remitting seronegative, symmetrical synovitis with pitting edema (RS3PE) is an inflammatory disease characterized by acute onset, symmetrical synovitis, bilateral pitting edema of the hands and feet, and seronegativity for rheumatoid factor (124). The etiology of RS3PE is unknown, but IL-6, produced by the synovial tissues, may play a central role in the development of RS3PE, either directly or through induction of VEGF production, which then leads to synovial hypervascularity and increased vascular permeability (125, 126). Although corticosteroids still constitute the preferred treatment for RS3PE, we reported that anti-IL-6RAb treatment for a case of RS3PE refractory to corticosteroids resulted in improved clinical symptoms and inflammatory laboratory findings such as CRP and matrix metalloproteinase-3 as well as a marked reduction in uptake of

gallium citrate in the bilateral shoulders and hands (127). Serum VEGF levels decreased from 127 pg ml^{-1} to 59 pg ml^{-1}. These case reports suggest that IL-6 blockade may become a novel treatment strategy for PMR and RS3PE.

BD: Behçet's disease

Spondyloarthritides

Spondyloarthritides are a group of disorders characterized by rheumatological manifestations such as axial involvement, peripheral arthritis and enthesopathy, extra-articular features, negativity for rheumatoid factor, and a genetic background with a higher-than-normal positivity for HLA-B27 (128). They include reactive arthritis, ankylosing spondylitis, psoriatic arthritis, and inflammatory bowel disease–related arthritis.

Reactive arthritis is a disease comprising the clinical triad of arthritis, urethritis, and conjunctivitis (129). The onset of the disease is often preceded by bacterial infections in either the urogenital or GI tract; these infections trigger systemic immunoreactions, and overproduction of proinflammatory cytokines including IL-6 contributes to disease development (130, 131). A 24-year-old woman with sustained active reactive arthritis resistant to conventional treatment regimens for 4 years was treated with anti-IL-6RAb (132). Serum CRP and SAA levels normalized after one injection of anti-IL-6RAb; two injections resulted in the disappearance of joint swelling and pain and complete resolution of symptoms; and after five injections, gallium citrate scintigraphy showed a marked reduction in uptake for the joints.

The clinical benefits of IL-6 blockade with murine anti-IL-6Ab or anti-IL-6RAb for patients with ankylosing spondylitis have also been demonstrated (133–137). It appears that anti-IL-6RAb can substantially improve clinical symptoms of patients with ankylosing spondylitis who were refractory to anti-TNFs, as was also found for the treatment of RA.

Behçet's Disease and Uveitis

Behçet's disease (BD), a systemic inflammatory disease of unknown etiology, is characterized by relapsing episodes of oral aphthous ulcers, genital ulcers, skin lesions, ocular lesions, and other manifestations including neurological, GI, and vascular involvement (138). IL-6 is involved in the pathological development of BD (139, 140). We used anti-IL-6RAb for the treatment of one patient with posterior uveitis who had been suffering from BD for 10 years and had been treated with colchicine, prednisolone, and cyclosporine. Treatment with infliximab, a chimeric anti-TNFα antibody for the recurrent posterior uveitis, brought the attacks of uveitis under satisfactory control. When a severe relapse of posterior uveitis occurred 16 months later, however, anti-IL-6RAb was initiated, and continuous treatment for 1 year reduced the number of ocular attacks and the BD Current Activity Form score (141).

Autoimmune and inflammatory uveitis constitute a group of potentially blinding intraocular inflammatory diseases that arise without a known infectious trigger. They often are associated with immunological responses to unique proteins (142) and often occur in conjunction with systemic diseases. Several studies of animal models indicate that both Th1 and Th17 can play a pathological role in these diseases (143). Blockade of IL-6 signaling was recently reported to suppress autoimmune uveoretinitis (144). The severity of uveoretinitis was similar for wild-type mice, IL-17KO, and IFN-γKO mice, but the inflammatory uveoretinitis was absent in IL-6KO mice. Antigen-specific Th17 and Th1 increased in the IFN-γKO mice and IL-17KO mice, respectively, whereas both populations were reduced in IL-6KO mice. However, Treg depletion in IL-6KO mice caused uveoretinitis, suggesting that the protective effects of IL-6 signaling blockade are mediated by Treg induction and by Th17 and Th1 inhibition. This indicates that anti-IL-6RAb may constitute a therapeutic option for uveitis.