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Ribosome inactivating protein B-chain induces osteoclast differentiation from monocyte/macrophage lineage precursor cells

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ABSTRACT

Human osteoclast formation from mononuclear phagocyte precursors involves interactions between lectins and their receptors. A type-2 ribosome inactivating protein consists of an A chain and a B chain. The glycosylated B chain binds specifically to galactose moieties of sugar molecules. In this study we showed that the recombinant ribosome inactivating protein B-chain (rRBC) could induce osteoclast formation from human monocytes and murine RAW264.7 macrophages. Tartrate-resistant acid phosphatase (TRAP) staining and bone resorption assays demonstrated that differentiation of osteoclast-like cells was induced in the presence of rRBC in a dose-dependent manner. The rRBC-induced osteoclast differentiation was independent of caspase activation and apoptosis induction activity; however, rRBC-induced osteoclastogenesis was dependent on activation of NF-κB, ERK1/2, and p38 MAP kinase. Thus, our data demonstrated that rRBC induced osteoclast differentiation through a non-apoptotic signaling pathway. In addition to triggering apoptosis, the rRBC also induced osteoclast differentiation. According to this study, a novel role is proposed for rRBC in regulating osteoclast differentiation and in osteoimmunology.

Introduction

It is known that normal differentiation of osteoclasts requires tumor necrosis family receptors, such as the receptor activator of nuclear factor-κB (RANK) [1–4]. There is substantial evidence that bone remodeling is a tightly regulated, finely balanced process influenced by subtle changes in proinflammatory and inhibitory cytokines that act primarily but not exclusively through the RANK/RANKL/osteoprotegerin (OPG) system [1–6]. Many of the cytokines and growth factors implicated in inflammatory processes in rheumatic diseases have also been demonstrated to affect osteoclast differentiation and function either directly, by acting on cells of the osteoclast-lineage, or indirectly, by acting on other cell types to modulate expression of the key osteoclastogenic factor, RANKL or its inhibitor, OPG or both [6–11]. Although macrophage-colony stimulating factor (M-CSF) and RANKL are the two essential cytokines for osteoclast differentiation, many other cytokines have positive or negative effects on osteoclastogenesis [6–9,12].

Ribosome inactivating proteins (RIPs) are widespread among higher plants of different taxonomic origins and belong to a group of proteins that inhibit protein synthesis by enzymatically damaging ribosomes [13]. RIPs are mainly divided into two structural families, type 1 and 2. Type 1 RIPs are single-chain proteins with N-β-glycosidase activity [14]. The larger type 2 RIPs consist of two distinct polypeptide chains, A and B. The A-chain of both types of RIPs selectively cleave an adenine from rRNA, which interrupts the interaction between elongation factor II of type 2 RIPs and ribosomes, thereby terminating protein synthesis. Held together with the A-chain by a disulfide bridge, the B-chain of type 2 RIPs is similar to lectin, preferentially binding galactose [15].

Abbreviations: M-CSF, macrophage colony-stimulating factor; RANKL, receptor for activation of NF-κB ligand; PBMC, peripheral blood mononuclear cell; TRAP, tartrate-resistant acid phosphatase; RIP, ribosome-inactivating protein; rRBC, recombinant RIP B-chain.

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Many lectin-like proteins not only induce osteoclast formation [16,17] but also inhibit osteoclast differentiation [18–20]. Moreover, the recombinant RIP B-chain (rRBC) induces apoptosis in Jurkat cells [21], in animal cells, and in tissues with virally-infected and stressed cells [22]. The rRBC binds to galactosyl-terminated residues on the surface of most animal cells [23] and enters the cell by receptor mediated endocytosis [24,25]. The biological roles of rRBC are still not clear. Therefore, this study sought to determine whether the recombinant RIP B-chain can induce osteoclast formation, and to identify the mechanism by which rRBC influences osteoclastogenesis. We found that in addition to triggering apoptosis, rRBC induces osteoclast differentiation in mononuclear phagocyte precursors. Our results indicate that this effect might play an important role in immune response-associated bone absorption.

Materials and methods

Cell lines

We used human peripheral blood mononuclear cells (PBMCs) and the RAW264.7 murine monocytic/macrophagic cell line as model systems of osteoclastogenesis. Both cell types differentiate into osteoclast-like cells in the presence of RANKL plus M-CSF [26,27]. RAW264.7 cells, which were derived from a tumor induced by the Abelson murine leukemia virus, were purchased from the American Type Culture Collection (ATCC; Rockville, MD). For osteoclast culture, RAW cells were seeded in a 96-well plate at a density of 2×10^4cells/well and cultured for 8 days in the presence of 25 ng/ml M-CSF and 50 ng/ml RANKL as the positive control, or 20 ng/ml rRBC. Human PBMCs from healthy donors were separated by gradient centrifugation with Ficoll-Hypaque reagent and were re-suspended in α-MEM supplemented with 10% heat-inactivated FBS. Human PBMCs were allowed to adhere for 3–5 days. The adherent PBMCs were positive for the monocytic/macrophagic markers CD14, CD36, and CD64 at this time point. The adherent PBMCs were then plated in 24-well plates at 5×10^6 cells/well and incubated in the absence or presence of 20 ng/ml rRBC for 30 days.

Osteoclast differentiation

For osteoclastic differentiation, cells were cultured in the presence of either 50 ng/mL human M-CSF (for PBMCs; Peprotech) or murine M-CSF (for RAW264.7; Peprotech), and 50 ng/mL human RANKL (for PBMCs, Peprotech) or murine RANKL (for RAW264.7, Peprotech), or rRBC purified from an *Escherichia coli* expression system. The purified rRBCs were further treated with Detoxi-GelTM Endotoxin Removing Gel (Pierce, Rockford, IL) to remove the lipopolysaccharide (LPS) during purification. In some experiments, cells were preincubated for 40 min with pharmacologic inhibitors of ERK1/2 (PD98059; 20 μM), JNK (SP600125; 10 ng/ml) and p38/MAPK (SB203580; 10 ng/ml) pathways (both from Calbiochem, La Jolla, CA), prior to the addition of rRBCs. In some experiments, caspase inhibitors were used. The general caspase inhibitor, Z-VAD-fmk (Bachem, Bubendorf, Sweden), the caspase-8 specific inhibitor, Z-IETD-fmk, the caspase-6 specific inhibitor, and a NF-κB inhibitor (NF-κB SN50, cell-permeable inhibitor peptides; Calbiochem, San Diego, CA) were applied at the concentration of 20 μM to the medium 30 min prior to treatment. Although we used an endotoxin removing gel to remove the LPS, it might be still contaminated by LPS. So we checked the LPS level in our rRBC samples. We have checked rRBC-induced osteoclast formation and signaling (NF-κB, p38, JNK, and ERK) using BMMs from C3H/HeN and C3H/HeJ to get rid of the LPS effect.

TRAP staining

Osteoclast formation was measured by quantifying cells positively stained with TRAP (Acid Phosphatase Kit 387-A; Sigma-Aldrich, St. Louis, MO). Briefly, specimens were fixed for 30 s and then stained with naphthol AS-BI phosphate and a tartrate solution for 1 h at 37 °C, followed by counterstaining with a hematoxylin solution. Control cells may positively stain red, so TRAP-positive multinuclear cells with three or more nuclei were regarded as osteoclasts and counted under an inverted-phase contrast microscope. We also used a higher concentration of 1 M tartrate (final 20 mM) instead of the tartrate provided in the kit, to suppress the background phosphatase activity [28]. By increasing the concentration of tartrate, the staining of the control cells is suppressed, and the rRBC- and M-CSF/RANKL-treated cells remain positive. The total number of TRAP-positive cells and the number of nuclei per TRAP-positive cell in each well were counted. The morphological features of osteoclasts were also photographed.

Bone resorption assay

In order to confirm the bone resorption ability of differentiated osteoclasts, RAW264.7 macrophages and human adherent PBMCs were plated onto 24-well plates coated with artificial bone slides (Bone Cell Culture System; OsteologicTM, BD) under the same culture conditions as described above. After 8 days of culture, each well was washed with DPBS, and the cells were detached in the presence of 5% sodium hypochlorite for 5 min. The area of resorption was measured using Multi Gauge 3.0 analysis software (FUJIFILM, Tokyo, Japan) [29]. The pits were also photographed.

Expression and purification of recombinant RIP B-chain protein, OPG, and soluble RANK-Fc proteins

The recombinant RIP B-chain protein (rRBC) was expressed in *E. coli* BL21. An overnight culture of each fresh transformant was diluted 1:100 in fresh Luria–Bertani medium (LB) containing kanamycin ($30\ \mu g\cdot mL^{-1}$) and incubated at 30 °C to an OD_{600} of 0.6–0.7. Isopropyl β-D-thiogalactoside (1.0 mM) was added, and the culture was incubated for an additional 3–4 h to induce expression. Cells were then harvested by centrifugation at 4000 *g*, resuspended in phosphate-buffered saline (PBS) at pH 7.4, and disrupted by sonication. The cell lysate was centrifuged at 20,000 *g*. The pellet and the supernatant were each analyzed by SDS-PAGE to detect rRBC. The refolding process was based on a modified protocol as previously described [30]. After the recovery of the rRBC inclusion bodies, the insoluble protein was dissolved in 100 ml of solubilization buffer (8 M urea, 10 mM NaCl, 10 mM Tris–HCl, pH 8.5). The same volume of reduction buffer (50 mM DTT in 50 mM Tris–HCl, pH 8.5) was added drop-wise to the sample, and the mixture was incubated at room temperature for 2 h. The solution was then diluted with oxidation buffer (4 M urea, 5 mM cysteine, 1 mM cystine, 5 mM D-Gal, 50 mM Tris–Cl, pH 8.5) and dialyzed against the same buffer containing half the urea concentration of the previous round, changing the dialysis buffer every 12 h. After the complete removal of urea, the solution was concentrated by dialo-filtration in a Centri-prep 10 apparatus (Amicon). Circular dichroism (CD) measurements and hemagglutination activity were used to check the extent of refolding. For biological assays, soluble rRBC was kept in 2.2% glycine buffer, pH 7.4 to maintain its stability. The level of contaminating LPS in the purified protein was determined using the Limulus Amebocyte Lysate Assay (Charles River Endosafe, Charleston, USA). The final concentration of LPS (<10 ng/mg protein) in cell cultures was never more than 1 pg/mL. To generate soluble recombinant OPG-Fc and RANK-Fc fusion molecules, the coding sequence of OPG/TRAIL-R5 and the extracellular domains of human RANK were isolated by RT-PCR as previously described [31]. The amplified products were ligated in-frame into a BamHI-cut pUC19-IgG1-Fc vector containing the human IgG1 Fc coding sequence. The fusion genes were then subcloned into a pBacPAK9 vector (Clontech, Palo Alto, CA). OPG-Fc and RANK-Fc fusion proteins were recovered from the filtered supernatants of

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the recombinant virus-infected Sf21 cells using protein G-Sepharose beads (Pharmacia, Piscataway, NJ). The bound OPG-Fc and RANK-Fc proteins were eluted with glycine buffer (pH 3) and dialyzed in PBS.

Activation of the MAP kinase pathway

The cell lysate of cells exposed to rRBC at different time points was run on SDS-PAGE and then immunoblotted. Activation of p38 MAP kinase, c-JNK, and ERK1/2 after rRBC stimulation was detected with a specific antibody to phospho-p38 MAP kinase, phospho-JNK, or anti-phospho-ERK (all from Cell Signaling, Beverly, MA) to identify the phosphorylation of these MAP kinase family members.

Preparation of RNA and real-time RT-PCR

Total RNA was prepared and real-time RT-PCR, using the SYBR Green incorporation technique, was performed as described previously [32]. Relative gene expression was calculated using the comparative cycle threshold method, using 18S rRNA as a housekeeping gene. The primers used for NFATc1 were 5′-CGAGCCGTCATTGACTGTGC-3′ (sense) and 5′-GAGCGCTGGGAGCATTCGAT-3′ (anti-sense); c-Fos were 5′-GGTGGAACAGTTATCTCCAG-3′ (sense) and 5′-TGTCTCCGCTTGGAGTGTAT-3′ (anti-sense); DC-STAMP were 5′-GGTTCACTTGAAACTGCACG-3′ (sense) and 5′-TAACACTGAGAGGAACCCAG-3′ (anti-sense); TRAcP were 5′-CTGTCCTGGCTCAAGAAACAG-3′ (sense) and 5′-CATAGTGGAAGCGCAGATAGC-3′ (anti-sense); CatK were 5′-GGCCAACTCAAGAAGAAAACTG-3′ (sense) and 5′-TCTCTGTACCCTCTGCATTTAGC-3′ (anti-sense); calcitonin receptor were 5′-GCGGTGGTATTATCTCTTGG-3′ (sense) and 5′-TTCCCTCATTTTGGTCACAAG-3′ (anti-sense).

Transfection and luciferase assay

RAW264.7 cells were plated on 24-well plates at a density of 7×10^4 cells/well 1 day before transfection. Plasmid DNA was mixed with Lipofectamine reagent and Lipofectamine Plus reagent in serum-free DMEM and transfected into the cells per the manufacturer's protocol. After 20 h of transfection, cells were treated with RANKL (100 ng/ml) or rRBC (100 ng/ml) for 12–16 h. Cells were washed twice with PBS and lysed in reporter lysis buffer (Promega, Madison, WI). Luciferase activity was measured with a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Western blot

To study the effects of rRBC on NF-κB activation, Western blot analysis was used to measure the expression of the p65 protein in nuclear extracts prepared from RAW264.7 cells. Briefly, RAW264.7 cells were washed with ice-cold PBS, scraped, and briefly centrifuged. The cell pellet was then resuspended in a hypotonic lysis buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.5 μg/ml leupeptin, and 6.4% Nonidet P-40 and incubated for 15 min on ice. After another brief centrifugation, the nuclear pellet was collected and suspended in nuclear extraction buffer containing 20 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM 4-(2-aminoethyl) benzenesulfonyl-fluoride, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin. After incubation on ice for 30 min, the nuclear extract was collected, boiled with 3× sodium dodecyl sulfate (SDS) sample buffer, and then subjected to SDS electrophoresis. The concentration of protein in the samples containing the NF-κB p65 subunit was measured using a DC Protein Assay kit (Bio-Rad Laboratories). Equal amounts of protein were size-fractionated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. The blot was blocked overnight in 5% non-fat dry milk in Tris-buffered saline with 0.5% Tween-20 (TBST), and incubated with primary mouse anti-NF-κB p65 antibody (1:200, Santa Cruz Biotechnology) on a rocking platform at 4 °C for 24 h. After washing in TBST, the blots were incubated with horseradish peroxidase conjugated anti-mouse antibody (Santa Cruz Biotechnology) (diluted in 1:5000) for 1 h at room temperature. The membrane was then washed again and exposed to film following chemiluminescence reagent treatment with the ECL plus Western blotting reagents (Amersham). Bands were quantified using densitometry. Each blot was then stripped and reprobed with anti-β actin antibodies, thus allowing normalization of expression between samples. The results of this assay were confirmed by repeating the experiment 3 times.

Statistical analysis

Each experiment was performed in duplicate, and its average was included for quantification. Data are expressed as the mean ± SD of averages from at least three experiments. Differences in means were assessed by the Student's *t*-test. A p value of <0.05 was considered statistically significant.

Results

The rRBC induces osteoclast differentiation in human PBMCs and murine RAW264.7 cells

We first investigated rRBC-induced osteoclast differentiation in preosteoclastic precursors. Monocyte precursors can differentiate into osteoclast-like cells in the presence of RANKL (50 ng/ml) and M-CSF (25 ng/ml). This differentiation was verified not only by the appearance of multinuclear cells but also by the presence of TRAP staining. Surprisingly, we found that rRBC itself could mimic the effects of M-CSF and RANKL in a dose-dependent manner (Fig. 1). The stimulating effect of rRBC on osteoclast differentiation was observed in both human PBMCs and murine RAW264.7 cells. Within 30 days of culturing human adherent PBMCs on glass coverslips with increasing doses of rRBC, the formation of large, multinucleated $TRAP^+$ cells was observed, characterized by the appearance of TRAP-positive giant multinucleated cells with more than three nuclei (Fig. 1). Similarly, multinucleated $TRAP^+$ cells were found after 8 days in murine RAW264.7 cells when cultured with rRBC, and were characterized by the appearance of TRAP-positive giant multinucleated cells (Fig. 1).

To exclude the possibility of rRBC-induced osteoclast differentiation being due to the effects of LPS contamination during the rRBC purification process, we used polymyxin B gel (Detoxi-GelTM Endotoxin Removing Gel) to remove the LPS, and similar effects were observed. Moreover, to further confirm that the osteoclastogenic effects were due to direct binding of rRBC to its receptors, we added *N*-acetyl-galactosamine [13,21,23,24] to the cultures to neutralize rRBC. According to the results shown in Fig. 1, the osteoclast differentiation ability was significantly reduced after adding *N*-acetyl-galactosamine to the cultures, indicating that the effect of osteoclast differentiation induced by rRBC was via direct interaction between rRBC and its receptors.

To determine whether differentiated osteoclast-like multinuclear cells induced by rRBC, RANKL and M-CSF have similar characteristics to those of osteoclasts, functional identification was confirmed in an in vitro culture system. Using commercial calcium phosphate apatite as a resorption substrate, the degree of pit formation caused by rRBC- and cytokine-treated human PBMCs and RAW264.7 cells was compared to cultures of cells without differentiation agents (Fig. 2). Lacunar resorption was observed in human PBMCs and RAW264.7 cells treated with rRBC for 8 days on calcium phosphate apatite plates. Our results clearly demonstrated that multinucleated TRAP-positive cells possessed major characteristics of functional osteoclasts. In addition, the rRBC-induced multinucleated TRAP-positive cells expressed CD51/61 (the vitronectin receptor), a specific

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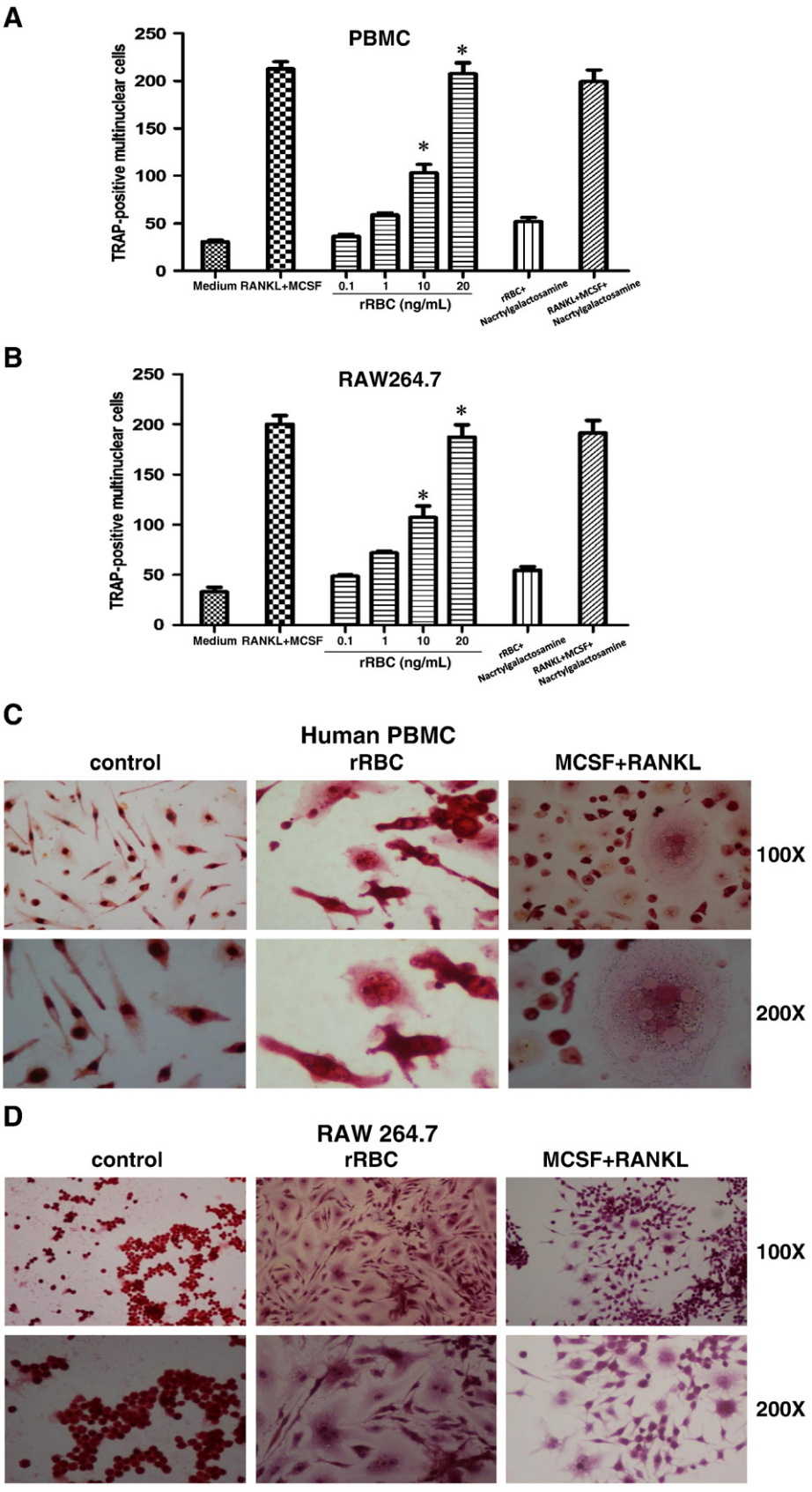

Fig. 1. The rRBC-induced formation of osteoclast-like multinucleated cells from human monocytes and RAW264.7 macrophages. (A) Human peripheral blood mononuclear cells (PBMCs) were plated in 96-well plates at 1.5×10^5 cells/well, and the next day the adherent monocytes were treated with rRBC at the concentrations indicated, as well as M-CSF (200 ng/ml), and RANKL (100 ng/ml) for 30 days. After incubation, cells were subjected to the TRAP assay. Cell morphology was examined by light microscopy, and the number of TRAP-positive multinuclear cells was quantified. (B) RAW264.7 cells were seeded at 2×10^3 cells/well in 96-well plates and incubated for 7 days with rRBC at the concentrations indicated, as well as M-CSF (20 ng/ml) and RANKL (50 ng/ml). (C and D) Images represent 100× and 200× magnifications. The rRBC-induced formation of osteoclast-like multinucleated cells from human monocytes (C) and RAW264.7 macrophages (D). Data in (A) and (B) represent the mean ± SD from three to six independent experiments. $^*p<0.005$ compared with the control.

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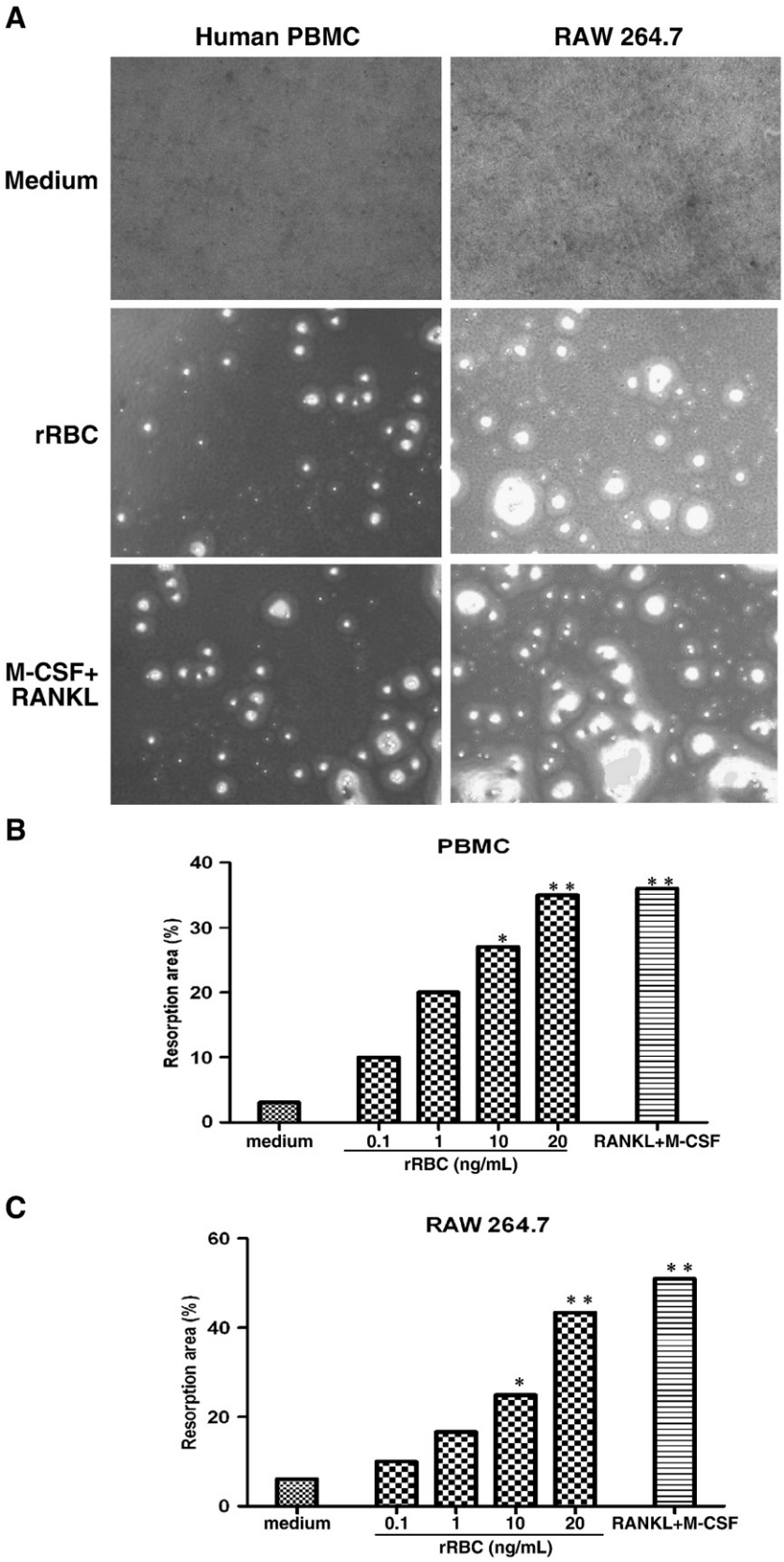

Fig. 2. The rRBC-induced osteoclast bone resorption activity. (A) After treatment with different agents, RAW264.7 macrophages and human monocytes were plated on an artificial bone matrix slide (BD BioCoat™ Osteologic™ Bone Cell Culture System) and then cultured with rRBC or cytokines. Cells were detached after 2 weeks (in the human monocyte group) or 8 days (in the RAW264.7 group) of culture. The area of resorption was measured by image analysis using Multi Gauge 3.0 analysis software (FUJIFILM, Tokyo, Japan) [29]. The pits were also photographed. (B) and (C), rRBC-induced osteoclast bone resorption activity from human monocytes (B) and RAW264.7 macrophages (C). Data in (B) and (C) represent the mean ± SD from five independent experiments. $^{*}p<0.05$, $^{**}p<0.005$, compared with the medium only control. (D). Expression of osteoclast markers on osteoclasts differentiated by the rRBC. RAW264.7 cells were treated with vehicle, rRBC (20 ng/ml), or M-CSF (20 ng/ml) plus RANKL (50 ng/ml) for 5 days. After incubation, cells were treated with anti-CD51/61-FITC and analyzed by flow cytometry. Similar results were obtained from three separate experiments.

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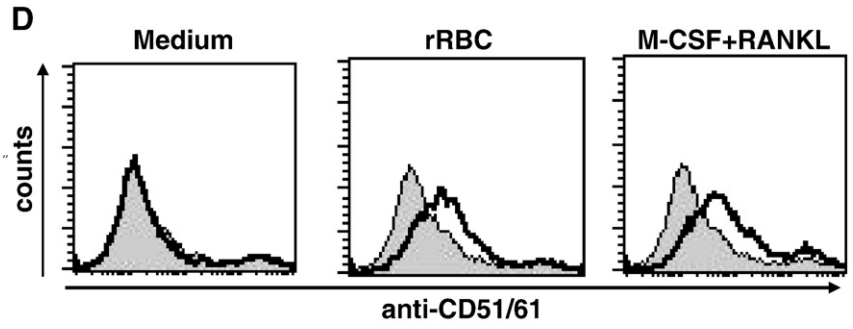

Fig. 2 (*continued*).

marker of osteoclasts [33] (Fig. 2D). Taken together, the large multinucleated $TRAP^+$ cells formed in rRBC-treated cultures had similar morphologic features as those formed following RANKL stimulation.

The rRBC-induced osteoclast formation was neutralized by osteoprotegerin (OPG), but was independent of RANKL

To investigate whether rRBC-induced osteoclast formation occurs via the RANKL/RANK pathway, OPG and RANK-Fc were added to rRBC-treated RAW264.7 cell cultures. The addition of excess molar concentrations of RANK-Fc demonstrated no significant decrease in osteoclast formation in cultures that had been treated with rRBC alone (Fig. 3). However, the osteoclast differentiation ability induced by rRBC was completely abolished after adding soluble OPG proteins to the cultures, indicating that OPG can bind to rRBC and can neutralize the effect of osteoclast differentiation induced by rRBC. In contrast, the osteoclast differentiation ability induced by RANKL plus M-CSF was completely inhibited by adding excess molar concentrations of either soluble RANK-Fc or OPG proteins to the cultures (Fig. 3).

The rRBC-induced osteoclast differentiation activity is independent of apoptosis induction and caspase activation, but is dependent on NF-κB activation

The rRBC engagement induces activation of caspase pathways and NF-κB [24]. It is not clear whether these signaling pathways are involved in rRBC-induced osteoclast differentiation. We examined whether the rRBC-induced osteoclast differentiation activity is dependent on the activation of caspase and induction of apoptosis after rRBC engagement. As seen in Fig. 4, our results demonstrated that the osteoclast precursors were resistant to rRBC-induced apoptosis and that there was no difference in the ability to induce osteoclast differentiation in the presence or absence of the pan-caspase inhibitor, Z-VAD-fmk, or the caspase-8 specific inhibitor, Z-IETD-fmk, indicating that rRBC-induced osteoclast differentiation activity is independent of the activation of caspases. In contrast, the rRBC-induced osteoclast differentiation ability was abolished after adding the NF-κB inhibitor to the cultures, indicating that rRBC-induced osteoclast differentiation activity is dependent on NF-κB activation (Fig. 4). To further examine rRBC- and RANKL-induced NF-κB activation, we used a reporter assay involving transient transfection into RAW264.7 cells. An NF-κB luciferase reporter plasmid was transfected in the presence or absence of RANKL or rRBC. RANKL or rRBC stimulation induced NF-κB transcriptional activity (Fig. 4C). Medium alone had no effect on nuclear NF-κB-p65 levels in RAW264.7, but adding the RANKL or rRBC to the RAW264.7 culture media caused a 3-fold increase in NF-κB activation within 30 min (Fig. 4D).

Activation of MAP kinases in rRBC-induced osteoclastogenesis

Three members of the MAP kinase family, namely ERK, JNK, and p38 MAPK, have been implicated in the mediation of cytokine-regulated osteoclastogenesis [34–39]. To elucidate the signaling

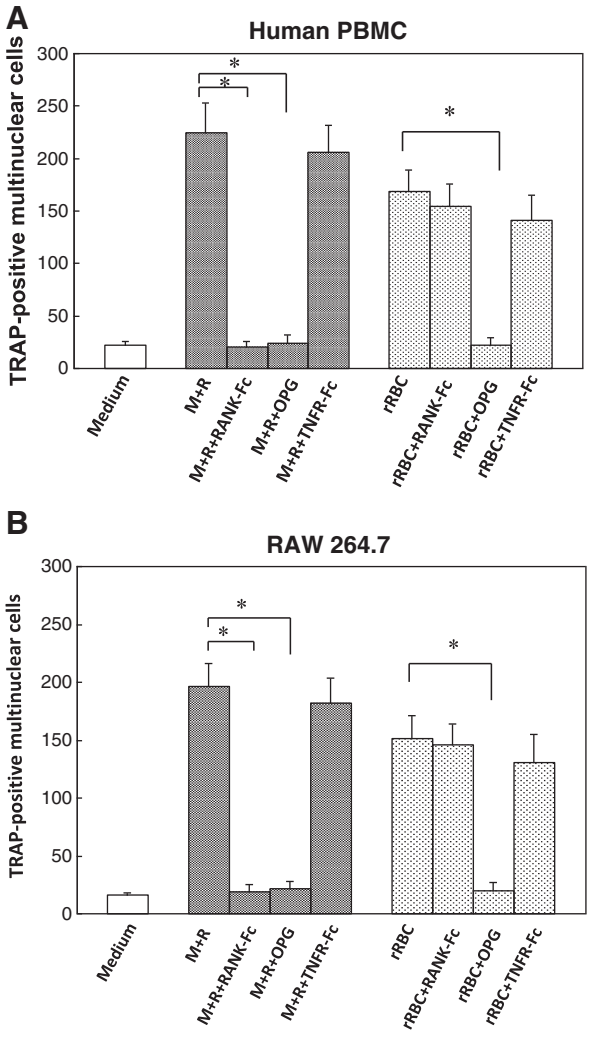

Fig. 3. The rRBC-induced osteoclast formation was neutralized by osteoprotegerin (OPG), but was independent of RANKL and tumor necrosis factor (TNF). Human PBMCs (A) and RAW264.7 macrophages (B) were treated with rRBC (15 ng/ml) and RANKL plus M-CSF, in the presence or absence of soluble receptors (OPG, RANK-Fc, or TNFR-Fc) (300 ng/ml). After a 7-day (RAW264.7 cell) or 30-day (human PBMC) incubation, cells were subjected to the TRAP assay. The data represent the mean ± SD from at least three independent experiments. $^*p<0.05$.

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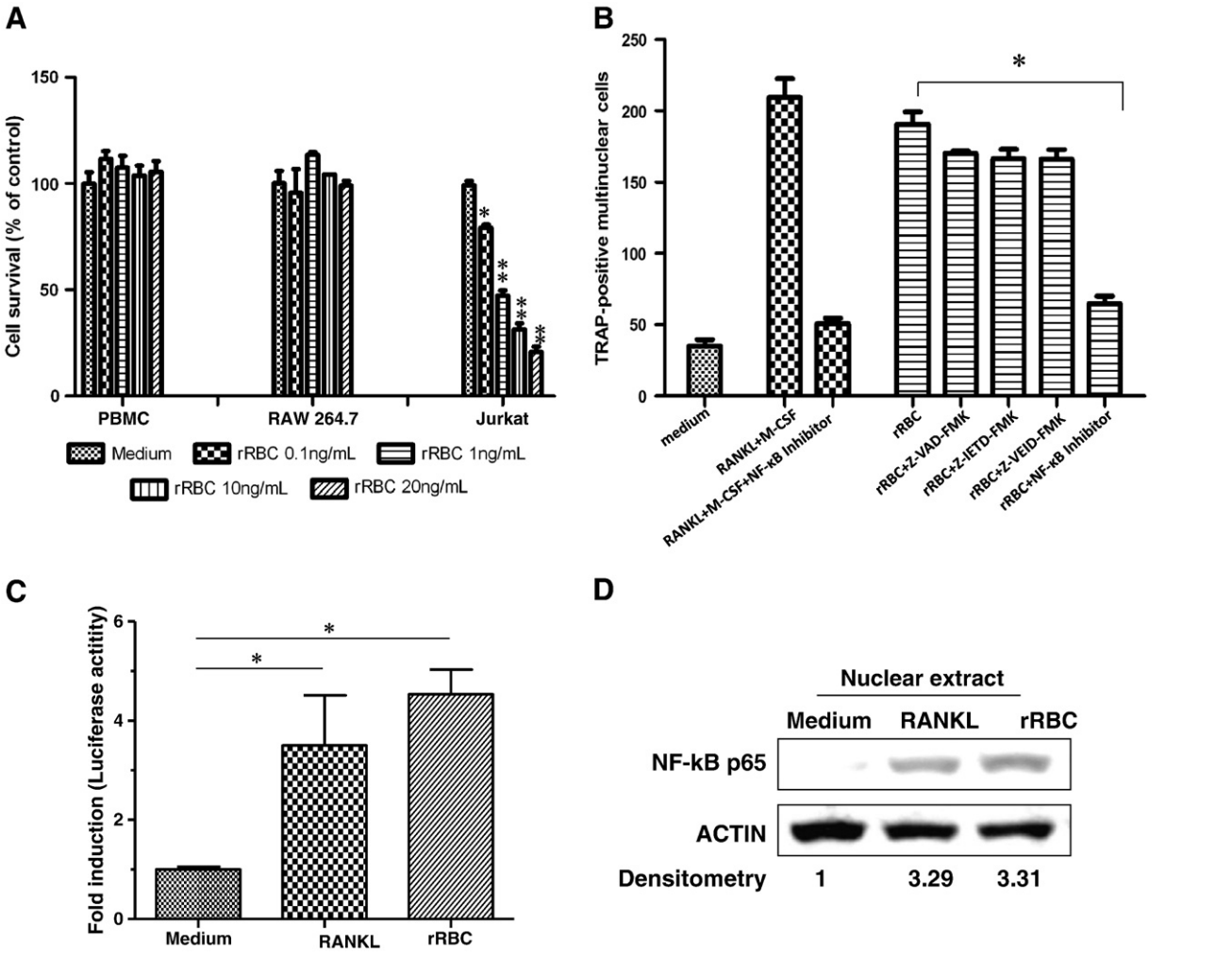

Fig. 4. The rRBC-induced osteoclast differentiation activity is independent of caspase activation and induction of apoptosis, but is dependent on NF-κB activation. (A). Human adherent PBMCs, murine RAW264.7 cells, and the human T cell line, Jurkat, were incubated with rRBC proteins for 18 h. Cell survival was assayed by the MTT reduction assay (Chemicon International Inc., Temecula, CA, USA). The data represent the mean ± SD from at least three independent experiments.*$p<0.05$, **$p<0.005$ compared with the medium alone control. (B). RAW264.7 cells were treated with rRBC (20 ng/ml) and RANKL plus M-CSF, in the presence or absence of the pan-caspase inhibitor, Z-VAD-fmk, the caspase-8 specific inhibitor, Z-IETD-fmk, the caspase-6 specific inhibitor, Z-VEID-fmk, and an NF-κB inhibitor (NF-κB SN50, cell-permeable inhibitor peptides; Calbiochem, San Diego, CA). After an 8-day incubation, cells were subjected to the TRAP assay. The data represent the mean ± SD from at least three independent experiments. *$p<0.05$. (C). RAW264.7 cells were transfected with NF-κB luciferase reporter. After 24 h of transfection, cells were further cultured with RANKL (100 ng/ml) or rRBC (100 ng/ml) for 12–16 h and then lysed for luciferase assay. Luciferase activity was measured using a dual-luciferase reporter assay system. Data represent means ± SD of triplicate samples. Results are representative of at least three independent sets of similar experiments. *$p<0.05$. Panel (D) shows the effects of RANKL (100 ng/ml), rRBC (100 ng/ml) on nuclear NF-κB-p65 levels in RAW264.7 cells after 30 min of treatment. RANKL activated NF-κB in RAW264.7 cells. Medium alone had no effect on NF-κB activation. The densitometry reading of each band was normalized by its β-actin value. The readings shown at the bottom of the panel are the relative fold changes over the values of control RAW264.7 cells treated only with M-CSF.

pathways underlying the action of rRBC, we examined the activation of ERK, JNK, and p38 MAPK in RAW264.7 cells treated with rRBC, RANKL, and M-CSF by immunoblotting. Similar to the stimulation by RANKL and M-CSF alone, rRBC markedly induced the phosphorylation of ERK, JNK, and p38 MAPK (Fig. 5A). Meanwhile, the data presented in Fig. 5A indicate that rRBC induces prolonged activation of JNK, ERK and p38. So, we examined further the similarities and differences between rRBC and RANK pathways in Fig. 5B. The prolonged activation of MAP kinases results in different expression patterns of key transcription factors, namely c-fos and NFATc1, and may further explain why the cells need longer exposure to rRBC than to RANKL to differentiate into osteoclasts. PD98059 [a selective mitogen-activated protein/ERK kinase (MEK) inhibitor], SP600125 [a selective (JNK) inhibitor], as well as SB203580 (a selective p38 MAPK inhibitor) were used to further confirm the involvement of ERK, JNK and p38 MAPK in the actions of rRBC and RANKL. As shown in Fig. 5C, differentiation of RAW264.7 macrophages into TRAP-positive multinuclear cells was inhibited by these kinase inhibitors. These results suggest that ERK, JNK and p38 MAPK signaling pathways are critical to the osteoclastogenic effect of the rRBC. We next used real-time RT-PCR to examine the expression levels of downstream osteoclast-specific genes, including osteoclast-specific transcription factors (NFATc1, c-fos), genes involved in osteoclast fusion (DC-STAMP), genes involved in osteoclast function (TRAcP, CatK), and genes involved in osteoclast regulation (calcitonin receptor) (Fig. 5D). The results revealed no significant difference in expression of downstream osteoclast-specific genes between cells exposed to rRBC and RANKL.

Discussion

In this study, we demonstrated that the rRBC induced the differentiation of monocyte/macrophage lineage precursor cells into osteoclast-like cells in both human monocytes and murine RAW264.7 macrophages. Osteoclast formation from mononuclear phagocyte precursors involves interactions between TNF ligand superfamily

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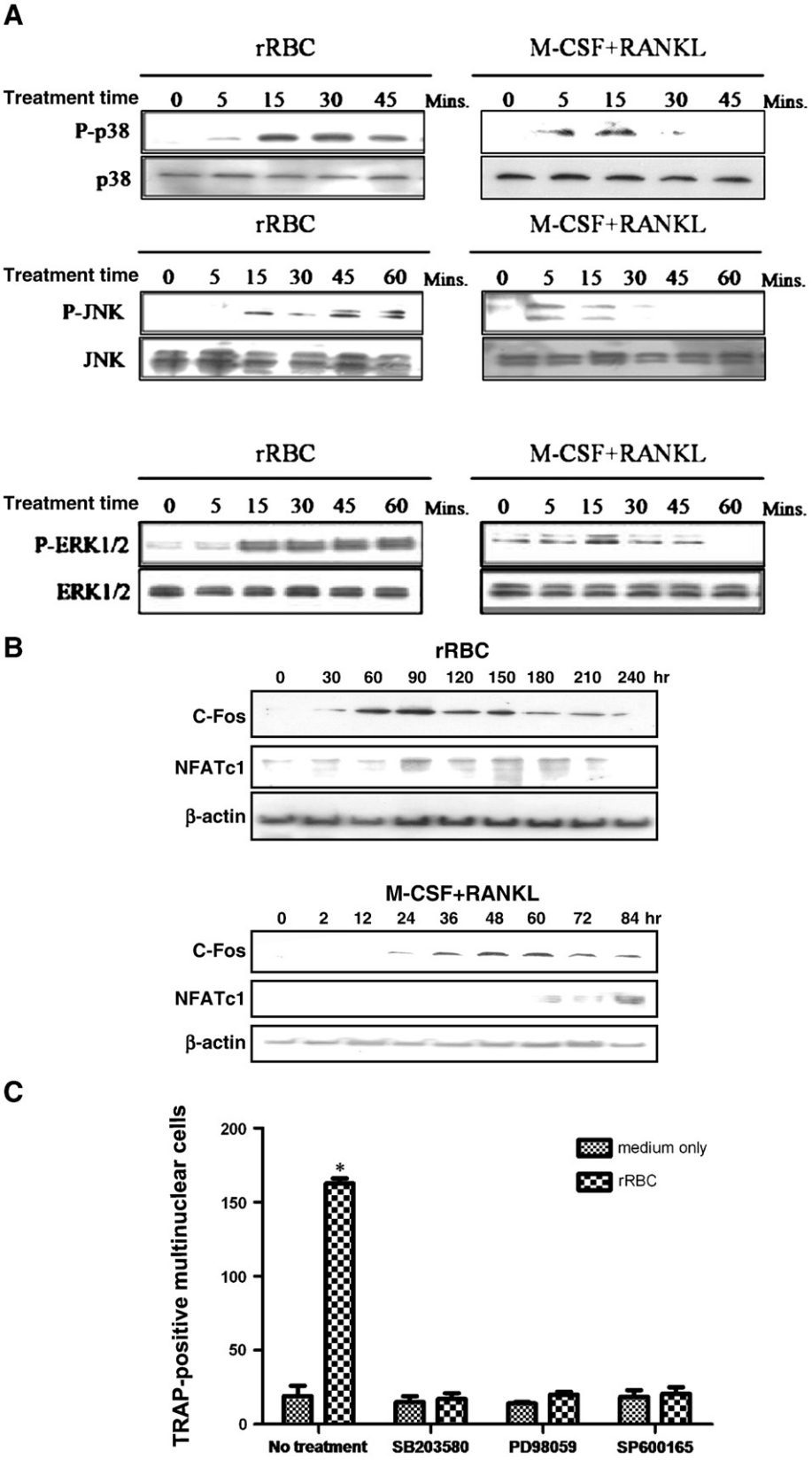
members and their receptors [1,3–7]. In this study, we demonstrate a novel and unique action of rRBC in osteoclastogenesis, and provide new insights into the molecular mechanism which may implicate osteoimmunology in the immune response associated with bone absorption. The rRBC-induced osteoclast differentiation is independent of caspase activation and apoptosis signaling. Thus, our data demonstrate that rRBC induces osteoclast differentiation via direct engagement with the rRBC through a signaling pathway distinct from apoptosis. The present observation that rRBC can enhance osteoclast differentiation from monocyte lineages through activation of NF-κB,



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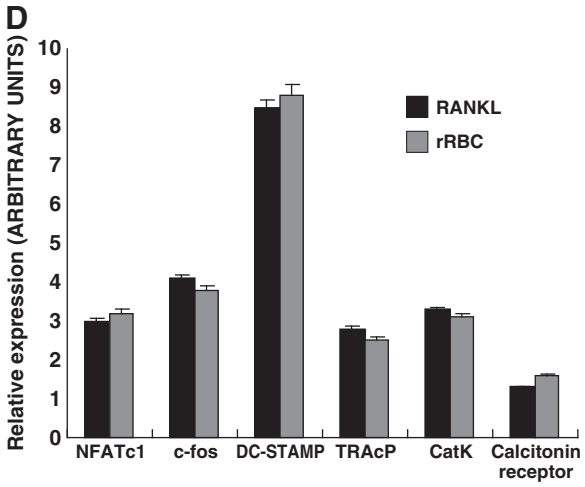

Fig. 5. The rRBC induces activation of MAP kinases. (A). RAW264.7 cells were treated with vehicle, rRBC (20 ng/ml), M-CSF (20 ng/ml), or RANKL (50 ng/ml) for the indicated time periods. After stimulation, cells were solubilized, and cell lysates were subjected to Western blot analysis of p38 MAPK, JNK, and ERK1/2 protein expression. The trace shown in the top panel for each group indicates the immunoreactivity of the phosphorylated kinase. The same membrane was then stripped and reprobed with the kinase antibody recognizing the total protein level of kinase (bottom panel). The results are representative of three separate experiments. (B) Whole cell lysates were harvested from cultured cells and analyzed by Western blot using Abs specific for NFATc1 (Santa Cruz Biotechnology), c-Fos (Calbiochem, San Diego, CA), and actin (control). (C). The rRBC-induced osteoclast formation is dependent on ERK, JNK and p38 MAPK activation. RAW264.7 cells were pretreated with vehicle, 0.5 ng/ml PD98059, 10 ng/ml SP600165, or 10 ng/ml SB203580 for 20 min prior to stimulation with rRBC (20 ng/ml) or RANKL plus M-CSF. After an 8-day culture in RAW264.7 macrophages, cells were subjected to the TRAP assay. The data represent the mean ± SD from at least three independent experiments. $^{*}p<0.05$ compared with the corresponding control. (D) Effect of rRBC and RANKL on human osteoclast gene expression. PBMCs were isolated and cultured in MEM supplemented with 10% charcoal/dextran-treated FCS containing RANKL (50 ng/ml) and M-CSF (25 ng/ml) or rRBC (50 ng/ml). Total RNA was extracted after an 8-day culture and real-time RT-PCR was performed for NFATc1, c-fos, DC-STAMP, TRAcP, CatK, and calcitonin receptor. Expression was normalized to that of 18S rRNA. Data are means ± SD of triplicate wells.

JNK, ERK, and p38 MAPK further strengthens the significance of distinct signaling to modulate cell function in addition to inducing apoptosis. Aims for our future experiments include identifying the signaling pathways in more detail.

The soluble TNF receptor superfamily molecule, OPG, can bind to RANK [40]. OPG has been demonstrated to be capable of neutralizing the osteoclastogenesis activity of RANKL and is considered to be a natural RANKL inhibitor. In this study, our results showed that rRBC-induced osteoclast differentiation was significantly reduced by OPG but not by RANK-Fc, indicating that OPG can inhibit osteoclast differentiation activity induced by both RANKL and rRBC. This observation further supports the role of OPG as a natural antagonist in suppressing osteoclastogenesis activity in vivo. The distinct antagonism of RANK-Fc and OPG suggests that rRBC and M-CSF/RANKL primarily use distinct pathways to elicit osteoclastogenesis.

In conclusion, using an in vitro culture system, we provide evidence that rRBC is a novel effector molecule that enhances osteoclast-like cell formation. We also define the crucial role of NF-κB, ERK, JNK and p38 MAPK signaling in inducing osteoclastogenesis. The novel function of rRBC demonstrated in this study indicates a new role for the rRBC in regulating osteoclastogenesis, and will be helpful in developing better strategies for treating inflammation-induced bone resorption in the future.

Disclosure statement

The authors have nothing to disclose.

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