1) the effect of IL-6 in the study by Duplomb et al was evaluated in a culture system with exogenous addition of RANKL, and 2) TNFα was not added to the system used in that study. Similarly, the combination of IL-1β and TNFα was reported to induce the differentiation of osteoclasts in a RANKL-independent manner (15). However, in our system, the addition of anti–IL-1β antibody revealed that IL-1β was not involved in the differentiation of osteoclast-like cells.

We investigated the molecular mechanisms driving differentiation of the cells and observed that the addition of IL-6 has little impact on the NF-κB pathway but augments c-Fos at the protein level. The expression level and activity of NF-ATc1 are critical for the differentiation of osteoclast-like cells, and we observed both to be elevated in response to the combination of TNFα and IL-6. In addition, the conditional knockout of *Stat3* did not significantly reduce the efficiency of the differentiation of TRAP-positive multinucleated cells. This result was unexpected given the important role of STAT-3 in IL-6 signal transduction. Thus, another important IL-6 signaling pathway downstream of JAK, namely the MEK/ERK pathway, is likely to play a crucial role in the induction of c-Fos (28). Indeed, the osteoclast-like cells were more susceptible to MEK inhibitors than were conventional osteoclasts (see Figure 5).

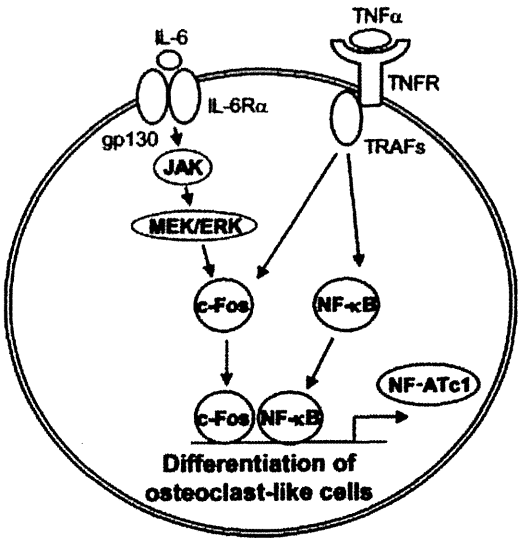

Figure 5. Schematic model of the differentiation of osteoclast-like cells induced by TNFα and IL-6. IL-6 signaling induces the up-regulation of c-Fos expression at the protein level, leading to the expression of *Nfatc1*. This induction is dependent on JAK but independent of STAT-3; the MEK/ERK pathway is likely to play an important role. TNFR = TNF receptor; TRAFs = TNFR-associated factors (see Figure 1 for other definitions).

MTX has been reported to inhibit IL-6 production under a variety of in vitro and in vivo conditions (30,31). Thus, we speculate that the inhibition of production and/or the activity of IL-6 by MTX may be one of the mechanisms by which the combination of a TNFα blocker and MTX almost completely blocks the periarticular bone destruction that occurs in RA (3). Considering the wide range of pharmacologic effects of MTX, however, it cannot be ruled out that it may also contribute to bone protection through other mechanisms.

The contribution of proinflammatory cytokines to bone resorption has been established both clinically and experimentally (32). In terms of the mechanism underlying the bone damage, most of the studies have focused on the capacity of the proinflammatory cytokines to induce RANKL (33,34). The aforementioned report by Kim et al (15) is noteworthy in that it claimed that the combination of IL-1 and TNFα induced the differentiation of osteoclasts in a RANKL-independent manner. There are also reports that TNFα alone is able to induce osteoclastogenesis independent of RANKL (35,36). Interestingly, Kobayashi and colleagues reported that such osteoclasts have bone-resorbing activity only in the presence of IL-1 (35). In a study by Sabokbar et al, human macrophages derived during surgery performed for aseptic loosening of hip implants were used as the osteoclast precursors. In that study, too, the addition of IL-1 further promoted osteoclast differentiation and function compared with TNFα alone (36). Moreover, a substantial number of TRAP-positive multinucleated cells was observed, even in the absence of exogenous TNFα. Thus, it is likely that the macrophages derived from periprosthetic sites were already activated and thus released various proinflammatory cytokines involved in osteoclastogenesis.

Taken together, the results of these studies imply that several combinations of proinflammatory cytokines, which are likely to coexist in the sites affected by arthritis, can induce the differentiation of bone-resorbing cells. These cells may have characteristics different from those of conventional osteoclasts that are induced by RANKL. For example, conventional osteoclasts are susceptible to an anti-RANKL antibody (37) or OPG. However, compared with differentiation of conventional osteoclasts, the differentiation of osteoclast-like cells induced by TNFα and IL-6 is more easily inhibited by ERK inhibitors and tofacitinib, although tofacitinib did not affect TRAP positivity. In this sense,

such cells might well be called "inflammatory osteoclast-like cells."

We expect that a greater understanding of the relative contribution of inflammatory osteoclast-like cells and conventional osteoclasts to RA can be gained through close scrutiny of the effects of novel antirheumatic drugs, including tofacitinib, on bone destruction. It is hoped that as a result, new combinations of drugs that are more efficient in preventing bone damage and have fewer side effects will become available in the near future.

ACKNOWLEDGMENTS

We are grateful to N. Murai, N. Shiraishi, N. Koga, Y. Yamada, N. Kurosawa, Y. Aizaki, and T. Ishibashi (Saitama Medical University) for technical assistance. We also thank S. Hida (Shinshu University), M. Asagiri (Kyoto University), and U. Sato (Tokyo Hitachi Hospital) for helpful discussions.

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. Sato 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. Yokota, Sato, Mimura.

Acquisition of data. Yokota, Sato, Miyazaki, Kitaura, Kayama, Takeda.

Analysis and interpretation of data. Yokota, Sato, Miyoshi, Araki, Akiyama, Mimura.

REFERENCES

1. Lam J, Takeshita S, Barker JE, Kanagawa O, Ross FP, Teitelbaum SL. TNF-α induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. J Clin Invest 2000;106:1481–8.
2. Ochi S, Shinohara M, Sato K, Gober HJ, Koga T, Kodama T, et al. Pathological role of osteoclast costimulation in arthritis-induced bone loss. Proc Natl Acad Sci U S A 2007;104:11394–9.
3. Klareskog L, van der Heijde D, de Jager JP, Gough A, Kalden J, Malaise M, et al, for the TEMPO (Trial of Etanercept and Methotrexate with Radiographic Patient Outcomes) study investigators. Therapeutic effect of the combination of etanercept and methotrexate compared with each treatment alone in patients with rheumatoid arthritis: double-blind randomised controlled trial. Lancet 2004;363:675–81.
4. Takeda K, Clausen BE, Kaisho T, Tsujimura T, Terada N, Forster I, et al. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. Immunity 1999;10:39–49.
5. Sato K, Suematsu A, Nakashima T, Takemoto-Kimura S, Aoki K, Morishita Y, et al. Regulation of osteoclast differentiation and function by the CaMK-CREB pathway. Nat Med 2006;12:1410–6.
6. Sato K, Suematsu A, Okamoto K, Yamaguchi A, Morishita Y, Kadono Y, et al. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. J Exp Med 2006;203:2673–82.
7. Kitaura H, Zhou P, Kim HJ, Novack DV, Ross FP, Teitelbaum SL. M-CSF mediates TNF-induced inflammatory osteolysis. J Clin Invest 2005;115:3418–27.
8. Kinugawa S, Koide M, Kobayashi Y, Mizoguchi T, Ninomiya T, Muto A, et al. Tetracyclines convert the osteoclastic-differentiation pathway of progenitor cells to produce dendritic cell-like cells. J Immunol 2012;188:1772–81.
9. Minkin C. Bone acid phosphatase: tartrate-resistant acid phosphatase as a marker of osteoclast function. Calcif Tissue Int 1982;34:285–90.
10. Nishimoto N, Kishimoto T. Interleukin 6: from bench to bedside. Nat Clin Pract Rheumatol 2006;2:619–26.
11. Tamura T, Udagawa N, Takahashi N, Miyaura C, Tanaka S, Yamada Y, et al. Soluble interleukin-6 receptor triggers osteoclast formation by interleukin 6. Proc Natl Acad Sci U S A 1993;90:11924–8.
12. Ishimi Y, Miyaura C, Jin CH, Akatsu T, Abe E, Nakamura Y, et al. IL-6 is produced by osteoblasts and induces bone resorption. J Immunol 1990;145:3297–303.
13. Mori T, Miyamoto T, Yoshida H, Asakawa M, Kawasumi M, Kobayashi T, et al. IL-1β and TNFα-initiated IL-6-STAT3 pathway is critical in mediating inflammatory cytokines and RANKL expression in inflammatory arthritis. Int Immunol 2011;23:701–12.
14. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998;93:165–76.
15. Kim N, Kadono Y, Takami M, Lee J, Lee SH, Okada F, et al. Osteoclast differentiation independent of the TRANCE-RANK-TRAF6 axis. J Exp Med 2005;202:589–95.
16. Teitelbaum SL. The osteoclast and its unique cytoskeleton. Ann N Y Acad Sci 2011;1240:14–7.
17. Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, et al. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev Cell 2002;3:889–901.
18. Franzoso G, Carlson L, Xing L, Poljak L, Shores EW, Brown KD, et al. Requirement for NF-κB in osteoclast and B-cell development. Genes Dev 1997;11:3482–96.
19. Iotsova V, Caamano J, Loy J, Yang Y, Lewin A, Bravo R. Osteopetrosis in mice lacking NF-κB1 and NF-κB2. Nat Med 1997;3:1285–9.
20. Johnson RS, Spiegelman BM, Papaioannou V. Pleiotropic effects of a null mutation in the c-fos proto-oncogene. Cell 1992;71:577–86.
21. Wang ZQ, Ovitt C, Grigoriadis AE, Mohle-Steinlein U, Ruther U, Wagner EF. Bone and haematopoietic defects in mice lacking c-fos. Nature 1992;360:741–5.
22. Higashi N, Kunimoto H, Kaneko S, Sasaki T, Ishii M, Kojima H, et al. Cytoplasmic c-Fos induced by the YXXQ-derived STAT3 signal requires the co-operative MEK/ERK signal for its nuclear translocation. Genes Cells 2004;9:233–42.
23. Williams NK, Bamert RS, Patel O, Wang C, Walden PM, Wilks AF, et al. Dissecting specificity in the Janus kinases: the structures of JAK-specific inhibitors complexed to the JAK1 and JAK2 protein tyrosine kinase domains. J Mol Biol 2009;387:219–32.
24. Burmester GR, Blanco R, Charles-Schoeman C, Wollenhaupt J, Zerbini C, Benda B, et al. Tofacitinib (CP-690,550) in combination with methotrexate in patients with active rheumatoid arthritis with an inadequate response to tumour necrosis factor inhibitors: a randomised phase 3 trial. Lancet 2013;381:451–60.
25. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res 1999;8:265–77.
26. Takeda K, Kaisho T, Yoshida N, Takeda J, Kishimoto T, Akira S. Stat3 activation is responsible for IL-6-dependent T cell proliferation through preventing apoptosis: generation and characterization of T cell-specific Stat3-deficient mice. J Immunol 1998;161:4652–60.
27. Schaper F, Gendo C, Eck M, Schmitz J, Grimm C, Anhuf D, et al.

Activation of the protein tyrosine phosphatase SHP2 via the interleukin-6 signal transducing receptor protein gp130 requires tyrosine kinase Jak1 and limits acute-phase protein expression. Biochem J 1998;335:557–65.
28. Sasaki T, Kojima H, Kishimoto R, Ikeda A, Kunimoto H, Nakajima K. Spatiotemporal regulation of c-Fos by ERK5 and the E3 ubiquitin ligase UBR1, and its biological role. Mol Cell 2006;24: 63–75.
29. Duplomb L, Baud'huin M, Charrier C, Berreur M, Trichet V, Blanchard F, et al. Interleukin-6 inhibits receptor activator of nuclear factor κB ligand-induced osteoclastogenesis by diverting cells into the macrophage lineage: key role of Serine727 phosphorylation of signal transducer and activator of transcription 3. Endocrinology 2008;149:3688–97.
30. Elango T, Dayalan H, Subramanian S, Gnanaraj P, Malligarjunan H. Serum interleukin-6 levels in response to methotrexate treatment in psoriatic patients. Clin Chim Acta 2012;413:1652–6.
31. Yoshida M, Kanno Y, Ishisaki A, Tokuda H, Hirade K, Nakajima K, et al. Methotrexate suppresses inflammatory agonist induced interleukin 6 synthesis in osteoblasts. J Rheumatol 2005;32:787–95.
32. Teitelbaum SL. Osteoclasts: culprits in inflammatory osteolysis. Arthritis Res Ther 2006;8:201.
33. Wei S, Kitaura H, Zhou P, Ross FP, Teitelbaum SL. IL-1 mediates TNF-induced osteoclastogenesis. J Clin Invest 2005;115:282–90.
34. Kitaura H, Sands MS, Aya K, Zhou P, Hirayama T, Uthgenannt B, et al. Marrow stromal cells and osteoclast precursors differentially contribute to TNF-α-induced osteoclastogenesis in vivo. J Immunol 2004;173:4838–46.
35. Kobayashi K, Takahashi N, Jimi E, Udagawa N, Takami M, Kotake S, et al. Tumor necrosis factor α stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. J Exp Med 2000;191:275–86.
36. Sabokbar A, Kudo O, Athanasou NA. Two distinct cellular mechanisms of osteoclast formation and bone resorption in periprosthetic osteolysis. J Orthop Res 2003;21:73–80.
37. Brown JP, Prince RL, Deal C, Recker RR, Kiel DP, de Gregorio LH, et al. Comparison of the effect of denosumab and alendronate on BMD and biochemical markers of bone turnover in postmenopausal women with low bone mass: a randomized, blinded, phase 3 trial. J Bone Miner Res 2009;24:153–61.