p38 MAPK in Myeloma Cells Regulates Osteoclast and Osteoblast Activity and Induces Bone Destruction

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Abstract

p38 mitogen-activated protein kinase (MAPK), which is constitutively activated in human myeloma, has been implicated in bone destruction by this cancer, but the processes it recruits are obscure. In this study, we show that p38 activity in myeloma inhibits osteoblast differentiation and bone formation, but also enhances osteoclast maturation and bone resorption. p38 regulated the expression and secretion of the Wnt pathway antagonist DKK-1 and the monocyte chemoattractant MCP-1. Attenuating p38, DKK-1, or MCP-1 were each sufficient to reduce bone lesions in vivo. Although it is well known that DKK-1 inhibits osteoblast differentiation, we found that together with MCP-1, it could also promote osteoclast differentiation and bone resorption. The latter effects were mediated by enhancing expression of RANK in osteoclast progenitor cells and by upregulating secretion of its ligand RANKL from stromal cells and mature osteoclasts. In summary, our study defined the mechanisms by which p38 signaling in myeloma cells regulates osteoblastogenesis, osteoclastogenesis, and bone destruction. Our findings, which may have implications for bone invasion by other cancers where p38 is elevated, strongly suggests that targeting p38 for inhibition may offer an effective therapeutic approach to treat osteolytic bone lesions in patients with myeloma. Cancer Res; 72(24): 1–10. ©2012 AACR.

Introduction

Bone destruction is a hallmark of multiple myeloma. More than 80% of myeloma patients have osteolysis, which is characterized by pathologic fractures, severe bone pain, spinal cord compression, and hypercalcemia. These symptoms can severely compromise a patient’s quality of life and performance status (1, 2). It has been proposed that myeloma cells activate osteoclast-mediated bone resorption and inhibit osteoblast-mediated bone formation (3–5), but the mechanism underlying the association of myeloma cells with bone lesions remains poorly elucidated.

Constitutive activation of p38 mitogen-activated protein kinase (p38 MAPK) has been found in benign bone diseases and malignant osteolytic tumors, including multiple myeloma (6–8). We recently discovered that p38 activity in myeloma cells is a master contributor to osteolysis in multiple myeloma (9). Our results show that the majority of established myeloma cell lines and primary myeloma cells from patients have high levels of phosphorylated p38 (p-p38). Injection of myeloma cells with high or detectable p38 levels into severe combined immunodeficient mice (SCID) and SCID-hu mice not only established myeloma but also caused severe lytic lesions in the murine and human bones; in contrast, injection of myeloma cells with no detectable p38 activity only established myeloma. Furthermore, disruption of p38 activity in myeloma cells by specific p38 shRNAs or inhibitors abrogated myeloma-induced bone lesions in mice, without affecting tumor growth, survival, or ability to home to the bones. In this study, we investigated the roles and mechanisms of activated tumor cell p38 in myeloma-mediated osteoblastogenesis and osteoclastogenesis.

Our results show that constitutive activation of p38 in myeloma cells leads to monocyte chemotactic protein-1 (MCP-1) and dickkopf-1 (DKK-1) expression and secretion. The p38-upregulated DKK-1 inhibits osteoblastogenesis, whereas p38-upregulated DKK-1 and MCP-1 promote osteoclast maturation and function via enhancing RANK/RANKL expression and activating NF-κB, p38, and ERK signaling pathways in their progenitor cells. These studies elucidate a novel mechanism of myeloma cell p38-induced osteolytic bone lesions and provide a strong rationale for developing new strategies targeting myeloma cell p38 activity for the treatment or prevention of myeloma bone disease.
Materials and Methods

Tumor cell lines and primary myeloma cells

The myeloma cell lines ARP-1 and MM.1S have been described previously (10). Other myeloma cell lines were purchased from American Type Culture Collection (ATCC). These cell lines were authenticated by short tandem repeat profiling and by matching with the profile published in ATCC. All myeloma cell lines were cultured in RPMI-1640 supplemented with 10% FBS (Invitrogen). Primary myeloma cells were isolated from bone marrow aspirates obtained from patients during routine clinic visits by magnetic bead sorting for CD138+ cells (Miltenyi Biotec GmbH). The study was approved by the Institutional Review Board at The University of Texas MD Anderson Cancer Center (Houston, TX).

Plasmids and reagents

Short hairpin RNAs (shRNA) for p38 3 isoforms including α, β, and γ were purchased from Santa Cruz Biotechnology and packed into the retroviral vector pSIREN-RetroQ (BD Biosciences, Clontech). Retroviral infections were conducted according to the manufacturer’s instructions. Retroviral vector supernatants of the p38 shRNAs were pooled and used to infect myeloma cells at 1:4 dilution. Stable cell lines were established in the presence of 1 µg/mL puromycin. In addition, siRNAs specific for p38 α, β, and γ were purchased from Santa Cruz Biotechnology. In the experiments, cells were harvested, plated on a 24-well plate at a concentration of 2 × 10^5 cells per well, and transiently transfected with pooled siRNAs or nonspecific/control siRNA at different doses using the Oligofectamine transfection reagent (Mirus) according to the manufacturer’s instructions. The p38 MAPK-specific inhibitors were purchased from Axon Medchem BV. Recombinant DKK-1 and MCP-1 were purchased from R&D Systems.

Mouse model, antibody treatment, and detection of osteolytic bone lesions by radiography

CB.17 SCID mice were purchased from Harlan Laboratories. All mice were maintained in the American Association of Laboratory Animal Care-accredited facilities, and the studies were approved by the Institutional Animal Care and Use Committee of MD Anderson Cancer Center. Six- to 8-week-old mice were inoculated intravenously with 1 × 10^6 myeloma cells. Serum was collected from the mice daily during the treatment and tested for myeloma-secreted M-proteins [human immunoglobulin (Ig)] or their light chains by using ELISA. For antibody treatment, the mice were injected intraperitoneally with neutralizing antibodies specific for MCP-1 or DKK-1 or with control IgG twice per week for 20 days. To measure lytic bone lesions, radiographs were obtained using a Faxitron X-ray cabinet (Faxitron X-ray).

For immunohistochemical analysis, Western blotting analysis, real-time PCR, identification of myeloma-derived cytokines in culture medium by cytokine array, measurements of soluble factors in culture medium and serum by ELISA, in vitro osteoclast formation and bone pit analysis, in vitro osteoblast formation and function assays, and statistical analysis, see Supplementary Experimental Procedures.

Results

Myeloma cell p38 upregulates myeloma cell production of osteolytic mediators DKK-1 and MCP-1

Recent studies have shown that myeloma cells release multiple cytokines or chemokines to the bone marrow microenvironment and regulate the process of bone remodeling (3). To elucidate the mechanisms underlying myeloma p38-mediated bone destruction, we specifically and stably knocked down p38 using shRNAs (9) and examined cytokines and chemokines that are regulated by p38 and play a role in bone remodeling in myeloma cells (ARP-1 and MM.1S). A cytokine array containing specific antibodies for 48 inflammatory cytokines and chemokines, the majority of which are known regulators of bone remodeling, was used. As shown in Fig. 1A, the levels of 10 osteoclastogenesis-associated cytokines were lower in p38 shRNA-treated ARP-1 (p38 shRNA-ARP-1) cells than in vector control-treated ARP-1 (vector-ARP-1) cells. Among these cytokines, MCP-1, which has recently been shown to induce osteoclast differentiation in prostate cancer (11), was highly expressed in vector-ARP-1 cells but significantly downregulated in p38 shRNA-ARP-1 cells (Fig. 1B; \( P < 0.01 \)). Real-time PCR (Fig. 1C) and ELISA (Fig. 1D) confirmed the results (\( P < 0.001 \)). Similar results were obtained with MM.1S cells (data not shown). Because DKK-1 and RANKL, 2 important cytokines involved in bone resorption, were not included in the cytokine array, we examined their expression in the myeloma cells. Both real-time PCR (Fig. 1C) and ELISA (Fig. 1E) showed that the expression and secretion of DKK-1 are downregulated in p38 shRNA-ARP-1 and p38 shRNA-MM.1S cells (\( P < 0.001 \), compared with controls). RANKL (data not shown) was not expressed by the cells. Consistent with these results, DKK-1 and MCP-1 were the main cytokines that were highly secreted by primary myeloma cells with high or detectable pp38 as compared with primary myeloma cells with undetectable pp38 (Fig. 1F; \( P < 0.01 \)). These results clearly suggest that DKK-1 and MCP-1 are the products of p38 in myeloma cells and might be involved in the formation of bone lesions.

On the basis of these results, we focused on DKK-1 and MCP-1 in subsequent studies. First, to verify the relationship between p38 and the expression of DKK-1 and MCP-1 in myeloma cells, the p38-specific inhibitor SB203580 (SB20) or siRNAs were used to inhibit or knockdown p38 in myeloma cells. Western blot analysis showed that treatment of ARP-1 or MM.1S (data not shown) cells with SB20 significantly downregulated, in a dose-dependent manner, the levels of phosphorylated MAPKPK-2 (pMK2), a downstream kinase of p38, DKK-1, and MCP-1, but not p-p38 (Fig. 2A). Because SB20 does not inhibit the phosphorylation of p38 but inhibits phosphorylation of its downstream kinase (12), slightly elevated levels of pp38 were observed in SB20-treated cells. Moreover, siRNAs specific for p38 (Fig. 2B) or the p38 upstream kinase MKK3/6 (Fig. 2C), but not control siRNAs (data not shown), also significantly inhibited, in a dose-dependent manner, the expression of DKK-1, MCP-1, p-p38, and p38 or pMKK3/6 and...
MKK3/6, respectively, in ARP-1 and MM.1S cells. ARP-1 and MM.1S secretion of DKK-1 (Fig. 2D) and MCP-1 (Fig. 2E) into culture supernatant was also inhibited by treatment with SB20 or siRNAs specific for p38 or MKK3/6 (\(P < 0.01\) to \(P < 0.001\)). More importantly, SB20 treatment also significantly inhibited secretion of DKK-1 (Fig. 2F) and MCP-1 (Fig. 2G) by primary myeloma cells (\(P < 0.01\)).

To examine the role of these cytokines in myeloma cell-induced bone lesions in vivo, we measured and compared the levels of circulating DKK-1 and MCP-1 in SCID mice injected with the myeloma cells. Before tumor inoculation, the levels of human DKK-1 (Fig. 3A) and MCP-1 (Fig. 3B) in the mouse sera were undetectable. Eight weeks after tumor injection, high levels of human DKK-1 and MCP-1 were detected in mice injected with control ARP-1 or MM.1S (data not shown) cells, and significantly lower levels of the 2 cytokines were observed in mice injected with p38 shRNA-ARP-1 cells (\(P < 0.001\)). Immunohistochemical staining revealed positive staining for DKK-1 and MCP-1 in vector-ARP-1 but not p38 shRNA-ARP-1 cells in the bone marrow of myeloma-bearing mice (Fig. 3C). Similar results were also obtained in ARP-1- or MM.1S-xenografted SCID mice (data not shown) and in primary myeloma cell-xenografted SCID-hu mice treated with or without the p38 inhibitor SD-169 (Fig. 3D). However, knocking down p38 activity in myeloma cells did not affect their growth in the mice (9).

Neutralizing antibodies against DKK-1 or MCP-1 were used to confirm their roles in the formation of bone lesions in vivo. SCID mice were injected intravenously with the parental ARP-1 or MM.1S cells, and beginning at week 4 (when tumors were established, without bone destruction) received intraperitoneal injections of anti-DKK-1 (10 mg/kg) and/or anti-MCP-1 (20 mg/kg) antibodies twice a week for a

Figure 1. Identification of osteolytic mediators DKK-1 and MCP-1 as the targets of myeloma cell p38 activity. Representative images (A) and densitometric data (B) of a cytokine array showing the profile of cytokine expression in the conditioned medium of vector- and p38 shRNA-ARP-1 and MM.1S (data not shown) cells. Detection of mRNA expression in ARP-1 and MM.1S cells (C) by using real-time PCR and secreted MCP-1 (D) and DKK-1 (E) in conditioned medium of the cells by using ELISA. F, real-time PCR showing elevated mRNA levels of MCP-1 and DKK-1, but not other cytokines, in primary tumor cells isolated from patients with myeloma with high or detectable p38 activity (p-p38; \(n = 8\)) compared with primary tumor cells with undetectable p38 activity (p-p38; \(n = 6\)). Representative results of 3 experiments are shown. **, \(P < 0.01\); ***, \(P < 0.001\).
total of 8 injections. Injections of equal amounts of IgG or PBS served as controls. Our results showed that treatment with antibodies against MCP-1 or DKK-1, but not control IgG, significantly reduced lytic bone lesions (Fig. 3E; \( P < 0.01 \)) without affecting tumor burdens in the mice (data not shown). Interestingly, treatment with antibodies against both DKK-1 and MCP-1 almost prevented the development of osteolytic bone lesions in most (8/10) of the myeloma-bearing mice. The neutralizing antibodies were also used in primary myeloma-bearing SCID-hu mice, with similar results (data not shown). Taken together, these findings clearly indicate that myeloma cell-derived DKK-1 and MCP-1 play a critical role in mediating the development of osteolytic bone lesions in vivo.

Myeloma cell p38 inhibits osteoblast differentiation and function via DKK-1

It is well known that bone remodeling is regulated by osteoblast-mediated bone formation and osteoclast-induced bone resorption (13). We have shown that myeloma cell p38 inhibits osteoblastogenesis and enhances osteoclastogenesis in vivo in myeloma-bearing SCID and SCID-hu mouse models. In this study, we examined the ability of and mechanism by which tumor cell p38 downregulates osteoblast differentiation and bone formation activity via DKK-1 and/or MCP-1. The differentiation and maturity of osteoblasts can be monitored by their production of alkaline phosphatase (ALP) in culture medium and their expression of bone morphogenetic protein-2 (BMP-2) and osteocalcin.

The results showed that vector-ARP-1 cells (data not shown) or their conditioned medium suppressed the differentiation of osteoblasts, as evidenced by large numbers of poorly differentiated cells that were BMP-2- and osteocalcin-negative (Fig. 4A), low production of ALP (Fig. 4B; \( P < 0.001 \)), and compromised bone formation activity, as determined by Alizarin Red-S staining assay (Fig. 4C; \( P < 0.01 \)). In contrast, cultures containing conditioned medium from p38 shRNA-ARP-1 cells generated BMP-2- and osteocalcin-positive osteoblasts, with production of high levels of ALP and bone formation activity. Similarly, conditioned medium of MM.1S cells (data not shown) or primary myeloma cells (Fig. 4D; \( P < 0.001 \)) with high or detectable p38 activity (Pt 9) suppressed osteoblast differentiation, as evident by the low production of ALP, whereas primary myeloma cells with low or undetectable p38 activity (Pt 10) had no such effects (Fig. 4E). Further studies showed that DKK-1, but not MCP-1, secreted by myeloma cells is mainly responsible for inhibited osteoblastogenesis, because the addition of neutralizing antibodies specific for DKK-1, but not MCP-1 or control IgG (data not shown), to the conditioned medium of vector-ARP-1 or MM.1S (data not shown) cells restored the generation of functional osteoblasts (Fig. 4F; \( P < 0.001 \)). Similarly, the addition of exogenous DKK-1, but not MCP-1, to conditioned medium from p38 shRNA-ARP-1 cells also inhibited osteoblastogenesis (Fig. 4G; \( P < 0.001 \)). Taken together, these results clearly
Myeloma cell p38-mediated inhibition of osteoblast differentiation and activity. Myeloma cell p38 activates osteoclast differentiation and bone resorption activity via DKK-1 and MCP-1

Next, we investigated the impact and mechanism of tumor cell p38 activity on osteoclast differentiation and bone resorption in vitro. To mimic the in vivo bone marrow microenvironment, a Transwell coculture system was used in which 1 × 10^5 osteoclast precursors/mL were seeded on the bottom of the wells and 10-fold numbers of mesenchymal stem cells (MSC) were planted in the Transwell inserts. The cells were cocultured in osteoclast medium, with or without RANKL. In some experiments, conditioned media of different ARP-1 or MM.1S cells were added to the cocultures. Mature multinuclear osteoclasts were generated in cocultures with addition of RANKL and in cocultures without RANKL in the presence of conditioned medium of vector-ARP-1 and/or DKK-1 and/or MCP-1 expression in established primary myeloma cells in SCID-hu mice treated without (vehicle) or with the p38-specific inhibitor SD-169 at 8 weeks after tumor inoculation. D, immunohistochemical staining for DKK-1 (top) and MCP-1 (bottom) expression in established primary myeloma cells in SCID-hu mice treated without (vehicle) or with the p38-specific inhibitor SD-169 at 8 weeks after tumor inoculation. Scale bar, 20 μm; scale bar for insets, 100 μm. E, representative radiographs showing reduced osteolytic bone lesions in the femurs of ARP-1-bearing mice (5 per group) treated with antibodies against MCP-1 and/or DKK-1 compared with control IgG. Representative results of 3 independent experiments are shown. **, P ≤ 0.01; *** , P ≤ 0.001.

demonstrate that DKK-1 was responsible for myeloma p38-mediated inhibition of osteoblast differentiation and activity. Myeloma cell p38 activates osteoclast differentiation and bone resorption activity via DKK-1 and MCP-1

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Furthermore, osteoclasts generated in the cocultures with conditioned medium of ARP-1 cells resorbed efficiently mineralized matrices (Fig. 6A; top) and bone slices (bottom). Analysis of the resorbed area indicates that the osteoclasts formed larger resorption areas (Fig. 6B; P < 0.001) and deeper resorbed bone pits (Fig. 6C; P < 0.001) and secreted more soluble CTX-1 (Fig. 6D; P < 0.001) in comparison with cells generated in cocultures in medium devoid of RANKL or with the addition of conditioned medium from p38 shRNA-ARP-1 cells.

Because myeloma cells secreted DKK-1 and MCP-1, we examined whether these cytokines were involved in osteoclastogenesis. Antibodies specific for DKK-1 and/or MCP-1 were added to the coculture of osteoclast precursors and MSCs in the presence or absence of conditioned medium of parental ARP-1 cells. After a 7-day culture, the telomeric repeat amplification protocol (TRAP)-positive, multinuclear osteoclasts were counted and the levels of TRAP5b in the medium were measured by ELISA. As shown in Fig. 6E, the addition of either anti-DKK-1 or anti-MCP-1 antibodies partially inhibited the generation of mature osteoclasts, and the addition of both
antibodies had a greater effect \((P < 0.001)\). Alternatively, the addition of exogenous DKK-1, MCP-1, and especially both, to the conditioned medium of p38 shRNA-ARP-1 cells restored the generation of mature osteoclasts in the cocultures devoid of RANKL (Fig. 6F; \(P < 0.001\)). Taken together, these results clearly indicate that DKK-1 and MCP-1 are responsible for myeloma p38-mediated activation of osteoclast differentiation and activity in vitro and in vivo.

**Myeloma cells activate RANK/RANKL signaling in osteoclast precursors via p38-upregulated DKK-1 and MCP-1**

Next, we examined signaling pathways in osteoclast activation in relation to tumor cell p38 activity. We focused on NF-κB, PI3K/Akt, and MAPKs, such as ERK, p38, and \(c-jun\)-NH2-kinase (JNK), because these pathways have been reported to be involved in the regulation of osteoclast differentiation (14). In the experiments, osteoclast precursors were treated with conditioned medium of ARP-1 or MM.1S (data not shown) cells for 3 days. The cells were then collected, and phosphorylation of IκBα, ERK, and p38 were detected by using Western blot analysis. As shown in Fig. 7A, the addition of tumor conditioned medium from vector-ARP-1, but not p38 shRNA-ARP-1 cells, upregulated the phosphorylation of IκBα, p38, and Akt during osteoclast differentiation. The levels of nonphosphorylated kinases (data not shown) and β-actin were unchanged. These signaling pathways were essential for osteoclast differentiation, because the addition of kinase inhibitors specific for IκBα, ERK, or p38 to the cultures significantly
abrogated the generation of osteoclasts induced by the tumor-conditioned medium (Fig. 7B; \( P < 0.001 \)).

Recent studies have shown that IkB\(\alpha\), ERK1/2, and p38 signaling pathways are activated by RANKL, an osteoclast activator, which binds to its receptor RANK on osteoclast precursors. However, as myeloma cells express low levels of RANKL (data not shown), we hypothesized that myeloma cells activate RANK/RANKL signaling pathways in osteoclast precursors via secreted DKK-1 and MCP-1. By using Western blot analysis, we examined the expression of RANK in CD14\(^+\) monocytes and in M-CSF–induced osteoclast precursors treated with or without conditioned medium of vector- or p38 shRNA-ARP-1 cells. The conditioned medium of vector-ARP-1 cells significantly increased RANK expression in both monocytes and osteoclast precursors, whereas the medium of p38 shRNA-ARP-1 cells only slightly upregulated RANK expression (Fig. 7C and 7D). Our studies also showed that conditioned medium of vector-ARP-1, but not p38 shRNA-ARP-1, cells significantly upregulated the expression (Fig. 7E) and secretion (Fig. 7F; \( P < 0.001 \)) of RANKL by MSCs and mature osteoblasts. Hence, these results support our hypothesis that myeloma cells modulate the expression of RANK and RANKL in osteoclasts and marrow stromal cells.

To determine whether DKK-1 and MCP-1 mediate the myeloma p38-induced increase of RANK/RANKL expression, neutralizing antibodies against these cytokines were used. As shown in Fig. 7G, antibodies against MCP-1, but not DKK-1, significantly abrogated conditioned medium-induced expression of surface RANK on CD14\(^+\) monocytes (\( P < 0.01 \)). On the other hand, anti-DKK-1 antibody remarkably inhibited tumor-induced RANKL secretion in MSCs (Fig. 7H; \( P < 0.001 \)). These results indicate that tumor-derived DKK-1 upregulates RANKL expression and secretion from stromal cells, and tumor-derived MCP-1 enhances RANK expression in the progenitors of osteoclasts, both of which activate RANK/RANKL-mediated signaling pathways in the progenitor cells, leading to osteoclast differentiation and activation.

**Discussion**

The p38 MAPK is an important signaling pathway involved in cell growth, survival, differentiation and inflammatory response, and can be activated by a variety of cellular stresses, including inflammatory cytokines, endotoxin, ultraviolet light, and growth factors (6–8). Functionally, p38 mediates the production of proinflammatory cytokines and other factors such as TNF-\(\alpha\) (15, 16) and soluble RANKL (17, 18). Preclinical studies indicate that inhibition of p38 suppresses the induction of inflammatory, angiogenic, and proapoptotic cytokines from bone marrow stromal cells (19). Moreover, p38 signaling plays an important role in the pathogenesis of multiple myeloma, including myeloma growth and survival and drug resistance (20, 21). Our previous study has shown that activated p38 signaling in myeloma cells induces osteolytic bone lesions in myeloma by inhibiting osteoblastogenesis and enhancing osteoclastogenesis (9). The current study further elucidates that myeloma cells express and secrete DKK-1 and MCP-1 as a result of constitutive activation of myeloma p38 signaling. Consequently, p38-upregulated DKK-1 and MCP-1 enhance RANK/RANKL expression and activate NF-\(\kappa\)B, p38, and ERK signaling pathways in the progenitors of osteoclasts, leading to...
osteoclast activation and bone resorption, whereas p38-upregulated DKK-1 inhibits osteoblast differentiation and bone formation, all of which result in bone destruction (Supplementary Fig. S1).

DKK-1 is a well-known inhibitor of osteoblast differentiation. Elevated levels of DKK-1 have been reported to induce bone loss in patients with inflammatory rheumatoid arthritis (21) and myeloma (13, 26). Previous studies have shown that DKK-1, produced by myeloma cells, inhibits osteoblast differentiation (22–24) and may enhance osteoclast differentiation (25) and bone lesions (26) by upregulating RANKL expression on stromal cells. However, the mechanism by which myeloma cells produce DKK-1 is unclear. By the analysis of Western blotting, real-time PCR and ELISA, we for the first time show that constitutively activated myeloma cell p38 signaling upregulates DKK-1 expression and secretion. Inhibition or shRNA knockdown of myeloma p38 significantly downregulates DKK-1 secretion in vitro and in myeloma-bearing mice. Furthermore, we also observe that myeloma cell p38 signaling induces the expression and secretion of MCP-1, a chemokine that recruits monocytes, T cells, and dendritic cells to sites of infection or tissue injury. MCP-1 is upregulated during osteoclastogenesis, promotes the fusion of hematopoietic precursors to mature osteoclasts (27), and determines osteoclast behavior in inflammatory osteoporosis via binding to receptor CCR2 (28). MCP-1 is mainly released from bone marrow stromal cells (29). However, our results show that myeloma cells also secrete MCP-1 in vitro and in myeloma-bearing mice, as a result of constitutive activation of myeloma cell p38. Knocking down myeloma cell p38 reduces MCP-1 expression by myeloma cells.

Figure 6. Activated tumor cell p38 enhances osteoclast bone resorption. Osteoclasts were generated in cocultures of osteoclast precursors with MSCs in osteoclast medium without (medium) or with RANKL, or in osteoblast medium in the absence of RANKL with addition of conditioned medium (CM) of vector- or p38 shRNA-ARP-1 cells. A, resorption pit analysis showing pit formation on mineralized matrices (top) or dentin slices (bottom) by mature osteoclasts generated in osteoclast medium devoid of RANKL with the addition of conditioned medium of vector-ARP-1 (Vector), but not of p38 shRNA-ARP-1 (p38 shRNA) cells. Color contours represent the depth of the resorption pits on dentin slices. Summarized data of resorption area per osteoclasts on mineralized matrices (B), average pit depth on dentin slices (C), and the levels of bone degradation product CTX-1 in culture medium (D). Representative results of 5 experiments are shown. E, effects of DKK1- and MCP-1-neutralizing antibodies on the differentiation of mature osteoclasts in cocultures with addition of conditioned medium from vector-ARP-1 cells (CM-V), and effects of exogenous DKK-1 and MCP-1 on the differentiation of mature osteoclasts in cocultures with addition of conditioned medium from p38 shRNA-ARP-1 cells (CM-p38sh; F). The levels of secreted TRAP5b were used as indicators of osteoclast differentiation and maturation. Representative results from 4 independent experiments are shown. ***, P < 0.01; ****, P < 0.001.
In addition, our results for the first time observe that p38-increased DKK-1 not only inhibits osteoblastogenesis, but also induces osteoclast differentiation and bone resorption by enhancing RANKL secretion from bone marrow stromal cells. On the other hand, p38-increased MCP-1 upregulates RANK expression in the progenitors of osteoclasts and activated RANK/RANKL signaling pathways such as NF-κB, ERK1/2, and p38 in the cells, leading to osteoclast activation and bone resorption. Neutralizing both DKK-1 and MCP-1 reduces myeloma-induced osteoclast activation in vitro and bone lesions in vivo. These results provide an alternative explanation of RANKL/RANK perturbation in myeloma.

Taken together, our findings indicate that the development of myeloma-induced bone lesions is initiated and regulated by activated p38 in myeloma cells, but is sustained through the interactions between myeloma cells and surrounding bone marrow cells via p38 upregulating cytokines or chemokines by myeloma cells. These results shed light on the regulation of osteolytic cytokine (such as DKK-1 and MCP-1) production by myeloma cell p38 activity and identify novel targets for treating myeloma-induced osteolytic bone lesions in patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: Z. Liu, Z. Cai, Q. Yi, J. Yang
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