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MKP-1 Signaling Events are Required for Early Osteoclastogenesis in Lineage Defined Progenitor Populations by Disrupting RANKL-Induced NFATc1 Nuclear Translocation

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Abstract

Cytokine-directed osteoclastogenesis is initiated in response to macrophage colony stimulating factor (M-CSF) and receptor activator of NF- κ B ligand (RANKL), to drive formation of osteoclasts (OC), large bone resorptive cells of hematopoietic origin. RANKL-induced signaling activates the MAPK pathways, which initiates nuclear translocation of the master regulator of osteoclast formation, transcription factor NFATc1. Proper control over these signaling events is essential to normal OC formation response to stimuli. MAPK phosphatase 1 (MKP-1), a serine and tyrosine phosphatase encoded by the gene *Dusp1*, functions to dephosphorylate and subsequently inactivate MAPK (p38 and JNK) signaling essential in osteoclastogenesis. Here, we explored the role of MKP-1 during RANKL-driven osteoclastogenesis from defined (B220/CD45⁺ GR1⁺CD11b^{lo}/CD115⁺) OC progenitor (dOCP) populations using WT and *Dusp1*^{-/-} global knockout mice. Sorted cells were driven to OC by M-CSF pre-treatment followed by RANKL stimulation for 3 days. OC formation and qPCR products were analyzed for maturation. Results indicate that *Dusp1*^{-/-} dOCP form less numerous, significantly smaller and less functional OC compared to WT controls. These data were corroborated by mRNA expression of the key OC genes, *Nfatc1* and *Tm7sf4* (DC-STAMP), which were significantly reduced in early osteoclastogenesis in OC progenitor from *Dusp1*^{-/-} mice. Intriguingly, our data reveals that MKP-1 may positively control OC formation in response to RANKL by regulating NFATc1 nuclear translocation. Collectively, this report supports the idea that MKP-1 signaling is essential in early osteoclastogenesis in response to RANKL-induced signaling.

Keywords

MAPK phosphatase; osteoclastogenesis; RANKL; cytokine; progenitors

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1. Introduction

Bone turnover and remodeling requires the formation of osteoclasts (OC), large multinucleated cells with the capacity to resorb bone matrix [1–5]. Bone marrow-derived OC arise from hematopoietic stem cell (HSC) progenitor populations late in monocyte differentiation and have been distinctly defined as CD45R⁺GR-1⁺CD11b^{lo/-}CD115⁺ (defined here as defined OCP or dOCP) [6–8]. One of the key elements to OC formation is the ability of cells to respond to cytokines that drive their differentiation. Macrophage-colony stimulating factor (M-CSF), a cytokine that stimulates survival, primes these cells for lineage commitment to either a macrophage or to a pre-OC by increasing expression of the RANK receptor [4, 9, 10]. Receptor activator of NF-κB ligand (RANKL), produced by proximal osteoblasts/stromal lineage cells and lymphocytes within the bone microenvironment, signals a temporal process through the RANK receptor where progenitors begin lose their macrophage-like phenotype and develop into multinucleated, active OC, approximately 72-hours post RANKL exposure through modulation of signal transduction pathways within the pre-OC[11].

At the sub-cellular level, RANKL-induced signaling activates transcription factors (TF) NF-κB and AP-1 (c-fos/c-Jun) through phosphorylation of pathway intermediates, IκB (NF-κB) or p38 and JNK MAPK, respectively [12, 13]. These intermediates direct the recruitment, dephosphorylation and translocation of nuclear factor of activated T cell (NFATc1), the master regulator of osteoclastogenesis in response to RANKL [5, 14, 15]. NFATc1 controls OC formation through transcriptional regulation where NFATc1 oligomerizes with other TF and binds to promoter regions of osteoclast-specific genes including; *Nfatc1*, tartrate resistant acid phosphatase (TRAP/*Acp5*), dendritic cell-specific transmembrane protein (DC-STAMP/*Tm7sf4*), calcitonin receptor (*CR*) and cathepsin K (*Ctsk*) [16].

While several signaling pathways are engaged during osteoclastogenesis, the precise mechanisms controlled by the signaling events are poorly understood. Distal RANKL-induced signaling events include activation of the MAPK and NF-κB signaling cascades whose activity is controlled through kinases and phosphatases. One family that controls MAPK phosphorylation is the dual-specific serine phosphatases (*Dusp*), which function to dephosphorylate serine and tyrosine residues [17–19]. *Dusp1*, the family archetype encodes for MAP Kinase Phosphatase-1 (MKP-1), that preferentially dephosphorylates phospho(p)-p38 and p-JNK. We have reported that in response to periodontal pathogen constituents, including bacterial lipopolysaccharide (LPS), MKP-1 ablation leads to enhanced and sustained activation of the MAPK, contributing to an inflammatory phenotype and bone loss [20, 21] and forced expression of MKP-1 reduces inflammation and bone loss in LPS-induced models. Together, these reports support the assertion that MKP-1 functions to negatively regulate inflammatory driven OC formation through signaling pathways including p38 and JNK MAPK. However, while much is known about the role of MKP-1 in LPS-driven OC formation, little is known about the role MKP1 signaling in the RANKL-driven systems.

Given their common lineage, most studies have evaluated OC formation in progenitors defined by the macrophage lineage (CD11b⁺) [22]. In contrast, further isolation of enriched, dOCP populations, may uncover subtle differences in OC progenitors within the bone marrow HSC population [6]. Our objective in the present study was to delineate the role of MKP-1 in RANKL-stimulated osteoclastogenesis from defined OC progenitor populations. Based on previous observations which show that MKP-1 is a negative regulator of OC formation in response to LPS challenge, we hypothesized that MKP-1 will also negatively regulate RANKL-induced OC formation. Surprisingly, we report that MKP-1 deletion

resulted in fewer, smaller and less functional OC in defined OCPs following RANKL stimulation. These results provide evidence for MKP-1 signaling as a permissive regulator of OC formation in response to RANKL, likely through interaction between the MAPK family and NFATc1 activation.

2. Materials & Methods

2.1 Animals

Mixed genetic background B6:129P WT and *Dusp1*^{-/-} mice were obtained from Bistol-Myers Squibb Pharmaceutical Research Institute and bred at the Medical University of South Carolina Animal Facility and maintained in accordance with NIH guidelines. Mice were subject to food and tap water *ad libitum* and maintained under normal 12-hour light cycles. The Institutional Animal Care and Use Committee (IACUC) at the Medical University of South Carolina approved all subsequent experimental protocols.

2.2 Selection of Osteoclast Progenitor Populations

Osteoclastic cell progenitor populations were isolated from WT and *Dusp1*^{-/-} female bone marrow using lineage-specific and progenitor markers as B220/CD45⁻GR1⁻CD11b^{lo/-}CD115⁺ as previously defined [6]. Briefly, hematopoietic stem cells (HSCs) were enriched initially by using the suspension populations after 24 hrs of whole marrow culture. Using the AutoMACS sorter (Miltenyi Biotec), bone marrow HSCs were sorted into three distinct groups based on expression of CD11b: CD11b^{hi} (macrophage lineage), CD11b^{lo} (dOCP^{lo}) and CD11b⁻ (dOCP⁻). Defined osteoclast progenitor (dOCP) populations were immunophenotyped using commercially available antibodies from Miltenyi Biotec (Auburn, CA) including anti-B-cell lineage (mAb): anti-CD45R/B220 (RA3-6B2), anti-T-cell lineage (mAb): anti-CD3e (17A-2), anti-Granulocyte lineage (mAb): anti-Gr-1/Ly-6G (R86-8C5), anti-monocyte/macrophage lineage (mAb): anti-CD11b (M1/70) and anti-monocyte progenitor antibody (mAb): anti-cfms/CD115 (AFS98; R&D Systems, Minneapolis, MN). All antibodies were directly conjugated fluorochromes or biotinylated from the manufacturer. Isolated cells were labeled for flow cytometric analysis following standard procedures. Dead cell exclusion was performed using propidium iodide (PI) staining. Flow cytometry analysis was performed on at least 30,000 events per sample using the Miltenyi MACSQuant (Auburn, CA). Data analysis was performed using the MACSQuant Software provided with the machine.

2.3 Osteoclast differentiation assays

CD11b^{hi} and dOCP populations were plated in 96-well plates at a density of 5×10^4 cells/well in priming media (10ng/ml M-CSF) overnight. Once seated, cells stimulated with fresh control media (25ng/ml M-CSF) or treatment media (25ng/ml M-CSF and 50ng/ml RANKL) for two days. Fresh control and treatment media were replaced every two days and used to stimulate cells for 24 hours, for a total of 3 days exposure to RANKL. Treated cells were fixed with 10% glutaraldehyde and TRAP stained as previously described [23]. For proof of principle, OC formation assays described above were repeated in the presence of p38 (SB508250) and JNK (SP600125) inhibitors. Cells were treated with inhibitors thirty minutes prior to stimulation with M-CSF and RANKL. An investigator blinded to the sample identity quantified osteoclasts based on the following criteria; 1) number of OC per field of view, 2) number of nuclei per OC (≥ 3) and 3) size of OC. OC were measured using Image J software. In addition, the density, distribution and the percentage of TRAP positive cells using 0–3 arbitrary scales was evaluated (0 indicated low density/no TRAP⁺, up to 3 indicating density greater than 75% of the well and highly TRAP⁺). To measure OC activity, sorted OCP were seeded onto bovine cortical bone slices and treated with M-CSF and RANKL as described previously. Bone slices were harvested eight-days following initial

priming to allow OC function to occur. OC function was measured following cell removal with a 5% hypochlorite solution for 10 min and then counterstaining lacunae with using 0.1% Toluidine blue solution in water. Resorption lacunae were enumerated based on field of view at 20× magnification under an inverted microscope. As a secondary surrogate measure of OC function, the pNPP-based TRAP enzyme activity assay kit was utilized according to manufacturer's protocol (Takara Bioscience, Japan) at the day of harvest. Activity was measured by absorbance at 405nm and normalized to total protein.

2.4 Immunoblotting

Sorted dOCP populations were plated on 35mm dishes at 2×10^6 cells/dish. Cells were primed with M-CSF (10ng/ml) for two days prior to stimulation. Cells were serum starved with 2% serum-supplemented α -MEM for 2 hours, then treated with RANKL (100ng/ml) for indicated time points. Cells were lysed by RIPA buffer and quantified using the Lowry assay (BioRad, Hercules, CA). 20 μ g of total protein was loaded onto 10% TGX gels (BioRad, Hercules, CA) and electrophoresed. Separated proteins were then transferred onto nitrocellulose membranes using the wet-transfer method and blocked in 5% milk for one hour prior to probing with rabbit-anti-mouse derived antibodies including; phospho(p)-p38, total p38, p-JNK (p46/54), total JNK, p-ERK (p42/44), total ERK and GAPDH. All primary antibodies were diluted in 5% BSA (in TBS-T) and incubated at 4°C overnight. Anti-rabbit and anti-biotin secondary, each conjugated to HRP were used to visualize the blots in the presence of West Pico ECL (Pierce).

2.5 RT-qPCR

mRNA was isolated from M-CSF or M-CSF/RANKL treated cells, three days following stimulation (1×10^6 cells/well) using the TriZOL reagent (Invitrogen) according to the manufacturer's specifications. Total extracted mRNA was quantitated using Nanodrop spectrophotometry (BioRad, Hercules, CA). cDNA was synthesized using TaqMan reverse transcriptase reagent kit (Applied Biosystems, Foster City, CA) and 500–1000ng of total RNA. Amplified cDNA was then probed using amplicon primers obtained from Applied Biosystems including; RANK (*Tnfrsf11a*), Traf6 (*Traf6*), cFOS (*cfos*), M-CSFR (*Csf1r*), NFATc1 (*Nfatc1*), DCSTAMP (*Tm7sf4*), OCSTAMP (*Ocstamp*) TRAP (*Acp5*), Cathepsin K (*Ctsk*) and Calcitonin Receptor (*Calcr*). Amplicon concentration was determined using threshold cycle values in comparison with endogenous loading control for each primer. GAPDH was used as endogenous loading control for normalization. Normalized amplicon values were expressed as fold change compared to the M-CSF only controls.

2.6 Confocal Microscopy

dOCP were seeded onto 8-well chamber slides at 1.5×10^5 cells per well. All cells were primed with M-CSF (10ng/ml) for 36 hours. Mitosis was synchronized by starvation in low serum media for 2 hours. Cells were then stimulated with 100ng/ml of RANKL. Cells were harvested at 0, 24 and 72hrs following RANKL stimulation. Wells were washed in PBS and cells were fixed with 4% PFA for 10 min at RT. Cells permeabilized with 0.5M Triton x-100 and blocked with 5% goat serum. Probing was done using antibodies against phospho- and total NFATc1, as well as Hoescht nuclear dye. The Image J plug-in Jacop determined nuclear localization, where Pearson's correlation coefficient (*r*) values were analyzed between NFATc1 fluorescence and nuclear counterstain. Zero time point correlation values from each group were used to normalize the plotted data.

2.7 TransAM Nuclear Translocation ELISA

dOCP were harvested as described above were seeded at a density of 8×10^6 onto 60mm dishes in 3ml of media with 10ng/ml MCSF. Cells were serum starved in 2% FBS for 1hr

then treated with vehicle (DMSO), p38 inhibitors (SB203580) and pJNK inhibitor (SP600125) treatments, made up in low serum media, at a final well concentration of 5mM and added to corresponding wells for 1 hour. Cells were then stimulated with RANKL at a final well concentration of 100ng/ml. Cells were harvested after 24 and 72 hours of stimulation by gentle scraping in PBS containing phosphatase inhibitors (PIB buffer). Cells were lysed using the Nuclear Extraction Kit provided by ActiveMotif (San Jose, CA). Briefly, proprietary mixtures of hypotonic buffers and detergents were used to lyse cell membranes, separating cytoplasmic and nuclear fractions. Nuclear fractions were further extracted using a detergent and complete lysis buffer provided in the kit. Total nuclear lysate was resuspended in this buffer and protein concentration was determined using the Bradford assay. For NFATc1 translocation, the ActiveMotif NFATc1 TransAM kit was employed. Briefly, a plate containing consensus-binding sequence of NFATc1 was purchased along with proprietary binding buffers and antibodies. 1–2ug of total nuclear extract, positive/negative control lysates were loaded into wells containing binding buffer at a ratio of 1:4. Samples were then probed with NFATc1 primary and HRP-conjugated secondary according to kit specifications. Additionally, we controlled for non-specific binding using competitive WT or mutant oligonucleotides loaded with WT vehicle treatments. Colorimetric development following assay was measured using a spectrophotometer at 450nm. Samples were normalized to negative and zero time point controls.

2.8 Statistical Analysis

Data were analyzed with GraphPad Prism software, version 5.0. To compare values between two groups, a paired t-test and subsequent Mann-Whitney test were used. Comparison of values between multiple groups was measured using one-way ANOVA analysis followed by a Bonferroni multiple comparison test. Results were expressed as mean $\pm$ SD or mean $\pm$ 95% confidence interval (CI) of at least three (n=3) biological replicates for each experiment. Six mice per condition were used in qPCR experiments for NFATc1, DC-STAMP, and OCSTAMP. Within each of hi, low and negative cell populations, resulting Δ Ct values were analyzed using generalized estimating equations (GEEs) with identity link function and exchangeable correlation structure, an approach akin to linear regression that accommodates the lack of independence of measures obtained from treated and untreated samples from the same mouse. Δ Ct was modeled as a function of genotype, treatment, and their interaction. Group comparisons were conducted using model-based linear contrasts. GEE analyses were performed using SAS version 9.3. For all analyses, a *P* value of less than 0.05 was considered to be statistically significant.

3. Results

3.1 *Dusp1* does not influence lineage distribution in dOCP^{lo} and dOCP^{hi} populations

Using flow cytometry, whole HSC populations were immunophenotyped to reveal the dOCP (CD45R-GR-1⁻CD11b^{lo/-}CD115⁺) through a hierarchical gating strategy. Cells were stained for expression of B220 (CD45R), Gr1 and CD11b and gated out based upon negative selection. Removal of CD45R⁺, GR1⁺ and CD11b^{hi} from analysis yielded CD11b^{lo/-}CD115⁺ cells, the dOCP. Further investigation within the lineage dOCP, revealed that both CD11b^{lo} and CD11b^{hi} had a subset of CD115⁺ cells. Data presented in Figure 1 shows no significant differences in CD11b/CD115 on macrophage (CD11b^{hi}), dOCP^{lo} or dOCP^{hi} between WT and *Dusp1*^{-/-} mice (Figure 1A and B). Subsequently, enrichment of each of these defined populations using magnetic beads resulted in distributions of surface markers, which were similar to the gated populations. Interestingly however, physical sorting resulted in 45% less CD11b^{lo} from *Dusp1* deficient cells (Supplemental Figure 1). While important to the phenotype of the mouse, in our *in vitro* analysis of OC formation, all experiments were normalized by cell number between genotypes.

Finally, whole bone marrow, spleen, thymus, and peripheral/mesenteric lymph nodes were immunophenotyped to identify potential inherent differences in lineages or if *Dusp1* ablation resulted in sub-clinical infection. The results from this pre-experimental screening revealed that there were neither inherent differences in lineage distribution between genotypes nor any evidence of sub-clinical infections or immune response (data not shown).

3.2 *Dusp1* deficiency increases RANKL-induced MAPK signaling in dOCP

Previous reports have shown that MKP-1 deficient cells have sustained and enhanced activation of MAPK pathway intermediates (p-p38, p-JNK and to a lesser extent p-ERK) under pathogenic stimuli [17, 21]. To determine its role in response to RANKL stimulation, lysates from dOCP deficient in *Dusp1* were analyzed by western blot. Results show that *Dusp1* deficient dOCP exhibit sustained activation of p-p38, p-JNK and surprisingly pERK early and throughout the two-hour time course in response to RANKL (Figure 2A). Blots were then probed with total MAPKs for normalization. Due to variable expression of all family members, quantitation of p-p38, p-JNK and p-ERK was normalized relative to GAPDH at 30, 60 and 120min post-RANKL stimulation in *Dusp1* deficient OCP compared to WT counterparts ($P < 0.05$ each) (Figure 2B).

3.3 MKP-1 controls early RANKL-induced osteoclastogenesis in dOCP^{lo} and dOCP⁻ populations

Our initial observations show that no inherent differences exist between genotypes prior to stimulation, yet *Dusp1* deficiency revealed marked differences in MAPK signaling in response to RANKL. Based on this, we tested whether *Dusp1* deficiency influenced early OC formation following exposure to RANKL. Figure 3A reveals significant differences in morphology exist between genotypes and sorted sub populations. Interestingly and in agreement with our rationale for sorting, our results indicate that macrophage lineage (CD11b^{hi}) cells consistently formed little to no OC following RANKL stimulation. Conversely, our data shows that the dOCP^{lo} and surprisingly the dOCP⁻ populations readily formed OC after RANKL stimulation. Similarly, enumeration (shown in Figure 3B) indicates that *Dusp1*^{-/-} dOCP^{lo} and dOCP⁻ formed significantly less OC (nearly 4 times less each) compared to matched WT counterparts ($P = 0.01$ and $P = 0.001$, respectively). Figure 3C shows that there were significantly less nuclei from the *Dusp1*^{-/-} dOCP⁻ fraction ($P = 0.05$) compared to WT control (Figure 3C). Data from CD11b^{hi} and dOCP^{lo} also followed a similar trend but results did not reach significance. Finally, dOCP^{lo} and dOCP⁻ fractions yielded significantly smaller OC from *Dusp1* deficient cell compared to WT controls ($P = 0.01$ each population; Figure 3D).

To determine the effect of MKP-1 on OC function, the “pit” assay, TRAP activity assay and *Acp5* (TRAP) gene expression was measured. Figure 3E reveals that significantly less pits formed in the *Dusp1*^{-/-} dOCP^{lo/-} group compared to matched WT controls ($P = 0.05$). Interestingly, but in accordance with our observations, the CD11b^{hi} population did form resorption lacunae, however no significant difference was found between genotypes. Figure 3F and G depicts expression of genes; *Acp5* (TRAP) as well as a surrogate marker for function (TRAP activity), measured in each of sorted population treated with M-CSF and M-CSF+RANKL for validation of function outcomes. Figure 3F reveals that TRAP gene expression is significantly reduced by more than 50% in all RANKL-treated, sorted populations ($P < 0.01$, $P < 0.05$, $P < 0.05$, respectively for CD11b^{hi}, dOCP^{lo} and dOCP⁻ populations). Figure 3G reveals a similar picture for TRAP activity. All *Dusp1*^{-/-} populations had a significantly reduced activity compared to matched counterparts. Specifically, the CD11b^{hi} population was reduced 53% ($P = 0.01$), the dOCP^{lo} population decreased activity by 46% ($P = 0.05$) and the dOCP⁻ fraction was diminished to only 42% of WT counterpart ($P = 0.0001$). Interestingly, our data consistently shows an increase in TRAP

activity in the WT CD11b^{hi} fraction, which has been shown to be the least osteoclastogenic. These data indicate that at both the genetic and functional protein level, there are significant reductions in the OC-dependent functional enzyme TRAP with *Dusp1* ablation.

3.4 *Dusp1* status controls expression of genes involved early in RANKL-induced osteoclastogenesis

RANKL-induced osteoclastogenesis is dependent upon activation and up-regulation of genes that control the formation, fusion and function of OC as they move from progenitors to functional cells. Given this necessity, we aimed to determine the role of *Dusp1* in RANKL-induced OC gene expression in our dOCP-enriched populations. Figure 4 A–D depicts mRNA expression patterns of genes involved in early RANKL-dependent signaling including; *Tnfrsf11r* (RANK), *Traf6*, *cfos*, *Nfatc1*, *Tm7sf4* (DCSTAMP) and *Ocstamp* after exposure to M-CSF alone or M-CSF and RANKL for 3 days. Results from Figure 4A show that no significant differences in *Tnfrsf11r* (RANK) mRNA expression exist between WT vs *Dusp1*^{−/−} cells (in any sub-population) following three-day stimulation with M-CSF or M-CSF+RANKL. Figure 4B reveals that at baseline *Dusp1* deficiency results in a 2.5-fold and 3-fold increase in TRAF6 in dOCP^{lo} and dOCP[−] populations ($P < 0.01$ and $P < 0.0001$, respectively). Interestingly, upon RANKL stimulation this trend persisted across all sorted populations lacking *Dusp1*, however the values failed to reach significance. Figure 4C data reveals that the expression of the transcription factor subunit c-Fos was significantly reduced (nearly 3-fold each) in each *Dusp1*^{−/−} dOCP populations at baseline ($P = 0.05$ each). Interestingly and like the TRAF6 data, treatment with RANKL suppressed the genotype differences, normalizing them compared to matched WT controls.

3.5 *Dusp1* regulates NFATc1-dependent gene expression

NFATc1 is the master-regulator of RANKL-induced OC formation. RANK signaling events elicit NFATc1 nuclear translocation, auto-regulation and induction of NFATc1 dependent genes[24]. Figure 4D shows that *Nfatc1* expression from *Dusp1*^{−/−} sorted populations (CD11b^{hi}, dOCP^{lo} and dOCP[−]) was reduced to 50, 48 and 49%, respectively ($P = 0.00001$ for each group) compared to WT counterparts. Given the observation that *Nfatc1* was significantly reduced in *Dusp1* deficient dOCP yet genes upstream of where not affected by RANKL, we therefore investigated expression of genes dependent upon NFATc1. Based on differences in size between WT and *Dusp1*^{−/−}, DC-STAMP and its newly identified isotype OC-STAMP were selected, as they are directly dependent upon *Nfatc1* and function in OCP fusion/formation. Figure 4E shows that expression of *Tm7sf4* (DCSTAMP) was reduced in all *Dusp1*^{−/−} populations compared with WT controls, with the dOCP^{lo} and dOCP[−] being reduced, respectively, at 51 and 68% ($P = 0.00001$ each). Expectedly, Figure 4F shows that *Ocstamp* gene expression followed similar trends to that of *Tm7sf4* where the all sorted populations were reduced 50, 55 and 56%, between WT and *Dusp1* counterparts ($P < 0.001$, $P < 0.00001$, $P < 0.00001$), respectively. In addition to function, markers of OC maturation were measured following exposure to M-CSF and M-CSF+RANKL. In Figure 4G, Cathepsin K (*Ctsk*) was reduced by 85, 45 and 64% in CD11b^{hi}, dOCP^{lo} and dOCP[−] *Dusp1* deficient populations compared to WT counterparts ($P < 0.0001$ for each). Finally, Figure 4H shows calcitonin receptor (*Calcr*) expression was reduced by 92, 59 and 47% in *Dusp1* deficient CD11b^{hi}, dOCP^{lo} and dOCP[−] compared to WT counterparts ($P < 0.0001$, $P < 0.001$, $P < 0.01$, respectively).

3.6 *Dusp1* Controls Early RANKL-Induced OC Formation by Regulation of NFATc1 Nuclear Translocation

During OC formation, one of the most important early physiological cues promoting OC progenitors towards mature functional OC is RANKL-induced NFATc1 activation and

nuclear translocation. Based on OC enumeration and gene expression, we conclude that the dissection of dOCP into dOCP^{lo} and dOCP⁻ did not highlight any major differences in OC formation or gene expression and as a consequence, all subsequent mechanistic studies utilized the dOCP^{lo/-} combined populations. Confocal analysis depicted in Figure 5 shows that there was a significant reduction in co-localization of NFATc1 and its nuclear counterstain within the *Dusp1* deficient dOCP^{lo/-} populations at the early, 1h ($P<0.05$) and later, 24h ($P<0.05$) and 72h ($P<0.01$) time points. These findings were also corroborated using the NFATc1 transcription factor ELISA kit data presented in Figure 5B. Our results indicate that over each of the early time points (15, 30 and 60 min) there was a significant reduction in NFATc1 bound to the DNA construct from *Dusp1* deficient dOCP ($P<0.05$, $P<0.001$, $P<0.01$, respectively). In agreement with presented confocal data, our results indicate that at each later time points (24, 48 and 72h), *Dusp1* deficient dOCP had significantly less NFATc1 nuclear accumulation. Together these results indicate that *Dusp1* deficiency results in reduced nuclear accumulation at all time points in response to RANKL.

3.7 Inhibition of NFATc1 Nuclear Translocation is Rescued and Enhanced By p38 and JNK Specific Inhibitors

In order to further investigate the signaling pathways needed for MKP-1 controlling NFATc1 nuclear translocation in response to RANKL, a series of experiments were designed along with p38 and JNK inhibitors to “rescue” the observed defect in NFATc1 nuclear translocation. Using specific DNA binding through the TransAM assay, we studied NFATc1 activation in the presence or absence of pharmacological inhibitors. Results shown in Figure 6 indicate that a p38 inhibitor significantly increased the ability of NFATc1 to translocate to the nucleus in *Dusp1* deficient dOCP. More interestingly, the use of JNK inhibitors significantly attenuated the blunting of NFATc1 at every time point ($P<0.001$) (Figure 6B). Together these results indicate that the reduced NFATc1 nuclear activation observed in response to *Dusp1* deficiency is directly related to the sustained activation of the both p38 and JNK.

To verify that OC formation is directly dependent upon proper p38 and JNK signaling, the TRAP assay was performed using the same treatments as described previously, but in the presence of a p38 and JNK inhibitor. Results shown in Figure 6C reveal that *Dusp1*^{-/-} dOCP^{lo/-} cells pretreated with each inhibitor normalized the number of OC formed relative to WT controls. Interestingly, these data suggest that JNK may be a more important signaling molecule in RANKL-induced NFATc1 nuclear translocation and subsequent OC formation. Together these data indicate that MKP-1 regulates NFATc1-driven OC formation by controlling the extent and intensity of p38 and JNK signaling.

4. Discussion

In this study, we made the observation that defined *Dusp1*^{-/-} dOCP formed fewer, smaller and less functional OC in response to RANKL through a mechanism whereby NFATc1 nuclear translocation is impaired. Previous reports show that ablation of NFATc1 eliminated OC formation in the presence of M-CSF and RANKL [24]. Conversely, over-expression of NFATc1 results in OC formation in the absence of MCSF and RANKL [24, 25]. In the current study, we observed a significant reduction in OC formation in *Dusp1* deficient dOCP, which was supported by reduced *Nfatc1* expression, transcriptional activity and NFATc1-dependent gene expression. This is the first study to report that MKP-1 regulates RANKL-induced OC formation through NFATc1 nuclear translocation.

One potential explanation for reduction in OC formation may be the enhanced p38 and JNK signaling observed in our western blot data from the RANKL-stimulated dOCP populations. Much of the current data regarding the role of MKP-1 in osteoclastogenesis has centered on

the anti-inflammatory response to bacterial challenges [20, 21, 26, 27]. Additionally, many studies have shown that p38 and JNK activation are required for normal physiological processes including cell differentiation [25, 28]. Aside from their necessity in RANKL-induced OC formation, other work has demonstrated that the intensity and duration of p38 and JNK activation could influence downstream events. In fact, JNK regulates the phosphorylation status of NFATc1 by inhibiting the target of phosphatase calcineurin, a component of NFATc1 nuclear translocation [29]. Moreover, specific hyper-activation of JNK leads to dysfunctional T cell activation and function by inhibiting NFATc1 translocation [30]. Consistent and prolonged activation of both p38 and JNK is followed by reduced OC formation in *Dusp-1* deficient dOCPs, reduced NFATc1 nuclear accumulation and reduced levels of *Nfatc1* mRNA expression. This evidence may provide a possible explanation for reduced OC formation and *Nfatc1* expression, as NFATc1 nuclear translocation is required for OC maturation. Data presented here indicates that sustained MAPK reduced NFATc1 nuclear translocation. Biochemical inhibitor data supports the idea that both p38 and JNK signaling rescue the ability of NFATc1 to translocate to the nucleus in *Dusp1* deficient dOCPs. In fact, this data showed that addition of these inhibitors to the WT cells significantly reduced their ability to allow for NFATc1 nuclear translocation over time, suggesting MAPK regulation in response to RANKL signaling in a critical component in osteoclast maturation. To corroborate the plausible mechanism, we utilized the TRAP assay to measure OC formation in response to p38 and JNK inhibition prior to stimulation with M-CSF and RANKL. Both p38 and JNK inhibitors independently normalized OC number relative to matched WT. These data are consistent with others showing that p38 signaling is crucial for RANKL-induced OC formation [25].

While our data does support the claim that MKP-1 controls OC formation through an NFATc1-dependent mechanism, an alternative explanation for reduced OC number and size observed in *Dusp1* deficient dOCP might be directly dependent upon the cytokine response. Previous studies from our group reported that in response to *Dusp1* ablation, *A. actinomycetemcomitans* LPS challenged cells exhibit enhanced and prolonged cytokine production including, TNF α , IL-1 β , IL-6 and interestingly, IL-10 [20, 31]. Consistent with our findings, several reviews summarizing multiple groups focused on MKP-1 biology have reported exacerbated inflammatory and anti-inflammatory cytokine production following challenge in *Dusp-1*-deficient mice [4, 32]. Of interest in these studies is the increased IL-10 production seen in response to *Dusp1* knockout. Other work has shown that IL-10 acts as a direct suppressor of OC in bone marrow progenitors stimulated with RANKL [33]. Moreover, IL-10 directly inhibited osteoclastogenesis by reducing *Nfatc1* expression and recruitment to the nucleus [34, 35]. Taken together, it is conceivable that the reduced number of OC seen in *Dusp1*^{-/-} OC progenitor in response to RANKL challenge may actually be a result of enhanced IL-10 production.

To rule out if any discrepancies in OC formation following RANKL stimulation were due to inherent differences in the lineage distribution or in *c-fms* (M-CSF receptor/CD115)/RANK receptor expression we performed immunophenotyping on populations found within the whole HSC and measured gene expression of each receptor. Results from the immunophenotyping whole HSC populations indicated that there were no inherent differences in nearly all of the monocyte or lymphocytic lineages within the bone marrow relative to *Dusp-1* (Supplemental Figure 1). Interestingly, the one difference noted between the genotypes was that CD11b^{hi} was significantly increased within the *Dusp1*^{-/-} population. Additionally, magnetic sorting for these cells revealed that there was more CD11b^{hi} and actually less dOCP^{lo} obtained post-sort (Supplemental Figure 1). However, this result was not significant in our studies since we plated the same number of cells for all experiments. Lastly, using the same immunophenotyping technique, we ruled out presence of any apparent sub-clinical infection or abnormalities in innate or adaptive immune cells from

spleen, thymus or lymph nodes (data not shown). In addition to lineage markers, we measured CD11b/CD115 expression on the surface of the naïve whole HSC population and found that there were no inherent differences in expression as a whole or within sorted populations. To determine expression of these surface receptors, we next measured the mRNA expression levels of *c-fms* in M-CSF only or M-CSF/RANKL stimulated cells at 3-days post sort. These results indicate a significant increase in *c-fms* mRNA occurs in the CD11b^{hi} (macrophage) dOCP⁺ populations both at baseline and in response to RANKL (data not shown). Interestingly, data from the dOCP sorted populations revealed significant differential expression of *c-fms* upon RANKL stimulation between genotypes. Conversely, given that NFATc1 activity is directly dependent on RANKL signaling through the RANK receptor, we measured the expression of *Rank* mRNA amongst sorted cells from each genotype. Since we show no inherent differences between genotypes, these data suggest that both WT and *Dusp-1*^{-/-} dOCP populations have the same capacity to respond to the cytokines. In addition, to surface receptor expression, key signaling intermediates (TRAF6 and c-Fos) were also analyzed as they are necessary for proper OC formation [36]. In the present study, there was increase in expression of TRAF6 in M-CSF treated cells from the *Dusp1*^{-/-} dOCP^{lo} and dOCP⁺ populations. However, this effect was marginalized upon treatment with RANKL, denoting the potential for normal signaling.

Previous work by others has established that *Dusp1* deficient mice have a smaller skeletal phenotype compared to matched WT littermates [37]. Interestingly, our group has also showed that deletion of *Dusp-1* resulted in less OC as a result of reduced Vitamin D-induced RANKL expression in bone marrow stromal populations due to impaired RXR/VDR heterodimer nuclear translocation [38]. Thus, direct effects on osteoclasts or indirect effects via coupled bone turnover may both be operative as manifested in the smaller skeletons of *Dusp1* deficient mice. Furthermore, these studies did not directly address RANKL signaling in dOCP populations nor explain how MKP-1 deficiency impacts RANKL-induced signaling and subsequent OC formation. Hence, the current work provides more mechanistic insight explaining these deficits by linking MKP-1 to its role in NFATc1 nuclear translocation and subsequent osteoclastogenesis.

In summary, the data provided in this study shows that in early response to physiological cues, defined OC progenitor cells (CD45R/B220⁺CD3⁺GR-1⁺CD11b^{lo}CD115⁺), deficient in *Dusp1* are less osteoclastogenesis than WT counterparts. More interestingly, we note that the differences seen in number, size and function in *Dusp1*^{-/-} are likely due to enhanced p38 and JNK MAPK activation leading to a blunting of activated NFATc1 nuclear translocation. These data support that MKP-1, historically shown to be a negative regulator of innate immunity, is essential for OC formation in response to RANKL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Utilizing defined osteoclast progenitor populations, major differences in early OC formation between WT and *Dusp1* deficiency were established where *Dusp-1*/MKP-1 is necessary for early OC formation.
- RANKL-induced NFATc1 nuclear translocation is inhibited in response to *Dusp1* deficiency, affecting downstream OC-specific gene transcription
- Chemical inhibition of p38 or JNK rescues NFATc1 nuclear translocation.

Figure 1. *Dusp1* does not control dOCP surface marker expression

Whole HSC population was analyzed to isolate the dOCP, defined as CD45R⁻GR-1⁻CD11b^{lo/-}CD115⁺. (A) Hierarchical gating showed no differences between WT versus *Dusp1* deficient subpopulations within the whole HSC. (B) Percentage distribution of dOCP^{lo} (CD45R⁻Gr1⁻CD11b^{lo}CD115⁺) and dOCP⁻ (CD45R⁻Gr1⁻CD11b⁻CD115⁺) from WT versus *Dusp1* deficient HSC. No significant differences exist between genotypes. Dot plots represent at least n=4 mice/group, each with base of 30,000 individual events recorded. Bar graphs are numerical representation of the various sub-fractions found within the gated subpopulations.

Figure 2. *Dusp1* controls RANKL-induced p38 and JNK activation

Cells sorted for expression of dOCP were plated, primed with M-CSF for 1 day and stimulated with RANKL (100ng/ml) for 0 to 120 min. (A) Western blot of total cell lysates were probed using antibodies (1:1000) against phospho(p)-p38, p38, pJNK (p-p46/54), JNK (p46/54), pERK (p-p42/44), ERK (p42/44) and GAPDH. Secondary antibodies (1:2000) were used to visualize proteins. (B) Densitometry analysis indicates that p-p38 and pJNK are activated earlier and remain activated throughout the time course in *Dusp1* deficient cells compared to controls. WB is a representative of n=3 independent observations. A p value <0.05 was considered significant.

Figure 3. *Dusp1* controls early RANKL induction of osteoclastogenesis in defined progenitor populations

CD11b^{hi} and dOCP populations were driven to become mature OC with M-CSF and RANKL as described previously up to seven days. OC were counted as TRAP positive cells with 3 nuclei (A–D) Day 3 OC enumeration and size data. (A) Representative images of TRAP assay performed 3-days post stimulation. (B) Day 3 enumeration of TRAP positive OC. dOCP^{lo} and dOCP^{neg} populations were significantly reduced in *Dusp1*^{-/-} samples (C) Day 3 average number of nuclei per OC. (D) Relative size of each formed OC at day 3. (E) OC activity (PIT) assay enumeration of pits per field of view. (F) *Acp5* (TRAP) gene expression normalized to *Gapdh* and plotted relative to WT M-CSF control. (G) TRAP enzyme activity normalized to total protein. Across sorted populations, *Dusp1*^{-/-} formed fewer, smaller and less functional OC compared to matched controls. CD11b^{hi} formed the fewest OC in both WT and *Dusp1*^{-/-} relative to all other sorted groups. Photographs are presented at 10× magnification. Relative size was determined by Image J software analysis. Data are plotted as mean ± SD (n=3, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001)

Figure 4. *Dusp1* controls expression of genes which drive OC formation and fusion

Sorted, defined populations were primed in the presence of M-CSF (10ng/ml) then stimulated with M-CSF (25ng/ml) and RANKL (50ng/ml) for up to 3 days. mRNA expression of Controls and RANKL stimulated cells were analyzed using qPCR. (A) RANK (*Tnfrsf11ra*) expression from WT and *Dusp1*^{-/-} sorted populations. (B) *Traf6* expression. *Dusp1*^{-/-} sorted populations express significantly more TRAF6 at basal (MCSF treatment) and following RANKL stimulation compared to matched WT (C) *c-fos* expression. *Dusp1*^{-/-} populations have significantly less *c-fos* expression from M-CSF treated controls but are not significantly different from WT once stimulated with RANKL. (D) Expression of

NFATc1 from control and RANKL treated populations. *Dusp1*^{-/-} dOCP^{lo} and dOCP⁻ were significantly reduced compared to WT treatments. (E) Expression of DC-STAMP from control and RANKL treated populations. *Dusp1*^{-/-} dOCP^{lo} and dOCP⁻ were significantly reduced compared to WT treatments. (F) Expression of OCSTAMP from control and RANKL treated populations. (G) Cathepsin K expression from control or RANKL stimulation. (H) Calcitonin receptor expression from control or RANKL stimulation. Data presented are representative of n = 3 independent observations run in triplicate. Data differentiates expression patterns between sorted populations relative to WT untreated controls from each group. Values are presented as mean ± 95% CI. (n=3, **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, ***** *P*<0.00001)

Figure 5. *Dusp1* Controls Activated NFATc1 Nuclear Translocation in Response to RANKL Stimulation

Primed WT and *Dusp1*^{-/-} dOCP were treated with 100ng/ml of RANKL for up to 72hrs. (A) Confocal analysis of NFATc1-Alexa488 and Hoescht (nuclear) stain at 24 and 72hrs post-RANKL stimulation. (B) Nuclear translocation of NFATc1 determine by Pearson's correlation coefficient (r) between NFATc1-Alexa488 and nuclear stain. Values are adjusted for time 0 and negative controls. (C) NFATc1 nuclear colocalization determined by the TransAM NFATc1 transcription factor ELISA kit. Data presented are OD 450 values adjusted for negative controls. Values are presented as mean $\pm$ SD. (n=3, * P <0.05, ** P <0.01).

Figure 6. NFATc1 Nuclear Translocation is Rescued in *Dusp1*^{-/-} OCP But Blocked in WT dOCP Treated with p38 and JNK Pharmacological Inhibitors

M-CSF primed WT and *Dusp1*^{-/-} dOCP were subject to 5mM each of SB203580 (p38) and SP600125 (JNK) inhibitors for 1hr prior to stimulation with RANKL. Cells were stimulated with 100ng/ml of RANKL for 24 and 72hrs post-RANKL. (A) Effect of p38 inhibitor (SB203580) on WT and *Dusp1*^{-/-} dOCP at 24 and 72hrs post-RANKL. (B) Effect of JNK inhibitor (SP600125) on WT and *Dusp1*^{-/-} dOCP at 24 and 72hrs post-RANKL. (C) Number of OC formed following p38 and JNK inhibitor (1μM each) prior to stimulation with M-CSF and RANKL. Values are presented as mean ± SD. (n=3, **P*<0.05, ***P*<0.01)