

Growth Factors, Cytokines, and Cell Cycle Molecules

Methotrexate Chemotherapy Promotes Osteoclast Formation in the Long Bone of Rats via Increased Pro-Inflammatory Cytokines and Enhanced NF- κ B Activation

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Cancer chemotherapy with methotrexate (MTX) is known to cause bone loss. However, the underlying mechanisms remain unclear. This study investigated the potential role of MTX-induced pro-inflammatory cytokines and activation of NF- κ B in the associated osteoclastogenesis in rats. MTX (0.75 mg/kg per day) was administered for 5 days, and bone and bone marrow specimens were collected on days 6, 9, and 14. Compared with a normal control, MTX increased the density of osteoclasts within the metaphyseal bone and the osteoclast formation potential of marrow cells on day 9. RT-PCR analysis of mRNA expression for pro-osteoclastogenic cytokines in the metaphysis indicated that, although the receptor activator of NF- κ B ligand/osteoprotegerin axis was unaffected, expression of tumor necrosis factor (TNF)- α , IL-1, and IL-6 increased on day 9. Enzyme-linked immunosorbent assay analysis of plasma showed increased levels of TNF- α on day 6 and of IL-6 on day 14. Plasma from treated rats induced osteoclast formation from normal bone marrow cells, which was attenuated by a TNF- α -neutralizing antibody. Indicative of a role for NF- κ B signaling, plasma on day 6 increased NF- κ B activation in RAW_{264.7} cells, and plasma-induced osteoclastogenesis was abolished in the presence of the NF- κ B inhibitor, parthenolide. Our results demonstrate mechanisms for MTX-induced osteoclastogenesis and show

that MTX induces osteoclast differentiation by generating a pro-osteoclastogenic environment in both bone and the circulation, specifically with increased TNF- α levels and activation of NF- κ B. (Am J Pathol 2012, 181: 121–129; <http://dx.doi.org/10.1016/j.ajpath.2012.03.037>)

Low bone mineral density (BMD) is associated with reduced bone strength and, thus, an increased fracture risk. Although bone remodeling (performed by bone-forming cell osteoblasts and bone-resorptive cell osteoclasts) and, thus, bone mass are chiefly regulated by genetic factors, they can also be influenced by other factors, including diet, hormones, and mechanical loading.¹ Although several of these factors can improve bone mass outcomes, several medical treatments, such as cancer chemotherapy, can disrupt bone remodeling and cause bone loss.

Recently, although increased use of chemotherapy drugs has promoted survivorship in patients with cancer, it has also highlighted significant ongoing bone-related adverse effects, including arrested bone growth in patients with pediatric cancer and significant reductions in BMD in both pediatric^{2–4} and adult^{5,6} patients with cancer after chemotherapy. Furthermore, increased incidences of fractures in patients with cancer and survivors

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have emerged, indicating long-term defects in BMD.⁷ In the experimental setting, animal models of chemotherapy-induced bone defects have demonstrated that single chemotherapeutic agents are capable of reducing bone mass and disrupting trabecular bone architecture,^{8–11} culminating in impaired bone mechanical strength.⁸

Methotrexate (MTX), a commonly used anti-metabolite, causes bone morbidity, including growth arrest and reduced BMD. At chemotherapeutic doses, MTX inhibits RNA/DNA synthesis via the inhibition of dihydrofolate reductase. After MTX treatment, bone formation is attenuated,¹² and bone synthesis (indicated by levels of circulating osteocalcin) and mineralization are depressed.^{4,9,13} These outcomes have been linked to several contributing factors, including reduced circulating levels of vitamin D₃,^{4,13} altered response of the bone cells to mechanical loading,¹⁴ and depression of the osteoblast precursor pool within the marrow.^{15,16} Although these studies imply that the decrease in BMD is, in part, caused by a reduction in total bone synthesis, there has also been evidence suggesting that the reduced BMD is also due to increased bone resorption, as indicated by increased osteoclast density^{9,11} and increased biochemical markers of bone resorption.¹⁷ Similarly, an increased density of osteoclast precursors and an elevated marrow potential to form mature osteoclasts have increased after short-term MTX treatment in rats.¹⁵ Consistently prolonged administration of MTX at a low dose causes osteopenia, which is possibly associated with enhanced osteoclast recruitment.¹⁰ These studies suggest that increased osteoclast density and resorption after MTX chemotherapy may be due to the promotion of osteoclast differentiation.

Osteoclasts are differentiated from osteoclast precursors of monocyte lineage after stimulation with macrophage colony-stimulating factor (M-CSF) and receptor activator of NF- κ B ligand (RANKL).^{18,19} RANKL, expressed by nearby osteoblasts and lineage cells, stimulates osteoclast maturation and activity. Osteoprotegerin (OPG), synthesized by osteoblastic lineage cells, is a soluble decoy receptor of RANKL, which inhibits osteoclastogenesis.¹⁹ Although RANKL and M-CSF remain the critical factors for osteoclast formation, several pro-inflammatory cytokines are known to enhance osteoclast formation and activity, including tumor necrosis factor (TNF)- α , IL-1, and IL-6.^{18,20,21} These cytokines also have significant roles in promoting localized osteoclast activity and bone erosion in inflammatory disorders, such as rheumatoid arthritis.^{22,23} Ultimately, the interaction of RANKL with its receptor, RANK, and its augmentation by pro-inflammatory cytokines lead to activation of the transcription factor, NF- κ B, and subsequent expression of genes promoting osteoclast differentiation, activation, and survival.^{24,25}

Although the role of osteoclasts in MTX chemotherapy-induced bone loss has been highlighted by increased osteoclast presence within areas of high bone turnover and, more recently, by increased osteoclast precursors, it remains unknown how MTX chemotherapy increases osteoclastogenesis. In this study, we examined this question using a rat model of short-term MTX chemotherapy. Herein, we confirmed the increased presence of osteoclasts in tibial metaphysis bone and the elevated local expression of pro-osteoclastogenic factors. Furthermore, we observed the systemic response to MTX

chemotherapy in terms of increased levels of osteoclastogenic factors (particularly TNF- α) in the circulation, which promoted osteoclastogenesis involving increased NF- κ B activation.

Materials and Methods

Animals and MTX Administration

Male Sprague-Dawley rats (aged 7 weeks) were s.c. injected with MTX at 0.75 mg/kg per day for 5 days and were euthanized by CO₂ overdose at days 4, 6, 9, 14, and 21 ($n = 6$ per group). A control group ($n = 6$) received saline injections. After CO₂ overdose, a blood sample was obtained from which plasma was collected and stored at -80°C . The left tibia was fixed in 10% formalin for 24 hours and decalcified using Immunocal (Decal Corporation, Brooklyn, NY) for 7 days before processing for paraffin embedding, from which sections ($4\ \mu\text{m}$ thick) were obtained for histological analysis. Metaphysis bone (0.3 cm) from the right tibia was frozen for gene expression studies. The remaining diaphysis, femurs, and humeri were used for obtaining bone marrow samples for cell culture studies, as described later. These protocols were approved by the Animal Ethics Committee of Child, Youth and Women's Health Service, South Australia.

Analysis of Osteoclast Density

Osteoclasts in the metaphysis were morphologically identified by their large size, multinucleation, and bone surface localization with H&E-stained sections. Their numbers were quantified as osteoclasts per millimeter trabecular lining in the primary spongiosa region (the woven bone region with limited bone marrow) and in the secondary spongiosa (containing distinct longitudinally arranged trabecular bone interspaced with marrow), as previously described.¹⁵

Ex Vivo Osteoclastogenic Potential of Marrow Cells

An *ex vivo* osteoclastogenesis assay was used to assess the potential of bone marrow samples in producing osteoclasts.²⁶ Bone marrow was flushed out with basal minimal essential medium containing 10% fetal bovine serum, 50 $\mu\text{g}/\text{mL}$ Pen-Strep (Sigma, Sydney, Australia), and 15 mmol/L HEPES buffer (Sigma) (pH 7). After an overnight incubation, the nonadherent fraction was collected and reseeded at 1×10^6 cells per well in basal media containing 10 ng/mL M-CSF. On the following day, media were further supplemented with 30 ng/mL RANKL and the culture was maintained for 7 days before fixation in 10% formalin. Osteoclasts were identified by tartarate-resistant acidic phosphatase (TRAP) staining and multinucleation (three nuclei or more), as previously described.²⁷

Quantitative RT-PCR Analysis of Pro-Osteoclastogenic Cytokines

Real-time RT-PCR was used to examine mRNA expression of pro-osteoclastogenic cytokines within the metaphysis. Total RNA was extracted with TRIzol reagent (Sigma) from frozen metaphysis and was further purified using a minicol-

Table 1. Forward and Reverse Primer Sequences Used for mRNA Expression Analysis

Gene	Forward primer	Reverse primer
OPG	5'-AGCTGGCACACGAGTGATGAA-3'	5'-CACATTTCGCACACTCGGTTGT-3'
RANKL	5'-TGGGCCAAGATCTCTAACATCAC-3'	5'-TCATGATGCCTGAAGCAAATG-3'
TNF- α	5'-ATGGCCCAGACCCCTCACACTCAGA-3'	5'-CTCCGCTTGGTGGTTTGCTACGAC-3'
IL1 β	5'-GTTTCCTCCCTGCCTCTGACA-3'	5'-GACAATGCTGCCTCGTGACC-3'
IL6	5'-GATACCCACAACAGACCAG-3'	5'-GCCATTGCACAACCTTTTCTC-3'
CYPA (cyclophilin A)	5'-CGTTGGATGGCACGCCTGTG-3'	5'-TGCTGGTCTTGCCATTCCTG-3'

umn with on-column DNase digestion.²⁸ Because of a small amount of RNA that could be obtained and the number of genes to be analyzed, RNA was pooled from two rats within the same group ($n = 3$ pools per group) before cDNA synthesis using Stratascript 5.0 (Stratagene, Sydney, Australia) and 5 μ g of pooled RNA. The expression of osteoclastogenesis regulators RANKL, OPG, TNF- α , IL-1, and IL-6 was analyzed in 20- μ L reactions with 100 ng/mL of cDNA and 300 nmol/L primers, except for RANKL at 900 nmol/L (sequences given in Table 1). The amplification of targets was measured by SYBR fluorescence²⁸ and expression calculated in relation to endogenous control cyclophilin A using the $2^{-\Delta C_T}$ method. As a measure of amplified product specificity, each PCR was run including one reaction in the absence of cDNA template (for each target gene), and a melt curve was also analyzed for single product formation. Furthermore, products were visualized by running 9 μ L of amplified product in a 3% agarose gel and visualized by ethidium bromide.

Analyses of Plasma Pro-Osteoclastogenic Cytokine Levels

Enzyme-linked immunosorbent assay (ELISA) was used to measure circulating levels of pro-osteoclastogenic factors RANKL, TNF- α , IL-1, and IL-6 in plasma, using the following specific assay kits: TNF- α (BD OptEIA; BD Biosciences Australia, Sydney, Australia), IL-6 (Invitrogen), and IL-1 (R&D Systems, Minneapolis, MN). Because mouse and rat RANKL is homologous,^{29,30} the mouse RANKL capture and detection antibodies (R&D Systems) were used to determine rat plasma-free RANKL levels.^{29,31}

Plasma-Induced Osteoclastogenesis

To determine whether circulating factors were capable of inducing osteoclastogenesis, bone marrow nonadherent cells from normal rats were cultured overnight in the presence of M-CSF, as previously described, and then were used to examine whether plasma from MTX-treated rats can induce formation of osteoclasts. After the overnight culture, the adherent cells were stimulated with a positive control medium with 10% fetal calf serum, 10 ng/mL M-CSF, and 30 ng/mL RANKL, or a medium containing 10% plasma from MTX-treated rats or control rats in place of fetal calf serum ($n = 5$ rats per group, triplicate wells per treatment). After culture for 7 days, cells were processed for TRAP staining to identify osteoclasts. To aid visualization, fluorescent staining of F-actin and nuclei

was performed with 50 μ g/mL tetramethylrhodamine isothiocyanate-labeled phalloidin (Sigma) for 10 minutes and with 1 μ g/mL DAPI (Sigma) for 5 minutes, respectively. To investigate the role of TNF- α in osteoclast formation when stimulated with MTX-treated plasma, a TNF- α -neutralizing antibody was used in the assay. In these experiments, cells were cultured as previously described in 0, 0.5, or 5 μ g/mL of TNF- α antibody (R&D Systems). The concentrations of antibody used were based on the manufacturer's recommended median narcotic dose 50 and plasma concentration of TNF- α , as established by ELISA.

Plasma-Induced NF- κ B Activation in RAW_{264.7} Cells

To investigate whether MTX induced a pro-osteoclastogenic environment in the plasma of treated rats, an NF- κ B activation luciferase reporter assay was used using RAW_{264.7} cells, stably transfected with NF- κ B luciferase reporter gene.^{32,33} Briefly, 1.5×10^5 transfected cells per well were allowed to settle overnight before the addition of MTX-treated or control rat plasma (1:5) in the presence or absence of RANKL for 8 hours before cell harvest. Luciferase activity was measured with the Promega Luciferase Assay System (Promega, Sydney, Australia).

Statistical Analysis

All data are represented as mean \pm SEM and analyzed by one-way analysis of variance. If statistical significance (at $P < 0.05$) was achieved, a Tukey's post hoc analysis was performed.

Results

MTX Chemotherapy Increases Osteoclast Number and Marrow Osteoclastogenic Potential

After MTX treatment, numbers of osteoclasts were significantly elevated on day 9 in the secondary spongiosa ($P < 0.05$ versus control) (Figure 1, A versus B and D) and were similarly elevated on days 9 and 11 in the primary spongiosa ($P < 0.05$ and $P < 0.01$ versus normal control, respectively) (Figure 1C). *Ex vivo* osteoclastogenesis assays with bone marrow mononuclear cells (BMMCs) revealed that multinucleated TRAP⁺ osteoclasts were apparent in cultures (Figure 2, A and B); however, the potential of the marrow to form osteoclasts was greatest in

cultures of day 9 MTX-treated rats ($P < 0.01$ versus normal control) (Figure 2, A versus B and C).

MTX Increases Expression of Pro-Osteoclastogenic Cytokines within the Metaphysis

The mRNA expression profiles of pro-osteoclastogenic cytokines after MTX administration were examined in metaphysis bone. Levels of RANKL and OPG increased over time, albeit not significantly (data not shown); consistently, the ratio of their expression (RANKL:OPG) did not change significantly (Figure 3A). However, the expression of pro-inflammatory cytokines IL-1, IL-6, and TNF- α (Figure 3, B–D, respectively) had a similar pattern, exhibiting a gradual increase that was significantly higher on day 9 when compared with normal controls (IL-1 at $P < 0.01$ and IL-6 and TNF- α at $P < 0.05$), before returning to normal levels by day 14.

MTX Administration Results in a Systemic Pro-Osteoclastogenic Response

To investigate whether MTX chemotherapy generated a systemic pro-osteoclastogenic response, the osteoclastogenic potential of the plasma from treated rats was assessed in cultured normal BMMCs in media that had the 10% fetal calf serum replaced with 10% plasma collected from normal rats or MTX-treated rats and in the absence of exogenous RANKL. The cultures stimulated with plasma from MTX-treated rats from days 4 and 6 fostered significantly more osteoclasts than those from untreated plasma ($P < 0.01$ and $P < 0.001$, respectively) (Figure 4, A versus B and D). Osteoclast-like TRAP⁺ multinucleated cells were apparent in all cultures and were confirmed by DAPI and phalloidin staining (Figure 4, B and C).

To ascertain which pro-osteoclastogenic factors may be promoting plasma-induced osteoclast formation, cytokine concentrations in plasma samples were analyzed

Figure 1. MTX administration increases the number of osteoclasts lining the bone surface within the metaphysis. **A** and **B:** Representative images of H&E-stained secondary spongiosa of a control and a day 9 MTX-treated rat, respectively, with multinucleated osteoclasts (**white arrows**) lining the trabecular bone (Tb). Original magnification, $\times 40$. **C** and **D:** Quantification of osteoclasts/mm² of Tb. area in both the primary and secondary spongiosa, respectively. $n = 6$. * $P < 0.05$, ** $P < 0.01$.

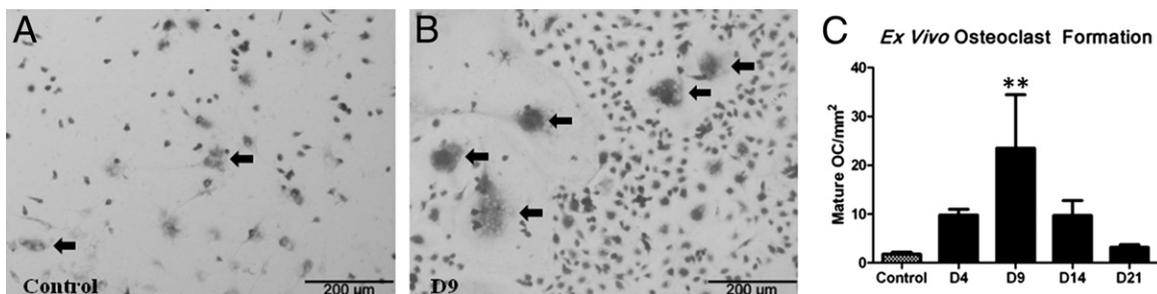


Figure 2. MTX chemotherapy increases the osteoclastogenic potential in the bone marrow. **A:** *Ex vivo* osteoclast formation of control rat plastic nonadherent BMMCs exposed to 10 ng/mL of M-CSF and 30 ng/mL of RANKL. **B:** TRAP⁺ osteoclasts formed from BMMCs from a day 9 MTX-treated rat (**arrows**). **C:** Quantification of *ex vivo* osteoclast formation demonstrating significantly greater osteoclast formation from D9 MTX-treated rats (TRAP⁺ cells/mm² culture area). $n = 6$. ** $P < 0.01$.

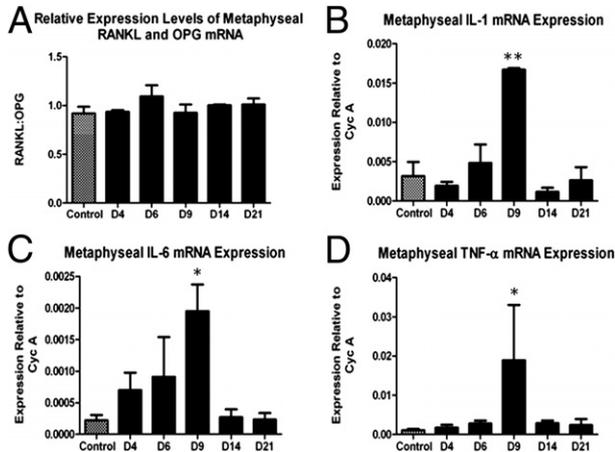


Figure 3. MTX chemotherapy does not affect the RANKL:OPG expression ratio but enhances mRNA expression of pro-inflammatory cytokines in the metaphysis. **A:** RANKL:OPG mRNA expression ratio illustrating no changes, although levels of both RANKL and OPG expression are increased on day 9 (data not shown). **B–D:** Quantitative RT-PCR analysis of IL-1, IL-6, and TNF- α mRNA expression, respectively. $n = 3$ pools of two rats per pool per time point. * $P < 0.05$, ** $P < 0.01$. Cyc, cyclophilin.

using ELISA. The RANKL level had a slight, yet insignificant, increase after MTX chemotherapy (Figure 5A). Interestingly, although IL-1 β levels were unaffected (Figure 5B), IL-6 remained unchanged before a significant increase on day 14 (Figure 5C, $P < 0.05$). Noticeably, TNF- α concentrations increased significantly on day 6 (Figure 5D, $P < 0.01$), a time that coincided with increased plasma-induced osteoclast formation (Figure 4A). To confirm if TNF- α was playing a specific role in plasma-induced osteoclast formation, BMMCs were cultured in the presence of MTX-treated day 6 plasma in the presence or absence of a TNF- α -neutralizing antibody (Figure 6). Osteoclast formation was attenuated with the

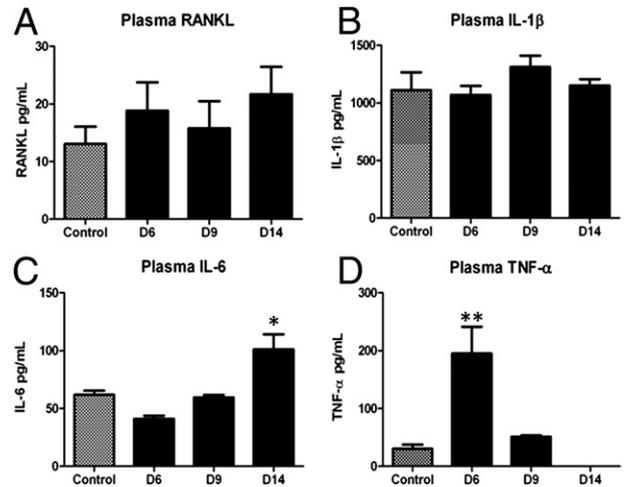


Figure 5. MTX administration enhances circulating levels of IL-6 and TNF- α in plasma, as measured by ELISA. Quantification of plasma RANKL (**A**), plasma IL-1 β (**B**), plasma IL-6 (**C**), and plasma TNF- α (**D**). $n = 4$ to 6. * $P < 0.05$, ** $P < 0.01$.

addition of a TNF- α -neutralizing antibody (Figure 6, A versus B and C). Consistently, cultures in the presence of day 6 plasma alone fostered significantly greater osteoclast formation when compared with normal controls (Figure 6C, $P < 0.001$).

MTX-Treated Rat Plasma Promotes Osteoclastogenesis through NF- κ B Activation

To investigate the involvement of NF- κ B activation in MTX chemotherapy-induced increased osteoclastogenesis, NF- κ B activation in osteoclast precursor RAW_{264.7} cells, stably transfected with an NF- κ B-luciferase reporter gene, was examined after stimulation by plasma col-

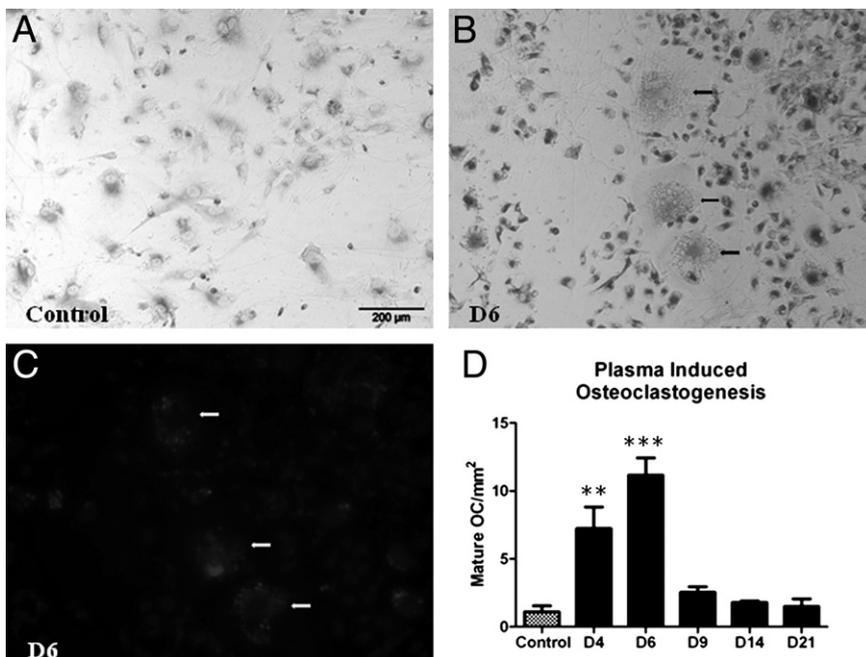


Figure 4. Osteoclast formation from normal rat nonadherent BMMCs *in vitro*, as supported by plasma obtained from MTX-treated rats. **A:** Formation of cells cultured in 10% control rat plasma. **B:** BMMCs exposed to plasma from a day 6 MTX-treated rat that formed TRAP⁺ multinucleated osteoclasts (black arrows). **C:** Fluorescent image corresponding to **B** of DAPI (blue) and phalloidin (red), confirming multinucleation of osteoclasts (white arrows). **D:** Quantification of TRAP⁺ osteoclasts (OC) formed/mm² of culture area, showing significantly greater osteoclast formation from cultures exposed to plasma from days 4 and 6 MTX-treated rats. $n = 3$. ** $P < 0.01$, *** $P < 0.001$. Scale bar = 200 μ m.

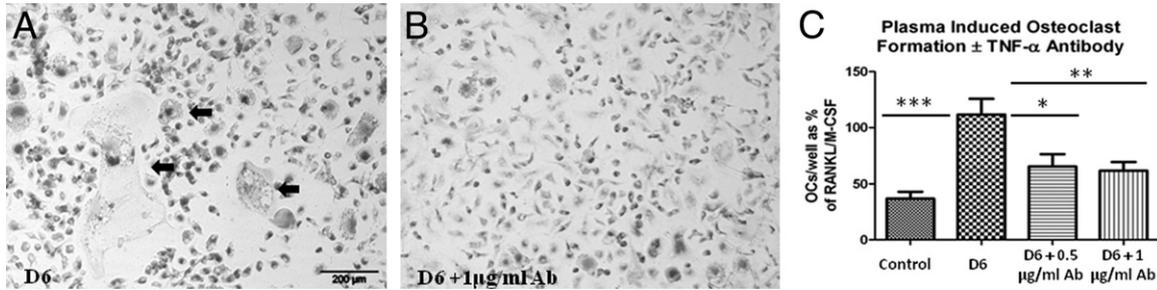


Figure 6. Plasma induction of osteoclast formation is diminished by a TNF- α -neutralizing antibody. **A:** TRAP⁺ multinucleated osteoclasts (arrows) formed from normal rat nonadherent BMMCs exposed to plasma from a D6 MTX-treated rat. **B:** Inhibition of osteoclast formation from normal BMMCs co-exposed to D6 MTX-treated plasma in the presence of a TNF- α -neutralizing antibody (Ab; 1 μ g/mL). **C:** Quantification of TRAP⁺ multinucleated osteoclasts formed from normal BMMCs when exposed to D6 MTX-treated plasma, with or without the addition of TNF- α -neutralizing antibody at 0.5 or 1 μ g/mL, expressed as a percentage of osteoclasts (OCs) formed from RANKL and M-CSF-positive control. $n = 6$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bar = 200 μ m.

lected from MTX-treated or control rats. NF- κ B activation was significantly elevated in cells cultured with plasma collected from day 6 rats when compared with untreated animals ($P < 0.05$) (Figure 7A). Furthermore, to test if activation of the NF- κ B was required for day 6 plasma to induce osteoclast formation, normal BMMCs were cultured in the presence of 10% plasma from normal or day 6 MTX-treated rats in the presence of parthenolide, a known NF- κ B inhibitor.³³ Parthenolide at both 0.5 and 1 μ g/mL inhibited the formation of osteoclasts, as induced by day 6 plasma from MTX-treated rats in these cultures (Figure 7B). To examine the potential roles of TNF- α in the plasma-induced NF- κ B activity, the assay was also conducted in the presence of the TNF- α -neutralizing anti-

body (at the same concentration previously found to inhibit osteoclast formation). However, this antibody did not block the plasma-induced NF- κ B activation (data not shown).

Discussion

Intensive use of cancer chemotherapy is known to reduce BMD in patients with cancer and survivors, and animal model studies have indicated altered bone remodeling as a primary cause of this adverse effect. Although bone loss caused by the most commonly used anti-metabolite, MTX, may be associated with suppressed osteogenesis (because of reduced osteoblast differentiation and activity, paired with enhanced adipogenesis)^{16,34} and elevated osteoclast presence and bone resorption,¹⁵ mechanisms for the increased osteoclastogenesis have remained unclear.^{35,36} The current study showed that MTX induces osteoclast differentiation by generating a pro-osteoclastogenic environment in the bone and in circulation, particularly with an increased TNF- α level, which can induce NF- κ B activation, promoting osteoclast formation.

Localized Effects of MTX within the Metaphysis

Consistent with our previous observations, MTX treatment increased osteoclast density within the metaphysis of treated rats.^{15,16} Although the activity of the osteoclasts was not measured in this study, MTX administration has enhanced bone erosion on the trabecular bone.⁹ Furthermore, studies^{2,11,17} have suggested that the increased number of osteoclasts correlates with the increased markers of bone resorption in patients receiving chemotherapy.

At the site of resorption, terminally differentiated osteoclasts are stimulated by a variety of factors to undergo bone resorption.¹⁸ *In vitro* studies have indicated that RANKL, TNF- α , IL-1, and IL-6 are capable of inducing active resorption by mature osteoclasts.^{18,37} We found that the mRNA expression of RANKL within the metaphysis increased after MTX administration, which, however, coincided with an equal shift in expression of osteoclastogenesis inhibitor, OPG, suggesting that, despite the

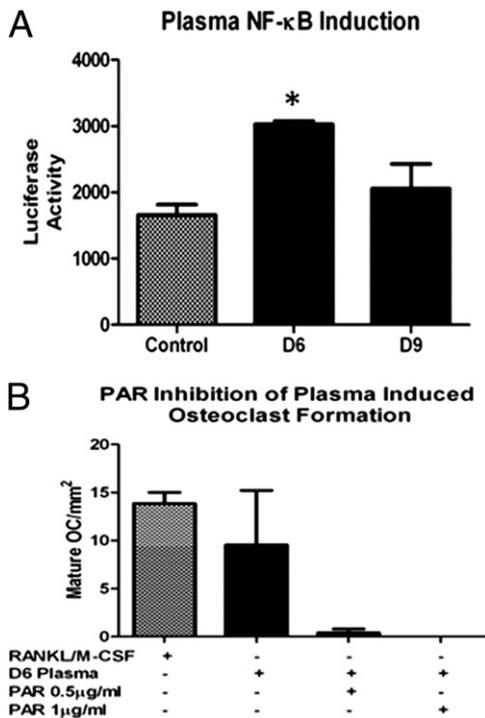


Figure 7. Plasma from MTX-treated rats enhances activation of NF- κ B. **A:** Quantification of NF- κ B activity when RAW_{264.7} cells, stably transfected with a *luc*-NF- κ B construct, are exposed to D6 MTX-treated plasma, as measured by luciferase activity. **B:** Quantification of osteoclast formation induced by D6 MTX-treated rat plasma in the presence or absence of NF- κ B inhibitor parthenolide (PAR). $n = 4$. * $P < 0.05$.

increased osteoclast density, the RANK-RANKL-OPG axis was largely unaffected by MTX chemotherapy in the bone. However, there was a significant increase in the expression of IL-1, IL-6, and TNF- α , which corresponded with the increased osteoclast density. IL-1, IL-6, and TNF- α can stimulate osteoclasts at multiple levels of differentiation; indeed, their pro-osteoclastic effects are clearly noticeable in localized bone destruction associated with rheumatoid arthritis.^{22,38}

MTX Increases the Osteoclastogenic Potential of Bone Marrow

Osteoclasts are differentiated from monocyte lineage cells in the blood and marrow.^{18,20} This process is started when early precursors expressing c-Fms begin expressing receptor RANK when stimulated by M-CSF.³⁹ Critically, this allows for commitment to the osteoclast lineage via stimulation by RANKL,^{39,40} resulting in cell fusion and multinucleation, cytoskeletal rearrangement, and expression of osteoclast-specific genes that typify the mature osteoclast.¹⁸ Recently, in the same model of MTX treatment, we found an increase in the number of osteoclast precursor cells in the bone marrow expressing early osteoclast precursor marker, CD11b,^{41,42} suggesting that treatment with MTX increased the osteoclast precursor pool. Consistently, we found that, after MTX treatment, the number of bone marrow cells forming osteoclast-like cells *ex vivo* was increased, indicating the increased marrow potential to form osteoclasts after MTX treatment.

Circulating TNF- α Increases after MTX Treatment

Although RANKL is known to be the critical osteoclastogenic factor, we showed that circulating RANKL levels were unaltered after MTX chemotherapy. On the other hand, because the mRNA expression of several pro-inflammatory/pro-osteoclastogenic cytokines was increased within the metaphysis and given that increased circulating inflammatory cytokines have been reported in patients receiving chemotherapy⁴³ and from cells exposed to chemotherapeutics *in vitro*,^{43,44} we investigated the levels of circulating IL-1, IL-6, and TNF- α in rats. Although osteoclast precursors exposed to IL-1 and IL-6 *in vitro* are capable of undergoing differentiation,^{45,46} the unaltered concentrations of IL-1 and the significant increase in IL-6, albeit on day 14, which is much later than the observed increase in osteoclasts, suggests that these two cytokines may not be the candidates supporting the increased osteoclast density seen after MTX chemotherapy.

In comparison, an elevated plasma TNF- α concentration on day 6 may represent a key mediator in MTX-induced increases in osteoclast formation and density in the metaphysis. TNF- α has supported osteoclast differentiation both directly^{20,37} and indirectly via enhanced RANKL synthesis by stromal cells.^{45,47} Incidentally, TNF- α has increased the number of spleen-derived CD11b⁺ osteoclast precursors,⁴² which may provide a potential mechanism for the increased levels of circulat-

ing osteoclast precursors apparent in rats after receiving MTX in our recent study.¹⁵ In this study, the enhanced formation of osteoclasts from normal bone marrow cells exposed to day 6 plasma indicated that, after MTX administration, the plasma had become pro-osteoclastogenic. More important, TNF- α was identified as a key contributing molecule to this effect because the addition of a TNF- α -neutralizing antibody attenuated the plasma-induced osteoclast formation. Interestingly, a higher dose of neutralizing antibody failed to further reduce the formation of osteoclasts, suggesting that the plasma contains other factors that support osteoclast development/maturation after MTX administration.

Plasma from MTX-Treated Rats Increases NF- κ B Activation

Activation of NF- κ B is essential for osteoclast differentiation after stimulation by RANKL and cytokines.^{24,48} Consistent with the ability of the plasma from day 6 MTX-treated rats in supporting osteoclast differentiation, the same plasma elevated the levels of NF- κ B activation in osteoclast precursor cells stably transfected with an NF- κ B luciferase reporter. This suggested that NF- κ B activation is a likely pathway for the systemic effect of MTX in inducing osteoclastogenesis, which was confirmed by our observation of abolished osteoclast formation from day 6 plasma with the addition to the cultures of parthenolide, an NF- κ B inhibitory compound.^{33,49} Surprisingly, although plasma-induced osteoclast formation from normal bone marrow cells suggested a role of TNF- α in supporting osteoclast formation, the addition of a TNF- α -neutralizing antibody did not dampen the NF- κ B response to MTX-treated plasma (data not shown). The overlap of the TNF- α and NF- κ B signaling pathways suggested that elevated levels of TNF- α would contribute to activation of this crucial pathway.^{18,50} Nonetheless, the heightened NF- κ B activity within cells exposed to day 6 plasma further supports the idea that there are other remaining factors in the plasma that could contribute to osteoclast formation via stimulation of this pathway.

In conclusion, MTX treatment increases osteoclast presence within the metaphysis and osteoclastogenic potential of the bone marrow. Local changes in the production of pro-osteoclastic cytokines within the metaphysis may contribute to osteoclast density, survival, and activity after MTX treatment. Moreover, we highlighted the role of elevated systemic circulating levels of TNF- α in MTX-treated rat plasma, which can promote osteoclast formation *in vitro*. Critically, MTX treatment-induced systemic changes in the plasma are capable of increasing activation of osteoclastogenic transcription factor NF- κ B, and NF- κ B activation has been critical for this plasma-induced osteoclastogenesis. Noticeably, the inability of the TNF- α -neutralizing antibody to absolutely inhibit plasma-induced osteoclast formation and reduce the plasma-induced NF- κ B activity indicates that other factors are present that may also contribute to the ability of plasma from MTX-treated rats to cause increased osteoclast formation. Nevertheless, TNF- α still represents a cytokine of

interest in MTX-induced osteoclast formation and bone loss; as such, its role in *in vivo* MTX-induced bone loss is worth further investigation. The results from this study have revealed novel mechanisms of elevated osteoclastogenesis in MTX-induced bone loss, implying that anti-osteoclastogenic interventions may benefit bone health during and after MTX chemotherapy.

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