Skeletal homeostasis is dynamically influenced by the immune system (1–3), and lymphocyte- or macrophage-derived cytokines are among the most potent mediators of osteoimmunological regulation (3–7). Therefore, the effect of individual cytokines on bone cells has been extensively studied (3–7), but the subset of immune cells with selective cytokine production that specifically affects bone cell differentiation has not been well characterized. Upon activation, CD4\(^+\) T cells undergo distinct developmental pathways to the specialized effector subsets: Th1 cells produce IFN-\(\gamma\) and regulate cellular immunity, whereas Th2 cells produce IL-4 and IL-5 and mediate humoral immunity (8). In addition, accumulating evidence suggests that newly recognized IL-17–producing T (Th17) cells have a crucial role in autoimmune inflammation (9, 10). CD4\(^+\)CD25\(^-\)Foxp3\(^+\) regulatory T (T reg) cells also constitute a distinct subset that prevents immune pathology through suppression of pathogenic T cells (11).

In autoimmune arthritis, traditionally classified as a T helper (Th) type 1 disease, the activation of T cells results in bone destruction mediated by osteoclasts, but how T cells enhance osteoclastogenesis despite the anti-osteoclastogenic effect of interferon (IFN)–\(\gamma\) remains to be elucidated. Here, we examine the effect of various Th cell subsets on osteoclastogenesis and identify Th17, a specialized inflammatory subset, as an osteoclastogenic Th cell subset that links T cell activation and bone resorption. The interleukin (IL)–23–IL-17 axis, rather than the IL-12–IFN-\(\gamma\) axis, is critical not only for the onset phase, but also for the bone destruction phase of autoimmune arthritis. Thus, Th17 is a powerful therapeutic target for the bone destruction associated with T cell activation.
been shown to be expressed by T cells, indicating that RANKL is a molecule that bridges the skeletal and immune systems (4).

In autoimmune arthritis, bone destruction is attributable to excessive bone resorption by osteoclasts, the formation of which is directly and indirectly regulated by CD4+ T cells infiltrating into the lesion (2, 3, 13, 14). Indirect effects are mainly mediated by inflammatory cytokines produced by macrophage-like synovial cells such as TNF-α and IL-1 that induce RANKL on synovial fibroblasts (14–16), but it is poorly understood how T cells exert direct effects (3). Although T cells express RANKL, the T cell–mediated positive effect is not easily observed because T cells also produce IFN-γ, which counterbalances the effect of the RANKL, making the net effect on osteoclastogenesis inhibitory (3, 13, 14). Although autoimmune arthritis has traditionally been assumed to be a Th1 disease (17, 18), there is controversy over the role of Th1 cells in the onset phase of the disease based on the observations that typical Th1 cytokines, such as IFN-γ, are not always highly expressed in the lesion (19, 20), and that collagen-induced arthritis is exacerbated in mice lacking IFN-γ signaling (21, 22). Therefore, neither bone destruction nor inflammation may be attributable to Th1 cells. It is a critically important issue to determine the type of T cells linked to the activated osteoclastogenesis under such inflammatory conditions.

Recently, it has been reported that the IL-23–IL-17 axis, rather than the IL-12–IFN-γ axis, is critical for the onset of autoimmune arthritis (23, 24). It is also reported that IL-17 is detectable in the synovial fluid from rheumatoid arthritis (RA) patients and enhances osteoclastogenesis by inducing RANKL on mesenchymal cells (25). Here, we explored the effects of various CD4+ T cell subsets on osteoclast differentiation and identified Th17 cells as the exclusive osteoclastogenic T cell subset among the known CD4+ T cell lineages. The importance of the IL-23–IL-17 axis in the bone destruction phase was underscored by the observations in mice lacking either IL-17 or IL-23 (p19). We also found that the mRNA expression of RANKL correlates with that of IL-23 (IL23A), but not that of IL-12 (IL12A), in the synovial tissues of RA patients. Collectively, these results suggest that autoimmune arthritis can be deemed a Th17-type disease in terms of both the onset and destruction phases and provide a molecular basis for targeting the IL-23–IL-17 axis in the treatment of RA.

RESULTS
Effects of Th1, Th2, and T reg cells on osteoclastogenesis
Although the effects of activated T cells on osteoclastogenesis have been documented in previous reports (13, 14, 26), these

![Figure 1. Effects of Th1, Th2, and T reg cells on in vitro osteoclastogenesis.](https://journals.jci.org/)
T cells were only stimulated by anti-CD3 antibody or PMA and the characterization of the T cells was not strictly performed. In this study, to investigate the effects of effector Th cell subsets on osteoclastogenesis, we added Th subsets, which were strictly developed under Th1 or Th2 conditions. Purified CD4+ T cells were stimulated with anti-CD3/CD28 mAbs in the presence of either IL-12 (with anti-IL-4 mAb) or IL-4 (with anti-INF-γ mAb) for Th1 or Th2 polarization. The Th cells were added to the two types of in vitro osteoclast differentiation systems: osteoclast precursor cells derived from BM cells (BMCs) were stimulated with recombinant RANKL and M-CSF (the RANKL–M-CSF system), or co-cultured with osteoblasts in the presence of 1,25(OH)2 vitamin D3 (VitD3) and prostaglandin E2 (PGE2) (the co-culture system), and the formation of MNCs stained for tartrate-resistant acid phosphatase (TRAP), a marker enzyme for osteoclasts, was evaluated (Fig. 1 A). When Th1 or Th2 cells were added to the RANKL–M-CSF system at the same time as RANKL, both subsets had a marked inhibitory effect on the formation of TRAP+ MNCs and the inhibitory effects were dependent on the number of added T cells (Fig. 1, B and D). These inhibitory effects were significantly enhanced by restimulation with soluble anti-CD3 mAb, suggesting that restimulation of T cell receptor augments the polarized cytokine secretion and the inhibitory effects on osteoclastogenesis. If Th1 or Th2 cells were added to the co-culture system 2 d after BMC addition, the inhibitory effects of both subsets on osteoclastogenesis were exerted only by T cells restimulated with anti-CD3 mAb (Fig. 1, C and D). Th1 and Th2 cells both had less suppressive effects in the co-culture system, possibly because osteoblasts provide protection against the inhibitory effects through costimulatory signals (27) (see Discussion). As expected, the Th1 and Th2 subsets used in these experiments produced a significant amount of IFN-γ and IL-4, respectively (Fig. 1 E). The inhibitory effects of Th1 cells on osteoclastogenesis were completely abrogated on the BM-derived monocyte/macrophage precursor cells (BMMs) derived from IFN-γ receptor–deficient (Ifngr1−/−) mice (28), indicating that IFN-γ is responsible for the Th1 cell–mediated inhibition of osteoclastogenesis (Fig. 1 F). We further analyzed the effects of CD4+CD25+ T reg cells on osteoclastogenesis in both systems, but they were found to have neither an enhancing nor an inhibitory effect (Fig. 1 G), suggesting that T reg cells are not directly related to the T cell–mediated regulation of bone resorption.

Characterization of MNCs induced by Th2 cells and IL-4

It has been reported that the inhibitory effect of IFN-γ on osteoclastogenesis is reduced if the osteoclast precursor cells encounter RANKL before IFN-γ stimulation, suggesting that RANKL–prestimulated preosteoclasts are resistant to such inhibitory cytokines (29). To test whether the inhibitory effects of Th cells on osteoclastogenesis are also dependent on the differentiation stage of the osteoclast precursor cells, we added the Th cells to the osteoclast formation systems 1 d later than in the previous experiment. Interestingly, the inhibitory effects of Th cells were less (Fig. 2 A). Although Th1 cells inhibited the formation of TRAP+ MNCs under this condition, Th2 cells induced a normal number of TRAP+ MNCs in the RANKL–M–CSF system and even an increased number in the co-culture system (Fig. 2 A).
These results appeared to suggest that Th2 cells increase osteoclastogenesis under certain conditions, but the MNCs induced in the presence of Th2 cells were only weakly stained for TRAP (TRAP<sup>dim</sup>) and were incapable of bone resorption (Fig. 2, B and C). Even in the presence of Th2 cells, TRAP<sup>+</sup> MNCs with bone-resorbing activity were formed from BMMs derived from mice deficient in Stat6, which is an essential mediator of IL-4 signaling (30), suggesting that IL-4 is involved in the formation of TRAP<sup>dim</sup> MNCs. Consistent with this, the addition of IL-4 to the RANKL–M–CSF system at the same time as RANKL strongly inhibited TRAP<sup>+</sup> MNC formation, and the addition of IL-4 1 d later induced TRAP<sup>dim</sup> MNCs (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20061775/DC1). The effects of IL-4 were abrogated if added to Stat6-deficient cells, which generated TRAP<sup>+</sup> MNCs that were able to resorb bone.

To further characterize the TRAP<sup>dim</sup> MNCs induced by Th2 cells through IL-4, we performed a genome-wide microarray screening of the genes expressed in the TRAP<sup>dim</sup> MNCs (31). TRAP<sup>dim</sup> MNCs induced by IL-4 expressed a high level of genes characteristic of activated macrophages, including chemokine ligands and enzymes involved in allergic responses (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20061775/DC1). The expression of most of the genes important for osteoclast differentiation or functions was decreased (Fig. 2 D). The expression of NFATc1, an essential transcription factor for osteoclastogenesis (31, 32), was also revealed to be down-regulated by immunostaining (Fig. 2 E). Thus, the TRAP<sup>dim</sup> MNCs induced by Th2 cells are not authentic osteoclasts but rather should be classified as macrophage polykarions.

**Th17 cells stimulate osteoclastogenesis through osteoclastogenesis-supporting cells**

Because the above results show that neither Th1, Th2, nor T reg cells enhance osteoclastogenesis, we next focused on a newly identified CD4<sup>+</sup> T cell subset producing IL-17 called Th17 (33, 34). We suspected the Th17 subset to be a good candidate for the osteoclastogenic Th subset because it has been reported that IL-17 induces RANKL on mesenchymal cells and promotes osteoclastogenesis in vitro (25). Moreover, Th17 cells, which produce IL-17 (IL-17A) and its related cytokines such as IL-17F, but not IFN-γ or IL-4, are responsible for a variety of autoimmune inflammatory effects (9, 10). Recent studies suggest that TGF-β and IL-6 are essential for the initiation of Th17 differentiation and IL-23 is critical for expanding the population (35, 36). IL-23 is one of the IL-12 family cytokines and is a heterodimer consisting of the subunits p40 and p19 (9, 10). Even though IL-23 shares a p40 subunit and one of its receptor subunits (IL-12β1) with...
IL-12, IL-23 and IL-12 selectively play critical roles in the regulation of Th1 and Th17 polarization, respectively.

To obtain the Th17 cells, we stimulated CD4⁺ T cells with anti-CD3/CD28 mAbs in the presence of IL-23, anti–IFN-γ mAb, and anti–IL-4 mAb. In the presence of Th17 cells, TRAP⁺ MNCs were efficiently formed in the RANKL–M-CSF system (Fig. 3 A) and possessed bone-resorbing activity (not depicted), although the efficiency is a little less than in the control culture without the T cells. Moreover, in the co-culture system, the Th17 cells significantly enhanced the formation of TRAP⁺ MNCs (Fig. 3 A). Consistent with the previous reports, Th17 cells used in the above experiments produced a large amount of IL-17 but little IFN-γ, but Th1 cells did the opposite (Fig. 3 B). When Th17 cells were added to the co-culture system even in the absence of VitD₃ and PGE₂, the formation of TRAP⁺ MNCs was observed (Fig. 3 C). The osteoclastogenic effects of Th17 cells in the co-culture system was greatly reduced when we used Th17 cells derived from Il17−/− mice (37), indicating that the IL-17 produced from Th17 cells is mainly responsible for the osteoclastogenic effects of Th17 cells. IL-23 or IL-17 had no effect on osteoclastogenesis in the RANKL–M-CSF system, but IL-17 promoted osteoclastogenesis in the co-culture system, suggesting that Th17 cells does not directly act on osteoclast precursor cells but rather on osteoclastogenesis-supporting cells (Fig. 3 D). This is consistent with the previous report that IL-17 promotes osteoclastogenesis through the induction of RANKL on osteoblastic cells (25). These results show that Th17 is the only osteoclastogenic Th subset according to the currently accepted categorization of CD4⁺ T cells, and that Th17 cells facilitate osteoclastogenesis, possibly through IL-17–mediated induction of RANKL on osteoblastic cells.

We evaluated the expression level of RANKL on the surface of Th cells and found that Th17 cells express a significant amount of RANKL, but Th1 cells express only a minimal amount (Fig. 3 E). Neither subset, however, exhibited promotive effects on osteoclastogenesis in the RANKL–M-CSF system (Fig. 3 A) or induced any TRAP⁺ cells when added to the BMM culture in the absence of exogenous soluble RANKL (not depicted). Thus, it is evident that the RANKL expressed by Th cells alone is not sufficient to activate osteoclastogenesis (see Discussion).

The IL-23–IL-17 axis plays a critical role in inflammation-induced bone destruction in vivo

To clarify the role IL-17 and IL-23 play in bone metabolism in vivo, we investigated the phenotype of Il17−/− and Il23a−/− (lacking p19) (38) mice. There was no significant difference in bone mineral density as evaluated by dual-energy x-ray absorptiometry (Fig. 4 A). Microradiography also

![Figure 4](https://jem.rupress.org/article-pdf/203/6/2677/2677.pdf)
Coordinated activation of the innate and adaptive immune systems is essential for the efficient eradication of pathogens, revealing no obvious abnormality in skeletal development (Fig. 4 B). Bone morphometric analyses revealed the parameters of bone resorption and formation to be normal even in the mutant mice (Fig. 4 C), indicating that neither IL-17 nor IL-23 is involved in the physiological regulation of bone homeostasis.

To further investigate the role of IL-17 and IL-23 in the disease conditions characterized by enhanced osteoclastogenesis associated with T cell activation, we used an LPS-induced model of inflammatory bone destruction, which is not induced by an autoantigen but is T cell dependent (14, 39). Because it is well documented that IL-17 and IL-23 play an important role in the development of autoimmune arthritis (23, 24), we used this inflammatory bone destruction model to evaluate their role in the osteoclast-mediated destruction phase. LPS injection into the calvarial bone results in severe bone destruction associated with aberrant formation of osteoclasts in WT mice, but the level of bone destruction was much less pronounced and the osteoclast formation was significantly reduced in both the Il17−/− mice and Il23a−/− mice (Fig. 4 D). These results suggest that the Th17 cells expanded through IL-23 stimulation are involved in the T cell–mediated osteoclastogenesis in vivo. In contrast, the bone destruction was enhanced and a greater number of osteoclasts were formed in Il17−/− Stat6−/− mice, which are deficient in the response to both IFN-γ and IL-4 (Fig. 4 D), suggesting that IFN-γ and IL-4 may play a protective role against bone destruction by suppressing osteoclastogenesis associated with inflammation.

The above results suggest that IL-23–stimulated proliferation of Th17 cells, a major osteoclastogenic Th subset, plays a pivotal role in inflammatory bone destruction by inducing RANKL through an IL-17 effect on mesenchymal cells. Consistent with this, it has been reported that RANKL is abundantly expressed in the synovial fibroblasts of RA patients (16, 40) and the IL-17 concentration is elevated in the synovial fluid of RA patients (25). To explore the role of IL-23 in the induction of RANKL in RA, we investigated whether IL-23 was detected in the synovium of RA patients. Quantitative RT-PCR analysis revealed the mRNA level of RANKL with that of IL23A (p19), IL12A (p35), or IL12B (p40) in the synovium of RA patients. The relative expressions of RANKL, IL23A, IL12A, and IL12B were all standardized using that of GAPDH (Fig. 5 A).

As summarized in Fig. 5 B, the results in this study show that Th1 and Th2 cells inhibit osteoclastogenesis by acting on the precursor cells, mainly through IFN-γ and IL-4, respectively, but aberrant or prolonged activation under certain pathological conditions, such as autoimmune inflammation, results in tissue damage through the activation of effector cells. In autoimmune arthritis, it has long been a challenging question as to how the abnormality of the immune system induces the skeletal damage, although the infiltration of CD4+ T cells in the RA synovium is a pathogenetic hallmark and is undoubtedly linked to the bone destruction that ensues (3, 13, 14, 20). After RANKL was cloned and the high RANKL expression in the synovium was brought to light (16, 40), the importance of bone-resorbing osteoclasts came into general acceptance (3). Based on recent reports using genetically modified mice, the crucial role of osteoclasts in the inflammatory bone loss has been established (41, 42), but which CD4+ T cells cause the induction of osteoclasts, and by what mechanism, has remained elusive.

As RANKL is expressed in activated T cells, T cells may have the capacity to induce osteoclast differentiation by directly acting on osteoclast precursor cells (13, 26). However, because T cells also secrete a variety of cytokines and express membrane-bound factors other than RANKL, the effects of T cells on osteoclastogenesis should be dependent on the balance of positive and negative factors expressed by the T cells. As summarized in Fig. 5 B, the results in this study show that Th1 and Th2 cells inhibit osteoclastogenesis by acting on the precursor cells, mainly through IFN-γ and IL-4, respectively.
The inhibitory effects of these cytokines were less observed in the co-culture system than in the RANKL–M-CSF system (Figs. 1, B and C, and 2 A). We infer that osteoblasts may provide membrane-bound RANKL and stimulate costimulatory signals for RANKL simultaneously, enabling the strong cell–cell contact between osteoblasts and osteoclast precursor cells and preventing the access of T cells or inhibitory cytokines to osteoclast precursor cells.

Previous observations that IL–12 and IL–18, which drive Th1 differentiation, both inhibit osteoclastogenesis via IFN-γ or GM–CSF (43, 44), and that IL–10, which is released from Th2 cells, also negatively regulates osteoclastogenesis (45) further support the negative role of Th1 and Th2 cells on osteoclastogenesis. In contrast, Th17 cells stimulated by IL–23 promote osteoclastogenesis mostly through production of IL–17 (Fig. 1, B and C, and 2 A). We infer that osteoblasts may provide membrane-bound RANKL and stimulate costimulatory signals specifically by osteoblasts (12, 27) are missing in T cells; and (b) costimulatory signals provided specifically by osteoblasts (12, 27) are missing in T cells; and (c) cell adhesion induces specific signals including those mediated by integrins, which are also important for osteoclastogenesis (49).

In our study, T reg cells had no apparent effect on osteoclastogenesis in vitro (Fig. 1 G). However, their function in the regulation of bone metabolism should be investigated in vivo considering the recent finding that the development of Th17 cells and T reg cells is coordinately regulated (10, 35, 36).

The importance of the IL–23–IL–17 axis in the autoimmune inflammation has been demonstrated in a variety of models of autoimmune diseases such as arthritis and encephalomyelitis (23, 24, 38). In arthritis models, IL–17−/− mice were protected from the development of destructive arthritis (24), whereas collagen-induced arthritis is exacerbated in IFN-γ receptor–deficient mice (21, 22). The specific role of IL–23 compared with IL–12 in the development of arthritis has been clearly demonstrated by a genetic study using mice deficient in p19 and p35 (23). Based on these observations, the IL–23–IL–17 axis inducing Th17 cells, rather than the IL–12–IFN-γ axis inducing Th1 cells, is critical for the development of autoimmune arthritis. Our study also provides evidence that the IL–23–IL–17 axis plays a critical role even in a model of bone loss induced by local inflammation that is independent of autoimmunity (Fig. 4 D), suggesting that the IL–23–IL–17 axis is not only essential for the onset phase, but also for the destruction phase of autoimmune arthritis characterized by the T cell–mediated activation of osteoclastogenesis. Thus, Th17 cells, an osteoclastogenic subset, have profound relevance in the bone damage that takes place in autoimmune arthritis. The identification of T cell subsets in the synovium of arthritis is a challenging issue of great importance that should be pursued in a future study. Considering the strong inhibitory effects of Th1 cells on osteoclastogenesis, Th17 cells may be overwhelmingly dominant and the co-localization of Th1 cells is unlikely, at least under the microenvironments in which osteoclastogenesis efficiently occurs. The positive correlation between IL–23 and RANKL expression in the synovium of RA patients further suggests the importance of IL–23 in the regulation of local osteoclastogenesis through IL–17 (Fig. 5 A). Despite the importance of TGF-β and IL–6 in the initiation of Th17 development (10, 35, 36), Th17 cells can be obtained in an IL–23–stimulated culture system without adding exogenous TGF-β/IL–6, suggesting that the endogenous level of TGF-β/IL–6 may suffice for the initiation and that osteoclastogenic activity of Th17 cells is mainly determined by IL–23 under certain pathological conditions.

For the treatment of RA, there are several drugs available, most of which were developed to modulate immune reactions. The anti-rheumatic drugs are effective in treating pain and inflammation, but patients still frequently have to undergo joint replacement surgery because of the progressive bone damage despite long-term treatment with anti-rheumatic drugs. Therefore, it is clinically an urgent issue to establish a method to prevent such persistent bone destruction (3). Although rheumatologists are now aware of the great impact that anti-TNF therapy has had on the management of RA (50), it is still not determined whether all patients respond
to the therapy or, indeed, whether bone destruction will be completely prevented by it. Recent progress in understanding the mechanism of bone loss in RA has provided promising new strategies, one of which is an anti–RANKL antibody directly suppressing RANKL–mediated osteoclastogenesis (51). As we have demonstrated a new role of Th17 in the context of bone damage in RA, the significance of the IL-23–IL-17 axis extends beyond the simple initiation or development of the autoimmune disease. Because osteoclastogenic Th17 cells link the autoimmune inflammation to bone damage, inhibition of this axis has the potential of a doubly beneficial impact on RA, i.e., in the context of both the immune and skeletal systems, and thus appears to be an ideal therapeutic strategy for ameliorating the bone destruction associated with T cell activation.

MATERIALS AND METHODS

Mice. B6.AgR1<−/−> (28), Stat6<−/−> (30), B17<−/−> (37), and R23a<−/−> mice (38) were described previously. All the mice were maintained under specific pathogen-free conditions and were backcrossed to C57BL/6 mice. All animal experiments were performed with the approval of the Animal Study Committee of Tokyo Medical and Dental University and conformed to relevant guidelines and laws.

Analysis of bone phenotype and LPS-induced bone destruction. Mice were subjected to histomorphometric and microradiographic examinations as described previously (27). 8-wk-old mice were injected with 25 mg/kg body weight LPS (Sigma-Aldrich) subcutaneously in the calvarial bone. After 5 d, calvarial bones were analyzed as described previously using decalcified paraffin sections (14).

In vitro assays for osteoclast differentiation and function. In vitro osteoclast differentiation was described previously (27, 52). For the RANKL–M-CSF system, we cultured BMCs with 10 ng/ml M-CSF (R&D Systems) osteoclast differentiation was described previously (27, 52). For the RANKL–TRAP<sup>+</sup> system, we used a LightCycler (Roche), as described previously (52). We performed quantitative RT-PCR using a LightCycler (Roche), as described previously (52) with the following primers.

Statistical analyses. All data were expressed as the mean ± SEM (n = 4, unless otherwise indicated). Mann-Whitney U test was used for statistical analyses (*, P < 0.05; **, *P < 0.01), and comparisons were made between each sample and the control (not treated with T cells/cytokines or WT mice).

Online supplemental material. Fig. S1 shows the effect of recombinant IL-4 on osteoclast precursor cells derived from WT or Stat6<−/−> mice in the RANKL–M-CSF system. Fig. S2 shows the list of genes whose expression was increased by IL-4 in osteoclast precursor cells (GeneChip analysis). Figs. S1 and S2 are available at http://www.jem.org/cgi/content/full/jem.20061775/DC1.

The authors have no conflicting financial interests.

Submitted: 18 August 2006
Accepted: 12 October 2006

REFERENCES


