6-Methylprednisolone down-regulates IRAK-M in human and murine osteoclasts and boosts bone-resorbing activity: a putative mechanism for corticoid-induced osteoporosis

Alessandra Soares-Schanoski,*,[1] Vanesa Gómez-Piña,*,[1] Carlos del Fresno,*,[1] Alexandro Rodríguez-Rojas,* Felipe García,† Alberto Glaría,‡ Miriam Sánchez,* María Teresa Vallejo-Cremades,* Rosa Baos,* Pablo Fuentes-Prior,§ Francisco Arnalich,|| and Eduardo López-Collazo*,[2]

Research Unit, 'La Paz' Hospital, and ||Department of Medicine, 'La Paz' Hospital Medical School, Universidad Autónoma de Madrid, Madrid, Spain; †Discovery Unit, EMPIREO S.L., Madrid, Spain; ‡Instituto Madrileño de Oncología, Madrid, Spain; and §Cardiovascular Research Center, ICCC-CSIC, Sant Antoni Ma. Claret 167, Barcelona, Spain

Abstract: Osteoclasts are large, multinucleated cells, which originate from the fusion of macrophages. They play a central role in bone development and remodeling via the resorption of bone and are thus important mediators of bone loss, which leads to osteoporosis. IL-1R-associated kinase (IRAK)-M is a pseudokinase, which acts as a negative modulator of innate immune responses mediated by TLRs and IL-1R. Recently, it has been reported that IRAK-M also participates in the control of macrophage differentiation into osteoclasts. In addition, it was shown that IRAK-M knockout mice develop a strong osteoporosis phenotype, suggesting that down-regulation of this molecule activates osteoclast-mediated bone resorption. We studied the effect of the osteoporosis-inducing glucocorticoid, 6-methylprednisolone (6-MP), on IRAK-M expression in osteoclasts. Our results showed that osteoclasts, derived from THP-1 and RAW cells as well as human blood monocytes, differentiated into osteoclasts, express high levels of IRAK-M at mRNA and protein levels. In addition, 6-MP down-regulates IRAK-M expression, which correlates with an increased activation of bone resorption. These findings suggest a mechanism of corticosteroid-induced osteoporosis and open new avenues for treating this endemic disease of Western societies. *J. Leukoc. Biol.* 82: 700–709; 2007.

Key Words: *glucocorticoids · TLR · IL-1R*

INTRODUCTION

Osteoporosis, the most prevalent, metabolic bone disease, is characterized by compromised bone strength predisposing an increased risk of fractures in response to mild trauma [1–4]. Social and economic burden of osteoporosis is growing rapidly as a result of aging of the world population; the disease, therefore, represents a severe problem to current public health systems. Postmenopausal women [3–6] and patients under glucocorticoid treatment [4, 7–10] are the main groups of persons who suffer from osteoporosis. Despite several studies describing different aspects of disease onset and progression, we still lack a clear picture of the ultimate molecular mechanism(s) underlying osteoporosis in humans.

Normal bone turnover involves a balance between the processes of bone formation and resorption [3, 11–13]. Osteoclasts, multinucleated cells originating from the fusion of macrophages, are essential for bone development and remodeling as a result of their unique capacity for bone resorption [1, 13–15]. The mechanism of bone removal involves its acidification and proteolytic digestion of collagen and other proteins of the extracellular matrix and counteracts osteoblast secretion of osteoid into the resorption cavity [12, 16]. Therefore, the balance between osteoblast and osteoclast activity regulates bone mass critically. Accordingly, it has been shown that increases in osteoclast number and/or activity lead to several diseases associated with general and localized bone loss (e.g., osteoporosis and rheumatoid arthritis, respectively; refs. [1, 11, 15]).

A family of kinases, IL-1R-associated kinases (IRAKs), is essential for transducing signals downstream of TLR/IL-1R signaling into a potent inflammatory response. Four members of this family have been identified to date, of which IRAK-1 and IRAK-4 are catalytically active, and IRAK-2 and IRAK-M lack enzymatic activity as a result of single-point mutations of active site residues [1, 17–22]. In particular, strong evidence associates IRAK-1 and IRAK-4 with inflammatory responses elicited by TLR or IL-1R activation [1, 17–23]. In contrast, IRAK-M acts as a negative regulator of TLR/IL-1R signaling,

[1] These authors contributed equally to this work.

[2] Correspondence: Research Unit, 'La Paz' Hospital, Madrid 28046, Spain. E-mail: elopezc.hulp@salud.madrid.org

Received November 12, 2006; revised May 16, 2007; accepted May 16, 2007.

doi: 10.1189/jlb.1106673

apparently by preventing dissociation of IRAK-1 from the critical bipartite adaptor, MyD88, and maintaining the integrity of the complex among the kinases, TNFR-associated factor-6, and TLR [1, 17–23].

Expression of IRAK-M is not only restricted to myeloid cells such as monocytes and macrophages but is also conserved after the fusion process, which results in their multinucleation and differentiation into osteoclasts [1]. Recently, it has been reported that the pseudokinase plays a critical, regulatory role during osteoclast differentiation and activation and concomitantly, in the development of osteoporosis [1]. Indeed, IRAK-M knockout mice showed accelerated osteoclastogenesis, and their osteoclasts possessed increased half-life and hyperactivation of NF-κB and MAPK signaling pathways via engagement of IL-1R/TLR pathways. Consequently, animals developed severe osteoporosis, which started 4 months after birth [1]. In contrast, high levels of IRAK-M are expressed in osteoclasts from normal mice, which counteract their (hyper) activation [1]. Altogether, IRAK-M emerges as a key regulator of the molecular mechanism(s), underlying bone loss as a result of osteoclastic bone resorption, at least in mice.

As mentioned above, it is well known that glucocorticoids induce osteoporosis in humans by a mechanism that is currently not well understood. In particular, daily glucocorticoid treatment increases the risk of fracture [8, 24, 25]. Recently, it has been reported that fracture risk increases rapidly (as early as 3 months) after the start of steroid therapy but reverses sharply toward baseline after its discontinuation [8]. Furthermore, a strong correlation between glucocorticoid doses and loss of bone mineral density has been reported [10]. In vitro and in vivo studies have indicated that excess glucocorticoids promote osteoblast apoptosis while extending the lifespan of pre-existing osteoclasts [8–10, 26]. Several authors suggest that corticosteroid-induced ostoporosis results from direct and indirect actions of these drugs on bone and calcium homeostasis [7–9]. However, we still lack a complete picture of molecular mechanisms, which ultimately lead to this disease.

Taking into account all information discussed above, we hypothesized that the well-characterized glucocorticoid, 6-methylprednisolone (6-MP), could induce osteoporosis through selective down-regulation of IRAK-M expression in osteoclasts. To verify this hypothesis, we studied IRAK-M expression in three different models of osteoclast-like cells, two from human origin and one from mice, and analyzed the effect of 6-MP on IRAK-M levels and osteoclast activity.

MATERIALS AND METHODS

Reagents

All reagents were of the highest quality available and were obtained from Merck (Darmstadt, Germany), Boehringer (Mannheim, Germany), or Sigma-Aldrich (St. Louis, MO, USA). LPS (from *Salmonella abortus*) was generously provided by Dr. Chris Galanos (Max Planck Institut für Immunobiologie, Freiburg, Germany). Goat polyclonal antibodies against IRAK-M and β-actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The mAb against mouse ERK (p42, total and phosphorylated forms) were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA). The recombinant, soluble form of human receptor for activation of NF-κB (sRANKL) was obtained from PeproTech EC (London, UK); soluble 6-MP (Urbason) was from Aventis (Bridgewater, NJ, USA), and vitamin D3 [Vit-D3; Calcijex: $1\alpha,25(OH)_2D_3$] was generously provided by Dr. Llanos Soler (Departamento de Medicina Interna, Hospital La Paz, Madrid, Spain). Each vial of Urbason contains 40 mg 6-MP and the excipients, disodium phosphate and monosodium phosphate. The actual concentrations of 6-MP are indicated in all experiments (usually 40 ng/ml). The doses used were based on our own therapeutic experience.

Cell lines and culture conditions

THP-1 human monocytes and RAW 264.7 murine macrophage cells (American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI-1640 medium (Gibco, UK), supplemented with heat-inactivated 10% FBS and 1% L-glutamine in a 5% CO_2 environment at 37°C, and were seeded in 60 × 15 mm cell culture dishes at a concentration of 2×10^6 cells/well.

PBMCs were isolated from blood of healthy donors by centrifugation on Ficoll-Hypaque Plus (Amershan Biosciences, Netherlands). Cells were cultured initially for 2 h to a density of 10^6 cells/ml in DMEM supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). After this period, the supernatant was removed, and adherent cells (2×10^6 per well) were cultured in the same medium supplemented with 10% heat-inactivated FBS. These cultures exhibited 80% CD14+ cells on average.

For differentiation into osteoclasts, THP-1 and RAW cells were cultured for 6 days in the presence of 40 ng/ml Vit-D3 or sRANKL, respectively. Human monocytes from healthy volunteers were differentiated into osteoclasts by culturing in the presence of 30 ng/ml sRANKL and 25 ng/ml M-CSF for 7 days. In some experiments, 6-MP was additionally added to culture medium at indicated concentrations.

Tartrate-resistant acid phosphatase (TRAP) assay

After 6 days in culture, nonadherent cells were removed, adherent cells were stained for TRAP (Sigma-Aldrich), and nuclei were counterstained with hematoxylin. Cells were viewed by light microscopy using a Zeiss Axioplan 2 microscope (Carl Zeiss, Germany) and photographed with a Coolpix 3200 digital camera (Nikon, Tokyo, Japan), and images were finally transferred using Nikon View 5 software.

RNA and protein isolation

After treatment, cells were washed once in PBS, and their RNA was isolated using TRI-Reagent (IMICO, Cincinnati, OH, USA). Purified RNA was treated with RNase-free DNase I (Amersham Biosciences, Piscataway, NJ, USA), and cDNA was obtained by RT of 1 μg of this RNA using a poly(dT) oligonucleotide primer (Roche, Palo Alto, CA, USA). Protein fractions were isolated as recommended (Roche), and the final pellets were dissolved in a solution containing 1% SDS.

mRNA quantification

The expression levels of osteoclast-associated receptor (OSCAR), IRAK-M, and 18S were analyzed by real-time quantitative PCR (Q-PCR; LightCycler, Roche Diagnostics, Indianapolis, IN, USA), using cDNA obtained as described above. Real-time PCR was performed using a Fast-Start DNA master SYBR Green system (Roche) and specific primers (listed below). All results were normalized to the expression of the 18S gene, and the cDNA copy number of each gene of interest was determined using a seven-point standard curve, and standard curves were run with each set of samples; the correlation coefficients r^2 for the standard curves were >0.99. To confirm specificity of the reaction products, melting profiles were analyzed using the LightCycler. To this end, reaction products were kept at 80°C for 10 s and then heated to 95°C at a linear rate of 0.1°C/s while measuring the fluorescence emitted. Analysis of the melting curves demonstrated that each pair of primers amplifies a single product.

PCR products were separated in agarose gels and stained with ethidium bromide (0.5 μg/ml) to verify each case of amplification of a single fragment of the predicted size. Each LightCycler PCR run consisted of 45 cycles with an initial denaturation step of 5 min at 95°C.

The cycles used were: for human OSCAR, 95°C for 10 s, 48°C for 10 s, and 72°C for 19 s; for murine OSCAR, 95°C for 10 s, 54°C for 10 s, and 72°C for 19 s; for 18S, 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s; and for IRAK-M, 95°C for 10 s, 59°C for 10 s, and 72°C for 19 s.

Primers

The sequences of oligonucleotides used are: human OSCAR: sense 5′-AGCTTCTTTCCAGGCCTTTC-3′, antisense 5′-GAGAACAAAGCTCCCACAGC-3′; murine OSCAR: sense 5′-TGGCGGTTTGCACTCTTCA-3′, antisense 5′-GATCCGTTACCAGCAGTTCCAGA-3′; IRAK-M: sense 5′-TTTGAATGCAGCCAGTCTGA-3′, antisense 5′-GCATTGCTTATGGAGCCAAT-3′.

All primers were synthesized, desalted, and purified by IZASA (Barcelona, Spain). For 18S mRNA detection, we used the primers of QuantumRNA Classic 18S provided by Ambion, Inc. (Austin, TX, USA)

Western blot analysis

Cell extracts were denatured by boiling in Laemmli buffer, resolved on 12% SDS polyacrylamide gels in Tris/glycine/SDS buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS), and transferred to Immun-Blot polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA) at 300 mA for 1.5 h at 4°C. After blocking for 1 h in 20 mM Tris-HCl, 150 mM NaCl, 0.2% Tween 20 (TBST), containing 5% nonfat milk, membranes were washed three times in TBST alone and probed for 20 h with antibodies anti-IRAK-M, anti-ERK, antiphosphorylated ERK (anti-p-ERK) diluted in TBST. Following three washes in TBST, the membranes were incubated with a secondary HRP conjugate (diluted 1:4000) for 90 min and washed three times in TBST. Bound antibodies were detected using ECL Plus reagents, according to the manufacturer's instructions (Amersham Pharmacia Biotech, Little Chalfont, UK).

Immunohistochemistry

After treatment, adherent cells were fixed for 15 min in PBS containing 2% paraformaldehyde and then rinsed in PBS. Endogenous peroxidase activity was quenched by incubating cultures for 10 min in methanol containing 0.3% H_2O_2. Thereafter, cells were incubated for 1 h at room temperature with blocking solution (PBS containing 1% BSA, 0.5% Triton X-100, and 1.5% blocking serum from Vector Laboratories, Inc., Burlingame, CA. USA). Cultures were labeled by overnight incubation at 4°C with goat anti-IRAK-M polyclonal antibody (1:500 in PBS containing 0.5% Triton X-100, 1% BSA, and 1.5% blocking serum). After rinsing with PBS, primary antibodies were localized using Vectastain Universal Quick kit (Vector Laboratories) and developed using diaminobenzidine (substrate kit, Zymed Laboratories Inc., San Francisco, CA, USA). Cells were analyzed finally by light microscopy using an Axioplan 2 microscope (Zeiss).

In vitro bone-reabsorbing assay

To confirm osteoclast activation, calcified matrix-resorption activity of osteoclast-like cells was tested using BD BioCoat Osteologic calcium hydroxyapatite-coated slides (BD Biosciences, San Jose, CA, USA). Cultures were established on BioCoat slides for 14 days and then removed with trypsin-EDTA solution (Gibco); recovered cells were used for mRNA expression analysis. Slides were washed with PBS, air-dried, and stained with 0.5% (w/v) toluidine blue in 0.5% boric acid, and the surface of each dentine slice was then examined by light microscopy for evidence of lacunar resorption. Resorbed area (calcium phosphate resorption pits) was measured using Photoshop 7.0 software (Adobe Systems, San Jose, CA, USA), and the relative resorption area was calculated by dividing the total pitted area by the total surface area.

RESULTS

Differentiation of RAW and THP-1 cells into osteoclast-like cells

Differentiation of THP-1 and RAW cells into osteoclasts was verified by different methods. Incubation of these premonocytic and macrophage cell lines in the presence of low concentrations of Vit-D3 (THP-1 cells) or sRANKL (RAW cells) for 6 days induced their differentiation into osteoclast cells, as shown in **Figure 1**. Although nonstimulated cultures (control in Fig. 1) did not exhibit multinucleated cells, fusion was evident in THP-1 and RAW cells stimulated with 40 ng/ml Vit-D3 or sRANKL, respectively (Fig. 1A). This observation was corroborated with a TRAP assay; stimulated cells showed stained nuclei (Fig. 1A, right panel, and Fig. 1B). By contrast, cell nuclei from control cultures were not stained with this procedure. Finally, OSCAR mRNA expression levels were increased significantly in cells stimulated with Vit-D3 or sRANKL (Fig. 1C). Altogether, these data demonstrate that macrophages can be differentiated efficiently into osteoclast-

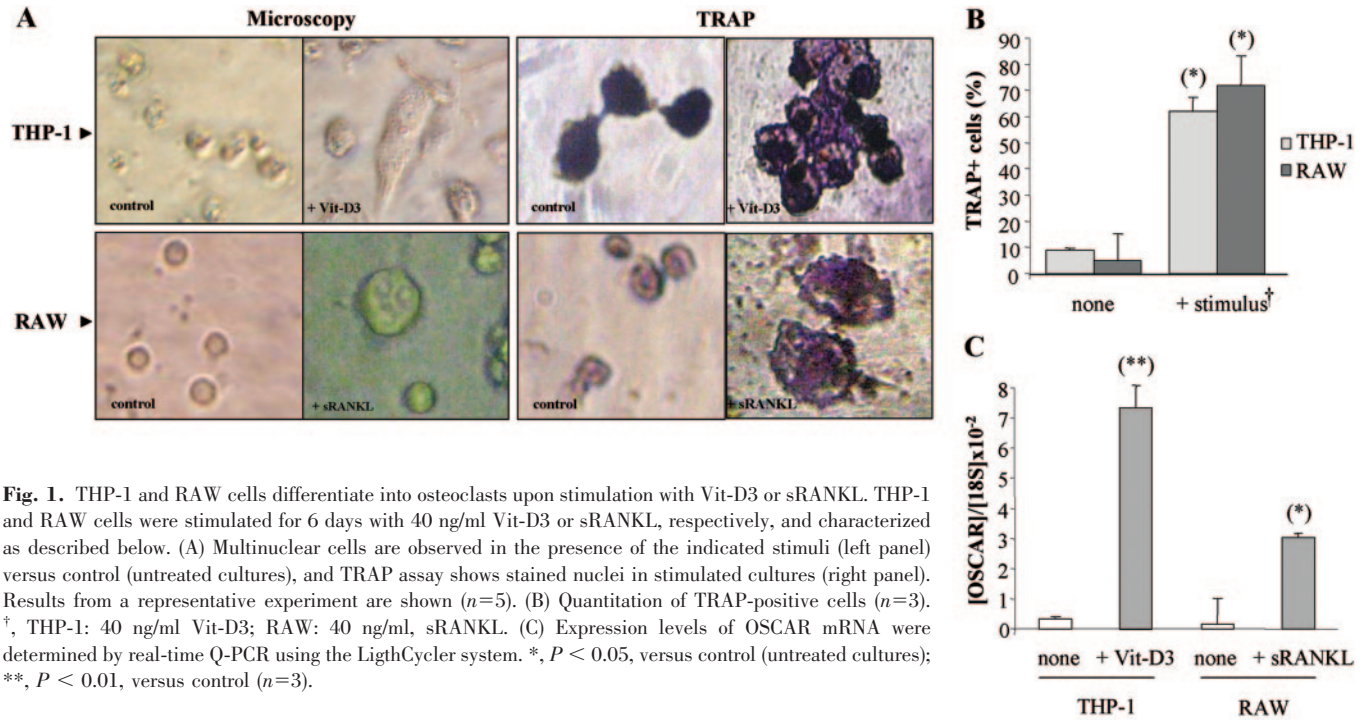

Fig. 1. THP-1 and RAW cells differentiate into osteoclasts upon stimulation with Vit-D3 or sRANKL. THP-1 and RAW cells were stimulated for 6 days with 40 ng/ml Vit-D3 or sRANKL, respectively, and characterized as described below. (A) Multinuclear cells are observed in the presence of the indicated stimuli (left panel) versus control (untreated cultures), and TRAP assay shows stained nuclei in stimulated cultures (right panel). Results from a representative experiment are shown ($n=5$). (B) Quantitation of TRAP-positive cells ($n=3$). †, THP-1: 40 ng/ml Vit-D3; RAW: 40 ng/ml, sRANKL. (C) Expression levels of OSCAR mRNA were determined by real-time Q-PCR using the LigthCycler system. *, $P < 0.05$, versus control (untreated cultures); **, $P < 0.01$, versus control ($n=3$).

like cells upon a 6-day treatment with the appropriate stimulus, in line with previous reports [27–31].

Osteoclast-like cells express IRAK-M

As previous work demonstrated that IRAK-M plays a pivotal role in the control of osteoclast differentiation and activation [1], we first determined IRAK-M mRNA expression levels in cultures of THP-1 and RAW cells stimulated with Vit-D3 or sRANKL, respectively. As shown in **Figure 2, A** and **B**, both cell lines exponentially up-regulated their basal levels of IRAK-M mRNA in a time-dependent manner. In both cases, after 3 days of culture in the presence of the appropriate stimulus, IRAK-M levels were significantly higher than in their respective controls (Fig. 2, A and B). This effect was also dose-dependent (Fig. 2, C and D). Our findings corroborate that murine osteoclast-like cells express IRAK-M, as reported [1], and extend this observation for the first time to human osteoclast-like cells.

6-MP reduces IRAK-M expression in osteoclast-like cells

Recent data establish a critical role for IRAK-M in osteoporosis onset and progression [1]. Given the well-characterized side-effect of 6-MP as an inducer of this important bone disease [28–30], we decided to study the effect of the glucocorticoid on IRAK-M expression. Results shown in **Figure 3** demonstrate two major, opposing effects of 6-MP treatment. First, the glucocorticoid strongly induced IRAK-M expression in untreated (e.g., nondifferentiated) THP-1 and RAW cells (Fig. 3, A and B, shaded bars), in agreement with its anti-inflammatory activity [9, 32]. It is interesting and in striking contrast that cells induced to differentiate into osteoclast-like cells showed a significant down-regulation of IRAK-M expression levels when costimulated with 0.4 mg/ml 6-MP during this process (Fig. 3, A and B, solid bars). The observed effect was dose-dependent, as illustrated in Figure 3, C and D.

In addition, we determined OSCAR levels in cultures stimulated with Vit-D3 or sRANKL in the presence of the glucocorticoid. 6-MP did not affect the expression of OSCAR, neither in original nor in differentiated cells, thus suggesting that differentiation into osteoclast-like cells was not influenced by the glucocorticoid (Fig. 3, E and F). This conclusion was also supported by the results of a TRAP assay (data not shown). Finally, we also verified that cell viability was not affected by any of these treatments (data not shown).

IRAK-M protein levels are reduced in osteoclast-like cells in the presence of 6-MP

Western blot analysis verified the presence of IRAK-M protein in osteoclast-like cells (**Fig. 4**). Furthermore and also in agreement with our observations at the mRNA level, 6-MP reduced IRAK-M protein expression in these cells (Fig. 4). Finally, expression of IRAK-M was verified by immunohistochemistry (**Fig. 5**); osteoclast-like cells expressed large amounts of IRAK-M, which was localized to the cytosol. However, in the presence of 6-MP, the immunochemical signal was reduced significantly (compare upper and lower panels in Fig. 5). Therefore, our findings strongly indicate that IRAK-M is expressed in osteoclasts, but its levels are reduced significantly in the presence of 6-MP. We have obtained similar results with

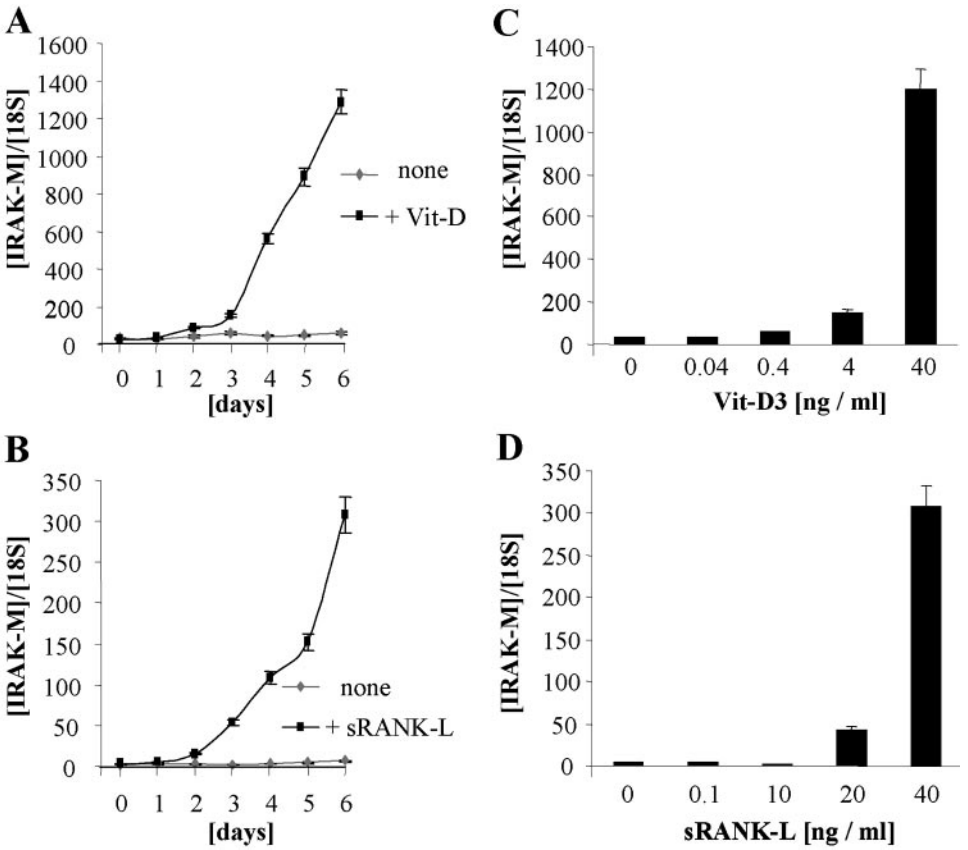

Fig. 2. IRAK-M is strongly overexpressed during osteoclast differentiation. (A and B) IRAK-M expression levels in cultures of THP-1 and RAW cells stimulated with 40 ng/ml Vit-D3 or sRANKL, respectively, were assessed by Q-PCR (LightCycler system) at indicated times. Cells were harvested, total RNA isolated, and cDNA synthesized as described in Materials and Methods. The ratio of IRAK-M:18S is shown ($n=3$). (C and D) THP-1 and RAW cells were cultured for 6 days with the appropriate stimulus at the indicated concentrations, and IRAK-M mRNA expression was analyzed by real-time Q-PCR using a LightCycler system ($n=2$).

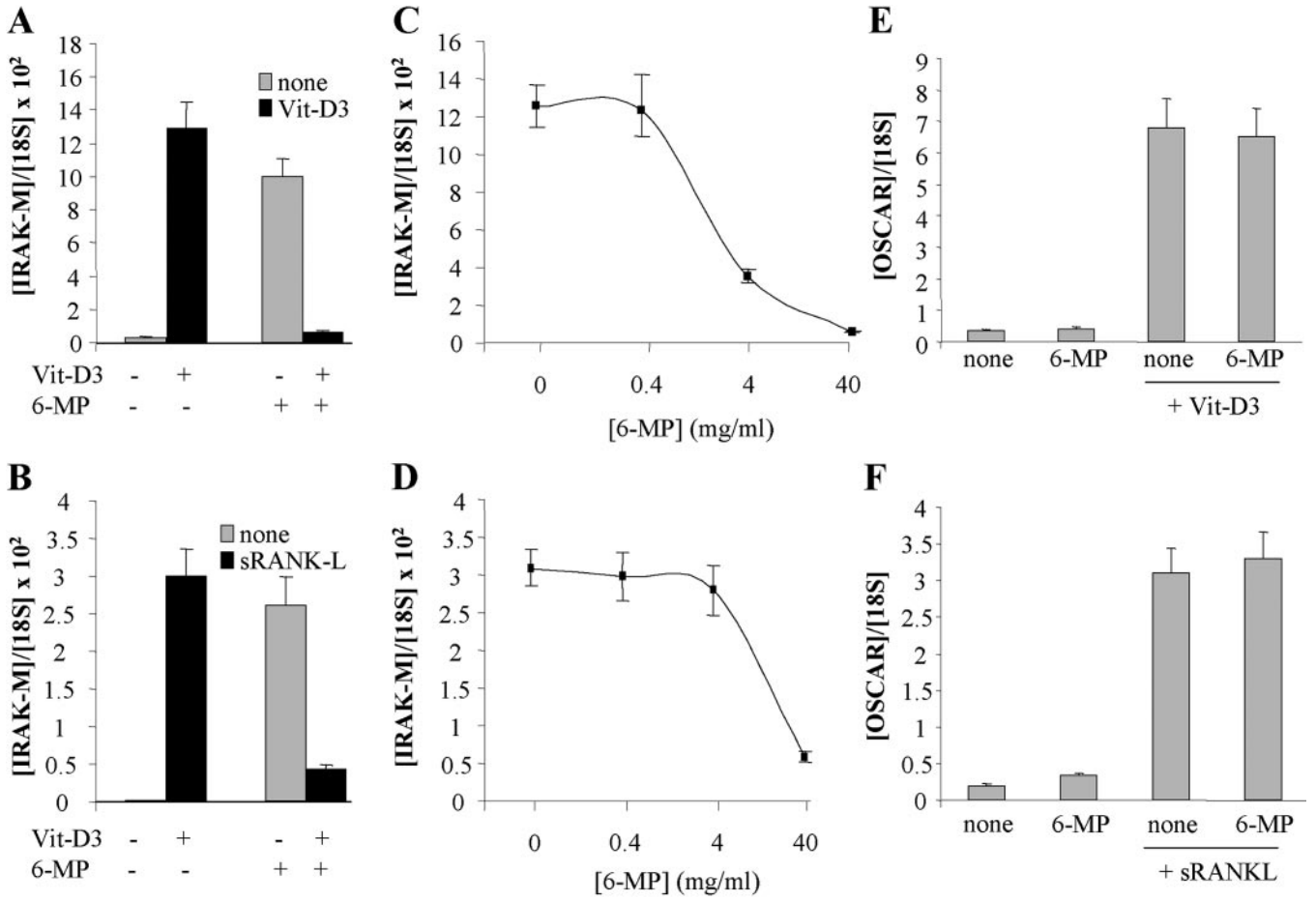

Fig. 3. 6-MP down-regulates IRAK-M expression during osteoclastogenesis. (A and B) Cultures of THP-1 and RAW cells were differentiated or not into osteoclasts in the presence of the appropriate stimulus (40 ng/ml Vit-D3 or sRANKL, respectively) for 6 days, in the additional presence or absence of 0.4 mg/ml 6-MP. After this period, cells were harvested, total RNA isolated, and cDNA synthesized. IRAK-M mRNA expression was assessed by real-time Q-PCR. The ratio of IRAK-M:18S is shown ($n=4$). (C and D) Cell lines were cultured under the same conditions as above but treated with various concentrations of 6-MP. IRAK-M mRNA expression was studied by real-time Q-PCR ($n=2$). (E and F) Expression levels of OSCAR mRNA were determined by real-time Q-PCR using a LigthCycler system in cultures of THP-1 and RAW cells stimulated for 6 days with the appropriate stimulus in the presence or absence of 0.4 mg/ml 6-MP ($n=4$).

other glucocorticoids such as dexametasone and hydrocortisone (data not shown), suggesting that the observed phenomenon represents a general response to glucocorticoids.

Activation of osteoclast-like cells is up-regulated by 6-MP and correlates with IRAK-M down-regulation

Data presented above showed a clear induction of IRAK-M in osteoclast-like cells at mRNA and protein levels. In addition, expression of the pseudokinase was down-regulated significantly by the glucocorticoid, 6-MP. Taking into account these results, we wondered whether 6-MP could also regulate the bone-resorbing ability of osteoclasts. Cultures of osteoclast-like cells derived from THP-1 and RAW cell lines were established on hydroxyapatite-coated wells for 14 days in the presence or absence of the glucocorticoid, and resorbed areas were measured after cell removal. We observed that total resorbed areas were significantly higher in osteoclast-like cells cultured in the presence of 6-MP (**Fig. 6, A** and **B**). By contrast, the glucocorticoid had no relevant effect on nondifferentiated THP-1 and RAW cells. Conversely, analysis of IRAK-M mRNA expression in osteoclast-like cells recovered from hydroxyapatite-coated wells showed a direct correlation between bone-resorbing capacity and degree of IRAK-M down-regulation (compare Fig. 6, B and C). Osteoclast-like cells treated with 6-MP exhibited high bone-resorbing capacity and low levels of IRAK-M expression, and untreated cells expressed IRAK-M, and their bone-reabsorbing ability was moderate. These findings suggest that the presence of 6-MP boosts bone-resorption by down-regulating IRAK-M expression in osteoclasts.

A human model of osteoclasts confirms 6-MP-mediated down-regulation of IRAK-M

Once established that 6-MP regulates IRAK-M expression in osteoclast-like cells differentiated from THP-1 and RAW cell lines and that its down-regulation correlates with osteoclast hyperactivation, we decided to test another human model of osteoclasts reported in the literature [12]. First, expression of IRAK-M was verified in osteoclast-like cells derived from human blood monocytes. These cells were stimulated simultaneously with 30 ng/ml human sRANKL and 25 ng/ml M-CSF

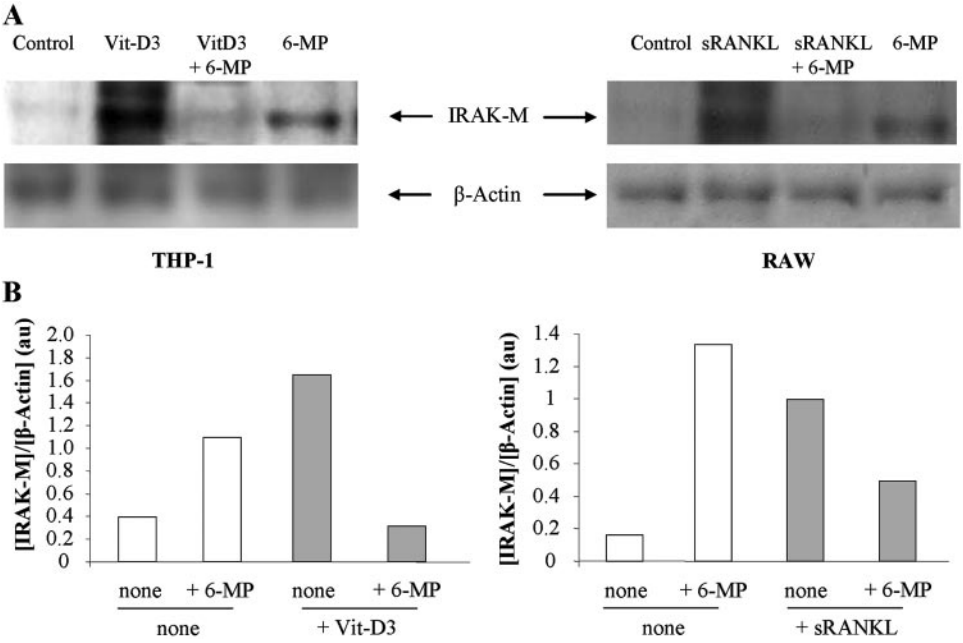

Fig. 4. 6-MP decreases protein levels of IRAK-M in osteoclasts. Expression of IRAK-M protein was analyzed by Western blot using 20 μg total proteins from cultures of THP-1 and RAW cells, treated as indicated in Figure 3, A and B. An anti-IRAK-M polyclonal antiserum was used for protein detection and an anti-β-actin mAb as a control for loading and transfer. (A) A representative experiment ($n=3$); (B) densitometric analysis of detected bands. au, Arbitrary unit.

for 7 days, following the reported protocol (ref. [12]) in the presence or absence of 6-MP. As **Figure 7** shows, although osteoclast-like cells expressed high levels of IRAK-M, incubation with the glucocorticoid reduced expression of the pseudokinase significantly. These findings confirm that corticoid-induced reduction of IRAK-M expression extends to osteoclast-like cells obtained from human monocytes cultured ex vivo.

6-MP induces hyperactivation of the ERK pathway

Recently published results indicate that the absence of IRAK-M leads to activation of MAPK as well as ERK1/2 pathways during osteoclast activation [1]. For this reason, we next examined whether the presence of 6-MP could lead to ERK activation in our models. We subjected cell lysates to Western blotting analysis with antibodies directed against ERK1/2 and their phosphorylated forms. Basal levels of p-ERK in THP-1 and RAW cells were not affected significantly by stimulation with Vit-D3 or sRANKL alone, respectively. It is interesting that additional stimulation with 10 ng/ml LPS for up to 2 h did not up-regulate the levels of p-ERK in osteoclasts. However, and in striking contrast, incubation with the glucocorticoid induced a considerable increase of p-ERK levels (**Fig. 8**). Furthermore, the presence of 6-MP, which inhibits

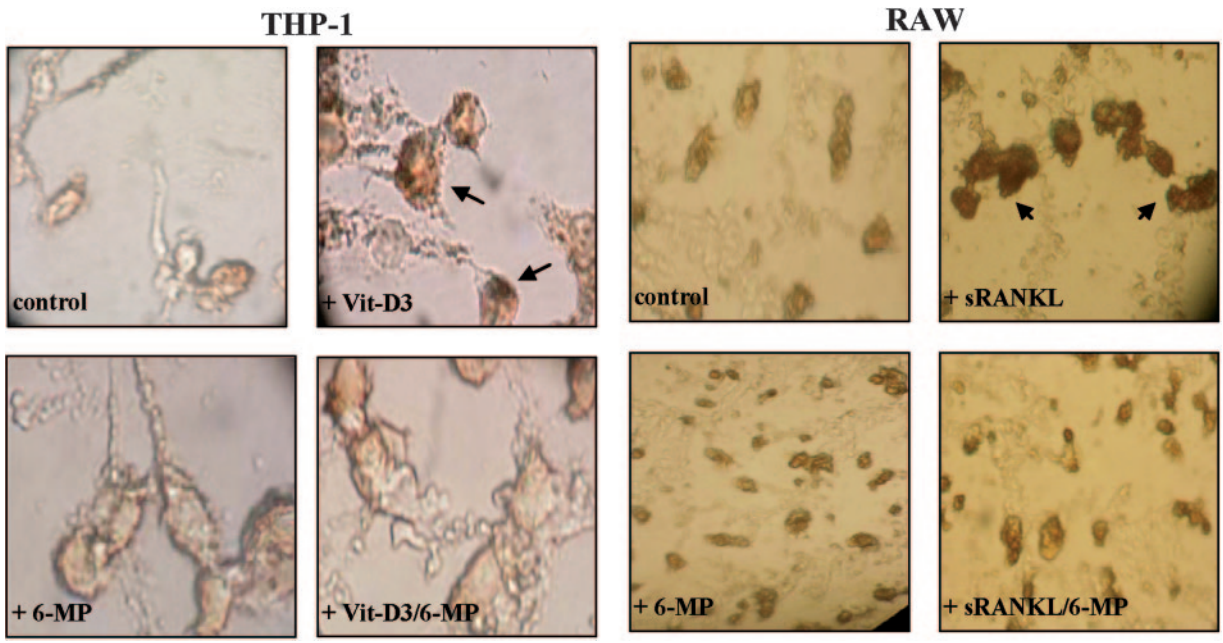

Fig. 5. Immunohistochemical analysis of IRAK-M expression. IRAK-M protein was detected in cultures of THP-1 and RAW cells treated with appropriate stimuli (see legend to Fig. 3, A and B), in the presence or absence of 0.4 mg/ml 6-MP. Note the brown, cytoplasmic staining with anti-IRAK-M polyclonal antibody (indicated with arrows).

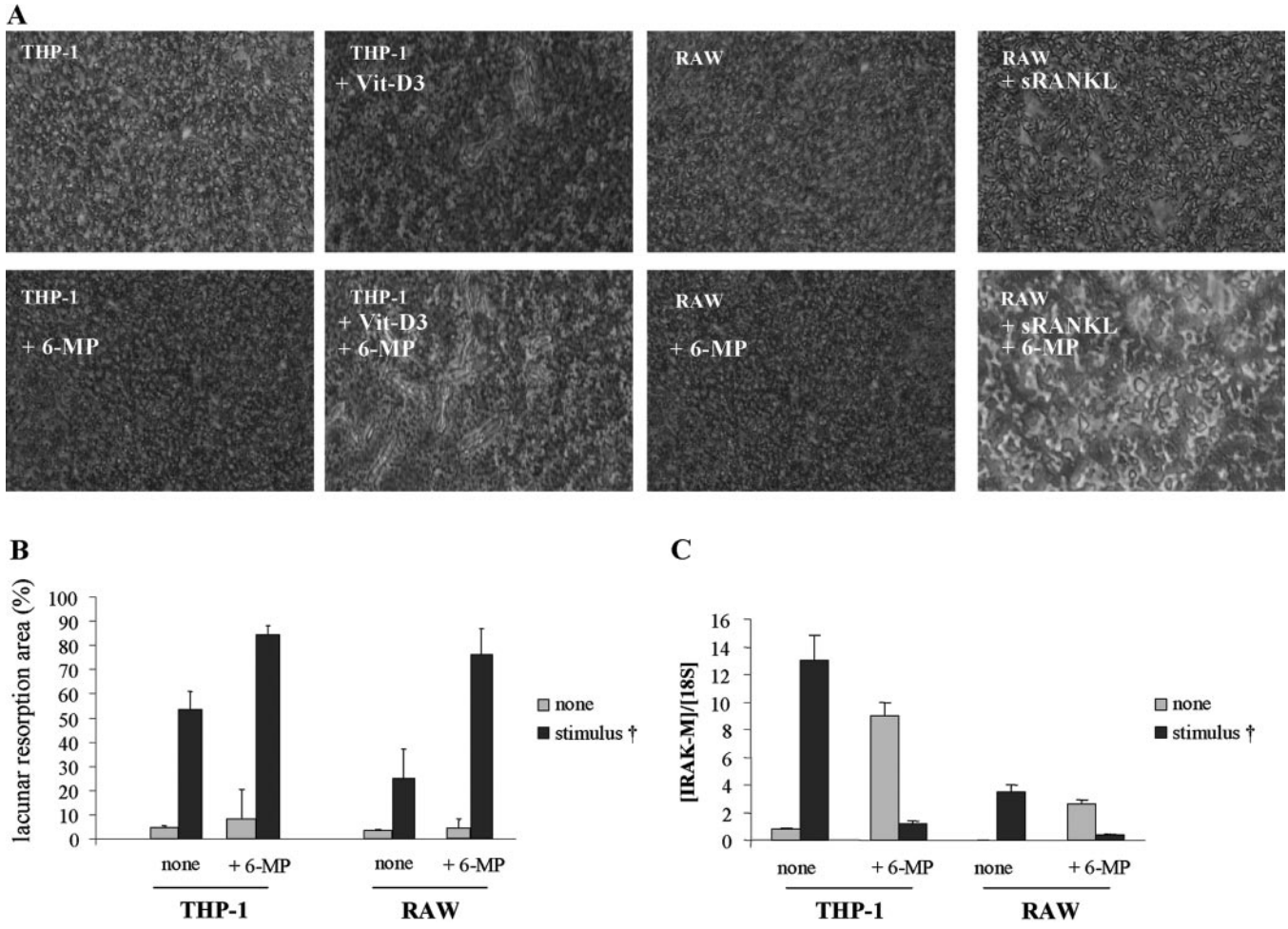

Fig. 6. 6-MP induces hyperactivation of bone resorption, which correlates with IRAK-M down-regulation. (A) THP-1 and RAW cells were cultured and stimulated with the appropriate differentiation stimulus on hydroxyapatite-coated wells for 14 days in the presence or absence of 0.4 mg/ml 6-MP. Adherent cells were then removed with trypsin-EDTA; slides were labeled with toluidine blue and examined by light microscopy. Note the presence of lacunar resorption areas. A representative experiment is shown ($n=3$). (B) Relative amounts of lacunae area (%) were calculated for each condition. Bars represent the mean of three independent experiments. (C) Cells recovered from hydroxyapatite-coated disks were lysed, total RNA isolated, and cDNA synthesized. IRAK-M mRNA levels were determined using real-time Q-PCR (LightCycler system). The ratio of IRAK-M:18S is given. †, THP-1: 40 ng/ml Vit-D3; RAW: 40 ng/ml sRANKL.

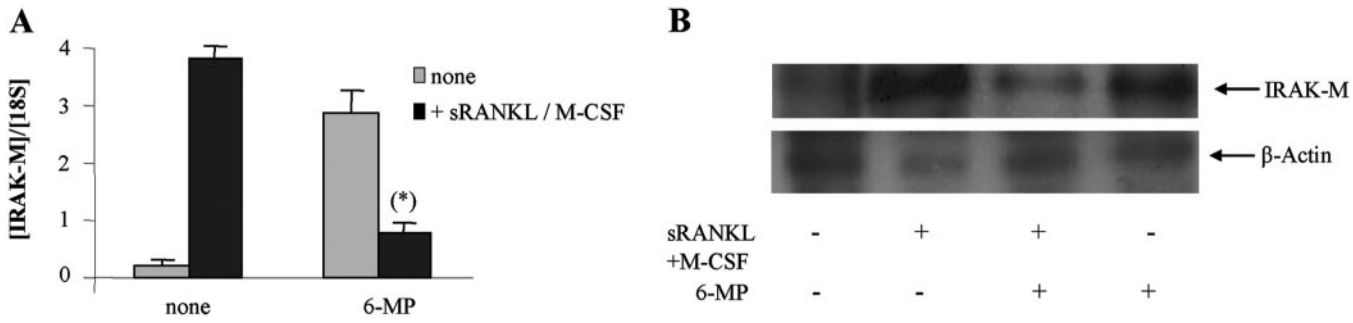

Fig. 7. Osteoclasts derived from human blood monocytes express IRAK-M, and its expression is down-regulated by 6-MP. Cultures of human monocytes/macrophages were stimulated with 30 ng/ml RANKL and 25 ng/ml M-CSF for 7 days in the presence or absence of 0.4 mg/ml 6-MP. After this time, cells were harvested, total RNA and protein were isolated, and cDNA synthesized. (A) Analysis of IRAK-M mRNA expression was carried out by real-time Q-PCR (LightCycler system). The ratio of IRAK-M:18S expression is shown ($n=4$). *, $P < 0.05$, versus control (untreated cultures). (B) Expression of IRAK-M protein was analyzed by Western blot using 20 μg total protein from cultures. An anti-IRAK-M polyclonal antiserum was used for detection of the pseudokinase, and an anti-β-actin mAb was used as a control for loading and transfer. A representative experiment is shown ($n=4$).

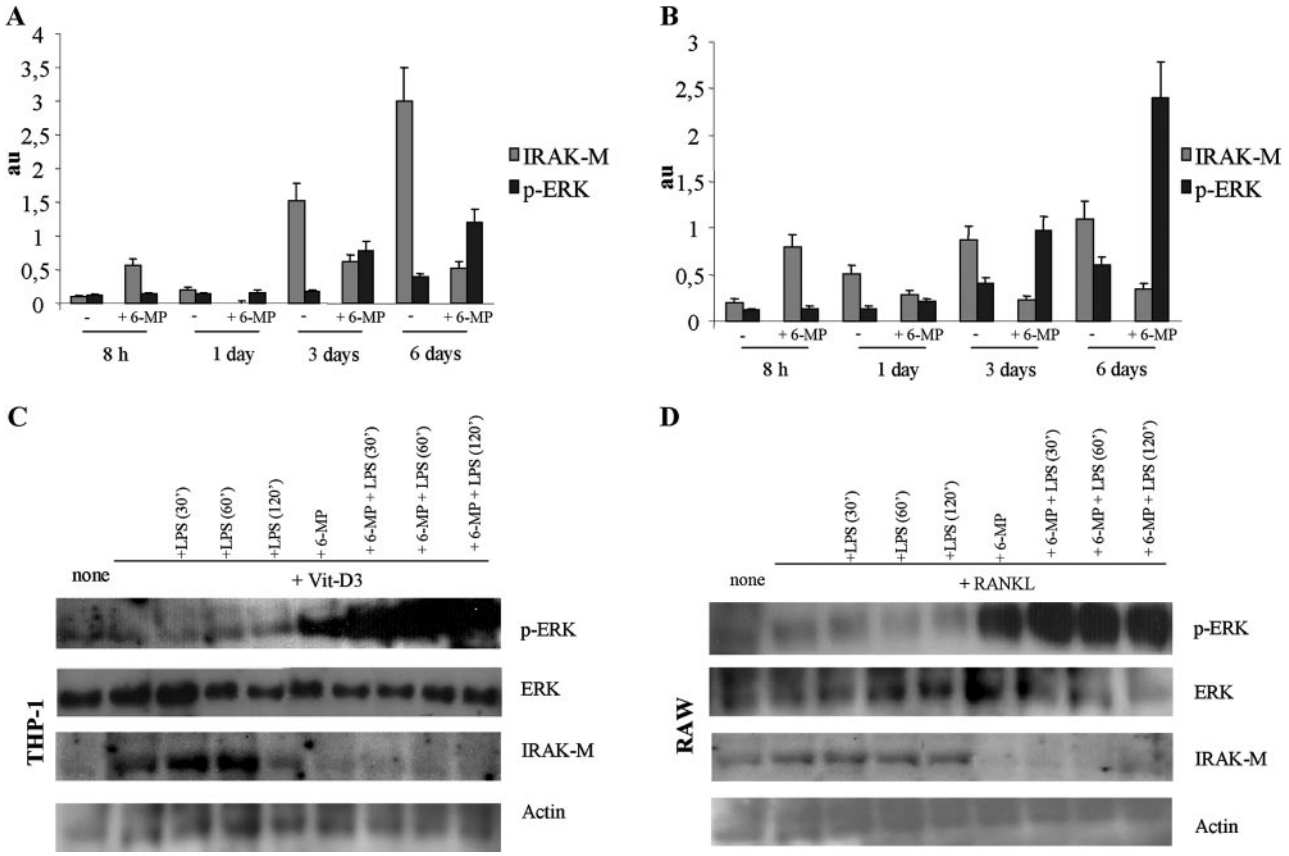

Fig. 8. 6-MP activates the ERK pathway in osteoclasts. (A and B) Cultures of THP-1 (A) and RAW cells (B) were treated with 40 ng/ml Vit-D3 (A) or sRANKL (B), alone or supplemented with 0.4 mg/ml 6-MP for indicated times. Levels of IRAK-M and p-ERK were analyzed by Western blot using 20 μg total proteins from cell lysates. A densitometric analysis of detected bands is shown (n=3). (C and D) Cultures of THP-1 (C) and RAW cells (D), differentiated previously into osteoclasts, were stimulated with 10 ng/ml LPS alone, 0.4 mg/ml 6-MP alone, or with the same concentration of both stimuli for indicated times. Levels of p-ERK, ERK, IRAK-M, and β-actin were analyzed by Western blot using 20 μg total proteins from cell lysates. A standard result from four different experiments is shown.

IRAK-M expression in these cells, results in rapid ERK hyperactivation upon LPS challenge (compare intensity of p-ERK bands in cells treated with 6-MP alone or with a combination of the glucocorticoid and endotoxin in Fig. 8, C and D). It is noteworthy that in osteoclasts derived from RAW cells, the presence of LPS induced a moderate down-regulation of p-ERK in the absence of 6-MP (Fig. 8D, top panel). Altogether, our findings suggest that down-regulation of IRAK-M by 6-MP leads to hyperactivation of the ERK pathway in osteoclast-like cells.

DISCUSSION

Osteoclasts, which originate from the fusion of mononuclear phagocytes, express high basal levels of IRAK-M in mice [1]. It is well established that this pseudokinase plays a crucial role in the regulation of several proinflammatory pathways [17–22]. In addition, a recent work revealed that IRAK-M is expressed in murine osteoclasts, and its expression is pivotal to prevent hyperactivation of osteoclast-mediated bone resorption and the subsequent osteoporosis phenotype [1].

Here, we could show that stimulation of THP-1 and RAW cell lines for 6 days with low concentrations of Vit-D3 and sRANKL, respectively, suffices to induce differentiation into osteoclast-like cells, in line with previous reports [27, 31]. Our data also indicate that IRAK-M levels increase in a time-dependent manner during the differentiation process, with similar kinetics in human and murine systems. Our observations represent the first evidence that osteoclasts of human origin also express considerable levels of this important pseudokinase. It is interesting that IRAK-M mRNA levels were significantly higher in differentiated THP-1 than in RAW cells (about fourfold), suggesting a tighter control of osteoclast activation in humans compared with mice. This is in line with several additional studies, which have demonstrated large differences in mRNA and protein levels between these two cell lines [33–35]. In addition, we have found that undifferentiated THP-1 expresses higher levels of IRAK-M than RAW upon LPS stimulation (data not shown).

We also note that 6-MP addition does not affect the differentiation process into osteoclasts, at least at the concentrations used in the current investigation. In particular, 6-MP alone did not induce osteoclastogenesis to any appreciable extent; THP-1 and RAW cell lines, stimulated solely with the glucocorticoid, showed the same morphology as the original macrophages. It has been reported that glucocorticoids compromise the viability of osteoblasts [8–10, 26] and increase the osteoclast lifespan [36]. Our findings are in line with these previous works, and THP-1 and RAW cells evolved from macrophages

into osteoclasts upon stimulation with Vit-D3 or sRANKL, respectively, irrespective of the presence or absence of 6-MP.

We could show, using different techniques, that mRNA and protein levels of IRAK-M were reduced significantly in the presence of the glucocorticoid in a dose-dependent manner. Further, using an in vitro assay of bone-resorbing ability, we demonstrated that osteoclasts obtained in the presence of 6-MP were hyperactive. In addition, the high bone-resorbing ability of cells recovered from this assay correlated with a significant down-regulation of their IRAK-M expression levels. By contrast, osteoclasts cultured in the absence of the glucocorticoid showed moderate bone-resorbing ability, and their levels of IRAK-M were high, in line with our previous results. In view of the role of IRAK-M as a negative regulator of osteoclast activation, our findings suggest that 6-MP induces osteoclast hyperactivation via down-regulation of the pseudokinase.

It is interesting that levels of IRAK-M expression in undifferentiated THP-1 and RAW cells stimulated with 6-MP were considerably higher than in controls. These findings are in agreement with previous observations of the anti-inflammatory action of glucocorticoids and with the IRAK-M role in the down-regulation of proinflammatory responses [17–21]. Taken together, our data suggest that 6-MP exhibits differential effects on macrophages and osteoclast-like cells. Although in macrophages, the corticoid up-regulates IRAK-M to control inflammatory responses, in osteoclasts, it acts as an inhibitor of IRAK-M expression, thus exacerbating bone-resorbing activity. We obtained similar results in a model of osteoclasts differentiated from human blood monocytes/macrophages using a combination of sRANKL and M-CSF. These cells exhibited similar responses to 6-MP as THP-1-derived osteoclasts, suggesting that our findings are valid for human osteoclasts, irrespective of source and stimulus used for their generation.

The molecular mechanism that governs the observed effect of glucocorticoids on osteoclasts is largely unknown; data presented here suggest that ERK activation might underlie this phenomenon, as there is a significant up-regulation of p-ERK1/2 during osteoclast activation by 6-MP. Li and coworkers [1] have shown a strong correlation among IRAK-M down-regulation, ERK1/2 expression, and osteoclastogenic activation in mice. Here, we extend these findings to human osteoclast-like cells and report for the first time that stimulation with the glucocorticoid, 6-MP, mediates osteoclast activation through this IRAK-M–pERK1/2 pathway.

Our results should contribute to a better understanding of the mechanism of corticosteroid-induced osteoporosis. Clinical data indicate that glucocorticoids have important actions on skeletal turnover, which eventually lead to the development of osteoporosis [4, 7–10, 37]. Significant bone loss occurs after the initial exposure to glucocorticoids, and even modest doses of these drugs, frequently considered within the physiological range, increase the risk of fractures [8]. A recent report suggests that glucocorticoids act directly on osteoclasts to increase their lifespan and reduce bone density [36]. Conversely, other authors had suggested that glucocorticoids have a direct effect on osteoblasts, increasing their expression of RANKL and CSF-1 and down-regulating levels of osteoprotegerin [26, 30]. Further, glucocorticoids enhance apoptosis of mature osteoblasts [8–10, 26]. Our findings demonstrate that 6-MP and other glucocorticoids influence osteoclast activation in addition via down-regulation of IRAK-M. Thus, strategies that counteract this effect might prove beneficial for treatment of osteoporosis in humans.

ACKNOWLEDGMENTS

This study was supported by grants from the 'Ministerio de Educación y Ciencia' (SAF 1256) and from 'Fundación Médica de la Mutua Madrileña de Automovilística' to E. L-C. We thank Drs. J. L. Bueno, E. Castro, and Ms. Prado from Cruz Roja and Dr. de la Rúa from the Department of Hematology, La Paz Hospital, for their help.

REFERENCES

1. Li, H., Cuartas, E., Cui, W., Choi, Y., Crawford, T. D., Zhu Ke, H., Kobayashi, K. S., Flavelland, R. A., Vignery, A. (2005) IL-1 receptor-associated kinase M is a central regulator of osteoclast differentiation and activation. *J. Exp. Med.* **201,** 1169–1177.
2. Cappellen, D., Luong-Nguyen, N. H., Bongiovanni, S., Wanke, O. G. C., Šuša, M. (2002) Transcriptional program of mouse osteoclast differentiation governed by the macrophage colony-stimulating factor and the ligand for the receptor activator of NFκB. *J. Biol. Chem.* **277,** 21971–21982.
3. Huang, H., Ryu, J., Ha, J., Chang, E-J., Kim, H. J., Kim, H.-M., Kitamura, T., Lee, Z. H., Kim, H.-H. (2006) Osteoclast differentiation requires TAK1 and MKK6 for NFATc1 induction and NF-κB transactivation by RANKL. *Cell Death Differ.* **13,** 1879–1891.
4. Chen, T. L. (2004) Inhibition of growth and differentiation of osteoprogenitors in mouse bone marrow stromal cell cultures by increased donor age and glucocorticoid treatment. *Bone* **35,** 83–95.
5. Halloran, B. P., Ferguson, V. L., Simske, S. J., Burghardt, A., Venton, L. L., Majundar, S. (2002) Changes in bone structure and mass with advancing age in the male C57BL/6J mouse. *J. Bone Miner. Res.* **17,** 1044–1050.
6. Susa, M., Luong-Nguyen, N-H., Capellen, D., Zamurovic, N., Gamse, R. (2004) Human primary osteoclasts: in vitro generation and applications as pharmacological and clinical assay. *J. Transl. Med.* **2,** 6.
7. Dempster, D. W., Moonga, B. S., Stein, L. S., Horbert, W. R., Antakly, T. (1997) Glucocorticoids inhibit bone resorption by isolated rat osteoclasts by enhancing apoptosis. *J. Endocrinol.* **154,** 397–406.
8. Iwamoto, J., Takeda, T., Sato, Y. (2005) Prevention and treatment of corticosteroid-induced osteoporosis. *Yonsei Med. J.* **46,** 456–463.
9. Leclerc, N., Luppen, C. A., Ho, V. V., Nagpal, S., Hacia, J. G., Smith, E., Frenkel, B. (2004) Gene expression profiling of glucocorticoid-inhibited osteoblasts. *J. Mol. Endocrinol.* **33,** 175–193.
10. Sivagurunathan, S., Muir, M. M., Brennan, T. C., Seale, J. P., Mason, R. S. (2005) Influence of glucocorticoids on human osteoclast generation and activity. *J. Bone Miner. Res.* **20,** 390–398.
11. Sato, K., Takayanagi, H. (2006) Osteoclasts, rheumatoid arthritis, and osteoimmunology. *Curr. Opin. Rheumatol.* **18,** 419–426.
12. Avnet, S., Lamolinara, A., Zini, N., Solimando, L., Quacquaruccio, G., Granchi, D., Maraldi, N. M., Giunti, A., Baldini, N. (2006) Effects of antisense mediated inhibition of cathepsin K on human osteoclasts obtained from peripheral blood. *J. Orthop. Res.* **24,** 1699–1708.
13. Schell, H., Lienau, J., Epari, D. R., Seebeck, P., Exner, C., Muchow, S., Bragulla, H., Norbert, P. H., Duda, G. N. (2006) Osteoclastic activity begins early and increases over the course of bone healing. *Bone* **38,** 547–554.
14. Vignery, A. (2005) Macrophage fusion: the making of osteoclasts and giant cells. *J. Exp. Med.* **202,** 337–340.
15. Teitelbaum, S. L. (2000) Bone resorption by osteoclasts. *Science* **289,** 1504–1508.
16. Rousselle, A-V., Heymann, D. (2002) Osteoclastic acidification pathways during bone resorption. *Bone* **30,** 533–540.
17. Del Fresno, C., Otero, K., Gómez-García, L., González-León, M. C., Soler-Ranger, L., Fuentes-Prior, P., Escoll, P., Baos, R., Caveda, L., García, F., Arnalich, F., López-Collazo, E. (2005) Tumor cells deactivate human monocytes by up-regulating IL-1 receptor associated kinase-M expression via CD44 and TLR4. *J. Immunol.* **174,** 3032–3040.

18. Del Fresno, C., Gómez-García, L., Caveda, L., Escoll, P., Arnalich, F., Zamora, R., López-Collazo, E. (2004) Nitric oxide activates the expression of IRAK-M via the release of TNF-α in human monocytes. *Nitric Oxide* **10,** 213–220.
19. Escoll, P., del Fresno, C., Gómez-García, L., Vallés, G., Lendínez, M. J., Arnalich, F., López-Collazo, E. (2003) Rapid up-regulation of IRAK-M expression following a second endotoxin challenge in human monocytes and in monocytes isolated from septic patient. *Biochem. Biophys. Res. Commun.* **311,** 465–472.
20. Lopez-Collazo, E., Fuentes-Prior, P., Arnalich, F., del Fresno, C. (2006) Pathophysiology of interleukin-1 receptor-associated kinase-M: implications in refractory state. *Curr. Opin. Infect. Dis.* **19,** 237–244.
21. González-León, M. C., Soares-Schanoski, A., del Fresno, C., Cimadevila, A., Goméz-Piña, V., Mendoza-Barberá, E., García, F., Marín, E., Arnalich, F., Fuentes-Prior, P., López-Collazo, E. (2006) Nitric oxide induces SOCS-1 expression in human monocytes in a TNF-α dependent manner. *J. Endotoxin Res.* **12,** 296–306.
22. Del Fresno, C., Soler-Rangel, L., Soares-Schanoski, A., González-León, M. C., Gómez-García, L., Gómez-Piña, V., Mendoza-Barberá, E., García, F., Fuentes-Prior, P., Arnalich, F., López-Collazo, E. (2006) Inflammatory responses associated with acute coronary syndrome up-regulate IRAK-M and induce endotoxin tolerance in circulating monocytes. *J. Endotoxin Res.* **13,** 39–52.
23. Dauphinee, S. M., Karsan, A. (2006) Lipopolysaccharide signaling in endothelial cells. *Lab. Invest.* **86,** 9–22.
24. Van Staa, T. P., Leufkens, H. G. M., Cooper, C. (2002) The epidemiology of corticosteroid-induced osteoporosis: a meta-analysis. *Osteoporos. Int.* **13,** 777–787.
25. Van Staa, T. P., Laan, R. F., Barton, I. P., Cohen, S., Reid, D. M., Cooper, C. (2003) Bone density threshold and other predictors of vertebral fracture in patients receiving oral glucocorticoid therapy. *Arthritis Rheum.* **48,** 3224–3229.
26. Canalis, E. (2003) Mechanisms of glucocorticoid-induced osteoporosis. *Curr. Opin. Rheumatol.* **15,** 454–457.
27. Yu, X., Huang, Y., Collin-Osdoby, P., Osdoby, P. (2004) CCR1 chemokines promote the chemotactic recruitment, RANKL development, and motility of osteoclasts and are induced by inflammatory cytokines in osteoblasts. *J. Bone Miner. Res.* **19,** 2065–2077.
28. Berneis, K., Oehri, M., Kraenzlin, M., Keller, U. (1999) Effects of IGF-I combined with GH on glucocorticoid-induced changes of bone and connective tissue turnover in man. *J. Endocrinol.* **162,** 259–264.
29. Wang, Y., Ohtsuka-Isoya, M., Shao, P., Sakamoto, S., Shinoda, H. (2002) Effects of methylprednisolone on bone formation and resorption in rats. *Jpn. J. Pharmacol.* **90,** 236–246.
30. Kondo, N., Tokunaga, K., Arai, K., Amizuka, N., Mingi, L., Kitahara, H., Ito, M., Naito, M., Shu-Ying, J., Odas, K., Murai, T., Tkano, R., Ogose, A., Endo, N. (2006) High dose glucocorticoid hampers bone formation and resorption after bone marrow abaltion in rat. *Microsc. Res. Tech.* **69,** 839–846.
31. Jakob, F., Siggelkow, H., Homann, D., Köhrle, J., Adamski, J., Schütze, N. (1997) Local estradiol metabolism in osteoblast and osteoclast-like cells. *J. Steroid Biochem.* **61,** 167–174.
32. Nakamura, E., Kitagawa, Y., Ozawa, S., Suda, K., Ando, N., Ueda, M., Kitajima, M. (2006) Role of steroid administration to reduce inflammation after thoracotomy in a rat surgical stress model. *J. Surg. Res.* **135,** 364–369.
33. Matveev, S., van der Westhuyzen, D. R., Smart, E. J. (1999) Co-expression of scavenger receptor-BI and caveolin-1 is associated with enhanced selective cholesteryl ester uptake in THP-1 macrophages. *J. Lipid Res.* **40,** 1647–1654.
34. Fitzgerald, M. L., Moore, K. J., Freeman, M. W., Reed, G. L. (2000) Lipopolysaccharide induces scavenger receptor A expression in mouse macrophages: a divergent response relative to human THP-1 monocyte/macrophages. *J. Immunol.* **164,** 2692–2700.
35. Teissier, E., Nohara, A., Chinetti, G., Paumelle, R., Cariou, B., Fruchart, J-C., Brandes, R. P., Shah, A., Staels, B. (2004) Peroxisome proliferator-activated receptor α induces NADPH oxidase activity in macrophages, leading to the generation of LDL with PPAR-α activation properties. *Circ. Res.* **95,** 1174–1182.
36. Jia, D., O'Brien, C. A., Stewart, S. A., Manolagas, S. C., Weinstein, R. S. (2006) Glucocorticoids act directly on osteoclasts to increase their life span and reduce bone density. *Endocrinology* **147,** 5592–5599.
37. Van Staa, T. P. (2006) The pathogenesis, epidemiology and management of glucocorticoid-induced osteoporosis. *Calcif. Tissue Int.* **79,** 129–137.