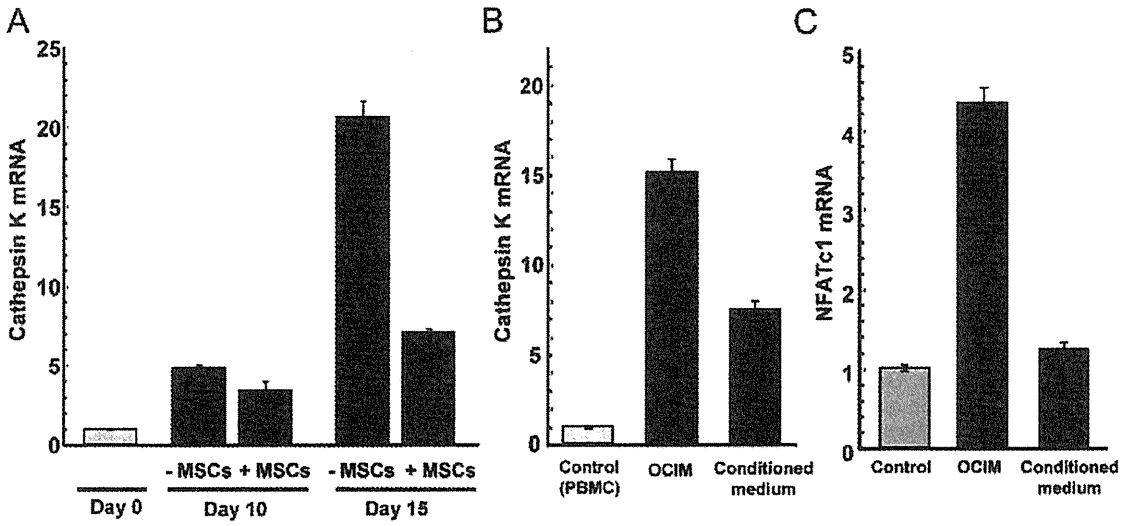

Figure 2. Human MSCs suppress induction of cathepsin K and expression of nuclear factor of activated T cells c1 (NF-ATc1). **A,** PBMCs (1×10^6/well) were cocultured for 10 or 15 days with human MSCs (2×10^3) in OCIM, using Transwells. After coculture, PBMCs in OCIM were lysed and total RNA was isolated. Cathepsin K mRNA expression compared with GAPDH was determined by real-time polymerase chain reaction (PCR). **B,** PBMCs (1×10^6/well) were cultured in OCIM or MSC-conditioned medium. After 15 days, cathepsin K mRNA expression was analyzed by real-time PCR. **C,** CD14+ cells isolated from PBMCs were cultured in OCIM or MSC-conditioned medium. After 7 days, NF-ATc1 mRNA expression was analyzed by real-time PCR. Levels of mRNA expression in control samples (day 0) were set at 1. Values are the mean ± SD of triplicate samples from 1 of 3 independent experiments. See Figure 1 for other definitions.

were observed in coculture wells on day 16, but osteoclast-like cells were not (Figure 1B).

Suppression of cathepsin K and NF-ATc1 expression by human MSCs. Cathepsin K is a cysteine protease that is essential for the bone-resorbing activity of osteoclasts. When PBMCs were cultured in OCIM, cathepsin K expression was induced and increased in a time-dependent manner, ultimately (on day 15) becoming 15–20-fold higher than expression in unstimulated PBMCs (Figure 2A). Cathepsin K expression on day 15 was, however, markedly suppressed when PBMCs were cocultured with human MSCs (Figure 2A). In addition, when PBMCs were cultured in MSC-conditioned medium, cathepsin K expression decreased in a similar manner (Figure 2B). To confirm the suppressive effect of MSCs on osteoclastogenesis, expression of NF-ATc1, known as the master transcription factor for osteoclast differentiation, was measured. NF-ATc1 mRNA expression increased in CD14+ cells cultured for 7 days with OCIM, whereas MSC-conditioned medium completely suppressed its expression (Figure 2C). These results therefore suggest that a soluble mediator from human MSCs plays an important role in inhibition of osteoclastogenesis.

Inhibition of osteoclastogenesis by OPG produced from human MSCs. The key role of RANKL in osteoclastogenesis has been established. In order to identify the soluble factor that inhibits osteoclastogenesis, we first considered the possibility of OPG, a decoy receptor for RANKL. Interestingly, human MSCs constitutively produced OPG, at both the mRNA and the protein levels (Figures 3A and B), which was also detectable in coculture wells but not in wells without human MSCs (Figure 3C). In addition, although neither TNFα nor prostaglandin E_2 (PGE_2) was detected in coculture wells (data not shown), IL-6, known as a modulator of osteoclastogenesis, was produced (~10–15 ng/ml) (Figure 3D).

To exclude the possible effect of $1\alpha,25(OH)_2D_3$ on osteoblast differentiation and OPG production, experiments without $1\alpha,25(OH)_2D_3$ were performed. Irrespective of the presence of $1\alpha,25(OH)_2D_3$, osteoclastogenesis was suppressed in a similar manner, resulting in reduced RANKL expression, alkaline phosphatase activity, and alizarin red staining (results not shown). In addition, OPG production levels were comparable between MSC culture supernatants and coculture supernatants (Figures 3A and C), indicating that constitutive

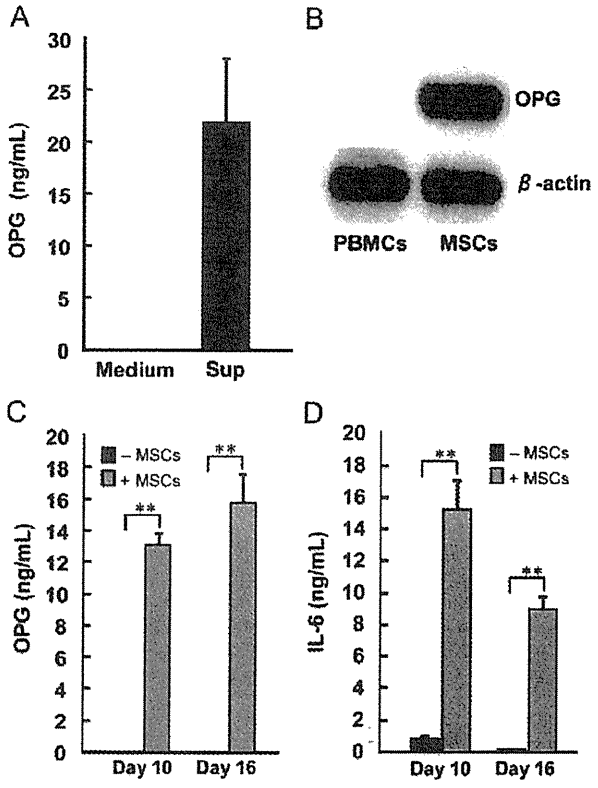

Figure 3. Constitutive production of osteoprotegerin (OPG) by human mesenchymal stem cells (MSCs). **A,** Concentrations of OPG protein in culture supernatant (Sup) of human MSCs in the confluent growth phase compared with concentrations in MSC growth medium were measured by enzyme-linked immunosorbent assay (ELISA). **B,** OPG mRNA expression in human MSCs and peripheral blood mononuclear cells (PBMCs) was determined by polymerase chain reaction (PCR). PCR products analyzed by electrophoresis are shown. **C** and **D,** PBMCs (1×10^6/well) and human MSCs (1×10^3/well) were cocultured in OCIM, using Transwells. Coculture supernatants were collected on days 10 and 16. Concentrations of OPG (**C**) and interleukin-6 (IL-6) (**D**) were measured by ELISA. Values in **A, C,** and **D** are the mean ± SEM of triplicate samples from 1 of 3 independent experiments. * = $P < 0.05$; ** = $P < 0.01$, by Student's *t*-test.

OPG production by MSCs inhibited osteoclastogenesis. As expected, addition of anti-OPG neutralizing monoclonal antibody to the coculture system increased the number of osteoclast-like cells compared to that obtained with control antibody (Figure 4).

To further confirm the inhibitory effect of OPG, human MSCs were transfected with OPG siRNA (OPG-knockdown [OPG-KD] MSCs). The production of OPG was reduced by 86% at both the mRNA level (data not shown) and the protein level (Figure 5B). When PBMCs were cocultured with OPG-KD MSCs, the number of osteoclast-like cells significantly recovered compared to negative siRNA–transfected MSCs (Figure 5A). Additionally, cathepsin K expression recovered when PBMCs were cocultured with OPG-KD MSCs (Figure 5C). However, recovery of osteoclast-like cell numbers and cathepsin K expression was only partial. These results show that OPG partially contributes to the inhibition of osteoclastogenesis by human MSCs, leaving open the possibility that other soluble factors are also involved. Accordingly, recovery of cathepsin K expression was lower compared to the recovery of osteoclast-like cells.

Offset of MSC-mediated inhibition of osteoclastogenesis by its specific blockade. To evaluate the function of bone-resorbing activity of osteoclast-like cells, a pit formation assay was performed. When CD14+ cells isolated from PBMCs were cultured in OCIM for 14 days, >200 osteoclast-like cells per square centimeter were observed in the 96-well plates, with pit formation making up 15.6% of the surface of dentin slices (Figure 6). In contrast, a mean of only 43

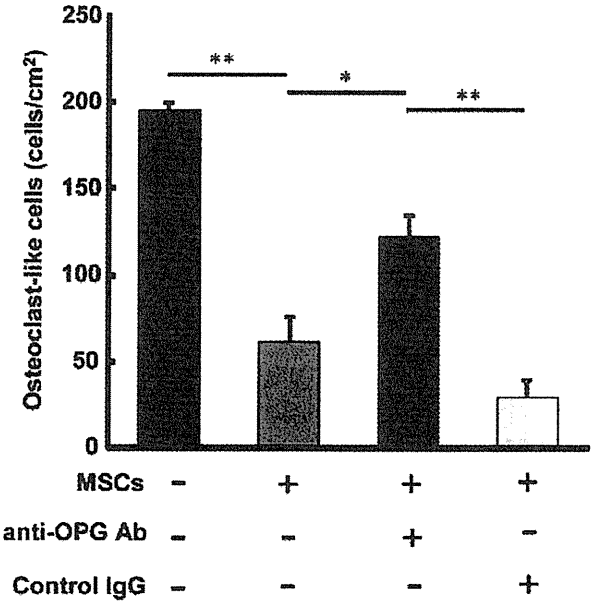

Figure 4. OPG blocking induces recovery of osteoclastogenesis. PBMCs (1×10^6/well) and human MSCs (1×10^3/well) were cocultured for 16 days in OCIM with anti-osteoprotegerin (anti-OPG) neutralizing monoclonal antibody (Ab) or mouse IgG1 isotype control. Osteoclast-like cells (recognized by TRAP positivity) and multinuclear cells were counted by microscopy. Values are the mean ± SEM of triplicate samples from 1 of 3 independent experiments. * = $P < 0.05$; ** = $P < 0.01$, by Tukey's test. See Figure 1 for other definitions.

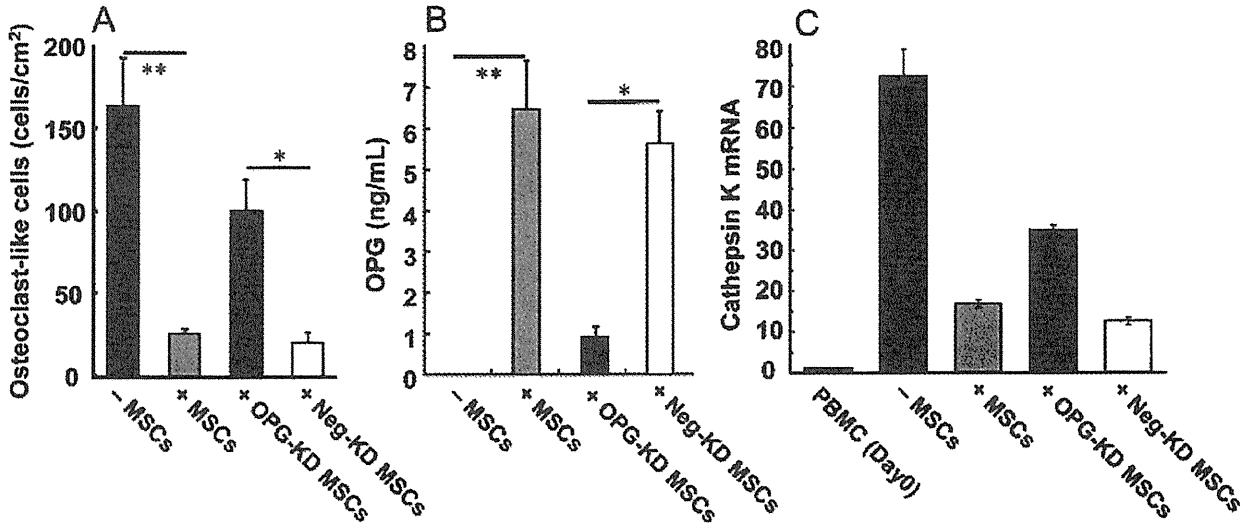

Figure 5. OPG small interfering RNA (siRNA)–transfected human MSCs induce partial recovery of osteoclastogenesis. Human MSCs (1×10^3/well) were transfected with OPG siRNA (OPG-knockdown [OPG-KD]) or negative control siRNA (Neg-KD), and PBMCs (1×10^6/well) were then cocultured for 14 days with OPG-KD or negative control MSCs (1×10^3/well) in OCIM. **A,** Tartrate-resistant acid phosphatase (TRAP)–positive and multinuclear cells were counted by microscopy. **B,** OPG concentrations in coculture supernatant were measured by ELISA. **C,** On day 14, PBMCs with OCIM were lysed and cathepsin K mRNA expression was quantified by real-time PCR. Values are the mean ± SEM (**A** and **B**) or the mean ± SD (**C**) of triplicate samples from 1 of 3 independent experiments. * = $P < 0.05$; ** = $P < 0.01$, by Tukey's test. See Figure 3 for other definitions.

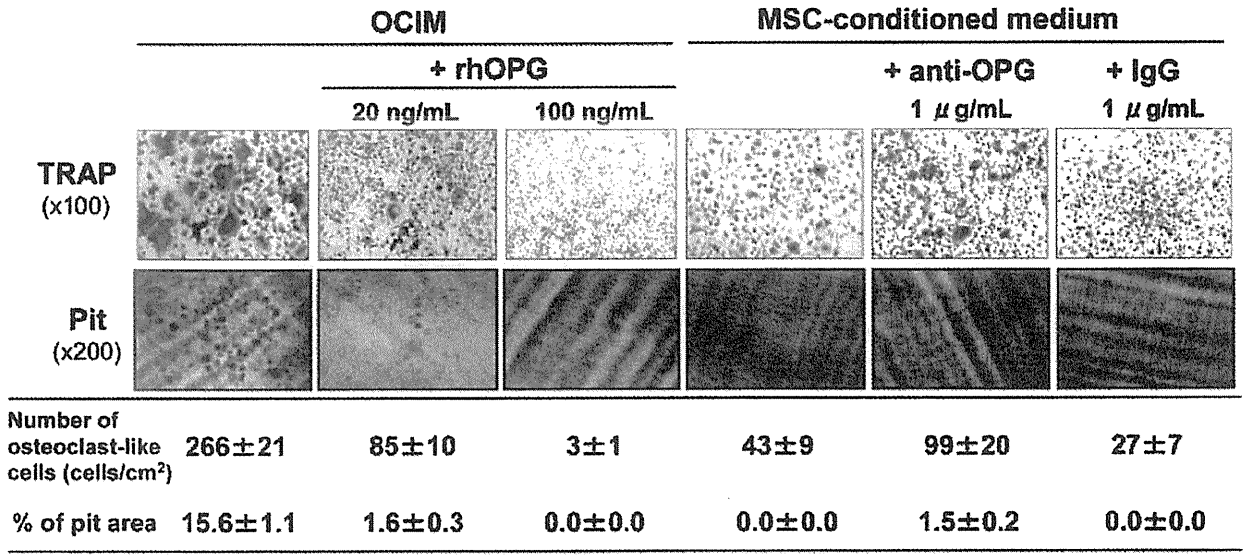

Figure 6. MSC-conditioned medium inhibits osteoclastogenesis and bone-resorbing activity of cultured osteoclast-like cells. Peripheral blood CD14+ cells were cultured in OCIM or MSC-conditioned medium in the presence of recombinant human OPG (rhOPG; 20 ng/ml or 100 ng/ml), anti-OPG antibody (1 μg/ml), or mouse IgG1 antibody (1 μg/ml). Pit formation assay was performed by culturing CD14+ cells on dentin slices. After 14 days, cells and dentins were stained with TRAP and hematoxylin, respectively. The number of osteoclast-like cells was counted, and the percentage of pit area in relation to the total surface area of dentin was calculated. Values are the mean ± SEM of triplicate samples from 1 of 3 independent experiments. Figure 1 for other definitions.

osteoclast-like cells per square centimeter was observed when cells were cultured in MSC-conditioned medium, with complete inhibition of pit formation. Osteoclastogenesis was partly recovered with addition of anti-OPG neutralizing antibody to MSC-conditioned medium, but the size and the number of osteoclast-like cells remained smaller compared to those in experiments with OCIM. In addition, pit formation was recovered with addition of anti-OPG antibody, but pit area was no more than 1.5% of dentin surface.

This partial recovery of pit formation by OPG blocking correlated with the recovery of expression of cathepsin K (Figure 5C), an indispensable protease for bone-resorbing activity of osteoclasts. In OCIM, both osteoclast-like cells and pit formation decreased with addition of OPG, in a concentration-dependent manner. Interestingly, the suppressive effects on osteoclast-like cell differentiation and pit formation in the presence of 20 ng/ml recombinant OPG were less than those observed in MSC-conditioned medium containing ~20 ng/ml OPG (Figure 3A). These results suggested that human MSCs can inhibit bone-resorbing activity not only by OPG secretion but also via other soluble mediators.

DISCUSSION

RA is a prototypical inflammatory disease that presents significant health and socioeconomic burdens. Even though biologic agents have made it possible to prevent joint destruction and also induce repair in some cases, improvement of physical function is still limited. MSCs are widely understood to exert trophic effects on various cells, which is considered to be a useful therapeutic avenue in a number of diseases. In fact, there are currently several ongoing clinical trials to evaluate their usefulness in graft-versus-host disease, autoimmune diseases, and autoinflammatory diseases (16). Given the known multipotency and immunosuppressive effects of MSCs, we considered that they might be a new source of RA treatment. Because the effect of MSCs on osteoclasts, which play an important role in bone resorption and joint destruction, remained unclear, we investigated the role of MSCs in osteoclastogenesis. We found that human MSCs markedly suppressed osteoclast differentiation and activation by constitutive production of OPG under cell–cell contact–free conditions. Our data suggest that a cell-based therapy using human MSCs would be expected to improve bone-erosive diseases including RA. In fact, blockade of RANKL has been shown to prevent progress of bone loss in an RA clinical study (17).

MSCs are able to differentiate into osteoblasts, chondrocytes, and adipocytes. Originally identified in bone marrow, MSCs can also be identified in adipose, synovium, umbilical cord, skin, and various other tissues (8,18). The MSCs used in the present study were derived from bone marrow donated by healthy donors. These cells have been characterized to differentiate into osteocytic, chondrocytic, or adipocytic lineages, and to be positive for SH2, SH3, CD29, CD44, CD71, CD90, CD106, and CD120a as surface proteins. On the other hand, hematopoietic markers, such as CD14, CD34, and CD45, are known to be negative on these cells (7). However, specific cell surface makers for MSCs have not been identified and moreover, differences between MSCs derived from different tissues remain unclear. As an example, although adipose-derived stem cells (ADSCs) are also known for their multipotency, of most interest is their powerful angiogenic potential rather than an immunosuppressive effect (8,19). Therefore, ADSCs may be useful for treatment of ischemic diseases rather than autoimmune diseases. Interestingly, OPG was constitutively produced not only by MSCs but also by ADSCs (data not shown), suggesting a common phenotype with MSCs.

MSCs are known for their strong immunosuppressive functions in vitro caused by soluble mediators such as antiinflammatory cytokines (20), and based on this intriguing property, they might be useful as a therapeutic application in autoimmune diseases, including RA. In fact, Augello et al have reported that a single intravenous injection of MSCs prevented the occurrence of severe, irreversible damage to bone and cartilage in experimental collagen-induced arthritis in mice (21). Although the effect of MSCs on T cells, dendritic cells, and NK cells has been reported by several groups, their effects on other immune cells and inflammatory cells remain to be fully elucidated. In particular, the effect of MSCs in differentiation and function of osteoclasts, which play an important role in bone resorption leading to joint destruction in RA, is still unclear. In the present study, we found that osteoclastogenesis was significantly inhibited by MSCs via constitutive production of OPG. However, experiments with anti-OPG neutralizing antibody and knockdown of OPG by its siRNA (Figures 4 and 5) suggested that not only OPG, but also other soluble mediators, are involved in inhibition of osteoclastogenesis by MSCs. Accordingly, MSC-conditioned medium containing ~20 ng/ml of OPG showed stronger

inhibition compared to that obtained with addition of 20 ng/ml recombinant OPG to OCIM (Figures 3 and 6).

It has been reported that IL-6, IL-8, PGE_2, and vascular endothelial growth factor are secreted from MSCs identical to the ones we used (8). Intriguingly, in our coculture experiments, IL-6 levels were increased to 10–15 ng/ml (Figure 3D). The role of IL-6 in osteoclastogenesis remains controversial. IL-6 has been indicated as a key molecule in driving osteoclastogenesis in various in vivo studies (22–24), and others have shown that IL-6 inhibited RANKL-dependent osteoclastogenesis (25,26). Our results suggest either that MSCs are able to inhibit osteoclastogenesis in the presence of IL-6 or simply that IL-6 did not induce osteoclastogenesis in our system. The incomplete recovery of osteoclast differentiation by inhibition of OPG can be related to an inhibitory effect of IL-6 on osteoclastogenesis. Further, although PGE_2 was previously reported to have an inhibitory effect on human osteoclast formation (27), the maximum concentration of PGE_2 in our coculture medium (on day 3) was only 1.5 ng/ml (data not shown). Based on previously reported findings (8), we speculated that PGE_2 production requires either interferon-γ or TNFα stimulation. Therefore, we believe inhibition of osteoclastogenesis is unrelated to PGE_2 in our coculture system.

Given the known immunosuppressive effect of MSCs, it is possible that immunosuppressive soluble mediators contribute to inhibition of osteoclastogenesis. Murine MSCs are able to induce apoptosis and cell cycle arrest of splenocytes stimulated with anti-CD3 and anti-CD28 antibodies under cell–cell contact–free conditions, and this is thought to be mediated by IL-10 (28). In fact, differentiation of human osteoclasts is reported to be suppressed by IL-10 due to inhibition of RANK signaling (29). However, IL-10 was undetectable in our experiments (data not shown). In recent years, indoleamine 2,3-dioxygenase and nitric oxide have also been reported to be important molecules suppressing the immune response of NK cells and proliferation of T lymphocytes (11,28,30). Further evaluation is needed to clarify what factors other than OPG are involved in the suppressive effect on osteoclastogenesis.

OPG is known to be an inhibitory molecule for RANKL-dependent osteoclast differentiation and function (31). In fact, OPG-deficient mice exhibit severe osteoporosis due to excessive bone resorption by osteoclasts (32,33). Accordingly, loss of trabecular bone density in OPG-deficient mice was recovered by intravenous injection of recombinant human OPG-Fc fusion protein, which decreased the number of osteoclasts (34). Moreover, it has been reported that short-term treatment with soluble OPG protein or OPG-Fc fusion protein significantly reduced osteoclast numbers and prevented bone erosion in rats with experimental arthritis, without affecting synovitis (35,36). Surprisingly, those effects were evident after subcutaneous injection of OPG-Fc for only 5–7 days. These results indicate that MSCs are an OPG producer, exerting an antierosive effect by decreasing osteoclasts.

Although we have demonstrated here that inhibition of osteoclastogenesis by human MSCs is independent of physical cell contact, the effect of cell contact is not certain. In a previous study, human MSCs were shown to induce osteoclastogenesis from CD34+ bone marrow hematopoietic stem cells (HSCs) in the presence of cell–cell contact, by up-regulation of IL-6, IL-11, and leukemia inhibitory factor production from MSCs (37). Interestingly, this effect was observed in the absence of osteoclastogenic stimulation such as OCIM, and under cell–cell contact–free conditions as well (37).

The mechanism of this effect is considered to involve the altered nature of MSCs, including production of cytokines, growth factors, and surface molecules. Accordingly, it may be that OPG expression by MSCs is reduced by contact with HSCs rather than by up-regulation of RANKL, unless osteoclastogenesis is driven by a RANKL-independent pathway such as via IL-6 and IL-11 (38). Additionally, use of PBMCs or HSCs can be involved in the different effect of MSCs on osteoclastogenesis. Although alteration of the characteristics of MSCs under conditions of coculturing with PBMCs in OCIM remains to be confirmed, RANKL mRNA expression was not detected in MSCs at the end of coculture (data not shown). Therefore, MSCs were at least not differentiated into osteoblasts expressing RANKL in our coculture system. However, further investigation is necessary to elucidate the effects of human MSCs on osteoclastogenesis from PBMCs in OCIM in the presence of cell–cell contact.

In conclusion, it is clear that human MSCs inhibit osteoclastogenesis without cell–cell contact, partly due to constitutive secretion of OPG. OPG can block RANKL–RANK interaction, which is essential for differentiation and activation of osteoclasts. In the synovium of patients with RA, activated T cells and synovial fibroblasts express RANKL and induce maturation and activation of osteoclasts instead of osteoblasts, resulting in characteristic bone erosion (39). Accordingly, OPG-producing human MSCs may prevent progression of bone destruction in RA through the production of OPG and subsequent suppression of osteoclastogenesis.

Moreover, because human MSCs are well known to have strong immunosuppressive potential, they should be capable of ameliorating synovial tissue inflammation in RA. We therefore suggest that cell therapy using human MSCs may be a novel strategy for the treatment of RA, which would be fully expected not only to suppress the autoimmune response but also to prevent excessive bone resorption by osteoclasts. Furthermore, development of a therapy taking advantage of the multipotency of MSCs, by which they are able to differentiate into osteoblasts and chondrocytes (a component of joint tissue), should enable regeneration of damaged joints in 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. Tanaka 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.

Study conception and design. Oshita, Yamaoka, Udagawa, Nakano, Saito, Okada, Chiba, Tanaka.

Acquisition of data. Oshita, Yamaoka, Fukuyo, Sonomoto, Maeshima.

Analysis and interpretation of data. Oshita, Yamaoka, Udagawa, Sonomoto, Kurihara, Nakano, Saito, Okada, Chiba, Tanaka.

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EDITORIAL

The new era of autoimmune disease research

Takao Koike*

See related research by Lee *et al.*, http://arthritis-research.com/content/13/2/R63

Abstract

Recent genome-wide association studies have advanced our understanding of genetic factors that underlie systemic lupus erythematosus (SLE), a multifactorial autoimmune disease characterized by various clinical manifestations. SLE also has an environmental component, which can trigger or exacerbate the disease. Despite extensive efforts aimed at elucidating the cellular and biological abnormalities that arise in the immune system of patients with SLE, its pathology remains unclear. Lee and colleagues recently carried out gene expression profiling of patients with SLE followed by bioinformatics analysis and discovered the existence of abnormal regulatory networks and potential key molecules. The authors found that ATP synthesis and DNA repair pathways may be involved in the pathogenesis, providing a potential explanation for photosensitivity experienced by patients with SLE.

Microarray and bioinformatics analyses

Microarray analysis and gene expression profiling allow patterns of gene expression in diseases and developmental processes to be assessed. Advances in biological databases have enabled the large-scale expression profiling data to be processed and the foundation for biological interpretation to be laid. Despite this, a major limitation involves the interpretation of massive amounts of microarray data. In microarray analysis, which is often used to identify differentially expressed genes, genes that are expressed at higher or lower levels than controls are of interest. In the previous issue of *Arthritis Research & Therapy*, Lee and colleagues [1] conducted gene expression and bioinformatics analyses between healthy individuals and patients with systemic lupus erythematosus (SLE) and provided insights into biological and functional abnormalities in SLE as well as abnormal regulatory networks. Such analyses – that is, gene ontology analysis, which is used to classify genes into functionally related gene groups, and network pathway analysis, which identifies relationships among these genes – provide an additional layer of insight that cannot be achieved by focusing on individual molecules [1].

Interferon signature

Lee and colleagues [2] previously demonstrated, by DNA microarray and bioinformatics analyses, that genes related to the immune response were differentially expressed in patients with SLE compared with healthy controls. Other studies have also reported increased expression of IFN-inducible genes (that is, the 'IFN signature') in peripheral blood cells from patients with SLE [2-4]. Many groups are currently looking into pathological roles of plasmacytoid dendritic cells (pDCs) and IFN-inducible genes in SLE since pDCs are major producers of IFN-α [4-7]. Given that SLE is a systemic disease that influences multiple organs, Lee and colleagues [1] emphasized the importance of assessing biological and cellular abnormalities associated with SLE other than those related to the immune response. To this end, the authors revealed not only that apoptosis-related genes are upregulated but also that genes related to sensory perception and response to radiation/light were downregulated.

Abnormalities in DNA repair and ATP synthesis

Downregulated genes associated with sensory perception and response to radiation/light included ATPase/ATPase domain-containing genes, two excision repair cross-complementing genes (*ERCC2* and *ERCC5*), and six mitochondrial DNA (mtDNA) encoded genes: ATP synthase 6 (*ATP6*), cytochrome c oxidases 1 (*COX1*) and 3 (*COX3*), cytochrome b (*CYTB*), and NADH dehydrogenase subunits 1 (*ND1*) and 2 (*ND2*). *ERCC2/XPD* and *ERCC5/XPG* are both involved in excision repair of UV-induced DNA damage. Patients with Xeroderma pigmentosum, Cockayne syndrome, or trichothiodystrophy harbor mutations in *ERCC* genes, and such patients exhibit photosensitivity. The process of UV-induced DNA damage repair is known to require ATP, and the main function of mitochondria is to generate ATP

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through oxidative phosphorylation. Accordingly, down-regulation of ATP-dependent *ERCC* genes and mtDNA-encoded genes implies that impaired DNA repair and ATP synthesis, or increased apoptosis, may contribute to the various manifestations of SLE. Consistent with this, a study by Fernandez and Perl [8] showed that mitochondrial hyperpolarization and ATP depletion predispose lupus T cells to necrosis. Via network pathway analysis, Lee and colleagues [1] demonstrated that cytokines IL-6, transforming growth factor-beta (TGF-β), and TNF play central roles as ATP synthesis-related molecules. Patients with SLE had significantly higher levels of TNF and IL-6, which are proinflammatory, whereas levels of the anti-inflammatory cytokine TGF-β were lower [9]. Furthermore, Pflegerl and colleagues [10] reported that epidermal loss of Jun B, which is linked to increased epidermal IL-6 secretion, is sufficient to induce an SLE phenotype in mice, and this suggests that defects in skin function may lead to systemic autoimmune diseases.

Conclusions

Lee and colleagues [1] revealed functional abnormalities in ATP synthesis and DNA repair in peripheral blood cells from patients with SLE. Further DNA microarray and bioinformatics analyses should provide interesting insights into the pathophysiology of autoimmune diseases.

Abbreviations

IFN, interferon; IL, interleukin; mtDNA, mitochondrial DNA; pDC, plasmacytoid dendritic cell; SLE, systemic lupus erythematosus; TGF-β, transforming growth factor-beta; TNF, tumor necrosis factor.

Competing interests

The author declares that he has no competing interests.

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EDITORIAL

Interferon-γ–Independent Suppression of Th17 Cell Differentiation by T-bet Expression in Mice With Autoimmune Arthritis

Takao Koike

Abundant evidence indicates that T cells are required for the initiation and/or chronicity of rheumatoid arthritis (RA) in humans as well as in mouse models of RA (1–3). Multiple animal models have demonstrated key roles of interleukin-17 (IL-17) and Th17 cells in the immunopathology and joint damage of arthritis (4). Further evidence of the role of IL-17 in RA is provided by its biologic properties in vitro and in vivo, as it induces monocyte- and fibroblast-derived proinflammatory cytokines (tumor necrosis factor α [TNFα], IL-1β, IL-8), mediators of bone and cartilage damage, such as matrix metalloproteinases and RANKL, neutrophil and monocyte recruitment, and osteoclastogenesis. Bone and synovial explants from RA joints exhibit increased production of functional IL-17, and IL-17+ CD4+ T cells can be found in RA synovial tissue. In early RA, the cytokine profile of synovial fluid is dominated by the presence of IL-17 as well as Th2 cytokines (5).

The differentiation of naive CD4+ T cells into effector populations is profoundly dependent on cytokines and specific transcription factors. Th1 cells are induced by IL-12 and interferon-γ (IFNγ) (including the transcription factor STAT-1) and by up-regulated expression of T-bet, which directly activates the IFNγ locus (6). Th17 cells in mice are induced by transforming growth factor β (TGFβ) and IL-6 (with STAT-3) or IL-21, which regulate the expression of the transcription factor retinoic acid receptor–related orphan nuclear receptor γt (RORγt) (7). Previous studies showed that these transcription factors negatively regulate the differentiation of other T cell subsets by direct co-interaction and/or by indirect effects of cytokines produced by each T cell subset. How the predominant differentiation of CD4+ T cells affects the development of autoimmune arthritis, however, remains unclear.

In this issue of *Arthritis & Rheumatism*, Kondo et al (8) provide evidence that overexpression of the T-bet gene on type II collagen (CII)–reactive CD4+ T cells regulates murine autoimmune arthritis in an IFNγ-independent manner. The investigators generated T-bet–transgenic (Tg) mice under the control of the CD2 promoter and then triggered collagen-induced arthritis (CIA) in them. They demonstrated complete suppression of CIA, a significant reduction in the level of anti-CII antibodies, and a decrease in CII-reactive IL-17 production in T-bet–Tg mice. Moreover, criss-cross coculture experiments using CII-reactive CD4+ T cells from T-bet–Tg mice and splenic dendritic cells from C57BL/6 (B6) mice showed the dysfunction of CII-reactive CD4+ Th17 cells. A consensus of immunologic findings in mice indicates that IFNγ overproduction suppresses Th17 cell differentiation, as demonstrated by the exacerbation of arthritis and the production of high levels of IL-17 in IFNγ-deficient B6 mice with CIA (9). In experiments performed under Th17-polarizing conditions, Kondo et al confirmed that CD4+ T cells in T-bet–Tg mice differentiate weakly to Th17 cells, and unexpectedly, this suppression was not reversed in T cells from T-bet–Tg/IFNγ$^{-/-}$ mice. Their experiments thus support the notion that the suppression of autoimmune arthritis in T-bet–Tg mice might be due to the direct inhibition of Th17 differentiation by T-bet overexpression in CD4+ T cells.

In contrast to their role in mouse models, the role of IL-17 and Th17 cells and in RA is less clear, and several differences have been noted. For example, while Th17/Th1 (IL-17+IFNγ+) cells have been clearly identified in humans, including arthritis patients, this population of cells is rarely seen in mice. Human Th17 cells are thought to be converted to Th17/Th1 cells, whereas Th1 cells are not (10). Recent studies also show that the levels of IL-17 and the number of Th17 cells with increased RORC expression (such as the human counterpart of RORγt) are related to disease activity in RA,

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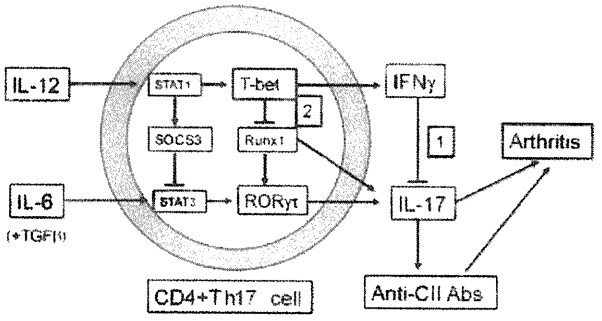

Figure 1. Possible mechanisms of T-bet regulation in autoimmune arthritis. Cytokines (interleukin-12 [IL-12] or IL-6 plus transforming growth factor β [TFGβ]) act as an activator of transcription factors, such as STAT-1 or STAT-3, resulting in the induced expression of T-bet or retinoic acid receptor–related orphan nuclear receptor γt (RORγt) for lineage commitment. **1**, T-bet may act on IL-17 through a direct suppressive pathway by affecting interferon-γ (IFNγ) production. **2**, T-bet may act on IL-17 through an indirect suppressive pathway by way of runt-related transcription factor 1 (RUNX-1) and/or RORγt down-regulation. SOCS-3 = suppressor of cytokine signaling 3; anti-CII = anti–type II collagen; Abs = antibodies.

whereas the level of IFNγ and T-bet is not (11). The mechanism of IFNγ-independent suppression of Th17 cells by T-bet identified by Kondo et al (8) seems to be an attractive target for regulating inflammatory Th17 cells in RA. Figure 1 illustrates the speculative mechanistic role of T-bet regulation.

Epigenetic regulation of T-bet and RORγt is undergoing intensive investigation. Previous studies have shown that RORγt expression is regulated positively by runt-related transcription factor 1 (RUNX-1) and STAT-3 (induced by IL-6 and TGFβ) (12). Moreover, Lazarevic et al (13) reported that expression of transfected T-bet under Th17-polarizing conditions (including neutralizing anti-IFNγ antibody) results in lower levels of RORγt expression. They also showed that T-bet inhibits RUNX-1 activity, and that RUNX-1 overexpression reverses the effect of T-bet on Th17 polarization. Therefore, T-bet inhibits IL-17 production in RORγt+ T cells by suppressing RUNX-1 (Figure 1). Several reports have suggested that single polymorphisms of RUNX-1 itself are associated with autoimmune diseases, including RA; therefore, the interaction of T-bet, RUNX-1, and RORγt should be intensively analyzed in future studies.

Kondo et al (8) further propose a new hypothesis that overexpression of T-bet transcription factors regulates autoimmune arthritis. This discovery should shed light on the molecular mechanisms of the generation of RA and assist in the development of new therapeutic strategies for RA.

AUTHOR CONTRIBUTIONS

Dr. Koike drafted the article, revised it critically for important intellectual content, and approved the final version to be published.

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Overexpression of T-bet Gene Regulates Murine Autoimmune Arthritis

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Objective. **To clarify the role of T-bet in the pathogenesis of collagen-induced arthritis (CIA).**

Methods. **T-bet–transgenic (Tg) mice under the control of the CD2 promoter were generated. CIA was induced in T-bet–Tg mice and wild-type C57BL/6 (B6) mice. Levels of type II collagen (CII)–reactive T-bet and retinoic acid receptor–related orphan nuclear receptor γt (RORγt) messenger RNA expression were analyzed by real-time polymerase chain reaction. Criss-cross experiments using CD4+ T cells from B6 and T-bet–Tg mice, as well as CD11c+ splenic dendritic cells (DCs) from B6 and T-bet–Tg mice with CII were performed, and interleukin-17 (IL-17) and interferon-γ (IFNγ) in the supernatants were measured by enzyme-linked immunosorbent assay. CD4+ T cells from B6, T-bet–Tg, or T-bet–Tg/IFNγ$^{-/-}$ mice were cultured for Th17 cell differentiation, then the proportions of cells producing IFNγ and IL-17 were analyzed by fluorescence-activated cell sorting.**

Results. **Unlike the B6 mice, the T-bet–Tg mice did not develop CIA. T-bet–Tg mice showed overexpression of *Tbx21* and down-regulation of *Rorc* in CII-reactive T cells. Criss-cross experiments with CD4+ T cells and splenic DCs showed a significant reduction in IL-17 production by CII-reactive CD4+ T cells in T-bet–Tg mice, even upon coculture with DCs from B6 mice, indicating dysfunction of IL-17–producing CD4+ T cells. Inhibition of Th17 cell differentiation under an in vitro condition favoring Th17 cell differentiation was observed in both T-bet–Tg mice and T-bet–Tg/IFNγ$^{-/-}$ mice.**

Conclusion. **Overexpression of T-bet in T cells suppressed the development of autoimmune arthritis. The regulatory mechanism of arthritis might involve dysfunction of CII-reactive Th17 cell differentiation by overexpression of T-bet via IFNγ-independent pathways.**

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by autoimmunity, infiltration of the joint synovium by activated inflammatory cells, and progressive destruction of cartilage and bone. Although the exact cause of RA is not clear, T cells seem to play a crucial role in the initiation and perpetuation of the chronic inflammation in RA.

The Th1 cell subset has long been considered to play a predominant role in inflammatory arthritis, because T cell clones from RA synovium were found to produce large amounts of interferon-γ (IFNγ) (1). Recently, interleukin-17 (IL-17)–producing Th17 cells have been identified, and this newly discovered T cell population appears to play a critical role in the development of various forms of autoimmune arthritis in experimental animals, such as those with glucose-6-phosphate isomerase–induced arthritis (2) and collagen-induced arthritis (CIA) (3). Conversely, IFNγ has antiinflammatory effects on the development of experimental arthritis (4,5). IL-17 is spontaneously produced by RA synovium (6), and the percentage of IL-17–positive CD4+ T cells

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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.

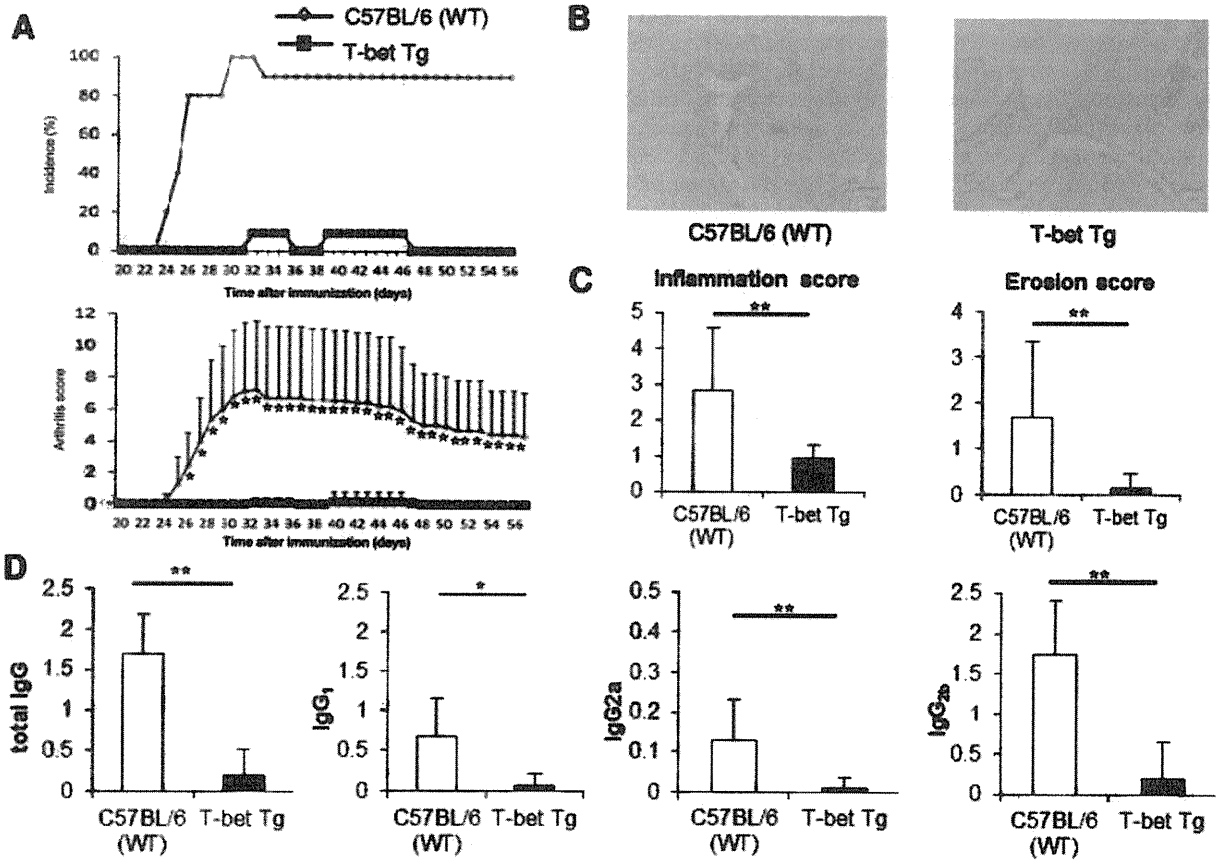

Figure 1. Significant suppression of collagen-induced arthritis (CIA) and type II collagen (CII)–specific IgG production in T-bet–transgenic (Tg) mice. On days 0 and 21, mice were immunized intradermally at several sites at the base of the tail with chicken CII emulsified with Freund's complete adjuvant. **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

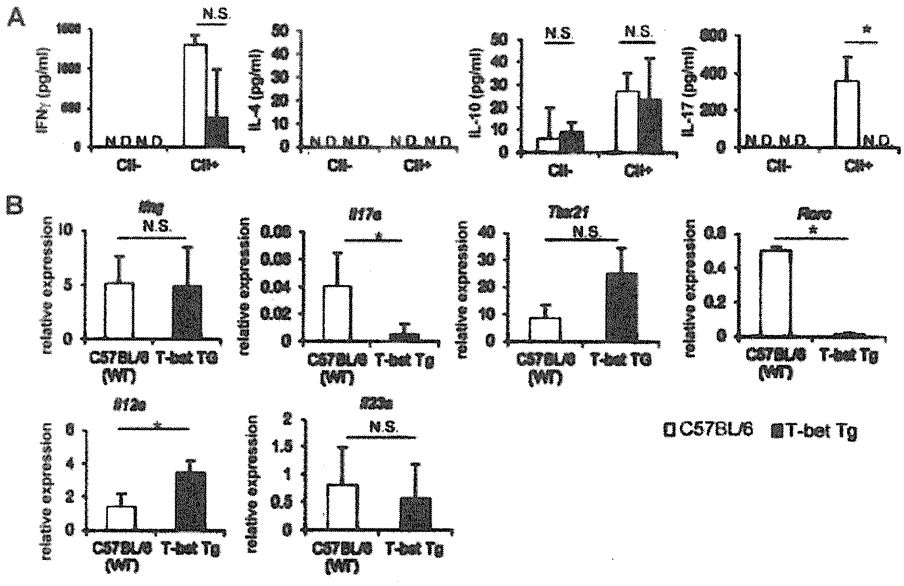

Figure 2. No production of interleukin-17 (IL-17) and low production of interferon-γ (IFNγ) in type II collagen (CII)–reactive CD4+ T cells. **A**, Ten days after the first CII immunization, lymphocytes derived from the draining lymph nodes of C57BL/6 (wild-type [WT]) mice and T-bet–transgenic (Tg) mice 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

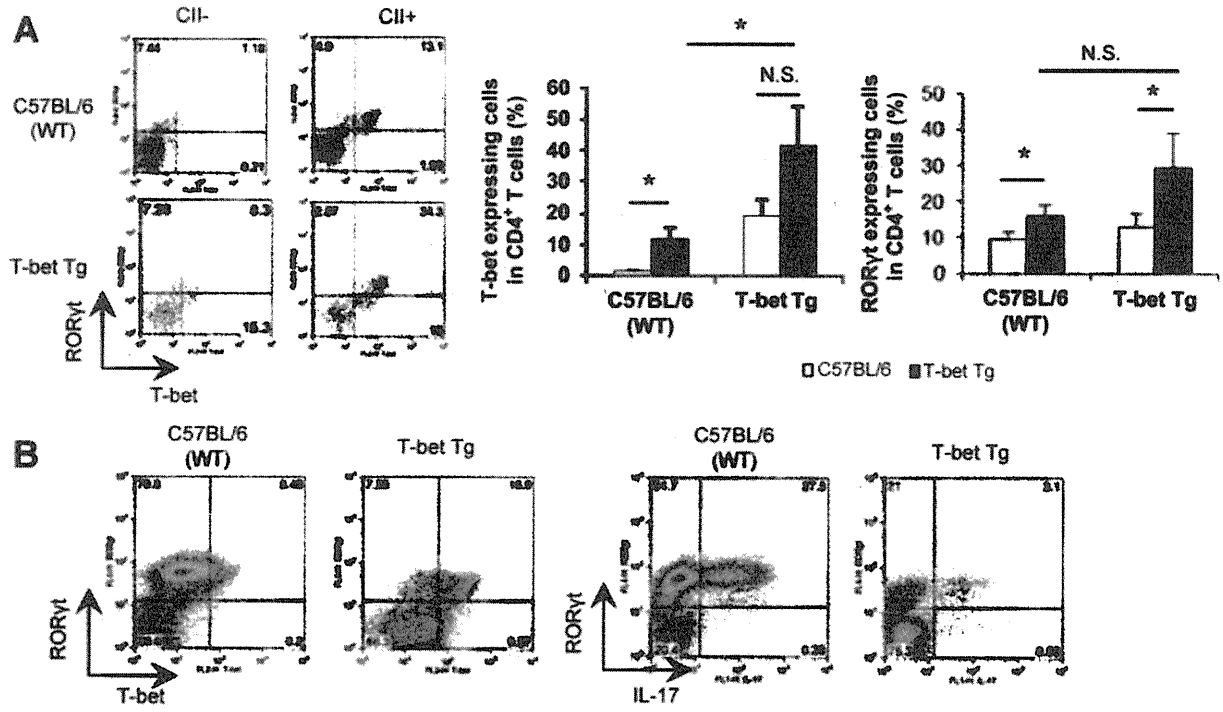

Figure 3. Suppression of Th17 cell differentiation by enforced expression of T-bet in T cells despite expression of retinoic acid receptor–related orphan nuclear receptor γt (RORγt). **A,** Ten days after the first type II collagen (CII) immunization, lymphocytes derived from the draining lymph nodes 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

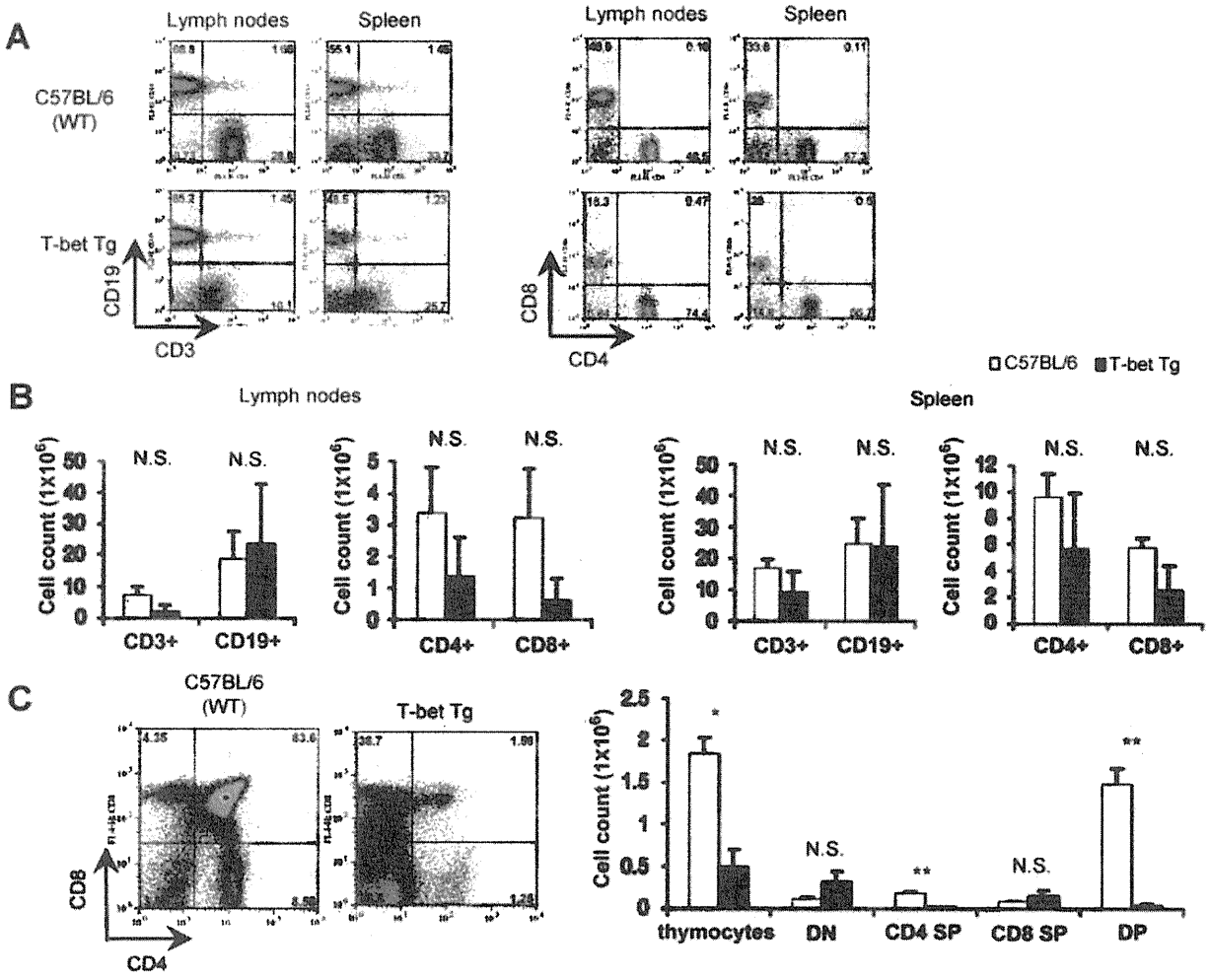

Figure 4. Decreased number of CD3+ T cells in spleen and lymph nodes and abnormal development of T precursor cells in the thymus in T-bet–transgenic (Tg) mice. **A,** Ten days after first immunization, the proportion of lymphocytes in draining lymph nodes and spleen were analyzed by fluorescence-activated 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

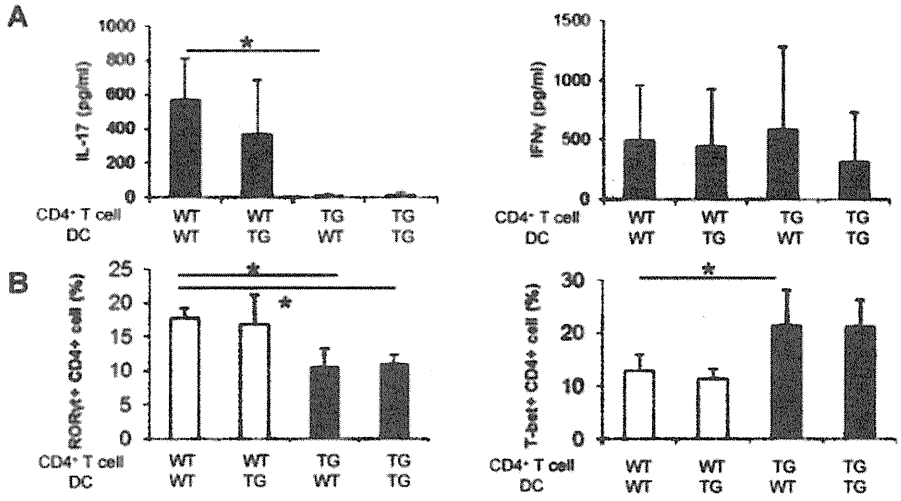

Figure 5. Impaired antigen-specific Th17 cell responses in T-bet–transgenic (Tg) mice with collagen-induced arthritis (CIA). Ten days after the first type II collagen (CII) immunization, CD4+ cells were isolated from draining lymph nodes of C57BL/6 (wild-type [WT]) mice and T-bet–Tg (TG) mice by positive 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/IFNγ$^{-/-}$ 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.

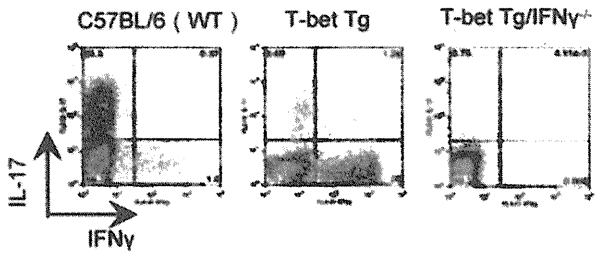

Figure 6. Suppressed expression of interleukin-17 (IL-17) by T-bet overexpression independently of interferon-γ (IFNγ) in T-bet–transgenic (Tg) mice. CD4+ T cells were isolated from the spleen of C57BL/6 (wild-type [WT]), T-bet–Tg, and T-bet–Tg/IFNγ$^{-/-}$ mice by magnetic-activated cell sorting and 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