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Molecular Basis of Requirement of Receptor Activator of Nuclear Factor κB Signaling for Interleukin 1-mediated Osteoclastogenesis*[S]

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Background: Interleukin 1-mediated osteoclastogenesis requires permissive levels of RANKL or RANKL pretreatment.
Results: Interleukin 1 can only activate the expression of osteoclast markers and osteoclastogenic transcription factor NFATc1 with permissive levels of RANKL or RANKL pretreatment.
Conclusion: RANKL is involved in interleukin 1-mediated osteoclastogenesis by rendering osteoclast and NFATc1 genes responsive to interleukin 1.
Significance: This is a novel mechanism of interleukin 1-mediated osteoclastogenesis.

IL-1, a proinflammatory cytokine, is implicated in bone loss in various pathological conditions by promoting osteoclast formation, survival, and function. Although IL-1 alone can sufficiently prolong osteoclast survival and activate osteoclast function, IL-1-mediated osteoclastogenesis requires the receptor activator of NF-κB (RANK) ligand (RANKL). However, the molecular basis of the dependence of IL-1-mediated osteoclastogenesis on RANKL is not fully understood. Here we show that although IL-1 cannot activate the expression of the osteoclast genes encoding matrix metalloproteinase 9, cathepsin K, tartrate-resistant acid phosphatase, and carbonic anhydrase II in bone marrow macrophages (BMMs), RANKL renders these osteoclast genes responsive to IL-1. We further demonstrate that IL-1 alone fails to induce the expression of nuclear factor of activated T cell cytoplasmic 1 (NFATc1), a master transcriptional regulator of osteoclastogenesis), in BMMs but can up-regulate its expression in the presence of permissive levels of RANKL or with RANKL pretreatment. The RANK IVVY motif, which has been previously shown to commit BMMs to the osteoclast lineage in RANKL- and TNF α-mediated osteoclastogenesis, also plays a crucial role in IL-1-mediated osteoclastogenesis by changing the four osteoclast marker and NFATc1 genes to an IL-1-inducible state. Finally, we show that MyD88, a known critical component of the IL-1 receptor I signaling pathway, plays a crucial role in IL-1-mediated osteoclastogenesis from RANKL-primed BMMs by up-regulating the expression of the osteoclast marker and NFATc1 genes. This study reveals a novel mechanism of IL-1-mediated osteoclastogenesis and supports the promising potential of the IVVY motif to serve as a therapeutic target for inflammatory bone loss.

IL-1, a proinflammatory cytokine, plays a crucial role in immune and inflammatory responses (1). Nonetheless, IL-1 production is abnormally elevated in various pathological conditions, and deregulation of IL-1 expression has been implicated in the pathogenesis of postmenopausal osteoporosis (2) and bone loss in inflammatory conditions such as rheumatoid arthritis (3) and periodontitis (4). IL-1 induces bone loss by stimulating the formation, survival, and function of osteoclasts (5, 6). Although IL-1 can directly target mature osteoclasts to prolong survival (7) and activate function (8), this cytokine can only promote osteoclastogenesis in the presence of permissive levels of RANKL[2] (9, 10). However, the precise molecular mechanism underlying the requirement of RANKL for IL-1-mediated osteoclastogenesis has not been fully elucidated.

Osteoclasts, the sole bone-resorbing cells, differentiate from cells of the monocyte/macrophage lineage upon stimulation by two key factors: the macrophage/monocyte colony stimulating factor (M-CSF) and RANKL (11). Although M-CSF stimulates the proliferation and survival of osteoclast precursors, RANKL is the main differentiation-promoting factor. RANKL, a member of the TNF superfamily, exerts its functions by activating its receptor, RANK, which belongs to the TNF receptor superfamily (12). Members of the TNF receptor family lack intrinsic

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[2] The abbreviations used are: RANKL, receptor activator of nuclear factor-κB ligand; RANK, receptor activator of nuclear factor-κB; M-CSF, macrophage/monocyte colony-stimulating factor; TRAF, tumor necrosis factor receptor-associated factor; BMMs, bone marrow macrophages; IL-1RI, IL-1 receptor I; PBS, phosphate buffered serum; Ctsk, cathepsin K; FAS-AB, human Fas-activating antibody; Car2, carbonic anhydrase II; Ch1, chimeric receptor with normal RANK cytoplasmic domain; Ch2, chimeric receptor with mutated RANK IVVY535–538 motif (VY is replaced by AF); MMP9, matrix metallopeptidase 9; TRAP, tartrate-resistant acid phosphatase 5; NFATc1, nuclear factor of activated T cell cytoplasmic 1.

enzymatic activity and, thus, transduce downstream signals mostly by recruiting TNF receptor-associated factors (TRAFs) (8, 13). It has now been established that RANK possesses three cytoplasmic motifs ($PFQEP^{369-373}$, $PVQEET^{559-564}$, and $PVQEQG^{604-609}$) that can recruit TRAFs to activate six major signaling pathways (NF-κB, JNK, ERK, p38, NFATc1, and Akt) to regulate the formation, function, and survival of osteoclasts (14–17). Moreover, our group has identified a TRAF-independent motif ($IVVY^{535-538}$) in the RANK cytoplasmic domain that plays an essential role in committing BMMs, namely osteoclast precursors, to the osteoclast lineage *in vitro* (18). The IVVY motif has also been demonstrated to regulate osteoclast formation and function *in vivo* (19).

IL-1 exerts its functions by activating IL-1 receptor I (IL-1RI), its main signaling receptor (1). Activation of IL-1RI leads to the recruitment of IL-1 receptor-associated factor (IL1Racf), which forms a complex with myeloid differentiation factor 88 (MyD88), IL-1 receptor-associated kinases (IRAKs), and TRAF6 to transduce downstream signaling. Also, IL-1 has another receptor, IL-1RII, which contains only 29 amino acids in its cytoplasmic tail and is thus unable to transduce signaling. As a result, IL-1RII functions as a decoy receptor and inhibits IL-1 signaling by competing with IL-1RI for IL-1.

Notably, IL-1 shares many similarities in function and pathobiology with TNF, another potent proinflammatory factor (1, 20). Moreover, both IL-1 and TNF are concomitantly elevated in postmenopausal osteoporosis (5, 6) and are abundant in inflammatory conditions such as rheumatoid arthritis (21) and periodontitis (4). More importantly, these two cytokines are implicated in bone loss associated with these diseases. Particularly, although both IL-1 and TNF can activate TRAF-dependent signaling pathways (1, 20), they cannot promote osteoclastogenesis independently of RANKL (9, 10, 22, 23). We have shown recently that the RANK IVVY motif plays an essential role in TNF-mediated osteoclastogenesis (24). In this study, we investigate the molecular basis of the dependence of IL-1-mediated osteoclastogenesis on RANKL by examining the involvement of this RANK IVVY motif in IL-1-mediated osteoclastogenesis.

EXPERIMENTAL PROCEDURES

Chemicals and Biological Reagents—All chemicals were obtained from Sigma. Synthetic oligonucleotides were from Sigma-Genosys. Alexa Fluor-488 phalloidin (catalog no. A12379) and Hoechst-33258 (catalog no. H1398) were purchased from Invitrogen. Recombinant IL-1α (catalog no. 400-ML-005) was purchased from R&D Systems. Anti-human Fas-activating antibody was obtained from Millipore. Anti-human Fas antibody conjugated with phycoerythrin (catalog no. sc-21730PE) and anti-NFATc1 antibody (catalog no. sc-7294) were from Santa Cruz Biotechnology, Inc. Recombinant GST-RANKL was prepared as described previously (25). Mouse M-CSF was prepared from a M-CSF-producing cell line, CMG14-12, as described previously (26).

In Vitro Osteoclastogenesis Assays—BMMs were isolated from long bones of 4- to 6-week-old C3H, C57BL/6 (WT), or $MyD88^{-/-}$ mice as described previously (27) and were cultured in α-minimal essential medium containing 10% heat-inactivated FBS and 220 ng/ml M-CSF. The C3H and C57BL/6 mice were purchased from Harlan Industries (Indianapolis, IN), and $MyD88^{-/-}$ breeding pairs were obtained under a material transfer agreement from Dr. Shizuo Akira (Osaka University, Osaka, Japan). The experiments involving mice were performed in accordance with the regulations of the University of Alabama at Birmingham institutional animal care and use committee.

In vitro osteoclastogenesis assays were performed by treating BMMs (5×10^4 cells/well) in 24-well tissue culture plates with M-CSF (44 ng/ml) and different doses of GST-RANKL and/or IL-1 as indicated in individual assays. Cultures were then stained for TRAP activity with a leukocyte acid phosphatase kit (catalog no. 387-A) from Sigma. The assays were performed in triplicate and repeated at least twice.

In vitro osteoclast formation on bone slices was carried out by seeding BMMs (5×10^4 cells/well) on bovine cortical bone slices in 24-well tissue culture plates, and the cells were then cultured as indicated in individual experiments. Bone slices were fixed with 3.7% formaldehyde solution in PBS for 10 min at room temperature, then treated with 0.1% Triton X-100 in PBS for 8 min, and finally stained with Alexa Fluor 488 phalloidin and Hoechst-33258 for 15 min for actin ring and nucleic staining, respectively. Bone slices were analyzed and imaged using a Leica DMIRBE inverted UV SP1 confocal microscope system with Leica confocal software at the imaging facility of the University of Alabama at Birmingham. The assays were performed in triplicates, and a representative view from each assay was shown. Osteoclastogenesis assays were quantified by counting the multinucleated TRAP-positive cells (> 3 nuclei) in a representative area in each of three replicate samples.

In Vitro Bone Resorption Assays—BMMs, 5×10^4 cells/well, were seeded on bovine cortical bone slices plated in 24-well culture plates and treated under conditions indicated in individual experiments. Bone slices were then harvested, and cells were removed with 0.25 M ammonium hydroxide and mechanical agitation. Bone resorption pits were visualized and imaged by scanning electron microscopy using a Philips 515 scanning electron microscope in the Material Engineering Department at the University of Alabama at Birmingham. Bone resorption assays were performed in triplicates, and a representative area from each assay was shown. The data were quantified by measuring the percentage of resorbed areas in three random resorption areas. The percentage of the resorbed area was determined using ImageJ analysis software from the National Institutes o Health.

Retroviral Infection of Primary BMMs—The retrovirus packaging cells, 293GPG, were maintained in DMEM with 10% heat-inactivated FBS supplemented with tetracycline, puromycin, G418, and penicillin/streptomycin as described previously (28). The chimeric receptor constructs pMX-puro-hFas-RANK (Ch1), pMX-puro-hFas-PM3 (Ch2), and pMX-puro-GFP (GFP) were generated in previous studies (24, 29). 293GPG cells were transiently transfected with the constructs using Lipofectamine Plus reagent (Invitrogen), and the virus supernatant was harvested at days 2, 3, and 4 post-transfection. BMMs were then infected with virus for 24 h in the presence of M-CSF (220 ng/ml) and 8 μg/ml polybrene. Cells were further cultured with M-CSF (220

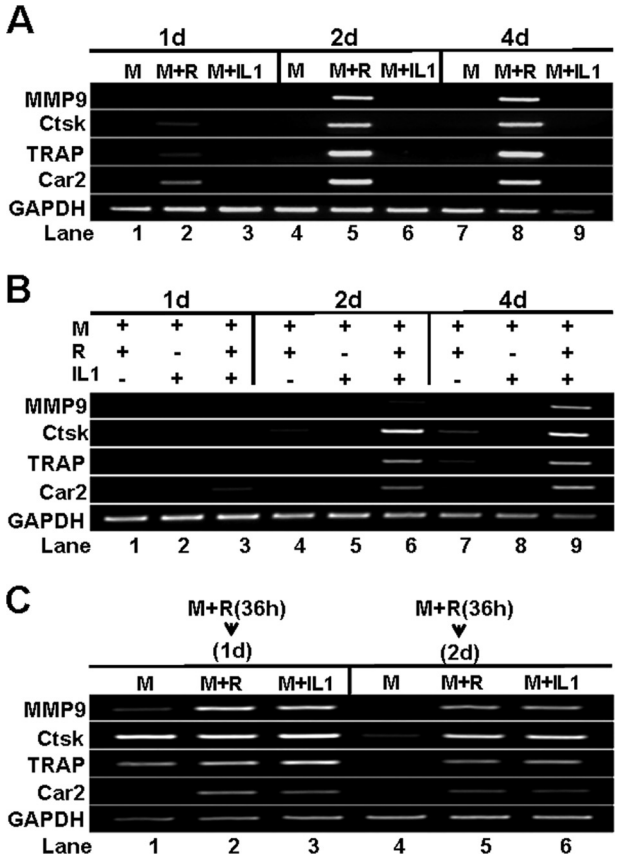

FIGURE 1. **RANKL renders osteoclast genes responsive to IL-1.** *A*, BMMs were cultured with M-CSF (*M*, 44 ng/ml), M (44 ng/ml) and RANKL (*R*, 100 ng/ml), or M (44 ng/ml) and IL-1 (IL1, 5 ng/ml) in tissue culture dishes for 1, 2 or 4 days (*d*). *B*, BMMs were treated with M (44 ng/ml) and R (10 ng/ml), M (44 ng/ml) and IL1 (5 ng/ml), or M (44 ng/ml) and R (10 ng/ml) plus IL1 (5 ng/ml) for 1, 2, or 4 days. *C*, BMMs were pretreated with M (44 ng/ml) and R (100 ng/ml) for 36 h, washed with PBS, and then continued with M (44 ng/ml), M (44 ng/ml) and R (100 ng/ml), or M (44 ng/ml) and IL1 (5 ng/ml) for 1 or 2 days. Gene expression was assessed by semi-quantitative RT-PCR using GAPDH as loading control. These gene expression analyses were repeated independently twice.

ng/ml) for 24 h, and then the cultures were continued with M-CSF (220 ng/ml) and puromycin (2 μg/ml) for selection and expansion of infected cells.

Flow Cytometric Analysis—1×10^6 infected BMMs were suspended in 200 ml α-minimal essential medium containing 10% heat-inactivated FBS supplemented with M-CSF (44 ng/ml) and then blocked with 1 μg of 2.4G2 antibody (30) for 30 mins at 4 °C. Under dim light, 10 μl human Fas antibody conjugated with phycoerythrin was added to each cell suspension, and cells were further incubated for 30 mins at 4 °C. Cells were then centrifuged at 2000 rpm for 5 min. Cells were subsequently washed three times by gently resuspending cells with α-minimal essential medium followed by centrifugation at 2000 rpm for 5 min. After the final wash, cells were suspended in 1 ml α-minimal essential medium for cytometric analysis using a BD Biosciences LSR II flow cytometer at the Center for AIDS Research at the University of Alabama at Birmingham.

Western Blot Analysis—Following treatments indicated in individual experiments, BMMs were washed twice with ice-cold PBS and then lysed in buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, 1× protease inhibitor mixture 1 (Sigma, catalog no. P-2850), and 1× protease inhibitor mixture 2 (Sigma, catalog no. P-5726). Lysates were then subjected to Western blot analysis as described in our previous study (31). Membranes were washed extensively, and an ECL detection assay was performed using a SuperSignal West Dura kit from Pierce.

Semi-quantitative RT-PCR Analysis—Total RNA was prepared from BMMs using TRIzol reagent from Invitrogen. 1 μg of total RNA was reverse-transcribed to cDNA with oligo(dT) in a 20 μl volume at 50 °C for 60 min using the SuperScript III RT-PCR system (Invitrogen). The RT reaction was then followed by enzyme inactivation and RNA H digestion. PCR amplification of the MMP9, Ctsk, TRAP, Car2, and GAPDH genes was performed as described previously (24). 20 μl of PCR product was loaded on 2% agarose gel for electrophoretic analysis. All semi-quantitative RT-PCR assays were independently repeated twice.

Real-time RT-PCR Analysis—Total RNA was prepared from BMMs using TRIzol reagent from Invitrogen. cDNA was synthesized from 1 μg of total RNA by reverse transcription using a QuantiTect RT kit (Qiagen). Real-time PCR was performed using a Lightcycler (Roche) with FastStart DNA Master SYBR Green I reagent (Roche) and PCR primers used in a previous study (24). Relative quantities of the tested genes were normalized to GAPDH mRNA. The normalized data were expressed using the comparative $2^{-\Delta\Delta CT}$ method. All real-time RT-PCR assays were independently repeated twice.

Statistical Analysis—Bone resorption and osteoclastogenesis data are expressed as mean ± S.D. of percent resorbed area (bone resorption) and numbers of TRAP-positive cells, respectively. Statistical significance was determined using Student's *t* test, and *p* values less than 0.05 were considered significant.

RESULTS

IL-1 Cannot Promote Osteoclastogenesis on Bone Slices without RANKL—It has been reported that although IL-1 alone cannot promote osteoclastogenesis in tissue culture dishes, it can do so in the presence of permissive levels of RANKL or with RANKL pretreatment (9, 10) (32). However, it is unclear whether this intriguing finding holds true for osteoclastogenesis on bone surface, which represents a physiological substratum for osteoclastogenesis. Before addressing this critical issue, we first confirmed the previous findings in our hands and found, consistent with the reported data, that IL-1 alone cannot mediate osteoclastogenesis in tissue culture dishes (supplemental Fig. 1*A*) but that it can do so with permissive levels of RANKL (supplemental Fig. 2, *A* and *B*) or RANKL pretreatment (supplemental Fig. 3*A*). We then investigated the role of IL-1 in osteoclastogenesis on bone slices. IL-1 fails to stimulate osteoclastogenesis on bone slices (supplemental Fig. 1, *B* and *C*), which was supported by the absence of actin ring and multinucleation, two important features of mature osteoclasts, and lack of resorption pits. Yet, IL-1 can mediate osteoclastogenesis on bone slices in the presence of permissive levels of RANKL

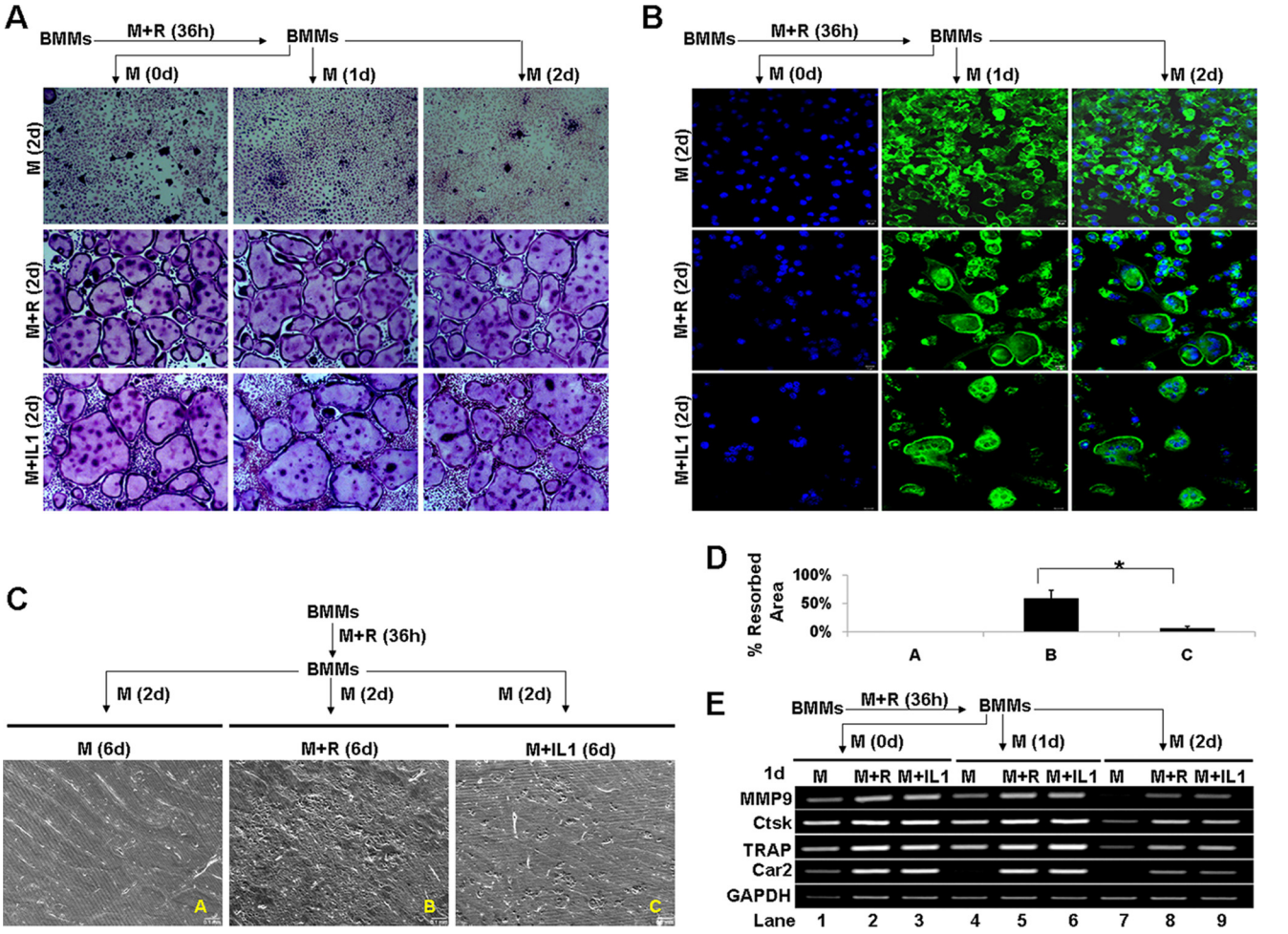

FIGURE 2. **IL-1 can promote osteoclastogenesis 2 days after 36-hour RANKL pretreatment.** *A*, BMMs were cultured with M-CSF (*M*, 44 ng/ml) and RANKL (*R*, 100 ng/ml) for 36 h, washed with PBS, and then continued with M for 0, 1, or 2 days (*d*) before treating with M (44 ng/ml), M (44 ng/ml) and R (100 ng/ml), or M (44 ng/ml) and IL-1 (5 ng/ml) for 2 days. The cultures were then stained for TRAP activity. *B*, the same set of assays as in *A* was repeated on bone slices, and cells were then stained with Hoechst-33258 (*Hoechst*) or Alexa Fluor 488-phalloidin (*phalloidin*) for actin ring and multinucleation, respectively. Cells were then analyzed by confocal microscopy. *C*, BMMs seeded on bone slices were cultured with M (44 ng/ml) and R (100 ng/ml) for 36 h, washed with PBS, and then continued with M (44 ng/ml) for 2 days before treating with M (44 ng/ml), M (44 ng/ml) and R (100 ng/ml), or M (44 ng/ml) and IL1 (5 ng/ml) for 6 days to promote bone resorption. Bone resorption pits were visualized by scanning electron microscopy. *D*, quantification of bone resorption assays performed in *C*. *Bars* show mean ± S.D. $p < 0.005$. *E*, BMMs were cultured with M (44 ng/ml) and R (100 ng/ml) for 36 h, washed with PBS, and then cultured with M (44 ng/ml) alone for 0, 1, or 2 days before treating with M (44 ng/ml), M (44 ng/ml) and R (100 ng/ml), or M (44 ng/ml) and IL1 (5 ng/ml) for 1 day. Gene expression was then accessed by semi-quantitative RT-PCR. The osteoclastogenesis assays were performed in three replicates and repeated independently twice. The gene expression analyses were repeated independently twice. For assays in *A–C*, a representative image from each assay condition is shown.

(supplemental Fig. 2, *C* and *D*) or with RANKL pretreatment (supplemental Fig. 3, *B–D*). However, the osteoclasts generated under the RANKL pretreatment have a low resorptive capacity (supplemental Fig. 3*D*). These data further indicate that IL-1-mediated osteoclastogenesis requires RANKL priming. Interestingly, although IL-1 cannot promote osteoclastogenesis in the absence of RANKL, IL-1-pretreatment can enhance osteoclastogenesis from BMMs treated with low levels of RANKL (supplemental Fig. 4*A*). Furthermore, IL-1 dose-dependently promotes osteoclastogenesis in the presence of permissive levels of RANKL (supplemental Fig. 4, *B* and *C*). These findings suggest that IL-1 is capable of sensitizing BMMs to RANKL treatment and enhancing the activation of certain common signaling pathways shared by IL-1 and RANKL.

IL-1 Cannot Activate Osteoclast Genes unless in the Presence of Permissive Levels of RANKL or with RANKL Pretreatment—RANKL stimulates osteoclastogenesis by inducing the expression of numerous genes, including MMP9, Ctsk, TRAP, and Car2 (33). To examine the molecular basis of the inability of IL-1 to mediate osteoclastogenesis, we determined the ability of IL-1 to activate these four osteoclast genes. RANKL activated the expression of these genes with time (Fig. 1*A*, *lanes 2*, *5*, and *8*), but IL-1 treatment for as long as 4 days failed to do so (*lanes 3*, *6*, and *9*), indicating that the inability of IL-1 to activate the expression of the osteoclast genes accounts for its failure to stimulate osteoclastogenesis. Given that IL-1 can mediate osteoclastogenesis in the presence of permissive levels of RANKL, we next addressed whether permissive levels of RANKL can make these genes responsive to IL-1. 10 ng/ml RANKL weakly activated gene expression (Fig. 1B, *lanes 1*, *4*, and *7*), and IL-1 completely failed to do so (*lanes 2*, *5*, and *8*). Nonetheless, 10 ng/ml RANKL plus 5 ng/ml IL-1 considerably activated the Ctsk, TRAP, and Car2 genes at day 2 (*lane 6*) and the MMP9 gene at day 4 (*lane 9*, Fig. 1*B*). These data demonstrate that the

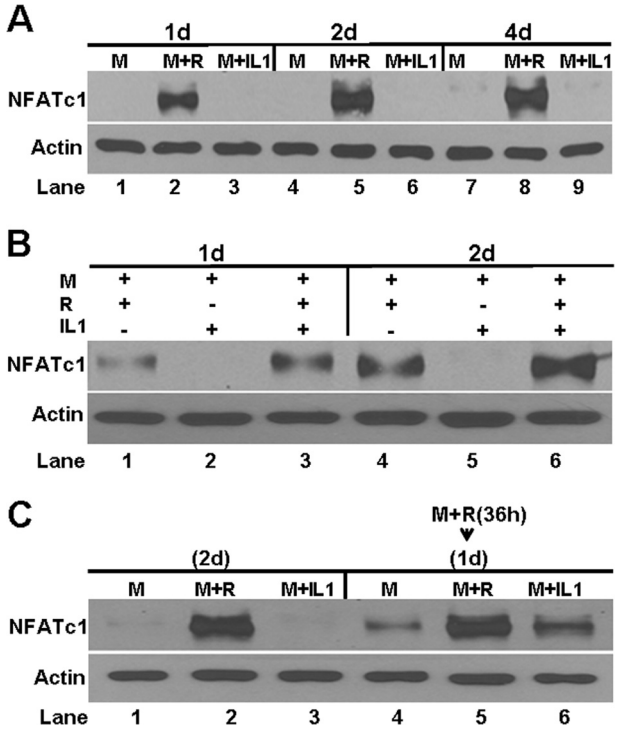

FIGURE 3. **RANKL renders the NFATc1 gene responsive to IL-1.** *A*, BMMs were treated with M-CSF (*M*, 44 ng/ml), M (44 ng/ml) and RANKL (*R*, 100 ng/ml), or M (44 ng/ml) and IL-1 (IL1, 5 ng/ml) for 1, 2, or 4 days (*d*). *B*, BMMs were treated with M (44 ng/ml) and R (10 ng/ml), M (44 ng/ml) and IL1 (5 ng/ml), or M (44 ng/ml) and R (10 ng/ml) plus IL1 (5 ng/ml) for 1 or 2 days. *C*, BMMs were treated with M (44 ng/ml), M (44 ng/ml) and R (100 ng/ml), or M (44 ng/ml) and IL1 (5 ng/ml) for 2 days. Also, BMMs were cultured with M (44 ng/ml) and R (100 ng/ml) for 36h, washed with PBS, and then cultured with M (44 ng/ml), M (44 ng/ml) and R (100 ng/ml), or M (44 ng/ml) and IL1 (5 ng/ml) for 1 day. NFATc1 expression was assessed by Western blot analysis using β-actin as a loading control. These assays were repeated independently twice.

RANKL-mediated priming, in part, involves rendering osteoclast genes responsive to IL-1.

We next determined whether RANKL pretreatment can also render the four osteoclast marker genes responsive to IL-1. 36-hour RANKL pretreatment rendered all four genes responsive to IL-1 (Fig. 1*C*, *lanes 3* and *6*). However, without subsequent stimulation with either RANKL or IL-1, the expression of these genes decreased over time (Fig. 1C, *lane 1 versus lanes 2* and *3* and *lane 4 versus lanes 5* and *6*). These data indicate that RANKL pretreatment primes BMMs partially by rendering the osteoclast genes responsive to IL-1.

IL-1 Can Promote Osteoclastogenesis 2 Days after RANKL Pretreatment—Because we have recently shown that TNF can stimulate osteoclastogenesis 2 days after RANKL pretreatment (24), we determined whether IL-1 can also do so. Our data indicate that IL-1 is able to promote osteoclastogenesis 2 days after 36-hour RANKL pretreatment both in tissue culture dishes (Fig. 2*A*) and on bone slices (*B*). The osteoclasts derived from this condition were functional but displayed a lower resorptive capacity than those of RANKL-differentiated osteoclasts (Fig. 2, *C* and *D*). This finding indicates that the RANKL-mediated lineage priming for IL-1-mediated osteoclastogenesis is not transient. Moreover, IL-1 strongly activated the expression of the four osteoclast genes 2 days after the 36-hour RANKL priming (Fig. 2*E*, *lanes 3*, *6*, and *9*). Without subsequent IL-1 or RANKL stimulation, the expression of the genes decreased over time following the RANKL priming (Fig. 2*E*, *lane 1 versus lanes 2* and *3*, *lane 4 versus lanes 5* and *6*, and *lane 7 versus lanes 8* and *9*). Collectively, these results reveal that the RANKL priming is durable and involves altering of osteoclast genes into an IL-1 inducible state.

IL-1 Cannot Induce NFATc1 Expression unless in the Presence of Permissive Levels of RANKL or with RANKL Pretreatment—To further delineate the molecular mechanism underlying the dependence of IL-1-mediated osteoclastogenesis on RANKL, we investigated the effect of IL-1 on the expression of NFATc1, a master transcriptional regulator of osteoclastogenesis (17), in the absence or presence of permissive levels of RANKL. As a positive control, 100 ng/ml RANKL potently stimulated NFATc1 expression over time (Fig. 3*A*, *lanes 2*, *5*, and *8*). In contrast, 5 ng/ml IL-1 failed to do so (Fig. 3*A*, *lanes 3*, *6*, and *9*). Moreover, although 10 ng/ml RANKL alone weakly induced NFATc1 expression at day 1 (Fig. 3*B*, *lane 1*), 10 ng/ml RANKL plus 5 ng/ml IL-1 strongly activated the expression of NFATc1 (*lane 3*). Intriguingly, 10 ng/ml RANKL treatment moderately induced NFATc1 expression over time (Fig. 3*B*, *lanes 1* and *4*), and 10 ng/ml RANKL plus 5 ng/ml IL-1 further enhanced the expression of NFATc1 (*lanes 3* and 6, Fig. 3*B*). These findings indicate that permissive levels of RANKL assist IL-1 in promoting osteoclastogenesis by compensating the inability of IL-1 to induce NFATc1 expression. We further addressed this issue by investigating whether RANKL pretreatment affects the ability of IL-1 to induce NFATc1 expression. We found that 36-hour RANKL pretreatment followed by 24-hour M-CSF treatment led to a low level of NFATc1 expression (Fig. 3*C*, *lane 4*), but 36-hour M-CSF/RANKL pretreatment followed by 24-hour M-CSF/IL-1 treatment significantly enhanced NFATc1 expression (*lane 6*). These data demonstrate that RANKL pretreatment facilitates IL-1-mediated osteoclastogenesis by rendering the NFATc1 gene responsive to IL-1.

The RANK IVVY Motif Plays a Crucial Role in IL-1-mediated Osteoclastogenesis—Next, we examined the role of the RANK cytoplasmic IVVY motif in IL-1-mediated osteoclastogenesis. To this end, we turned to two chimeric receptors that contain the human Fas external domain linked to transmembrane and cytoplasmic domains of normal mouse RANK (Ch1) or RANK bearing inactivating mutations in the IVVY motif (Ch2) (Fig. 4*A*) (24, 29). These chimeras can be activated by an anti-human Fas-activating antibody (FAS-AB) that does not recognize mouse Fas (29). BMMs expressing similar levels of Ch1 or Ch2 (Fig. 4*B*) were used to assess the role of the IVVY motif in IL-1-mediated osteoclastogenesis (*C–D*). BMMs expressing Ch1 or Ch2 were treated with M-CSF and 10 ng/ml FAS-AB, which leads to permissive levels of RANK signaling activation from the chimeric receptors, in the absence or presence of IL-1 for 5 days (Fig. 4*C*). Both Ch1- and Ch2-expressing BMMs failed to form osteoclasts in response to 10 ng/ml FAS-AB stimulation for 5 days (Fig. 4*C*, *top row*). The addition of IL-1 led to the formation of osteoclasts from Ch1-expressors, but not Ch2-expressors, in the presence of 10 ng/ml FAS-AB (Fig. 4*C*, *bottom row*). These findings indicate that the RANK IVVY motif is required for IL-1-mediated osteoclastogenesis.

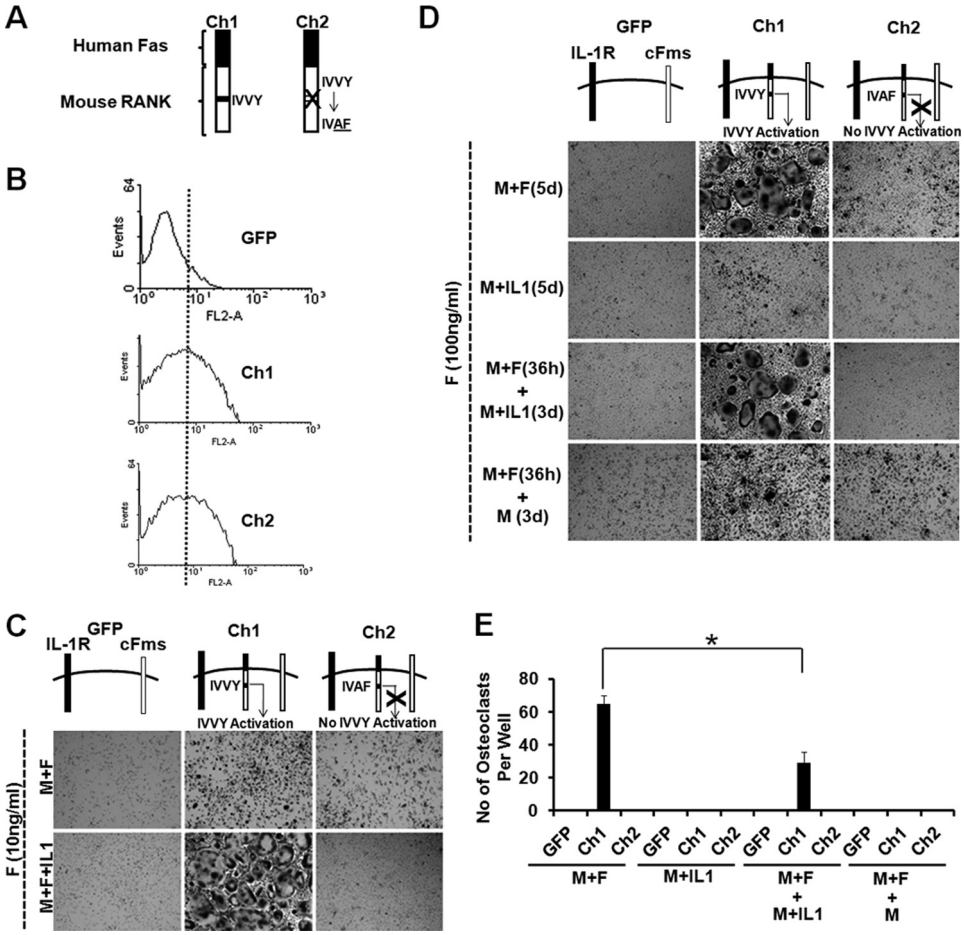

FIGURE 4. **The RANK IVVY motif is required for IL-1-mediated osteoclastogenesis.** *A,* schematic diagram of Ch1 and Ch2. *B*, flow cytometric analysis of BMMs infected with virus encoding GFP control, Ch1, or Ch2. *C*, BMMs expressing GFP, Ch1, or Ch2 were cultured with M-CSF (*M*, 44 ng/ml) and FAS-AB (*F*, 10 ng/ml) with or without IL-1 (*IL1*, 5 ng/ml) for 5 days (*d*). *D*, BMMs expressing GFP, Ch1, or Ch2 were treated with M (44 ng/ml) and F (100 ng/ml) or M (44 ng/ml) and IL1 (5 ng/ml) for 5 days. These infected cells were also treated with M (44 ng/ml) and F (100 ng/ml) for 36 h, washed with PBS, and then continued with M (44 ng/ml) or M (44 ng/ml) and IL1 (5 ng/ml) for 3 days. The cultures were then stained for TRAP activity. The assays were performed in three replicates and repeated independently twice. A representative image is shown. *E*, quantification of the osteoclastogenesis assays in *D* is shown in mean number of multinucleated TRAP-positive cells ($>$ 3 nuclei) per well. *Bars* show averages $\pm$ S.D. *, $p < 0.05$.

To assess the role of the IVVY motif in IL-1-mediated osteoclastogenesis from BMMs pretreated with RANKL, BMMs expressing comparable levels of GFP control, Ch1, or Ch2 were treated with M-CSF and 100 ng/ml FAS-AB, which is sufficient to drive optimal RANK activation in the chimeras, for 5 days or with M-CSF and IL-1 for 5 days (Fig. 4, *D* and *E*). Moreover, these cells were pretreated with M-CSF and 100 ng/ml FAS-AB for 36 h, and the cultures were then continued with M-CSF and IL-1 or with M-CSF alone for 3 days. BMMs expressing Ch1, but not Ch2, formed osteoclasts in response to M-CSF and FAS-AB for 5 days (Fig. 4, *D*, *top row*, and *E*), but neither Ch1-expressors nor Ch2-expressors formed osteoclasts in the presence of M-CSF and IL-1 for 5 days (*D*, *second row*, and *E*). Importantly, BMMs expressing Ch1 but not Ch2 formed osteoclasts when primed with FAS-AB for 36 h prior to 3-day IL-1 treatment (Fig. 4, *D*, *third row*, and *E*). As a negative control, BMMs expressing Ch1 treated with M-CSF only for 3 days after the 36-hour FAS-AB pretreatment failed to form osteoclasts (Fig. 4, *D*, *bottom row*, and *E*). These data further demonstrate that the RANK IVVY motif plays an essential role in IL-1-mediated osteoclastogenesis.

The RANK IVVY Renders the Osteoclast Marker and NFATc1 Genes Responsive to IL-1—To examine whether the RANK IVVY motif specifically renders the four osteoclast genes responsive to IL-1, BMMs expressing GFP, Ch1, or Ch2 were first treated with M-CSF and FAS-AB (10 ng/ml), M-CSF and IL-1 (5 ng/ml), or M-CSF and FAS-AB (10 ng/ml) plus IL-1 (5 ng/ml) for 3 days (Fig. 5*A*). As negative controls, 10 ng/ml FAS-AB or 5 ng/ml IL-1 alone could barely activate the osteoclast genes in the infected BMMs (Fig. 5*A*, *lanes 1–6*). However, 10 ng/ml FAS-AB plus 5 ng/ml IL-1 activated the expression of the four osteoclast genes in the Ch1 expressors (Fig. 5*A*, *lane 8*) but not the GFP (*lane 7*) or Ch2 (*lane 9*) expressors. We then determined the involvement of the IVVY motif in IL-1-induced expression of these genes in BMMs pretreated by RANKL. To this end, BMMs expressing GFP, Ch1, or Ch2 were treated with M-CSF and FAS-AB (100 ng/ml) or M-CSF and IL-1 (5 ng/ml) for 3 days (Fig. 5*B*). 100 ng/ml FAS-AB, but not IL-1, strongly activated the expression of these genes in BMMs expressing Ch1 (Fig. 5*B*, *lane 2 versus lane* 5) but not GFP (*lane 1 versus lane* 4) or Ch2 (*lane 3 versus lane 6*). In parallel, BMMs expressing GFP, Ch1, or Ch2 were pretreated with M-CSF and

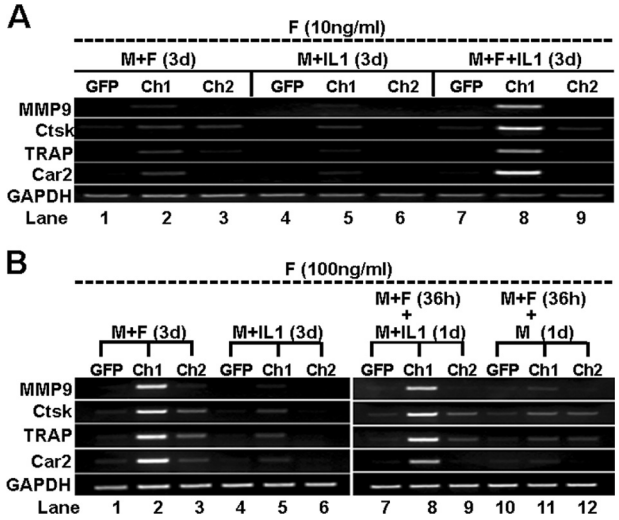

FIGURE 5. **The RANK IVVY motif is involved in rendering osteoclast genes responsive to IL-1.** *A,* BMMs expressing GFP, Ch1, or Ch2 were cultured with M-CSF (*M*, 44 ng/ml) and FAS-AB (*F*, 10 ng/ml), M (44 ng/ml) and IL-1 (*IL1*, 5 ng/ml), or M (44 ng/ml) and F (10 ng/ml) plus IL1 (5 ng/ml) for 3 days (*d*). *B*, BMMs expressing GFP, Ch1, or Ch2 were treated with M (44 ng/ml) and F (100 ng/ml) or M (44 ng/ml) and IL1 (5 ng/ml) for 3 days. These cells were also cultured with M (44 ng/ml) and F (100 ng/ml) for 36 h, washed with PBS, and then continued with M (44 ng/ml) or M (44 ng/ml) and IL1 (5 ng/ml) for 1 day. Gene expression was assessed by semi-quantitative RT-PCR using GAPDH as loading control. These gene expression analyses were repeated independently twice.

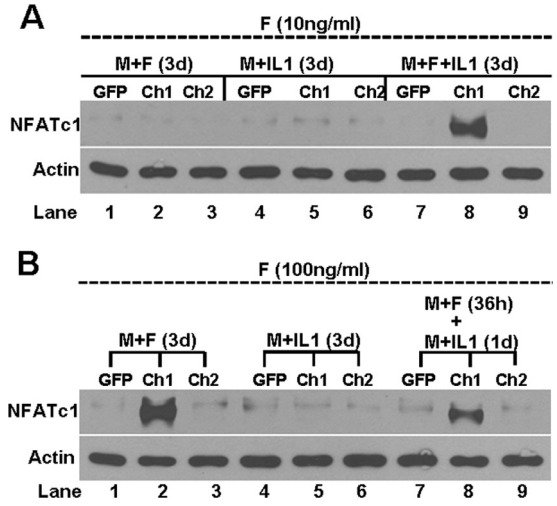

FIGURE 6. **The RANK IVVY motif is involved in rendering the NFATc1 gene responsive to IL-1.** *A,* BMMs expressing GFP, Ch1, or Ch2 were cultured with M-CSF (*M*, 44 ng/ml) and FAS-AB (*F*, 10 ng/ml), M (44 ng/ml) and IL-1 (*IL1*, 5 ng/ml), or M (44 ng/ml) and F (10 ng/ml) plus IL1 (5 ng/ml) for 3 days (*d*). *B*, BMMs expressing GFP, Ch1, or Ch2 were treated with M (44 ng/ml) and F (100 ng/ml) or M (44 ng/ml) and IL1 (5 ng/ml) for 3 days. These cells were also cultured with M (44 ng/ml) and F (100 ng/ml) for 36 h, washed with PBS, and then continued with M (44 ng/ml) and IL1 (5 ng/ml) for 1 day. NFATc1 expression was assessed by Western blot analysis with β-actin as a loading control. The assays were repeated independently twice.

FAS-AB (100 ng/ml) for 36 h, and the cultures were then continued with M-CSF and IL-1 (5 ng/ml) or M-CSF alone for 1 day. IL-1 was able to induce the expression of the genes after 36-hour FAS-AB pretreatment in BMMs expressing Ch1 (Fig. 5*B*, *lane 8*) but not Ch2 (*lane 9*). As negative control, these genes were only weakly activated in BMMs expressing Ch1 when the FAS-AB pretreated cells were cultured with M-CSF alone (Fig. 5*B*, *lane 11*). These findings indicate that the RANK IVVY play a critical role in IL-1 induced expression of osteoclast marker genes.

Next, we addressed whether the IVVY motif is involved in IL-1 induced expression of NFATc1 (Fig. 6). Consistent with the role of this motif in IL-1 induced expression of osteoclast marker genes, although BMMs expressing GFP, Ch1 or Ch2 treated with M-CSF and FAS-AB (10 ng/ml) or M-CSF and IL-1 (5 ng/ml) did not increase NFATc1 expression (Fig. 6*A*, *lanes 1–6*), treatment of the infected BMMs with M-CSF and FAS-AB (10 ng/ml) plus IL-1 (5 ng/ml) strongly activated NFATc1 in the Ch1-expressing cells (*lane 8*) but not in the GFP (*lane 7*) or Ch2 (*lane 9*) expressors. Moreover, 100 ng/ml FAS-AB, but not IL-1, strongly activated NFATc1 in BMMs expressing Ch1 (Fig. 6*B*, *lane 2 versus lane 5*) but not in GFP (*lane 1 versus lane 4*) or Ch2 (*lane 3 versus lane* 6). Notably, 36-hour pretreatment with M-CSF and FAS-AB followed by 1-day treatment with M-CSF and IL-1 activated NFATc1 expression in BMMs expressing Ch1 (Fig. 6*B*, *lane 8*) but not GFP (*lane 7*) or Ch2 (*lane 9*). Taken together, these data demonstrate that the RANK IVVY mediates IL-1-mediated osteoclastogenesis by rendering the osteoclast marker and NFATc1 genes responsive to IL-1.

MyD88 Is Involved in IL-1-mediated Osteoclastogenesis by Up-regulating the Expression of Osteoclast Marker and NFATc1 Genes—Finally, we investigated whether MyD88, a key component in the IL-1RI signaling pathway, plays a crucial role in IL-1-mediated osteoclastogenesis from RANKL-primed BMMs. WT or MyD88$^{-/-}$ BMMs were cultured with M-CSF and 10 ng/ml RANKL, M-CSF and 5 ng/ml IL-1, or M-CSF and 10 ng/ml RANKL plus 5 ng/ml IL-1 for 4 days (Fig. 7, *A* and *B*). IL-1 dramatically induced osteoclastogenesis in the presence of permissive levels of RANKL from WT BMMs compared with MyD88$^{-/-}$ BMMs. Moreover, WT or MyD88$^{-/-}$ BMMs were pretreated with M-CSF and 100 ng/ml RANKL for 36 h, and the cultures were then continued with M-CSF or M-CSF and 5 ng/ml IL-1 for 2 days (Fig. 7, *C* and *D*). IL-1 stimulated osteoclastogenesis from RANKL-pretreated WT but not MyD88$^{-/-}$ cells. These results indicate that MyD88 is critically involved in IL-1-mediated osteoclastogenesis from RANKL-primed BMMs.

We next investigated the role of MyD88 in regulating the IL-1-mediated expression of osteoclast genes and NFATc1 in RANKL-primed BMMs. WT or MyD88$^{-/-}$ BMMs were treated with M-CSF and 100 ng/ml RANKL for 36 h, and the cultures were then continued with M-CSF, M-CSF and RANKL, or M-CSF and 5 ng/ml IL-1 for 2 days (Fig. 8). The data indicate that although IL-1 induced the expression of osteoclast genes in RANKL-primed WT BMMs, IL-1-induced expression of osteoclast genes is reduced in RANKL-primed MyD88$^{-/-}$ BMMs (Fig. 8*A*). Furthermore, IL-1 activated NFATc1 expression in RANKL-primed WT BMMs (Fig. 8*B*, *lane 1 versus lane 3*) but not in RANKL-primed MyD88$^{-/-}$ BMMs (*lane 4 versus lane 6*). Thus, we conclude that MyD88 plays an important role in IL-1-mediated osteoclastogenesis by up-regulating the expression of NFATc1 and osteoclast genes from RANKL-primed BMMs.

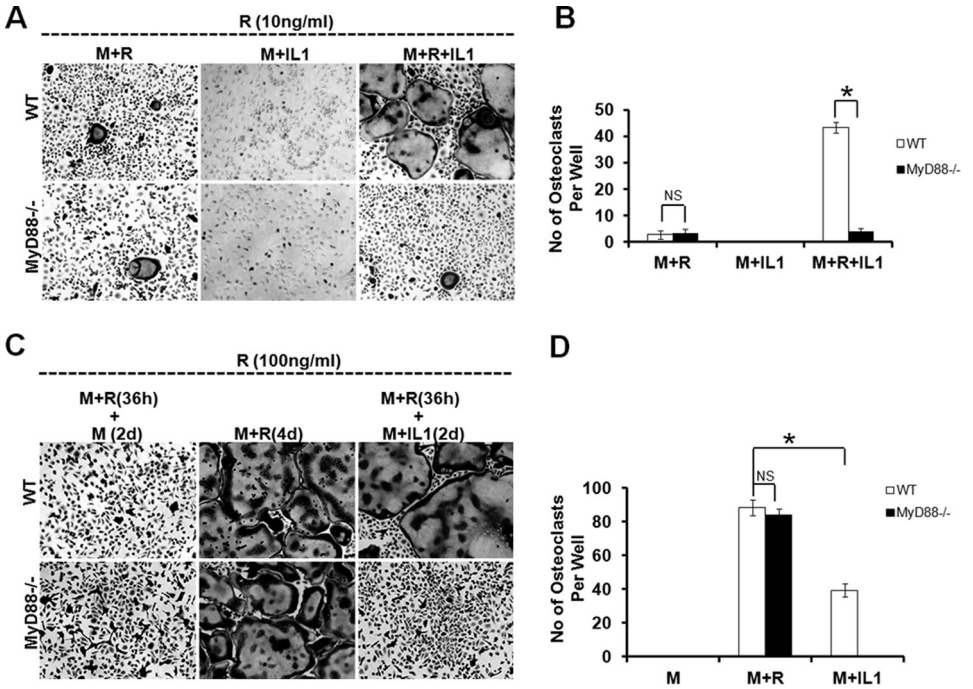

FIGURE 7. **MyD88 is central to RANKL-induced IL-1-mediated osteoclastogenesis.** *A*, BMMs from C57BL/6 (WT) or MyD88$^{-/-}$ mice were cultured with M-CSF (*M*, 44 ng/ml) and RANKL (*R*, 10 ng/ml), M (44 ng/ml) and IL-1 (*IL1*, 5 ng/ml), or M (44 ng/ml) and R (10 ng/ml) plus IL1 (5 ng/ml) for 4 days (*d*). The cultures were then stained for TRAP activity. The assays were performed in three replicates and repeated independently twice. A representative image is shown. *B*, quantification of the osteoclastogenesis assays in *A* is shown in mean number of multinucleated TRAP-positive cells (> 3 nuclei) per well. *Bars* show averages ± S.D. * $p < 0.05$. *C*, BMMs from WT or MyD88$^{-/-}$ mice were cultured with M (44 ng/ml) and R (100 ng/ml) for 36 h, washed with PBS, and then continued with M (44 ng/ml) or M (44 ng/ml) and IL1 (5 ng/ml) for 2 days. Cells treated with M (44 ng/ml) and R (100 ng/ml) were used as controls. The cultures were then stained for TRAP activity. *D*, quantification of the osteoclastogenesis assays in *C* is shown in mean number of multinucleated TRAP-positive cells (> 3 nuclei) per well. *Bars* show averages ± S.D. *, $p < 0.05$.

DISCUSSION

A stimulatory role for IL-1 in bone resorption was first discovered by several groups 25 years ago (34, 35). This initial finding prompted subsequent investigations demonstrating that human peripheral blood monocytes from postmenopausal osteoporosis patients and ovariectomized premenopausal women produce larger amounts of IL-1. This is an effect not seen in peripheral blood monocytes from either estrogen-treated postmenopausal women with osteoporosis or untreated premenopausal women (36, 37). IL-1 has been recognized as one of the key factors involved in the pathogenesis of postmenopausal osteoporosis (2). Moreover, as a proinflammatory cytokine, IL-1 is produced abundantly within inflammatory sites. Not only is IL-1 an important mediator of inflammatory responses, but it is also implicated in promoting bone loss in inflammatory conditions such as rheumatoid arthritis (3) and periodontitis (4). IL-1 causes bone loss in these pathological conditions primarily by promoting osteoclastic bone resorption via distinct mechanisms. First, IL-1 exerts a stimulatory effect on osteoclast survival (7) and plays a role in activating osteoclast function (8). Furthermore, IL-1 indirectly promotes osteoclastogenesis via up-regulation of RANKL expression in osteoblasts and stromal cells (10, 38). Interestingly, although IL-1 alone is unable to directly mediate osteoclastogenesis, it can do so in the presence of permissive levels of RANKL (9, 10). This intriguing finding has created interest in elucidating the molecular mechanism by which IL-1-mediated osteoclastogenesis requires permissive levels of RANKL.

In this study, we seek to investigate the molecular basis of the requirement for RANKL in IL-1-mediated osteoclastogenesis with a focus on a potential involvement of the RANK IVVY in the process for two reasons. First, the identification of the IVVY motif was prompted by earlier observations that IL-1 cannot mediate osteoclastogenesis in the absence of RANKL (18). Specifically, although it has been well established that both RANK and IL-1RI utilize TRAF6 to activate intracellular signaling pathways (39), administration of IL-1 to RANK$^{-/-}$ mice fails to promote osteoclastogenesis (40). Consistently, IL-1 fails to stimulate osteoclastogenesis *in vitro* in the absence of RANKL (41, 42). These findings suggest that RANK may also activate a TRAF-independent pathway(s) to regulate osteoclastogenesis. Consequently, we identified the RANK IVVY motif and revealed that this motif plays a vital role in osteoclastogenesis by committing BMMs to the osteoclast lineage (18). Secondly, we have recently shown that TNF-induced osteoclastogenesis depends on RANKL to commit BMMs to the osteoclast lineage and that RANKL regulates the lineage commitment through the IVVY motif (24). Mechanistically, the IVVY motif mediates the lineage commitment by changing osteoclast genes into an inducible state in which they can be activated by TNF (24). Our current work demonstrates that the IVVY motif also plays an essential role in IL-1-mediated osteoclastogenesis by mediating the RANKL-induced osteoclast lineage commitment. Similarly, the IVVY motif mediates the lineage commitment by rendering osteoclast genes responsive to IL-1.

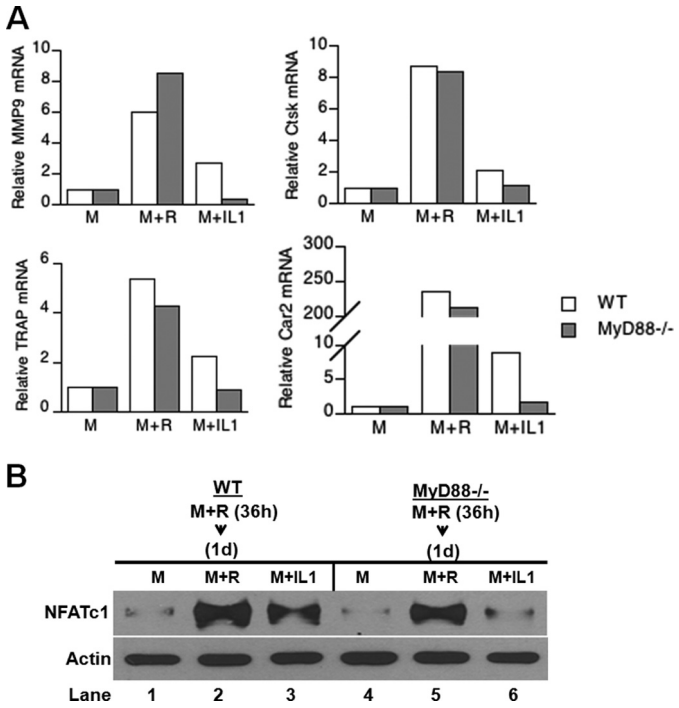

FIGURE 8. **MyD88 is required for IL-1-mediated expression of osteoclast and NFATc1 genes in RANKL-primed BMMs.** *A*, BMMs from C57BL/6 (WT) or MyD88$^{-/-}$ mice were cultured with M-CSF (*M*, 44 ng/ml) and RANKL (*R*, 100 ng/ml) for 36 h, washed with PBS, and then continued with M (44 ng/ml), M (44 ng/ml) and R (100 ng/ml), or M (44 ng/ml) and IL-1 (IL1, 5 ng/ml) for 2 days. Gene expression was assessed by real-time PCR using GAPDH as the endogenous control. The real time RT-PCR assays were repeated independently twice. *B*, BMMs from C57BL/6 (WT) or MyD88$^{-/-}$ mice were cultured with M (44 ng/ml) and R (100 ng/ml) for 36 h, washed with PBS, and then continued with M (44 ng/ml), M (44 ng/ml) and R (44 ng/ml), or M (44 ng/ml) and IL-1 (IL1, 5 ng/ml) for 1 day. NFATc1 expression was assessed by Western blot analysis with β-actin as a loading control. The assays were repeated independently twice.

Therefore, the next critical question is how exactly RANKL changes osteoclast genes into an IL-1-inducible state. It was proposed recently that a lack of IL-1 signaling potential, because of low levels of IL-1RI expression and high levels of IL-1RII expression in BMMs, causes the inability of IL-1 alone to directly stimulate osteoclastogenesis (32). Moreover, it was shown that overexpression of IL-1RI in BMMs increased TRAP mRNA levels (32). Thus, it is possible that RANKL-induced increase in IL-1 signaling potential plays a role in enabling IL-1 to induce the expression of these osteoclast genes. Notably, a previous study demonstrated that BMMs without prior exposure to RANKL exhibited considerable levels of basal responsiveness to IL-1 (43), but our current work has shown that IL-1 failed to activate the expression of the osteoclast genes in BMMs (Fig. 1*A*). These observations suggest that although RANKL-induced enhancement in IL-1 signaling potential is likely to be a contributing factor, another unidentified mechanism(s) may also account for the inability of IL-1 to activate the expression of the osteoclast genes.

To further address the issue, we investigated the potential involvement of NFATc1 in RANKL-induced alteration of osteoclast genes into the IL-1-inducibale state because NFATc1, a master transcriptional regulator of osteoclastogenesis (17), has been shown to play a role in activating the expression of the TRAP, Car2, and Ctsk genes during osteoclastogenesis (44, 45). Our data indicate that although IL-1 alone is unable to stimulate NFATc1 expression (Fig. 3*A*), it can do so in the presence of permissive levels of RANKL (*B*). Moreover, RANKL pretreatment activates NFATc1 expression, and subsequent IL-1 treatment further enhanced NFATc1 expression (Fig. 3*C*). Numerous previous studies have demonstrated that NFATc1 works in concert with a number of other transcriptional factors, including AP-1, PU.1, microphthalmia transcription factor (MITF), and cAMP response element-binding protein to regulate gene expression during osteoclastogenesis (17). Taken together, these observations support the notion that RANKL plays a crucial role in IL-1-mediated osteoclastogenesis by assisting IL-1 in inducing the expression of NFATc1, which in turn cooperates with other transcriptional factors to stimulate gene expression and thus osteoclastogenesis in osteoclast precursors.

Although IL-1 can stimulate osteoclastogenesis from RANKL-pretreated BMMs in tissue culture dishes (supplemental Fig. 3*A*) and on bone slices (supplemental Fig. 3*C*), osteoclasts derived from RANKL pretreatment followed by IL-1 stimulation gave rise to fewer resorption pits compared with control osteoclasts resulting from the continuous RANKL treatment (supplemental Fig. 3, *D* and *E*). It has been shown that osteoclastogenesis requires the activation of a large number of genes inducing the expression of numerous genes, including MMP9, Ctsk, TRAP, and Car2 by RANKL in osteoclast precursors (33). Despite this study showing that IL-1 induces the expression of all the four osteoclast genes tested, *i.e.* MMP9, Ctsk, TRAP, and Car2, it is unlikely that RANKL pretreatment followed by IL-1 stimulation is able to activate the complete set of genes normally activated by the continuous RANKL treatment. Specifically, although RANKL pretreatment is able to activate the expression of NFATc1, subsequent IL-1 stimulation may not be able to activate the same set of transcriptional factors that are activated by RANKL. Thus, osteoclasts derived from RANKL pretreatment followed by IL-1 stimulation do not possess normal resorptive machinery, which results in incomplete activation of the osteoclast genes and, hence, leads to the reduced capacity to resorb bone compared with those formed from the continuous RANKL treatment.

In conclusion, our major findings are as follows. 1) IL-1 cannot stimulate osteoclastogenesis in part because of its inability to activate the expression of osteoclast genes; 2) RANKL plays a crucial role in IL-1-induced osteoclastogenesis by rendering osteoclast genes responsive to IL-1; 3) RANKL renders osteoclast genes responsive to IL-1 in part by inducing the expression of NFATc1; 4) the RANK IVVY motif is specifically involved in rendering the osteoclast marker and NFATc1 genes responsive to IL-1; and 5) MyD88 plays an important role in IL-1-mediated osteoclastogenesis by up-regulating the expression of NFATc1 and osteoclast genes from RANKL-primed BMMs. These observations provide important new insights into the molecular basis of the dependence of IL-1-mediated osteoclastogenesis on RANKL. Significantly, the IVVY motif, because of its critical role in IL-1-mediated osteoclastogenesis, has the potential to serve as an attractive therapeutic target for bone disorders.

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