Tumor Necrosis Factor-α Increases Circulating Osteoclast Precursor Numbers by Promoting Their Proliferation and Differentiation in the Bone Marrow through Up-regulation of c-Fms Expression*

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Osteoclasts are essential cells for bone erosion in inflammatory arthritis and are derived from cells in the myeloid lineage. Recently, we reported that tumor necrosis factor-α (TNFα) increases the blood osteoclast precursor (OCP) numbers in arthritic patients and animals, which are reduced by anti-TNF therapy, implying that circulating OCPs may have an important role in the pathogenesis of erosive arthritis. The aim of this study is to investigate the mechanism by which TNFα increases this increase in OCP frequency. We found that TNFα stimulated cell division and conversion of CD11b+/Gr-1−/c-Fms+ to CD11b+/Gr-1−/lo/c-Fms− cells, which was not blocked by neutralizing macrophage colony-stimulating factor (M-CSF) antibody. Ex vivo analysis of monocytes demonstrated the following: (i) blood CD11b+/Gr-1−/lo cells but not CD11b+/Gr-1−/hi cells give rise to osteoclasts when they were cultured with receptor activator NF-κB ligand and M-CSF; and (ii) TNFα-transgenic mice have a significant increase in blood CD11b+/Gr-1−/hi cells and bone marrow proliferating CD11b+/Gr-1−/lo cells. Administration of TNFα to wild type mice induced bone marrow CD11b+/Gr-1−/lo cell proliferation, which was associated with an increase in CD11b+/Gr-1−/lo OCPs in the circulation. Thus, TNFα directly stimulates bone marrow OCP genesis by enhancing c-Fms expression. This results in progenitor cell proliferation and differentiation in response to M-CSF, leading to an enlargement of the marrow OCP pool. Increased marrow OCPs subsequently egress to the circulation, forming a basis for elevated OCP frequency. Therefore, the first step of TNFα-induced osteoclastogenesis is at the level of OCP genesis in the bone marrow, which represents another layer of regulation to control erosive disease.

Mature osteoclasts are essential effector cells for normal bone remodeling and pathologic bone loss seen in many forms of erosive diseases, such as rheumatoid arthritis (RA).1,2 The importance of osteoclastic resorption in this process has been proven in studies with various knock-out mice deficient in genes essential for osteoclastogenesis, which develop osteopetrosis because of the accumulation of un-resorbed bone matrix within the bone marrow cavity (1, 2). Similarly, these mice are completely resistant to bone destruction of affected joints when they are induced to develop erosive arthritis (3–5). Osteoclasts are derived from common osteoclast/monocyte precursors that are generated in bone marrow and travel to peripheral tissues through the bloodstream (6). In patients or animals with RA, these precursor cells constantly migrate to inflamed joints perhaps from the following two directions: "outside in," from blood to the pannus-bone interface, and "inside out," from epiphysial bone marrow to the subchondral bone. They then differentiate to mature osteoclasts in response to high levels of osteoclastogenic cytokines, including receptor activator NF-κB ligand (RANKL), macrophage-colony-stimulating factor (M-CSF), and tumor necrosis factor-α (TNFα), produced by inflammatory cells in the synovium (7, 8). Although the teams of mature osteoclasts mediate focal erosions via resorption of the periarticular and subchondral bone over long periods of time (months to years), the life span of individual osteoclasts is only a few weeks. Thus, mature osteoclasts must be constantly replaced by a perpetual supply of osteoclast precursors (OCPs). Currently, the molecular mechanism by which joint inflammation sustains the perpetual supply of OCPs is poorly understood.

OCPs are derived from c-Kit+ multipotent hematopoietic stem cells in the bone marrow through a series of differentiation processes (9). The first step of OCP ontogeny is the commitment of hematopoietic stem cells to the myeloid lineage under the control of the Ets transcription factor PU.1 (10). Then the myeloid progenitors survive, proliferate, and differentiate into various "downstream" lineages, including OCPs. These events have been characterized by changes in the surface expression of distinct markers. For instance, early myeloid progenitors do not express detectable levels of c-Fms, the receptor for M-CSF, an essential survival factor for OCPs. As such, they exhibit lower proliferation and differentiation potency. However, under the influence of hematopoietic factors such as stem cell factor and perhaps M-CSF itself, c-Fms− cells differentiate to c-Fms+ cells (11). The conversion of c-Fms− cells to c-Fms+ cells is an important landmark for the progression of myelopoiesis because the M-CSF signal at this stage is critical for cell survival (anti-apoptosis), proliferation, and differentiation. However, the regulation of this conversion in chronic inflammatory bone diseases has not been well studied.

TNFα is one of the most potent pro-inflammatory cytokines, and its role in RA has been formally established by the development of anti-TNF therapy. Additional proof comes from studies of TNF transgenic (TNF-Tg)
mice that develop erosive arthritis featured with intense synovial inflammation and destruction of cartilage and bone (12). These mice have increased numbers of OCPs in spleen and blood and increased numbers of mature osteoclasts in the affected joints (13). The role of TNFα in osteoclastogenesis has been studied extensively in the last decade. Administration of TNFα into wild-type (WT) mice greatly increases the number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts locally and systematically (13, 14). In vitro, TNFα directly stimulates mature osteoclast formation from OCPs in the absence of osteoblasts/stromal cells through activation of NFκB and nuclear factor of activated T cells pathways (15). It also promotes the production of M-CSF by T lymphocytes and RANKL by osteoblasts, thereby indirectly stimulating osteoclast formation (16, 17). However, whether TNFα promotes OCP generation from early myeloid precursors and the mechanisms involved in this process have not been investigated.

In this study, we test the hypothesis that TNFα increases OCP numbers through regulation of c-Fms expression. We demonstrate that TNFα increases the proliferation of OCPs in vivo and in vitro and promotes the differentiation of c-Fms+ cells to c-Fms+ cells. TNFα-induced OCP genesis is directly related to increased blood OCP frequency. Thus, our results reveal a new mechanism for TNFα in the control of peripheral osteoclast numbers and provide an additional regulatory step to control osteoclast formation and bone resorption in inflammatory erosive diseases.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant murine RANKL, TNFα, and human M-CSF (Invitrogen) were purchased from R & D Systems (Minneapolis, MN). Anti-murine CD11b (M1/70), M-Fms (AFS98), CD3 (145–2C11), B220 (RA3–6B2), and isotype controls were from eBioscience Inc. (San Diego, CA); anti-murine CD16/32 (FcγRII/II), c-Kit (2B8), Gr-1 (1A8), and isotype controls were from Pharmingen.

Animals—TNFα-Tg mice in a CBA × C57Bl/6 background (3647 TNF-tg line) were obtained from Dr. G. Kollias (12). Four-month-old TNFα-Tg mice were used because this is the age when they typically develop severe joint disease with elevated serum human TNFα concentrations (13). Acute TNFα in vivo treatment was performed as we described previously (13). In brief, 2-month-old C57/B6 male mice were randomly divided into TNFα and PBS groups. Murine TNFα (0.5 μg in 25 μl of PBS) or the same volume of PBS was injected four times/day for 3 days into the subcutaneous layer overlying the calvariae of WT mice. Bone marrow and PBMCs were collected for cell cycle analysis, as described below. The Institutional Animal Care and Use Committee approved all animal studies.

Generation of Osteoclasts—Cells from several sources were used to generate osteoclasts: 1) freshly isolated bone marrow cells; 2) M-CSF or TNFα pretreated nonadherent bone marrow cells; and 3) flow-sorted bone marrow or blood cells. Cells were cultured in α-modified essential medium (Invitrogen) with 10% fetal calf serum (Hyclone Laboratories, Logan, UT), RANKL (5 ng/ml), and M-CSF (10 ng/ml) for 3–5 days when multinucleated cells typically were observed under an inverted microscope. Cells were then fixed and stained for TRAP activity, and TRAP+ cells containing ≥3 nuclei were counted as mature osteoclasts as we described previously (13).

Fluorescence-activated Cell Sorting (FACS) Analysis and Cell Sorting—For FACS analysis, freshly isolated bone marrow, PBMCs or cultured OCPs were incubated with anti-murine CD16/32 to block Fc receptor-mediated antibody binding. Cells were then stained with various fluorescent-labeled antibodies. Data were acquired using a FACSCalibur flow cytometer (BD Biosciences) and analyzed using the Cellquest soft-

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total blood (72.4 ± 3.4%) or bone marrow (77.4 ± 2%) CD11b⁺ cells in adult C57/B6 mice. Because of this, OCP numbers may be overestimated if the frequency of CD11b⁺ cells is used to assess the number of OCPs in these compartments. In this study, we first characterized the osteoclastogenic potential of blood and bone marrow cells using CD11b⁺ and Gr-1 to eliminate CD11b⁺/Gr-1hi granulocytes (Fig. 1). Peripheral blood mononuclear cells (PBMC) and bone marrow cells from WT adult mice were stained with anti-CD11b and Gr-1 antibodies, and

FIGURE 1. Identification of cell populations with osteoclastogenic potential from peripheral blood and bone marrow cells. A, PBMCs or bone marrow cells (B) from a pool of five WT mice were stained with FITC anti-CD11b and PE anti-Gr-1 antibodies and subjected to FACS analysis. The living cells were gated using forward and side scatter (R1). The CD11b⁺/Gr-1lo (R2), CD11b⁺/Gr-1hi (R3), and CD11b⁺/Gr-1⁻ (R4) populations were sorted and cultured with M-CSF and RANKL to generate osteoclasts. Photography (×4) of the TRAP⁺ osteoclasts formed from purified PBMCs and from bone marrow are shown in the upper and lower panels, respectively. C, the number of TRAP⁺ osteoclasts formed from above cultures was quantified and presented as means plus S.E. of three wells. Experiments were repeated once with similar results.
three populations were fractionated by FACS on the basis of their surface expression: CD11b+/Gr-1lo (R2), CD11b+/Gr-1hi (R3), and CD11b-/Gr-1- (R4). The sorted cells were cultured with M-CSF and RANKL to generate osteoclasts that were assessed by TRAP staining. Although both CD11b+/Gr-1lo (R2) and CD11b-/Gr-1- (R4) marrow cells gave rise to osteoclasts (Fig. 1), the number of TRAP+ osteoclasts was much higher in the CD11b+/Gr-1lo (R2) fraction than that in the CD11b+/Gr-1- (R4) fraction (Fig. 1, B and C). The CD11b+/Gr-1hi cells did not form TRAP+ osteoclasts and died off under our culture conditions. Because it is likely that marrow CD11b+ cells differentiate to TRAP+ osteoclasts through the CD11b+/Gr-1- stage and only blood CD11b+/Gr-1lo cells give rise to osteoclasts, we reasoned that CD11b+/Gr-1lo cells include the majority of OCPs and can be used as OCP surface markers.

**TNF-Tg Mice Have Increased Blood and Bone Marrow OCPs—** By using CD11b+/Gr-1lo as OCP markers, we found that in TNF-Tg mice, the percentage of blood CD11b+/Gr-1lo OCPs increased 5-fold (Fig. 2A), and their absolute number increased 14-fold, compared with WT littermates. TNF-Tg mice also had a 1-fold increase in bone marrow CD11b+/Gr-1lo but not in CD11b+/Gr-1- cells (Fig. 2B). Consistent with this change, the percentage of CD11b+/Gr-1hi granulocyte numbers was reduced in these mice. However, the absolute number of granulocytes was unchanged both in blood (cell number (106/ml), 1.48 ± 0.21 in TNF-Tg mice versus 1.36 ± 0.16 in WT mice) and in bone marrow (cell number (106/femur), 3.53 ± 1.34 in TNF-Tg versus 3.32 ± 1.14 in WT mice, respectively). The osteoclast-forming potential of purified marrow CD11b+/Gr-1lo cells from TNF-Tg mice and WT littermates was determined by cell culture in the presence of different amounts of M-CSF and RANKL. In all cases, TNF-Tg CD11b+/Gr-1lo cells formed significantly more TRAP+ osteoclasts (Fig. 2C), indicating that TNF-Tg mice have more OCPs within the CD11b+/Gr-1lo population.

**TNFα Promotes the Proliferation of Bone Marrow OCPs—** To examine whether increased bone marrow CD11b+/Gr-1lo OCPs in TNF-Tg mice results from an alteration in proliferation, cell cycle analysis was performed using a combination of anti-CD11b and Gr-1 antibodies and Hoechst 33342 DNA dye staining. This approach allows us to assess cell cycle status in different fractions of CD11b+ and Gr-1-stained cells simultaneously and avoid cell sorting and in vitro labeling. Compared with CD11b+/Gr-1lo and CD11b+/Gr-1hi cells, the CD11b+/Gr-1lo population contained cells in the S/G2/M phase of the cell cycle in both TNF-Tg and WT mice (Fig. 3A). Furthermore, more TNF-Tg CD11b+/Gr-1lo cells were in the S/G2/M phase than those of WT littermates, and there was no difference in the frequency of apoptosis (Fig. 3B, upper panel). In contrast to marrow cells, PBMCs were not in

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**FIGURE 2. Increased blood and bone marrow OCP frequency in TNF-Tg mice.** A, PBMCs from 4-month-old TNF-Tg mice and WT littermates were stained with anti-CD11b and anti-Gr-1 antibodies and subjected to FACS analysis. Representative histograms show the gated population of CD11b+/Gr-1lo OCPs. The percentage of blood OCPs is shown as means plus S.E. from three pairs of TNF-Tg mice and WT littermates. B, bone marrow cells were stained with anti-CD11b and anti-Gr-1 antibodies and analyzed by FACS. The percentage of individual cell population is shown as means ± S.E. from three pairs of TNF-Tg mice and WT littermates. C, purified CD11b+/Gr-1lo cells from bone marrow of TNF-Tg mice and WT littermates were cultured with M-CSF and RANKL to generate osteoclasts. The number of TRAP+ osteoclasts was counted and shown as means ± S.E. of three wells. Similar results were obtained from two independent experiments. *p < 0.05 versus WT mice.
FIGURE 3. Increased cycling OCPs in bone marrow, but not in blood, of TNF-Tg and TNFα-treated WT mice. A, bone marrow cells from TNF-Tg mice and WT littermates were stained with anti-CD11b, anti-Gr-1 antibodies, and Hoechst 33342 DNA dye and subjected to FACS analysis. Representative histograms show cell cycle profiles of various gated cell populations. B, cell cycle analysis was performed for blood and bone marrow CD11b+/Gr-1âµ OCPs. The percentage of cells in various phases of cell cycles is shown as means ± S.E.
the cell cycle, and the majority of them were at the G0/G1 phase (Fig. 3, lower panel).

To investigate whether increased cycling of marrow OCPs leads to increased total numbers of OCPs in the bone marrow and subsequently in the peripheral blood, we injected TNFα (0.5 μg/injection, four times/day for 3 days) into WT mice and examined the cell cycle status of marrow OCPs, as well as the number of OCPs in blood and bone marrow by FACS analysis. A 3-day regimen was used because our preliminary experiments showed that it took 3 days for TNFα to increase blood OCPs (data not shown). TNFα significantly increased the percentage of cycling marrow OCPs, which started after the 2nd day and peaked after the 3rd day of injection. Correspondingly, total OCP numbers in bone marrow increased at 3 days. Blood OCPs were slightly elevated after 2 days and increased significantly after 3 days of TNFα treatment, in parallel with bone marrow cells (Fig. 3, C and D). Together, these findings suggest that TNFα may increase bone marrow OCP numbers by promoting their proliferation.

To determine the direct effects of TNF and M-CSF on OCP proliferation in vitro, we labeled WT bone marrow cells with 5-(and 6-)-carboxyfluorescein diacetate succinimidyl ester (CFSE) to monitor cell division. The labeled cells were then treated with PBS, M-CSF (10 ng/ml), TNFα (10 ng/ml), or M-CSF/TNFα for 24, 48, and 72 h, stained with anti-CD11b, and subjected to FACS analysis. The percentage of dividing cells in the CD11b+/Gr-1lo population was measured as an indicator of OCP division. Because our initial results demonstrated that 100% of CD11b+/Gr-1hi cells die off, leaving only CD11b+/Gr-1lo cells 24 h after culture (data not shown), Gr-1 staining was omitted in these experiments. TNFα alone had no effect on cell division at any time point, and M-CSF stimulated OCP division after treatment for 48 h (Fig. 4). In contrast, TNFα synergized with M-CSF to stimulate OCP division from 33% in M-CSF-treated cells alone up to 70% in TNFα plus M-CSF-treated cells (Fig. 4, A and B).

TNFα Increases Osteoclast Precursors through c-Fms Expression of Bone Marrow OCPs—Increased M-CSF-dependent proliferation after TNFα treatment suggests that
FIGURE 5. TNFα increases c-Fms expression in OCPs. A, bone marrow cells and PBMCs from TNF-Tg mice and WT littermates were stained with anti-CD11b, Gr-1, and c-Fms antibodies and subjected to FACS analysis. The percentage of c-Fms⁺ cells within the CD11b⁻/Gr-1⁻/c-Fms⁺ population was determined. Values are the means ± S.E. from four pairs of TNF-Tg mice and WT littermates. B, WT bone marrow cells were cultured with PBS or TNFα (10 ng/ml) for 3 days, and nonadherent cells were collected. The expression levels of c-Fms were determined by real-time RT-PCR using c-Fms- and β-actin-specific primers and are shown as mean fold changes of TNF-treated cells over PBS-treated cells (upper panels). The osteoclast forming potential was examined by culturing cells with M-CSF and RANKL and is shown as means ± S.E. of number of TRAP⁺ osteoclasts from three wells (lower panel). C, purified CD11b⁻/Gr-1⁻/c-Fms⁺ cells from WT bone marrow were treated with TNFα (10 ng/ml) for 24 h, and c-Fms expression levels were determined by real-time RT-PCR. D, WT bone marrow cells were incubated with PBS, M-CSF (10 ng/ml), or TNFα (10 ng/ml) in the presence of various doses of M-CSF neutralizing antibody for 3 days, and the percentage of CD11b⁻/c-Fms⁺ cells was determined by FACS analysis. *, p < 0.05 versus PBS-treated group. All experiments were repeated once with similar results.
TNFα may affect the factors essential for M-CSF signaling. Because the M-CSF receptor, c-Fms, is expressed on the surface of late stage of myeloid precursors, including OCPs (21), we hypothesized that TNF-Tg CD11b+/Gr-1−/lo OCPs may contain more c-Fms+ cells to account for the TNFα-induced M-CSF-dependent proliferation. To test this, we compared the percentage of c-Fms+ cells in the gated CD11b+/Gr-1−/lo population of TNF-Tg with that of WT mice, and we found that the TNF-Tg mice had significantly more c-Fms+ cells in CD11b+/Gr-1−/lo OCPs in both blood and bone marrow (Fig. 5A).

To determine whether TNFα promotes the differentiation of c-Fms− cells to c-Fms+ cells, we examined the induction of CD11b−/c-Fms− cells in vitro in response to TNFα. WT marrow cells were treated with PBS, M-CSF (10 ng/ml), TNFα (10 ng/ml), or M-CSF/TNFα, and the percentage of CD11b−/c-Fms− cells was examined after 24, 48, and 72 h in nonadherent and adherent fractions (Fig. 5). TNFα increased the percentage of CD11b+/c-Fms− cells at 24 h, and this peaked at 72 h. M-CSF decreased c-Fms expression over the first 12–24 h, which may reflect ligand-induced receptor internalization (22). However, after 48 h, M-CSF significantly stimulated c-Fms expression. TNFα alone increased the percentage of CD11b−/c-Fms− cells in nonadherent cells by 9–10-fold and synergized with M-CSF to stimulate c-Fms expression in those cells (Fig. 5B). TNFα also stimulated the percentage of CD11b+/c-Fms− cells in adherent cells, but to a lesser extent (by 3–4-fold, data not shown).

To confirm the functional consequence of increased c-Fms expression by TNFα in osteoclastogenesis, nonadherent TNFα- or PBS-treated cells were cultured to assess their osteoclast-forming potency. Cells primed by TNFα formed significantly more osteoclasts when they were subsequently cultured with M-CSF and RANKL (Fig. 5B). To examine if TNFα stimulates c-Fms gene transcription, mRNA expression levels of c-Fms were determined in TNFα-treated nonadherent cells or purified CD11b+/Gr-1−/lo OCPs. TNFα significantly increased the c-Fms expression levels in both cell preparations (Fig. 5C). Finally, we treated cells with TNFα in the presence of M-CSF neutralizing antibody to determine whether TNFα-induced c-Fms expression is mediated by M-CSF. This treatment completely prevented M-CSF-induced c-Fms expression in a dose-dependent manner but had little effect on TNFα-induced c-Fms up-regulation (Fig. 5D). These findings indicate that TNFα can directly stimulate c-Fms expression by OCPs.

**DISCUSSION**

TNFα is a pleiotropic cytokine that induces focal erosions in inflamed arthritic joints by several mechanisms. Recently, we have demonstrated that one of these mechanisms involves an increase in the frequency of peripheral blood CD11b+ OCP in arthritic patients and in TNF-Tg arthritic mice. We also found that this increased OCP frequency is reduced by and associated with a successful outcome of anti-TNF therapy (4, 13), implying that circulating OCP numbers may have an important role in the pathogenesis of inflammatory erosive arthritis. However, the mechanism by which systemic TNFα induces this increase in OCP frequency remains unknown.

Although it has been well established that osteoclasts are derived from myeloid precursors (23, 24), the identity, function, and regulation of OCPs in vivo remain poorly understood, partly because of the absence of unique cell surface markers to identify them. We and others have previously used CD11b+ as a single surface marker for spleen OCPs (13, 25). Recently, we established that many of these cells are blood granulocytes that express CD11b, and the number of OCP may be significantly overestimated by this method. Apart from CD11b, c-Fms and RANKL expression also has been used to estimate OCPs, based on the essential role of M-CSF and RANKL for osteoclastogenesis (11). However, CD11b−/c-Fms− and CD11b−/c-Fms+ cells as well as CD11b+/RANK− and CD11b+/RANK+ cells all can give rise to osteoclasts. Thus, they are also suboptimal OCP markers (13, 26). To try to improve on this, here we assessed CD11b+/Gr-1−/hi cells, excluding CD11b+/Gr-1−/lo granulocytes, which include both early (c-Fms− or/and RANK−) and late OCPs (c-Fms− or/and RANK+). We found that 60–70% of CD11b+/Gr-1−/hi cells can differentiate to TRAP+ mononuclear osteoclasts when cultured with M-CSF and RANKL, indicating that most of these cells have osteoclast-forming potential. However, it is important to realize that CD11b+/Gr-1−/lo cells are not composed of only OCPs because they can differentiate into macrophages and dendritic cells under the appropriate conditions. At present, it is not known if there are committed mono-potential OCPs that can be separated from other precursor cells. Recently, Geissmann et al. (27) proposed that blood monocytes can be divided into two functional subsets according to expression patterns of an array of surface proteins: CD11b+/Gr-1+/CX3CR1+/CCR2+/CD62L+ cells are the cells that are actively recruited to inflamed tissues, and CD11b+/Gr-1−/CX3CR1−/CCR2−/CD62L+ cells are resident monocytes that give rise to specialized cell types, including osteoclasts. According to this classification, our CD11b+/Gr-1−/lo cells appear to belong to resident monocytes. Although the significance of this kind of classification in vivo is controversial (28), it may be possible to define further OCPs by using multiple surface markers.

Using CD11b+/Gr-1−/lo as broad OCP markers, we demonstrated that TNFα promotes the differentiation of bone marrow CD11b+/Gr-1−/lo cells to CD11b+/Gr-1−/lo/c-Fms+ cells. Thus, the first step of TNFα-induced osteoclastogenesis is the conversion of c-Fms− OCPs to c-Fms+ OCPs. This results in enhanced sensitization of precursor cells to M-CSF and provides more M-CSF-requiring cells. TNFα-induced c-Fms mRNA expression has been reported in murine tissue macrophages (29). However, tissue macrophages are terminally differentiated cells that do not have osteoclast-forming potential (30), and the findings from macrophages may differ from those of osteoclasts or their precursors. Our findings provide the first experimental evidence of TNFα increasing c-Fms expression by OCPs, and the functional consequence of this increased c-Fms expression on osteoclastogenesis (Fig. 5). In addi-
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tion, the failure of neutralizing M-CSF antibodies to block TNFα-induced c-Fms expression indicates that this is a direct effect and is not secondary to M-CSF autoregulation (Fig. 5). Recently, Kitaura et al. (31) used chimical WT and TNF receptor knock-out mice to dissect the contribution of cells in the osteoclast lineage from that of stromal cells in the response to TNFα-mediated osteoclastogenesis in vivo. From the findings of this elegant study, they conclude that in conditions where TNFα concentrations are high, TNFα can fully exert its bone erosive effects by directly promoting the differentiation of osteoclast precursors independent of cytokine-responsive stromal cells and T lymphocytes (31). Our results are consistent with their findings and further point out that one of the mechanisms by which TNFα promotes OCP differentiation is through up-regulation of c-Fms expression.

We found that M-CSF alone increases c-Fms+ cell numbers (Fig. 5), indicating that M-CSF can induce its own receptor expression and thereby forms an autocrine loop to amplify M-CSF-mediated signals. This is not a surprising because c-Fms expression is controlled by PU.1, one of the Ets transcription factors that is stimulated by M-CSF treatment (32). Because M-CSF is constitutively expressed in the bone microenvironment in vivo, and TNFα stimulates osteoblasts and T lymphocytes to up-regulate their expression of M-CSF (16, 17), TNFα-induced c-Fms expression must work through both M-CSF-dependent and -independent mechanisms. We have reported previously that TNFα promotes mature osteoclast survival (33). However, we did not observe clear changes in OCP apoptosis. Thus, TNFα likely has a different effect on OCPs versus mature osteoclasts; it stimulates proliferation and differentiation of the former to provide more OCPs and increases survival of the latter to prolong the duration of bone resorption (Fig. 6).

TNFα administration stimulates bone marrow OCP proliferation 1 day before the increase in total OCPs numbers are observed in both bone marrow and blood (Fig. 3). This finding, along with similar findings in TNF-Tg mice, suggests that TNFα-induced OCP proliferation and the subsequent increase in the bone marrow OCP pool may lead to the increased OCP numbers in the peripheral blood. Based on these observations, we propose that in chronic inflammatory arthritis, where TNFα levels are elevated, TNFα increases marrow OCP numbers, which may directly result in a “push” of cells out of the bone marrow (marrow cell release) into the bloodstream because of the limited space available in the bone marrow cavity. Thus, chronic exposure to TNFα could increase the total turnover rate of OCPs. Unfortunately, we do not yet have experimental evidence to prove that the increase in blood OCP numbers in TNF-Tg mice is a direct consequence of an enlarged bone marrow pool. However, it is known that proliferation is not a pre-requisite for cell release from the bone marrow. In fact, the majority of common mobilizing factors stimulates marrow cell release without influencing their proliferation (18). Thus, TNFα-induced elevation in blood OCPs might result from different mechanisms than common mobilization and represent a unique response to chronic inflammation.

One important question regarding circulating OCPs is the role of these cells in normal bone remodeling, because this process occurs exclusively within the bone marrow cavity. Do OCPs need to travel in the blood to initiate physiological bone remodeling or do they simply receive signals from osteoblast/lining cells adjacent to them on or near bone surface identified for remodeling? We do not know the answers to these questions at this time. However, given that focal erosions develop from chronic inflammation over a number of years and the active osteoclasts that mediate these erosions have a half-life of only a few weeks, these cells must be replaced continuously. Because these osteoclasts are known to enter the joint through synovium as OCPs, our conclusion is that circulating OCPs serve as the pre-osteoclast reservoir. Identification of the mechanisms whereby OCP generation and trafficking are regulated should help answer these important questions.

In summary, our studies reveal a new mechanism by which TNFα stimulates osteoclast-mediated bone resorption in chronic inflammatory arthritis. We propose that the first influence of TNFα on osteoclastogenesis is to increase bone marrow OCP genesis through the following two mechanisms (Fig. 6): 1) TNFα directly stimulates the conversion of CD11b+Gr-1–/CD-Fms+ cells to c-Fms+ cells; and 2) it stimulates osteoblasts/stromal cells to produce M-CSF, which subsequently increases c-Fms+ cell proliferation. These CD11b+Gr-1–/CD-Fms+ cells are then released to the bloodstream, through a yet to be defined mechanism, and then home to inflamed joints where they differentiate in response to RANKL and other osteoclastogenic signals.

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