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Targeting Extracellular Signal-Regulated Kinase (ERK) Signaling Has Therapeutic Implications for Inflammatory Osteolysis

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Abstract

The extracellular signal-regulated kinase 1/2 (ERK) pathway, part of the mitogen-activated protein kinase (MAPK) family, is well-known for its role in cell differentiation and proliferation. In the context of osteoclastogenesis, macrophage colony stimulating factor (M-CSF) is an upstream activator of ERK, which signals for the survival of osteoclast precursors prior to their differentiation into multinucleated osteoclasts. In addition, many recent studies have revealed the involvement of ERK in promoting osteolysis. In this study, we extended these existing findings linking ERK and osteolysis by identifying the ERK pathway as the primary pathway for osteolysis in osteoclasts and macrophages. We also elucidated the pro-inflammatory capacity of osteoblasts using the ERK pathway. Moreover, the ERK inhibitor, PD98059, inhibited the inflammatory reaction propagated by all three cell types at both a local and systemic level. The importance of ERK signaling in previously known cell types mediating inflammatory osteolysis as well as the discovery of osteoblastic innate immunity involving ERK signaling enhances our understanding of inflammatory osteolysis and supports further future investigation of targeted therapies against the ERK pathway for treating osteolytic diseases.

Keywords

Extracellular signal-regulated kinase (ERK) signaling; osteoblasts; Macrophage-colony stimulating factor (M-CSF); inflammatory osteolysis; lipopolysaccharide (LPS)

Introduction

Inflammatory osteolysis is an immune response against bacterial products or artificial implants. Increased osteoclast activity leads to bone loss and severe morbidity that underlies a number

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of clinical conditions, including osteomyelitis, and wear particle-induced periprosthetic osteolysis [1-4]. Immune cells, such as macrophages [5] and T lymphocytes [6], initiate this pathologic process upon encountering inflammatory stimuli such as bacterial cell wall components and wear particles by producing pro-osteoclastogenic cytokines such as Receptor Activator for Nuclear Factor κ B (NF κ B) Ligand (RANKL) [7], Macrophage-Colony Stimulating Factor (M-CSF) [8-10], and Tumor Necrosis Factor- α (TNF- α) [11-13]. These cytokines subsequently promote differentiation of pre-osteoclasts recruited from the monocyte lineage into mature osteoclasts, the cellular effectors of bone resorption.

Osteoblasts, the effectors of bone deposition, are the most common cell type found in trabecular bone and the inner aspect of cortical bone. Osteoblasts directly encounter pathogens and foreign substances and have been implicated in inflammatory osteolysis as well [14,15]. Osteolysis is governed and mediated by complex cellular and molecular interactions and has been relatively impervious to therapeutic approaches targeting only a single pathway. Previous studies have unsuccessfully attempted to find a single target molecule to suppress osteoclastogenesis.

Extracellular signal-regulated kinase (ERK), a serine/threonine-specific end effector of the mitogen-activated protein kinase (MAPK) signaling pathway, is a well-known downstream effector of the M-CSF receptor (C-FMS) and signals for pre-osteoclast survival and proliferation. However, ERK signaling has never been studied as a therapeutic target for osteolysis. Here, we investigated the cellular mechanisms of ERK signaling in both *in vitro* and *in vivo* models of inflammatory osteolysis and the role of ERK in the inflammatory response of various cell types mediating inflammatory osteolysis.

Materials and Methods

Mice and cells

Animal experiments were approved by the Institutional Animal Care and Use Committee of Columbia University (Protocol No. 5162). Four day-old and 4-10 week-old male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). Primary osteoblasts were harvested from the calvaria of 4 day-old mice while bone marrow (BM) cells were derived from femora of 10 week-old mice. The murine pre-osteoblastic cell line MC3T3-E1 was purchased from American Type Culture Collection (ATTC, Manassas, MD).

Primary osteoblasts were harvested from the calvaria of 4 day-old male C57BL/6J mice by successive enzymatic (collagenase/trypsin) digestion. After washing with PBS, each side of the parietal bones were separated and placed in individual wells of a 24-well cell culture plate. They were cultured overnight in α -MEM containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), 100 U/ml penicillin G and 100 μ g/ml streptomycin at 37°C and 5% CO₂. The culture medium was replaced with low serum medium (1% FBS) 1 hour prior to LPS treatments at which time cells were pre-treated with the pre-determined doses of PD98059.

Bone marrow-derived monocytes (BMMs) were prepared from male C57BL/6J mice. Non-adherent bone marrow cells are cultured in Minimum Essential Medium (MEM) α Medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Gemini Bio), 1% antibiotic/antimycotic (Gemini Bio) and 10 ng/ml of M-CSF (R&D) to obtain only BMMs.

In vivo inflammation study

Mice calvarial bones were treated subcutaneously with 25 μ g LPS from *E. coli* 026:B6 (Sigma-Aldrich, St. Louis, MO) in 40 μ l PBS. The ERK inhibitor, PD98059 (Calbiochem, San Diego, CA), was injected intraperitoneally 24 hours prior to LPS treatment and everyday thereafter. Calvarial bones were harvested after 3 days, fixed with 4% paraformaldehyde at 4°C for 6

hours, and decalcified with 10% EDTA for 2 days, after which 5 µm thick paraffin embedded calvarial bones were prepared.

Immunohistochemistry (IHC)

The primary antibodies used were anti-pERK1/2 antibody (Cell Signaling Technology, Danvers, MA), anti-M-CSF antibody (Abcam, Cambridge, MA), and anti-RANKL antibody (Calbiochem, San Diego, CA) at a 1:50 dilution. Immuno-staining was performed using the HRP-ABC and HRP-DAB Cell & Tissue Staining Kits (R&D Systems, Minneapolis, MN). The calvarial sagittal suture line was stained using the Acid Phosphatase Leukocyte (TRAP) Kit (Sigma-Aldrich, St. Louis, MO), and TRAP positive multinucleated osteoclast cells were counted under 40× magnification.

Total RNA isolation and quantitative real-time PCR

Total RNA was prepared using the Qiagen RNeasy Mini kit (Valencia, CA) according to the manufacturer's directions. Approximately 2 µg of RNA were reverse transcribed by extension of random primers with 200 U of Superscript III (Invitrogen, San Diego, CA). The cDNA levels of murine M-CSF and GAPDH were quantified by real-time PCR with FastStart DNA Master^{PLUS} SYBR Green I (Roche Diagnostics, Indianapolis, IN) and the SmartCycler II System (Cepheid, Sunnyvale, CA). Amplification was achieved using an optimized protocol with an initial cycle of 94°C for 10 minutes, followed by 40 cycles of 94 °C for 10 seconds, 60 °C for 20 seconds, and 72 °C for 20 seconds. All cDNA levels during the linear phase of amplification were normalized against GAPDH as an internal control. The primer sequences used are as follows:

GAPDH: 5'-AGAACATCATCCCT-3', 3'-AGTTGCTGTTGAAGTCGC-5'; M-CSF: 5'-GGGGGGGGGTACCGTCCAGGCACACCAACCTTTC-3', 3'-GGGGGGGGCTCGAGCTTCGCTGGCCAGCCCTC-5'; RANKL: 5'-TTGCTTTCGGCATCATGAAACATCG-3', 3'-CAGGGAAGGGTTGGACACCTGAATG-5'; ERK1: 5'-TCTGCTACTTCTCTACCAG-3', 3'-TCGATGGATTGGTGTAGCC-5'; ERK2: 5'-TGCCATGGAACAGGTTGTTTC-3', 3'-GCTTCTCCTTAGGTAAGTCG-5'.

Protein isolation and analysis

Cells were washed in PBS and subsequently scraped in a cell lysis buffer (Qiagen, Valencia, CA). The Nuclear Extract Kit (Active Motif, Carlsbad, CA) was used to isolate nuclear and cytoplasmic extracts. These extracts were further homogenized by sonication for 15 strokes at a 10% duty cycle and 4 °C (Sonifier 250; Branson, Danbury, CT). The samples were centrifuged at 10,000×g for 10 minutes, and the supernatant was collected for analysis. Protein quantification was performed using the BCA Protein Assay (Pierce, Rockford, IL) reagents and 20 µg of protein extract underwent Western blotting. Samples were run in 4-20% SDS-polyacrylamide gels (Invitrogen, San Diego, CA) and electrotransferred to PVDF membranes which were blocked and probed with primary antibodies overnight at 4°C. Blots were washed of excess antibodies in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and with 10% FBS, probed with a HRP-labeled secondary antibody (Cell Signaling Technologies, Beverly, MA), and detected using the enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were stripped with Restore® Western blot stripping buffer (Pierce, Rockford, IL) and reprobed with GAPDH (Chemicon, Temecula, CA) or β-Actin (Abcam, Cambridge, MA) as loading controls.

Luciferase reporter assay

The M-CSF-promoter region (nt 451 – 1231, Accession #NT_039240) were PCR amplified from MC3T3-E1 genomic DNA, subcloned, and sequenced to confirm the fidelity of the product. The respective promoter regions were inserted into the multiple cloning site of the PGL4.10 luciferase vector (Promega, Madison, WI, USA) resulting in the PGL4-MCSF luciferase reporter plasmids. Transfection of the luciferase reporter plasmids was performed using the Nucleofector System (Amaxa, Cologne, Germany). For the luciferase activity study, the PGL4-MCSF luciferase reporter plasmids were co-transfected with Renilla as a normalizing control. After 24 hours post-transfection, samples were pre-treated for 1 hour with 40 μ M PD98059 (Calbiochem, San Diego, CA) in 1% FBS containing medium before the addition of 3 μ g/ml LPS (Sigma, St. Louis, MO). After an additional 24 hours of incubation, the Dual-Glo™ Luciferase Assay System (Promega, Madison, WI) was used to obtain luciferase and Renilla activity.

Flow cytometry analysis

Spleens were obtained from C57BL/6N mice sacrificed by cervical dislocation. Single cell suspensions of spleen cells were prepared by pressing the organs through a nylon mesh and removing red cells with lysis buffer. To determine the frequency of osteoclast and T cells in the spleens, FITC-CD62L, FITC-CD11b, PE-RANK, PE-CD44, PECY5-CD8 and APC-CD4 (ebioscience, San Diego, CA) were used. Isolated splenocytes were washed with a staining buffer (2% BSA and 0.1% sodium azide in PBS). Cells were incubated with antibodies at 4° C for 15 min and washed twice with the staining buffer. Data acquisition and analysis were performed using FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) and FlowJo software (TreeStar, Ashland, OR).

Statistical analysis

Statistical analysis was performed using the Student's *t*-test (**P* < 0.05, ***P* < 0.005), and all data were presented as mean $\pm$ S.E.M. Our preliminary data on the loss-of-function experiments using PD98059 revealed that 8 samples provided an adequate study power (β > 0.8; *P* = 0.05). Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software (version 10, Chicago, IL).

Results

Blockade of ERK signaling inhibits LPS-induced inflammatory bone loss in vivo

Inflammatory osteolysis in murine calvarial bone was induced *in vivo* by direct application of lipopolysaccharide (LPS), a bacterial cell wall component known to be one of the strongest biological stimulators of the innate immune response that is also responsible for periprosthetic bone loss associated with infection [16]. First, 25 μ g of LPS in 40 μ l of phosphate buffered saline (PBS) was injected subcutaneously onto the sagittal suture of murine calvarial bone. Staining for activated osteoclasts with tartrate-resistant acid phosphatase (TRAP) three days later revealed a TRAP-positive osteoclast count that was significantly increased in the LPS-treated group compared to control mice given PBS alone (Fig. 1A, B).

The immunoreactivities of RANKL and M-CSF, pro-osteoclastogenic cytokines essential for osteoclastogenesis [17], were both increased against LPS and suppressed by PD98059 (Fig. 1C, D), an upstream MAPK kinase inhibitor. PD98059 was also used to determine whether ERK could be a major signaling mediator of inflammatory osteolysis. Varying doses of PD98059 were injected intraperitoneally into mice one day prior to the subcutaneous administration of LPS, followed by daily injections of PD98059 until the animals were sacrificed. Histological specimens confirmed the inhibitory effect of systemically administered

PD98059 on ERK phosphorylation in the calvarial region. TRAP staining of the calvaria revealed that the number of LPS-induced TRAP-positive osteoclasts along the sagittal suture decreased significantly with PD98059 in a dose-dependent manner (Figure 1E). Immunohistochemistry also showed an increase in activated or phosphorylated ERK in the calvarium following LPS treatment (Figure 1F), further supporting the potential role of ERK as a downstream mediator of LPS signaling.

Osteoblasts upregulated RANKL and M-CSF expression via ERK signaling in response to LPS

To further examine the role of osteoblasts in inflammatory bone loss, we verified the association between ERK activation and RANKL/M-CSF expression in response to bacterial endotoxin by using both primary calvarial osteoblasts and the MC3T3-E1 pre-osteoblastic cell line. Upon LPS stimulation, we observed phosphorylation of ERK in MC3T3-E1 cells by immunoblotting (Fig. 2A). These findings were consistent with LPS activation of ERK *in vivo* (Fig. 1F). We then examined whether M-CSF and RANKL were produced by osteoblasts directly in response to LPS. We stimulated primary osteoblasts harvested from the calvarial bone of 4 day-old mice, a more physiologically relevant cell model given our earlier *in vivo* observations. Primary osteoblasts were found to be sensitive to LPS, with even 1 µg/ml of LPS eliciting robust RANKL and M-CSF mRNA expression (Fig. 2B, C).

Next, PGL4-M-CSF luciferase reporter plasmids were co-transfected into MC3T3-E1 cells with Renilla as a normalizing control. After 24 hours of incubation with 3 µg/ml LPS, the Dual-Glo™ Luciferase Assay System showed that the M-CSF promoter was highly activated. This activation, however, was inhibited by 40 µM PD98059 (Fig. 2D). The obligatory role of ERK signaling in eliciting the osteoblasts' response to LPS was further demonstrated by the dose-dependent suppression of M-CSF and RANKL mRNA expression by PD98059 (Fig. 2E, F). We further clarified that activated ERK pathway is directly mediating the upregulation of M-CSF by several experiments. First, we suppressed well-known inflammatory signaling activated by LPS, such as NFκB and Cox2 pathways to see whether the upregulation of M-CSF induced by LPS is affected by those pathways. However, those inhibitors did not significantly suppress M-CSF induction (Fig. 2G, H) compared to ERK inhibitor, PD98059.

Secondly, we activated ERK signaling through a different upstream stimulator, Phorbol 12-myristate 13-acetate (PMA). PMA is a potential inducer of protein kinase C and also induces activation of ERK signaling (Fig. 3A). We found PMA induced M-CSF expression, which was suppressed by ERK inhibitor, PD98059 (Fig. 3B).

Lastly, we examined whether loss of ERK has a distinct role in the regulation of M-CSF gene expression in MC3T3-E1 cells. Selective silencing of ERK1 and ERK2 mRNA was confirmed by semi-quantitative real time rt-PCR. ERK1 gene silencing resulted in significant reduction of LPS-induced M-CSF gene expression in MC3T3 cells (Fig. 3C).

PD98059 suppressed LPS-induced TNF-α and MIP-1α expression

We obtained bone marrow-derived macrophages (BMMs) from the femurs of 4 week-old male C57BL/6J mice. We treated the BMMs with LPS in the presence or absence of PD98059 and found that PD98059 selectively reduced LPS-induced expression of TNF-α (Fig. 4A) and macrophage inflammatory protein (MIP)-1α (Fig. 4B), a crucial mediator of osteoclast formation. ERK suppression, however, did not significantly affect the expression of other cytokines such as interleukin 1β (Fig. 4C) or interleukin 6 (Fig. 4D).

PD98059 suppressed osteoclastogenesis in bone marrow-derived macrophage *in vitro*

The osteoclastogenic potential of bone marrow-derived pre-osteoclasts in the presence of M-CSF and RANKL was assessed both in the presence and absence of PD98059. BMMs were differentiated into TRAP-positive multinucleated cells in 12-well dishes with various doses of PD98059. The number of TRAP-positive multinucleated cells was then counted in three different areas of a 400× field. PD98059 significantly reduced the number of TRAP-positive multinucleated cells in a dose-dependent manner (Fig. 4E).

Changes of inflammatory cells in the spleen of the experimental mice

To observe fractional changes in inflammatory cells in the spleen, the spleens of 6-8 week old male C57BL/6J mice were harvested 3 days after injecting 25µg of LPS in the calvarium. Localized LPS-induced inflammation in the mice calvaria was sufficient to increase the total lymphocyte count as well as the number of pre-osteoclasts in the spleen (Fig. 5A, B). Interestingly, PD98059 decreased the number of pre-osteoclasts in the spleen in a dose-dependent manner (Fig. 5A, B). We also analyzed the proportion of systemic lymphocytes using flow cytometry analysis. LPS-induced localized calvarial inflammation significantly increased the systemic proportion of CD8(+) cytotoxic T cells, which are known to promote inflammation and tissue damage (Fig. 5C). The addition of PD98059, however, decreased the number of cytotoxic T cells even in the presence of LPS (Fig. 5C). In contrast, the number of CD4(+) T helper cells decreased in the setting of LPS-induced calvarial inflammation and was not affected by PD98059 (Fig. 5D).

Discussion

In this study, we examined the potential of ERK inhibition in treating inflammatory osteolysis by analyzing the presence of ERK signaling in multiple cell types at both the local and systemic level. In doing so, we found that ERK was an important signaling molecule for many cell types involved in inflammatory osteolysis. Although some of these findings have already been evaluated in other *in vitro* studies, this is the first study to evaluate the therapeutic potential of an ERK inhibitor, PD98059, with regard to inflammatory osteolysis. Moreover, ours is the first study to demonstrate the crucial role of ERK in regulating M-CSF expression in osteoblasts. We observed that inflammatory stimuli promoted osteoblasts to increase expression of RANKL and M-CSF, the two most critical cytokines for osteoclastogenesis that have also been associated with bone implant failures and infection [9,18]. Kikuchi et al. previously demonstrated that ERK signaling is required for LPS-induced RANKL (or ODF) expression, which is consistent with our result [19]. ERK pathway thought to be linked to RANKL gene induction via ATF-4 [20], however, the mechanism of M-CSF expression was previously unclear. This study showed that ERK inhibition suppressed the LPS-induced over-expression of M-CSF. Thus, the same physiologic over-expression of RANKL and M-CSF observed with inflammatory osteolysis could potentially be mitigated by selective suppression of ERK signaling. The present study also highlighted the pronounced innate immune reaction mounted by osteoblasts in response to LPS, which is implicated in osteomyelitis and bone implant failures [2,3,19]. Although we showed LPS activated ERK signaling with a simultaneous increase of RANKL and M-CSF in calvarial tissues, we were not able to identify the specific cell types that play a major role in inflammatory osteolysis. Nevertheless, our *in vitro* experiments demonstrated that many cell types involved in osteolysis, namely osteoclasts, osteoblasts, bone marrow derived macrophages, and T lymphocytes, are independently able to promote osteolysis via ERK signaling, making ERK a potentially important target pathway for osteolysis therapy.

Inflammatory cells play a major role in osteolysis by expressing inflammatory cytokines such as TNF- α . Previous studies have reported ERK signaling to regulate TNF- α expression in

macrophage in response to inflammatory stimuli. TNF- α regulates the survival and proliferation of pre-osteoclasts necessary for osteoclastogenesis to subsequently occur. In the present study, LPS induced TNF- α and MIP-1 α expression in bone marrow derived macrophages, while PD98059 suppressed their expression. This mechanism also can be an important target for pharmacologic ERK inhibition in the specific context of inflammatory osteolysis.

It is well-known that the downstream effect of C-FMS activation, which includes proliferation and survival of pre-osteoclastic cells, is mediated by ERK signaling. We studied ERK-mediated C-FMS signaling in the context of *in vivo* inflammation by observing whether ERK signaling affected the pool of splenic pre-osteoclasts *in vivo*. Local injection of LPS not only induced local bone inflammation at the injection site, but also caused a systemic inflammatory reaction by increasing the number of circulating osteoclast precursors in the spleen. Even though we were not able to clarify the specific mechanism behind this phenomenon, ERK inhibition nevertheless suppressed the systemic inflammatory response in dose-dependent manner, demonstrating ERK signaling to be also crucial for regulating systemic inflammatory response.

More specifically, PD98059 also prevented systemic activation of T-cells induced by bone inflammation. Previous studies showed that activation of T cells promoted bone loss in autoimmune arthritis by inducing osteoclastogenesis via RANKL upregulation [21,22]. CD4 (+) T cells in arthritis have been known to enhance osteoclastogenesis [23]. However, CD4(+) T helper-cell subsets TH1 and TH2 release interferon gamma and interleukin 4, respectively, both of which are antiosteoclastogenic. Therefore, the role of CD4(+) T cells still remains unclear.

In investigating whether PD98059 affected the activity of CD8(+) T cells, we discovered that the number of CD8(+) T cells significantly increased in the spleen of mice treated with LPS on the calvarium and that ERK inhibition significantly reduced the number of CD8(+) T cells. While this experiment did not show the local effects of increased CD8(+) T cells on calvarial bone, it did demonstrate the involvement of ERK signaling in the systemic increase of CD8 (+) T cells in inflammatory osteolysis. Such a change in the CD4(+)/CD8(+) ratio has been previously postulated to be a cause of aseptic osteolysis [24]. Moreover, in that study, Landgraaber et al. found an inverse relationship between the value of this ratio and the severity of osteolysis, wherein a decrease in this ratio due to implant wear particle-induced CD4(+) T cells apoptosis increased the severity of osteolysis. Thus, our finding of ERK inhibition decreasing the CD8(+) T cell count can be interpreted to increase the aforementioned CD4(+)/CD8(+) T cell ratio, thereby decreasing the severity of osteolysis.

In summary, this study demonstrated using both *in vivo* and *in vitro* models that the ERK signaling pathway is a pro-inflammatory pathway present in many cell types, including osteoblasts. These cells are individually able to promote inflammatory osteolysis via cytokine production through ERK activation. The inhibition of ERK signaling, however, attenuated the inflammatory response in these cell types both at the local and systemic level. Thus, the ERK pathway may be a potentially important therapeutic target in the treatment of inflammatory osteolysis.

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Figure 1. LPS-induced osteoclastogenesis in the mouse calvarium

Tartrate-resistant acid phosphatase (TRAP) staining shows osteoclasts from sham (left), 25 μg lipopolysaccharide (LPS)-treated (middle), and LPS + PD98059 (5 mg/kg i.p.)-treated (right) mouse calvarium. Lipopolysaccharide (LPS) treatment increased the number of osteoclasts and induced the expression of macrophage-colony stimulating factor (M-CSF) and Receptor Activator for Nuclear Factor κB (NFκB) Ligand (RANKL) by sutural osteoprogenitor cells in murine calvaria (**A**, **B**). Immunohistochemical staining with anti-RANKL and anti-M-CSF antibodies shows increased RANKL and M-CSF expression after LPS treatment (**C**, **D**). A bar diagram summary of TRAP(+) multinucleated cells in the sagittal suture of LPS-treated mice with or without PD98059 ($n = 5$ for each group, $* p = 0.018$) (**E**). Phosphorylated-extracellular signal-regulated kinase (pERK) immunohistochemical staining of calvarial bone from sham or LPS-treated groups. Increased pERK signal was observed in the suture cells of LPS-treated mice, while little signal was seen in untreated mice (**F**) (Scale Bar; $\times 40$: 50μm, $\times 100$: 20μm).

Figure 2. The effect of LPS on ERK activation and cytokine expression in osteoblasts

Western blot detection of phosphorylated-extracellular signal-regulated kinase (pERK) in preosteoblastic MC3T3-E1 cells treated with lipopolysaccharide (LPS) alone and with LPS and PD98059 (A). PD98059 inhibited pERK while baseline total extracellular signal-regulated kinase (tERK) was not affected. Primary osteoblasts cultured from the calvaria of 4 day-old mice were sensitive to LPS, expressing both RANKL and M-CSF even with 1 μ g/ml of LPS ($n = 3$, $*p < 0.05$) (B, C). The PGL4-M-CSF luciferase reporter plasmids were co-transfected into MC3T3-E1 cells with Renilla. After 24 hours of incubation with 3 μ g/ml LPS, M-CSF promoter was highly activated, which was inhibited by 40 μ M PD98059 ($n = 3$, $*p < 0.05$) (D). PD98059 suppressed LPS-induced expression of both M-CSF and RANKL mRNA in a dose-dependent manner ($n = 3$, $*p < 0.05$) (E, F). 20 μ M SN50, a cell permeable peptide inhibitor of NF κ B did not significantly inhibit the upregulation of M-CSF in the presence of by LPS ($n=3$, $*p=0.058$) (G). Cox2 inhibitors (20 μ M NS398 and 20 μ M cell permeable selective COX2 inhibitor, CoxII) failed to suppress the induction of M-CSF by LPS ($*n=3$, $p > 0.05$).

Figure 3. Specific correlation between ERK signaling and M-CSF expression

Phorbol 12-myristate 13-acetate (PMA) stimulated ERK signaling in MC3T3-E1 cells (A). Both 100 μ M PMA and 3 μ g/ml LPS induced M-CS expression, which was suppressed by ERK inhibitor, PD98059 in MC3T3-E1 cells (B). MC3T3-E1 cells were transfected with siRNA designed to specifically inhibit the expression of ERK1 or ERK2. Control cells were treated with a non-silencing siRNA (sc-37007, Santa Cruz, CA). Real-time PCR experiments were performed 48 hours subsequent to the transfection to confirm the target gene knockdown. ERK 1 gene silencing significantly reduced LPS-induced M-CSF expression (* $n=3$, $p < 0.001$) (C).

Figure 4. The effect of LPS on cytokine expression in bone marrow derived macrophages
Lipopolysaccharide (LPS) alone induced upregulation of the osteoclastogenic cytokines tumor necrosis factor- α (TNF- α) and macrophage inflammatory protein (MIP)-1 α (A, B). The simultaneous addition of 20 μ M of the ERK inhibitor PD98059 significantly reduced the LPS-induced inflammation ($n = 3$, $*p < 0.05$) (A, B). LPS and PD98059 did not have a similar effect on the expression of other non-osteoclastogenic cytokines, namely interleukin 6 (IL-6) and interleukin 1 β (IL-1 β) (C, D). PD98059 inhibited osteoclastogenesis in a dose-dependent manner, with higher doses of PD98059 resulting in fewer multinucleated cells (MNCs; E)

Figure 5. Flow cytometry analysis of spleen inflammatory cells in the setting of LPS and PD98059 administration

Lipopolysaccharide (LPS) alone increased the total number of circulating lymphocytes while simultaneous administration of PD98059 significantly reduced this number ($n = 5$, $*p < 0.05$) (A). LPS alone also increased the number of circulating monocytes while PD98059 significantly decreased this effect ($n = 5$, $*p = 0.056$) (B). The LPS-induced increase in number of activated CD8(+) T lymphocytes was also significantly reduced by PD98059 in a dose-dependent manner ($n = 5$, $*p < 0.001$) (C). Neither LPS nor PD98059 had an effect on the quantity of circulating CD4(+) T lymphocytes (D).