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Marrow Stromal Cells and Osteoclast Precursors Differentially Contribute to TNF-α-Induced Osteoclastogenesis In Vivo

Hideki Kitaura,* Mark S. Sands,‡ Kunihiro Aya,* Ping Zhou,* Teruhisa Hirayama,‡ Brian Uthgenannt,§ Shi Wei,* Sunao Takeshita,* Deborah Veis Novack,* Matthew J. Silva,‡ Yousef Abu-Amer,‡ F. Patrick Ross,* and Steven L. Teitelbaum*‡

The marrow stromal cell is the principal source of the key osteoclastogenic cytokine receptor activator of NF-κB (RANK) ligand (RANKL). To individualize the role of marrow stromal cells in varying states of TNF-α-driven osteoclast formation in vivo, we generated chimeric mice in which wild-type (WT) marrow, immunodepleted of T cells and stromal cells, is transplanted into lethally irradiated mice deleted of both the p55 and p75 TNFR. As control, similarly treated WT marrow was transplanted into WT mice. Each group was administered increasing doses of TNF-α. Exposure to high-dose cytokine ex vivo induces exuberant osteoclastogenesis irrespective of in vivo TNF-α treatment or whether the recipient animals possess TNF-α-responsive stromal cells. In contrast, the osteoclastogenic capacity of marrow treated with lower-dose TNF-α requires priming by TNFR-bearing stromal cells in vivo. Importantly, the osteoclastogenic contribution of cytokine responsive stromal cells in vivo diminishes as the dose of TNF-α increases. In keeping with this conclusion, mice with severe inflammatory arthritis develop profound osteoclastogenesis and bone erosion independent of stromal cell expression of TNFR. The direct induction of osteoclast recruitment by TNF-α is characterized by enhanced RANK expression and sensitization of precursor cells to RANKL. Thus, osteolysis attending relatively modest elevations in ambient TNF-α depends upon responsive stromal cells. Alternatively, in states of severe periarthritis inflammation, TNF-α may fully exert its bone erosive effects by directly promoting the differentiation of osteoclast precursors independent of cytokine-responsive stromal cells and T lymphocytes.

We established that TNF-α directly induces mononuclear precursors to assume the osteoclast phenotype, but its capacity to do so is dependent upon pruning of bone marrow macrophages with permissive levels of stromal cell-produced RANKL (16). Thus, while constitutive production of RANKL is an essential component of direct TNF-α induction of osteoclast precursor differentiation, we do not know whether stromal cells, as a TNF-α target, are fundamental to inflammatory osteoclastogenesis. T lymphocytes, in inflammatory arthritis, also produce abundant RANKL (10). In this circumstance, the extracellular domain of the cytokine appears to be cleaved from the membrane and impact osteoclastogenesis as a soluble molecule (17).

Because of the central role TNF-α plays in inflammatory osteoclastogenesis, determination of the contribution made by its various target cells in vivo carries important therapeutic implications. However, resolution of this issue requires the capacity to individualize the response of the three principal osteoclastogenic cells, namely macrophages, marrow stromal cells, and activated T lymphocytes, to the cytokine. Although we established that osteoclast precursors are direct targets of TNF-α (16), the significance of stromal cell responsivity to the cytokine in inflammatory osteoclastogenesis is unresolved. We addressed this issue by generating T cell-deficient chimeric mice whose stromal precursors, but not stromal cells, respond to TNF-α. We find that optimal osteoclast recruitment in the face of moderate amounts of TNF-α, as likely attends chronic, low-grade inflammation, requires marrow stromal cells responsive to the cytokine. In contrast, profound periarticular inflammation-induced bone erosion, as in active rheumatoid arthritis, occurs independent of TNF-α-responsive stromal cells and T lymphocytes, and involves stimulated RANK expression by osteoclast precursors and their sensitization to RANKL.

Materials and Methods
Mice for transplantation
C57BL/6-Tnfrsf1btmMwm (p55, TNFR1-deficient), B6.129S2-Tnfrsf1btmMwm (p75, TNFR2-deficient), and C57BL6 wild-type (WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.129S2-Tnfrsf1btmMwm was backcrossed to C57BL6 for 10 generations. C57BL/6-Tnfrsf1btmMwm and B6.129S2-Tnfrsf1btmMwm were crossed to generate TNFR1+/−/TNFR2+/− on a C57BL6 background. These animals were crossed to produce TNFR1−/− (KO) mice. Mice were genotyped by PCR in 50 μl reaction mixture containing PCR supernox (Invitrogen Life Technologies, Carlsbad, CA), 100 μM each oligonucleotide primers and chromosomal DNA. The set of oligonucleotide primers used for detection of TNFR1 included 5′-GGAGTTTGCACGTCGGCCTGAAG-3′ (p60-B); 5′-TGGCAAGGACACGGT GTGTCGGC-3′ (p60-E); 5′-CTCCTGACGAGGATACATCCACT-3′ (p60-spe); and 5′-CCGGTGGATGCGAAATGTGTTG-3′ (pgk5′-66). Primers to detect TNFR2 included 5′-AGAGCTCAGCCAAAGGACGCC-3′ (p80-Kas); 5′-AAGGCGCCAGACCTCCGTGCT-3′ (p80-I); and 5′-CCGGTGGATGCGAATTGTCG-3′ (pgk5′-66). During the thermal reaction for detection of TNFR1- or TNFR2-deficient, 35 cycles of denaturation were conducted for 1 min at 94°C, annealing for 1 min at 65°C, and extension for 30 s at 72°C. The expected PCR products for TNFR1 were: +/+ 120 bp, +/− 120 bp and 155 bp, and −/− 155 bp. Those for TNFR2 were: +/+ 275 bp, +/− 275 bp and 210 bp, and −/− 210 bp. The PCR products were resolved in 5% agarose gels and stained with ethidium bromide.

Reagents
The following mAbs were obtained from BD Biosciences (San Diego, CA): purified hamster anti-mouse TNFR1 (559915), FITC-conjugated mouse anti-hamster IgG (554011), PE-conjugated hamster anti-mouse TNFR2 (550866), FITC-conjugated rat anti-mouse CD3 mouse monoclonal complex (555274), FITC-conjugated rat anti-mouse CD4 (553047), FITC-conjugated rat anti-mouse CD8 (553031), purified rat anti-mouse CD106 (VCAM-1; 553300), purified rat anti-mouse CD3 molecule complex (555273), FITC-conjugated rat anti-mouse CD11b (Mac-1) (553310), and CD3-ε antibodies. Goat anti-rat IgG microbeads for immunopurification were obtained from Miltenyi Biotec (Auburn, CA). Reombinant murine TNF-α was prepared in our laboratory as following. A TNF-α cDNA fragment encoding aa residues 83–235 was cloned by RT-PCR, using primers 5′-CAGGAAATTCCTCTTTCAATTTCTAAGGACC-3′ and 5′-ATT AGCGGCCGCTCAGACAAGCATAGCCTAAAG-3′. The PCR product was digested with EcoRI and NotI, and cloned into a pGEX-6P-1 (Amersham Biosciences, Piscataway, NJ) to generate a GST-fusion protein. GST-TNF-α was expressed in Escherichia coli BL21 (DE3) cells (Stratagene, La Jolla, CA). The cells were lysed under nondenaturing conditions and GST-TNF-α was purified over a glutathione-Sepharose column, followed by ion exchange chromatography. GST was cleaved off by PreScission Protease (Amersham Biosciences) by manufacturer’s directions and was removed by a glutathione-Sepharose column. Lack of endotoxin contamination was confirmed by Limulus amoebocyte lysate assay (BioWhitaker, Walkersville, MD). Recombinant human M-CSF was generously provided by Dr. D. H. Fremont (Washington University, St. Louis, MO).

Preparation of bone marrow cells
Mice were killed by CO₂ gas, and cells were flushed with culture medium from femoral marrow as previously described (13). VCAM-1 and CD3-positive cells were depleted from marrow cells by negative selection using MACS goat anti-rat IgG microbeads (Miltenyi Biotec, Auburn, CA). RBC were lysed with NH₄Cl (0.727%) and Tris-HCl (0.017%) at pH 7.2 at room temperature for 5 min. A total of 1 × 10⁸ cells/ml were resuspended in PBS (0.5%) plus EDTA (2 mM). The cells were incubated with rat anti-mouse CD3 and rat anti-mouse CD106 Abs (each 25 μg per 10⁶ cells) for 15 min at 4°C. After incubation, the cells were washed with PBS (0.5%) plus EDTA (2 mM) and resuspended in 800 μl of the same solution. Two hundred microliters of MACS goat anti-rat IgM microbeads were added to the suspension and incubated for 20 min at 4°C. The pellet was resuspended in 500 μl of 0.5% PBS-BSA 2 mM EDTA per 10⁶ cells. The sample was applied to the column and placed in a magnetic field, and the effluent was collected. Absence of VCAM-1 cells and CD3-positive cells was confirmed by FACS (data not shown).

Preparation of ascites
Female ICR-SCID mice (Taconic Farms, Germantown, NY) were primed by i.p. injection of 0.5 ml of IFA. After 7 days, the mice received i.p. injections of 5 × 10⁶ YTS cells which secrete anti-CD4 Abs or H35 cells which secrete anti-CD8 Abs (kindly provided by Dr. O. Kanagawa, Washington University). One to 2 wk later, ascites were recovered, incubated at 37°C for 1 h, and transferred to 4°C overnight. Cells and oil were removed by centrifugation and ascites were stored at −80°C. Mice were administered four weekly injections of 50 μl of anti-CD4 and anti-CD8 ascites to assure arrest of T cell generation in vivo.

Evaluation of blockage of T cell maturation in vivo by FACS analysis
Spleen cells were incubated with NaN₃ (0.1%) plus FBS (1%) for 30 min with FITC-conjugated rat anti-mouse CD4, FITC-conjugated rat anti-mouse CD8, or FITC-conjugated rat anti-mouse CD3 mAbs. The samples were diluted with the same solution and analyzed by FACS for CD4-, CD8-, and CD3-expressing cells.

Bone marrow transplantation
A total of 1 × 10⁶ CD3- and VCAM-1-depleted marrow cells in 100 μl of PBS were i.v. injected via the tail vein in 4- to 6-wk-old WT or KO male mice 1 day following 10 Gy of total body gamma-irradiation. To evaluate marrow engraftment, marrow cells were incubated for 30 min with anti-TNF1 mAb, and then for 30 min with FITC-conjugated anti-IgG mAb, washed, and diluted with NaCl, plus FBS. A second aliquot of marrow cells was incubated for 30 min with PE-labeled anti-TNF2 mAbs. TNF1 and TNF2 expression was analyzed by FACS.

Ex vivo osteoclastogenesis
Bone marrow cells (5 × 10⁶) were cultured in 200 μl of medium with recombinant human M-CSF (50 ng/ml) and specified doses of TNF-α in 96-well plates (Corning Glass, New York, NY). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ for 2 days and supplemented with fresh medium and cytokines on day 3.

RNA preparation and RT-PCR analysis
Total RNA from bone marrow cells was isolated by an RNeasy mini kit (Qiagen, Valencia, CA). For RT-PCR analysis, cDNA were synthesized from 1 μg of total RNA using reverse transcriptase and oligo-dT primers in a volume of 20 μl. PCR was performed with 2 μl of cDNA reaction mixture using PCR supernox (Invitrogen Life Technologies) and appropriate primers in a volume of 50 μl. The following primers were used:
GAPDH, 5′-ACTTTGTCAGCTATTCC-3′ and 3′-TGAAGCGAA CTATTGATG-5′; RANK, mouse RANK PCR Primer Pair (R&D Systems, Minneapolis, MN); and RANKL, human/mouse TRANCE/TNFSF11 PCR Primer Pair (R&D Systems). Samples were transferred to a programmable thermal cycler (Thermo Hybaid, Franklin, MA) preheated to 94°C, and incubated for 35 PCR cycles for RANK and 39 PCR cycles for RANKL. Each cycle consisted of a denaturation step at 94°C for 45 s, an annealing step at 55°C for 45 s, and an extension step at 72°C for 45 s. Ten-microliter aliquots of PCR products were separated by electrophoresis on a 2.0% agarose gel.

Serum transfer arthritis

KRN-TCR transgenic mice on a C57BL/6 background were kindly provided by D. Mathis and C. Benoist (Harvard University Medical School, Boston, MA). K/BxN mice, which spontaneously develop severe inflammatory arthritis (18) and whose serum Ig is arthrogenic in a T cell-independent manner (19), in other strains, were generated by breeding KRN-TCR with nontoxic diabetic mice (Taconic Farms). Serum was obtained from K/BxN mice (6-12 wk-old), pooled and stored in aliquots at −70°C. T cell-depleted WT→WT, WT→KO, and KO→KO mice were injected i.p. with serum or PBS. A single dose of 200 μg of serum was found to induce arthritis in all chimeric mice. Ankle thickness was measured by a caliper every day for 7 days, after which the animals were sacrificed.

Histological analysis

Osteoclast number was determined using the Bioquant System (BIOQUANT Image Analysis, Nashville, TN). Synovial inflammation was semiquantitatively analyzed using blinded histological sections scored as follows: 0, normal; 1, mild inflammation; 2, moderate inflammation; and 3, severe inflammation.

Statistics

All data are expressed as mean ± SD and statistical significance calculated by Student’s t test.

Results

TFN-α-responsive stromal cells mediate ex vivo osteoclastogenesis in a cytokine dose-dependent manner

Assessment of the role of stromal cell TNFRs in optimal TNF-α-induced osteoclastogenesis required individualizing their osteoclastogenic response to the cytokine. To this end, we generated three species of chimeric rodents. Each experiment involved transplantation of murine marrow, depleted of stromal cells and T lymphocytes, into lethally irradiated mice. In this situation, all osteoclasts generated in transplanted animals are donor derived (16).

In the first instance, stromal- and T cell-depleted WT marrow was transplanted into irradiated recipients deleted of both TNFR1 and 2 (WT→KO). As a positive control, WT marrow was transplanted into WT mice (WT→WT), and as negative control, marrow from mice lacking both TNFRs was transplanted into the same genotype (KO→KO). All WT and KO animals, which did not undergo marrow transplantation, died 10–12 days following irradiation (data not shown).

Our initial task was to assure the success of bone marrow engraftment. To this end, we assessed TNFR expression in each species of chimeric animals. Fig. 1 establishes that TNFR1 and 2 are detectable by FACS analysis in WT mice and undetectable in mice deleted of these moieties. Importantly, the same assay performed 4 wk after transplantation of WT marrow into irradiated KO mice demonstrates expression of both receptors to levels mirroring the nonirradiated, WT situation.

T lymphocytes, like marrow stromal cells, are a source of TNF-α-induced RANKL, and thus, influence osteoclastogenesis (10). Defining the specific contribution of stromal cells to TNF-α-stimulated osteoclastogenesis in vivo, therefore, requires elimination of T cells. To this end, we injected ascites containing anti-CD4 and anti-CD8 mAbs into WT animals and after 7 days, determined spleen cell expression of the T cell markers, CD4, CD8, and CD3. Although T lymphocytes are abundant in the spleens of untreated

FIGURE 2. T cell development is arrested in mice treated with anti-CD4 and anti-CD8 mAbs. Spleen cells recovered 7 days following a single injection of ascites containing anti-CD4 and anti-CD8 mAbs or vehicle were incubated with FITC-conjugated mAbs against CD4, CD8, and CD3 (dashed line), or FITC-conjugated isotype mAbs (solid line), and analyzed by FACS.

FIGURE 1. WT marrow engrafts into irradiated TNFR-deleted mice. WT, TNFR1 and 2−/−, and WT→KO bone marrow were incubated with purified anti-TNFR1 mAb or PE-conjugated anti-TNFR2 hamster mAb (hatched line). The cells exposed to anti-TNFR1 mAb were further incubated with FITC-conjugated anti-hamster IgG (hatched line). TNFR expression was determined by FACS. TNFR1 (solid lines) represent cells incubated with purified hamster IgG and FITC-conjugated anti-hamster IgG, and TNFR2 (solid lines) represent cells incubated with PE hamster IgG.
animals, they are essentially undetectable by FACS analysis 3 (data not shown) and 7 (Fig. 2) days following administration of the mAbs. Hence, we are assured that interpretation of our data is not confounded by the presence of osteoclastogenic T cells.

Having developed a T cell-deficient, TNFR chimeric model, positioned us to define the role of cytokine-responsive marrow stromal cells in optimal TNF-α-induced osteoclastogenesis. Our initial exercise was to determine whether TNF-α-responsive stromal cells are required to prime osteoclast precursors to directly respond to various doses of the cytokine. To address this issue, we isolated bone marrow cells from chimeric mice administered in incremental amounts of TNF-α in vivo for 5 days. The marrow was then cultured in 50 ng/ml TNF-α for an additional 5 days and stained for tartrate-resistant acid phosphatase (TRAP) activity. As seen in Fig. 3, ex vivo osteoclast recruitment is equally effective in high-dose TNF-α, irrespective of in vivo administration of the cytokine and whether or not osteoclast precursors are exposed to TNF-α-responsive stromal cells in the intact animal. As expected, the absence of TNFRs, in both populations (KO→KO), completely ablates TNF-α-induced osteoclastogenesis. Given that TNF-α enhances RANKL expression (16), these ex vivo data suggest that in states of abundant TNF-α, the cytokine has the capacity to

![Figure 3](image3.png)

**FIGURE 3.** High-dose TNF-α induces osteoclastogenesis ex vivo, independent of stromal cell responsivity. A, Bone marrow cells were recovered from WT→WT, WT→KO, and KO→KO mice after five sequential injections of increasing doses of TNF-α. The cells were cultured with M-CSF (50 ng/ml) and TNF-α (50 ng/ml) for 5 days, and the cells were stained for TRAP activity to identify osteoclasts. The number of osteoclasts generated in wells containing various amounts of TNF-α and WT→WT (B), WT→KO (C), or KO→KO marrow (D). Numbers 1–5 in B–D correspond to those in A.

![Figure 4](image4.png)

**FIGURE 4.** Low dose of TNF-α induction of osteoclastogenesis ex vivo requires stromal cell responsivity. A, Bone marrow cells were recovered from WT→WT, WT→KO, and KO→KO mice after five daily injections of increasing doses of TNF-α. The cells were cultured with M-CSF (50 ng/ml) and TNF-α (10 ng/ml) for 5 days and stained for TRAP activity to identify osteoclasts. Numbers of osteoclasts generated in wells containing various doses of TNF-α and WT→WT (B), WT→KO (C), or KO→KO marrow cells (D). Numbers 1–5 in B–D correspond to those in A.
species with 3 mal cells necessary? To this end, we treated the three chimeric KO or KO WT osteoclast precursors by more modest doses of TNF- directly of TNFR-bearing stromal cells, direct induction of the cytokine. Therefore, mice were injected again with progressive doses of TNF- in various osteolytic disorders (11, 14, 20), we next asked whether TNF-α-responsive stromal cells are necessary to prime osteoclast precursors in vivo to directly respond to lower concentrations of the cytokine. Because of the variance in the magnitude of TNF-α expression in the previous experiment. Although this relatively moderate dose of TNF-α induces brisk ex vivo osteoclastogenesis in cultures derived from WT>WT mice, particularly those treated with 1.5 or 3.0 μg/day TNF-α, no osteoclasts are present in wells containing WT>KO or KO>KO marrow (Fig. 4). Thus, while ex vivo osteoclastogenesis induced by high concentrations of TNF-α occurs independently of TNFR-bearing stromal cells, direct induction of osteoclast precursors by more modest doses of TNF-α requires priming by stromal cells responsive to the cytokine.

To further explore the mechanism by which TNF-α prompts osteoclastogenesis, we asked whether the cytokine enhances the osteoclast precursor number and if so, are TNF-α-responsive stromal cells necessary? To this end, we treated the three chimeric species with 3 μg of TNF-α, or vehicle, daily for 5 days. FACS analysis of macrophage differentiation, using anti-CD11b mAb, demonstrates that at sacrifice, TNF-α increases the number of marrow-residing osteoclast precursors from 57.2 to 82.0% of total bone marrow cells in WT>WT chimeric mice (Fig. 5). In contrast, the cytokine fails to generate additional osteoclast precursors in both chimeric mice whose stromal cells lack TNFRs. Thus, osteoclast precursor proliferation occurs in response to a modest dose of TNF-α and requires TNF-α-responsive marrow stromal cells.

TNF-α-responsive stromal cells mediate in vivo osteoclastogenesis in a cytokine dose-dependent manner

Having established the dose-dependent role of marrow stromal cells in TNF-α-induced osteoclastogenesis ex vivo, we used two strategies to ask if the same obtains in vivo. In the first instance, the cytokine was administered, once a day for 5 days, to each of the three groups of chimeric mice. Histological sections of calvariae were then stained for TRAP activity to identify osteoclasts.

These observations suggest that the role of stromal cell TNFRs, in the osteoclastogenic process, diminishes as ambient cytokine increases. If such is the case, one would expect the profound osteoclast formation and bone erosion occurring in clinical situations of overwhelming TNF-α production to be independent of stromal cell responsivity. To address this issue, we injected the three TNF chimeric strains with arthrogenic serum (18, 19). Seven days later, at the time of sacrifice, each serum-injected animal had developed ankle erythema (Fig. 7A) and swelling (Fig. 7B). Although these clinical features are indistinguishable among the WT>WT and WT>KO mice, they are less severe in the KO>KO

FIGURE 5. TNF-α-mediated increase in osteoclast precursor, in vivo, requires responsive marrow stromal cells. The percentage of osteoclast precursors in marrow recovered from WT>WT, WT>KO, and KO>KO mice after five daily injections of TNF-α (3 μg per day) or vehicle, was determined by FACS using FITC-conjugated anti-CD11b mAb (filled area under curve) and FITC-conjugated isotype mAb (open area under curve).
animals. Histological sections of ankles were semiquantitatively scored, in a blinded fashion, for synovial inflammation. As seen in Fig. 7C, such inflammation is profound in WT>WT (3.0 ± 0.0 < 0.003 vs KO>KO) and WT>KO (2.75 ± 0.7; p < 0.025 vs KO>KO), but much less so in KO>KO (1.4 ± 1.2) mice. All control animals which received PBS in lieu of arthrogenic serum exhibit no joint inflammation. As manifest by TRAP staining, the magnitude of osteoclastogenesis mirrors synovial inflammation in each circumstance with WT>WT and WT>KO arthritic mice containing numerous sites of bone erosion (Fig. 7, D and E). These data indicate that in states of severe periarticular inflammation, such as active rheumatoid arthritis, TNF-α may fully exert its bone erosive effects by directly targeting osteoclast precursors and promoting their differentiation independent of stromal cells and T lymphocytes.

**FIGURE 6.** Optimal TNF-α-induced osteoclastogenesis in vivo requires stromal cell responsivity. A, Histological sections of calvariae excised from WT>WT, WT>KO, and KO>KO mice after five daily supracalvarial injections of increasing doses of TNF-α were stained for TRAP activity (red reaction product). The percentage of bone/marrow interface covered by osteoclasts was histomorphometrically determined in specimens derived from WT>WT (B), WT>KO (C), and KO>KO (D) mice (*, p < 0.01, ***, p < 0.001 as compared with PBS injected control). Numbers 1–5 in B–D correspond to those in A.

**TNF-α enhances osteoclast precursor expression of RANK and sensitivity to RANKL**

TNF-α promotes RANKL expression by stromal cells accounting for their intermediary role in TNF-α-induced osteoclastogenesis (16). As we show, TNF-α is also capable of directly prompting marrow macrophages to undergo osteoclast differentiation in WT>KO mice without enhanced RANKL expression by bone marrow-derived cells (Fig. 8). In contrast, osteoclastogenesis depends upon RANK activation. Therefore, we asked whether the direct effect of the inflammatory cytokine on marrow macrophages reflects modulation of the RANKL/RANK axis within the context of osteoclast precursors, per se. In fact, administration of TNF-α to WT>WT chimeras induces marrow RANK mRNA, in vivo (Fig. 8). Furthermore, equivalent expression of RANK mRNA was obtained in WT>KO mice, validating that the increase in TNF-α-induced RANK is independent of TNF-α-responsive marrow stromal cells and reflects direct targeting by the cytokine, of osteoclast precursors.

If TNF-α-induced RANK expression on osteoclast precursors is physiologically significant, such cells, when pre-exposed to TNF-α, should exhibit increased sensitivity to RANKL. To determine whether this is so, WT marrow, lacking stromal and T cells, was cultured in M-CSF plus increasing doses of TNF-α. After 3 days, the medium was changed to that containing the same concentration of M-CSF plus 10 or 50 ng/ml RANKL, and the cells were cultured for an additional 2 days. Characteristic osteoclasts
form within this short duration of RANKL exposure, but do so only if the cells have been primed with at least 10 ng/ml TNF-α (Fig. 9). Thus, not only do TNF-α and RANKL act synergistically when added simultaneously to osteoclastogenic cultures (16), but each cytokine primes osteoclast precursors to respond to the other. Furthermore, in contrast to TNF-α-stimulated RANKL production, TNF-α-induced RANKL sensitization is a stromal cell-independent event.

Discussion

Physiological osteoclastogenesis depends upon expression of RANKL, by marrow stromal cells or their derivative osteoblasts, principally as a membrane-residing protein (1). This observation places this family of mesenchymal cells in the center of the osteoclastogenic process and prompted the discovery that related cells, such as inflamed synovites, are also capable of producing RANKL (7). In the context of inflammation, TNF-α induces RANKL synthesis by marrow stromal cells (16), and RANKL prompts TNF-α expression by osteoclast precursors (21). Both cytokines have profound effects in states of inflammatory osteolysis such as rheumatoid arthritis, periprosthetic implant loosening, and periodontitis.

We previously established that a high dose of TNF-α is capable of directly inducing osteoclast precursors to differentiate in face of marrow stromal cells able to produce only constitutive levels of RANKL (16). In contrast, in the authentic in vivo situation, stromal cells respond to TNF-α. Therefore, we turned to the important, yet unresolved, issue of the marrow stromal cell as a direct TNF-α target in inducing optimal osteoclastogenesis.

To delineate the contribution of marrow stromal cells to TNF-α-induced osteoclastogenesis, we generated chimeric mice in
which TNF-α-responsive osteoclast precursors exist in vivo, in association with stromal cells either capable or incapable of responding to TNF-α. Establishing donor engraftment, expression of both TNFRs in the marrow of these chimeric animals mirrors that of their WT counterparts. Because T lymphocytes, when activated, express RANKL (10), we eliminated these cells in the marrow graft and prevented their development during the course of the experiment with appropriate Abs. Thus, we were positioned to isolate the contribution of stromal cells as direct TNF-α targets in the osteoclastogenic process.

TNF-α expression varies profoundly in different pathological circumstances. For example, the cytokine is central to the loosening of orthopedic implants secondary to particularization of the prosthesis (20). On the one hand, the quantity of TNF-α expressed by periprosthetic macrophages is proportional to the load of implant-derived particles to which they are exposed (14, 20). On the other hand, one would expect a greater abundance of the inflammatory cytokine in the bone environment of advanced rheumatoid arthritis than in any state of periprosthetic osteolysis or periodontal disease (11). Thus, we asked if the mechanisms of TNF-α-stimulated osteoclastogenesis vary with the concentration of ambient cytokine. In this regard, we first determined that marrow cells exposed to 50 ng/ml TNF-α ex vivo, undergo effusive osteoclast differentiation regardless of stromal cell responsivity or the amount of preadministered cytokine. In contrast, osteoclast induction ex vivo, by a dose of TNF-α one-fifth of that used in the previous experiment, requires prior in vivo exposure to the same cytokine in the presence of TNF-α-responsive stromal cells. Thus, while TNF-α-stromal cell targeting is irrelevant in high-dose cytokine-induced ex vivo osteoclastogenesis, such is not the case at more modest TNF-α levels.

The abundance of inflammatory cytokines in disorders such as rheumatoid arthritis is responsible for mobilization of osteoclasts leading to the crippling consequences of periarticular osteolysis (9). Our next undertaking was to define the role of stromal cells in the osteoclastogenesis and bone erosion attending TNF-α excess in vivo. We find that direct, supracalvarial injection of TNF-α results in a progression in osteoclast number in circumstances in which both osteoclast precursors and stromal cells are cytokine responsive. In contrast, the number of resorptive cells does not increase at more modest doses of the cytokine administered to mice with WT osteoclast precursors and TNFR−/− stromal cells. Furthermore, when injected with 1.5–3.0 μg of TNF-α per day, osteoclast recruitment in the same chimeric animals lacking cytokine-responsive stromal cells, approximates 40% of the WT situation. Thus, while TNF-α has the capacity to induce direct osteoclast differentiation in vivo, in other instances, such as those described in this study, the presence of TNF-α-responsive stromal cells is critical for osteoclastogenesis in vivo.

**FIGURE 8.** TNF-α increases RANK expression in vivo. Bone marrow was obtained from WT>WT, WT>KO, and KO>KO mice after five daily injections of TNF-α (3 μg per day) or vehicle. RNA was isolated, and RANK and RANKL mRNA expression was measured by RT-PCR.

**FIGURE 9.** TNF-α sensitizes osteoclast precursors to RANKL. WT marrow, lacking stromal and T cells, was cultured with M-CSF (50 ng/ml) and various concentrations of TNF-α. After 3 days, the medium was changed to that containing the same concentration of M-CSF in the presence or absence of 10 or 50 ng/ml RANKL. The cells were maintained for an additional 2 days. A, Cells were stained for TRAP activity. B, Osteoclast number in each condition.
conditions of relatively moderate amounts of the cytokine, optimal osteoclast recruitment requires the participation of responsive stromal cells. This observation stands in contrast to the osteoclastogenesis occurring in the face of severe inflammatory arthritis in which abundant osteoclastogenesis and severe periarthritis bone erosion occur regardless of whether or not the mouse bears TNF-α-responsive stromal cells. Given the absence of T lymphocytes and responsive stromal cells, in WT >KO arthritic animals, it appears that in states of profound TNF-α excess, such as active rheumatoid arthritis, the osteoclast precursor is the sole relevant target of the cytokine.

RANKL and TNF-α enjoy an intimate and complex relationship in the osteoclastogenic process. Taken with the posture that RANKL, at least in permissive levels, is essential for TNF-α to directly induce osteoclast precursor differentiation (16), a reasonable hypothesis would hold that the osteoclastogenic capacity of TNF-α reflects its modulation of the RANK/RANKL signaling pathway, in vivo. In fact, RANK is enhanced in mice administered high-dose TNF-α independent of stimulated participation of marrow stromal cells. Thus, the capacity of TNF-α to directly prime osteoclast differentiation likely reflects, at least in part, its capacity to induce RANK expression by precursor cells. This posture is buttressed by the fact that RANKL-mediated signaling in these cells, is TNFR expression dependent (16). Moreover, WT marrow macrophages, in the absence of T lymphocytes and stromal cells, demonstrate accelerated differentiation in response to RANKL, only if pre-exposed to a sufficient amount of TNF-α. Thus, pre-treatment of osteoclast precursors with either RANKL (16) or TNF-α primes the cell to respond to the reciprocal cytokine with enhanced osteoclastogenesis.

Taken with those previously reported (16), our in vivo observations suggest a model for the relationship of osteoclast precursors and marrow stromal cells in TNF-α-induced osteoclastogenesis. Excess TNF-α is produced in inflammatory conditions and the macrophage may serve as both a source of the cytokine as well as osteoclast progenitor (21). In slowly progressive inflammatory osteolysis, the osteoclastogenic effect of TNF-α is mediated largely by the marrow stromal or related cells. In this circumstance, the cytokine’s principal effect is to induce RANKL (16) and M-CSF (22), eventuating in a moderately enhanced population of stromal cells, which is TNFR expression dependent (16). However, WT marrow macrophages, in the absence of T lymphocytes and stromal cells, demonstrate accelerated differentiation in response to RANKL, only if pre-exposed to a sufficient amount of TNF-α. Thus, pre-treatment of osteoclast precursors with either RANKL (16) or TNF-α primes the cell to respond to the reciprocal cytokine with enhanced osteoclastogenesis.

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