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IL-10 Suppresses Calcium-Mediated Costimulation of Receptor Activator NF-κB Signaling during Human Osteoclast Differentiation by Inhibiting TREM-2 Expression

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Induction of effective osteoclastogenesis by RANK (receptor activator of NF-κB) requires costimulation by ITAM-coupled receptors. In humans, the TREM-2 (triggering receptor expressed on myeloid cells 2) ITAM-coupled receptor plays a key role in bone remodeling, as patients with TREM-2 mutations exhibit defective osteoclastogenesis and bone lesions. We have identified a new rapidly induced costimulatory pathway for RANK signaling that is dependent on TREM-2 and mediated by calcium signaling. TREM-2-dependent calcium signals are required for RANK-mediated activation of calcium/calmodulin-dependent protein kinase (CaMK)II and downstream MEK and ERK MAPKs that are important for osteoclastogenesis. IL-10 inhibited RANK-induced osteoclastogenesis and selectively inhibited calcium signaling downstream of RANK by inhibiting transcription of TREM-2. Down-regulation of TREM-2 expression resulted in diminished RANKL-induced activation of the CaMK-MEK-ERK pathway and decreased expression of the master regulator of osteoclastogenesis NFATc1. These findings provide a new mechanism of inhibition of human osteoclast differentiation. The results also yield insights into crosstalk between ITAM-coupled receptors and heterologous receptors such as RANK, and they identify a mechanism by which IL-10 can suppress cellular responses to TNFR family members. The Journal of Immunology, 2009, 183: 2444–2455.

B one resorption in both physiological and pathological settings is dependent on specialized bone resorbing cells termed osteoclasts (1). Osteoclasts are hematopoietic lineage cells that derive from bone marrow myeloid precursors and circulating monocytes. The first key step in osteoclastogenesis is the generation of osteoclast precursors (pOCs)1 that express high levels of the RANK receptor that mediates differentiation in response to the major osteoclastogenic factor RANK ligand (RANKL). Generation of RANKhighpOCs is dependent on M-CSF that is expressed systemically and in multiple tissues including bone (2). The key event in subsequent osteoclastogenesis is activation of RANK by RANKL, which is expressed on osteoblast lineage cells under physiological conditions. Activation of RANK leads to TRAF6 (TNF receptor-associated factor 6)-dependent activation of signaling cascades that includes activation of IkB kinases, MAPKs, protein tyrosine kinases, and calcium signaling, with downstream activation of transcription factors of the NF-κB, AP-1, and CREB families (3). These cascades of signaling lead to expression and posttranslational activation of the calcium-dependent NFATc1 transcription factor that is a master regulator of the osteoclast differentiation program (4). NFATc1, working in conjunction with other transcription factors such as PU.1, Mitf (microphthalmia-associated transcription factor), and CREB, drives the expression of osteoclast-related genes (such as TRAP (tartrate-resistant acid phosphatase), cathespin K, DC-STAMP (dendritic cell-specific transmembrane protein), and αβ integrin) and terminal differentiation into multinucleated functional osteoclasts that resorb bone (5).

Recent work has identified a key role for ITAM-containing adaptor proteins DAPI2 (DNAX-activating protein of 12 kDa) and FcRγ in providing an essential costimulatory signal for RANKL-induced osteoclastogenesis (6–8). DAPI2-deficient mice exhibit mild osteopetrosis, whereas deficiency in both DAPI2 and FcRγ leads to severe osteopetrosis. A role in osteoclastogenesis for the DAPI2-associated receptors TREM-2 and signal regulatory protein (SIRP)β1, and the FcRγ-associated receptors OSCAR (osteoclast-associated receptor) and PIR-A (paired Ig-like receptor A; ILT7 in humans), has been proposed (6). Such a role is most clear for TREM-2 in humans, as loss of function of TREM-2 mutations phenocopy DAP12 mutations and lead to Nasu-Hakola disease (also known as polycystic lipomembranous osteodysplasia with sclerosing leuкоencephalopathy) that is characterized by abnormalities in bone remodeling and defective osteoclastogenesis (9–11). In mice, TREM-2 function in osteoclastogenesis appears to be more limited or redundant (12), suggesting a more prominent role

1 Abbreviations used in this paper: pOC, osteoclast precursor; CaMK, calcium/calmodulin-dependent protein kinase; ChIP, chromatin immunoprecipitation; DAPI2, DNAX-activating protein of 12 kDa; Mitf, microphthalmia-associated transcription factor; MMP9, matrix metalloproteinase 9; OSCAR, osteoclast-associated receptor; PIR-A, paired Ig-like receptor A; PLC, phospholipase C; RANK, receptor activator of NF-κB; RANKL, RANK ligand; RNAi, RNA interference; SIPR, signal regulatory protein; TRAP6, TNF receptor-associated factor 6; TRAP, tartrate-resistant acid phosphatase; TREM-2, triggering receptor expressed on myeloid cells 2.
for other DAP12-associated receptor(s) and highlighting the existence of significant differences in osteoclastogenesis and mechanisms of bone resorption between humans and mice that have been long appreciated but are not well understood (1, 13). Ligands for TREM-2, SIRPβ1, OSCAR, and PIR-A are not known, although semaphorin 6D may activate TREM-2 indirectly via the plexin-A1 coreceptor (14). Ligands for Fcγ-associated OSCAR and PIR-A appear to be expressed on osteoblasts, whereas ligands for DAP12-associated TREM-2 (and possibly SIRPβ1) are constitutively expressed on myeloid cells and osteoclast precursors. Thus, TREM-2 is continuously ligated in an autocrine manner and generates a tonic ITAM-mediated signal (7, 15, 16). Additionally, RANK ligation may inductibly augment ITAM-mediated signaling (6, 17). The key ITAM-mediated event important for osteoclastogenesis is calcium signaling that leads to the activation of NFATc1 (5, 6, 18).

Inflammation promotes osteoclastogenesis and bone resorption and can lead to inflammatory osteolysis that is a prominent feature and cause of morbidity in several diseases, including rheumatoid arthritis, periodontitis, and peri-prosthetic loosening (5, 19). A variety of inflammatory mediators, such as TNF, IL-1, IL-17, and prostaglandins, promote bone resorption by increasing expression of RANKL on stromal cells such as fibroblasts and osteoblast lineage cells, and by acting directly on osteoclast precursors to synergize with RANK in driving osteoclastogenesis. Importantly, immune cells also produce a panoply of homeostatic factors that suppress osteoclastogenesis and play a key role in limiting bone lysis and tissue damage associated with inflammation. Among the most important immune homeostatic factors that limit osteoclastogenesis are type I IFNs (IFNα/β), IFN-γ, and IL-10. Type I IFNs suppress osteoclastogenesis by inhibiting RANKL-induced Fos expression (20), and IFN-γ inhibits proximal RANK signaling by promoting degradation of TRAF6 (21). IL-10 is an immunosuppressive and antiinflammatory cytokine that is best known as a potent deactivator of myeloid lineage cells and is produced as part of the homeostatic response to infection and inflammation (22). IL-10 plays a critical role in limiting tissue injury during infections and in preventing autoimmunity by limiting the duration and intensity of immune and inflammatory reactions. An important role for IL-10 in suppressing inflammatory bone resorption in vivo has been demonstrated (23–27). Despite intensive study, mechanisms of the antiinflammatory function of IL-10 are poorly understood, but they include the induction of transcriptional repressors (28, 29) and mRNA decay. IL-10 can directly inhibit osteoclast lineage cells and suppress NFATc1 expression by an unknown mechanism (30, 31). Taken together, the biology of IL-10 in immunity and bone remodeling is well established, but the mechanisms of IL-10-mediated inhibitory actions are mostly unknown.

We are interested in identifying mechanisms by which osteoclastogenesis can be suppressed during human inflammatory diseases. Given the prominent homeostatic role of IL-10, its expression in many inflammatory disease settings, and its proven role as an inhibitor of osteoclastogenesis in vivo, we investigated mechanisms by which IL-10 inhibits osteoclastogenesis at the transition of myeloid lineage cells to osteoclast precursors. Given the species differences between humans and mice in the function of TREM-2 (12), and the somewhat different bone phenotypes of DAP12 mutations, RANK gain-of-function mutations, and deficiency of OPG (a decoy receptor that binds and sequesters RANKL) in humans and mice (discussed in Refs. 1, 13), we performed experiments using human cells to try to maximize the relevance of our findings to human disease. We discovered that IL-10 induced a defect in RANK signaling that preferentially affected calcium-dependent signaling pathways, including a rapidly activated calcium/calmodulin-dependent protein kinase (CaMK)-MEK-ERK pathway that we have newly identified. IL-10 suppressed RANK signaling and human osteoclastogenesis in part by inhibiting expression of TREM-2 by a transcriptional mechanism, thereby suppressing TREM-2-mediated costimulation. These results reveal a new mechanism of inhibition of osteoclastogenesis and identify an early step in osteoclast differentiation that can be effectively targeted for inhibition.

Materials and Methods

Cell culture, mice, and reagents

PBMCs were obtained from blood purchased from the New York Blood Center by density gradient centrifugation with Ficoll (Invitrogen) using a protocol approved by the Hospital for Special Surgery Institutional Review Board. Human monocytes were purified from PBMCs immediately after isolation by positive selection with anti-CD14 magnetic beads, as recommended by the manufacturer (Miltenyi Biotech), and cultured in α-MEM medium (Invitrogen) supplemented with 10% FBS (HyClone) and M-CSF (10 ng/ml) in the presence or absence of IL-10. IL-10 (100 ng/ml) was added at the same time as M-CSF at the initiation of cultures, unless otherwise specified in the figure legends. Monocyte-derived pOCs obtained after 1–2 days of culture with M-CSF were used unless otherwise noted in figure legends, and purity of monocytes/macrophages was >97%, as verified by flow cytometry. Murine bone marrow-derived macrophages were obtained by culturing bone marrow cells on petri dishes (Midwest Scientific) with 20 ng/ml recombinant murine M-CSF (PeproTech). Macrophages from synovial fluids of five rheumatoid arthritis patients and two patients with seronegative psoriatic arthritis were isolated by Ficoll density gradient centrifugation, followed by positive selection of CD14+ cells using magnetic beads, as previously described (32). The patients’ diagnoses were determined by their physicians, in each case a Board-certified rheumatologist at the Hospital for Special Surgery, and were either definite rheumatoid arthritis according to American College of Rheumatology criteria or seronegative arthritis associated with psoriasis. Recombinant human IL-10 was from R&D Systems, and human M-CSF and sRANKL were from PeproTech. KN-92, KN-93, BAPTA, W7, and picatannol were from CalBiochem. TT assays were performed using an MTT assay kit (Roche Diagnostics), according to the manufacturer’s instructions.

Osteoclast differentiation

Human CD14+ cells were incubated with 20 ng/ml M-CSF for 1 day to generate osteoclast precursors. Osteoclast precursors were further incubated with 20 ng/ml M-CSF and 40 ng/ml sRANKL for an additional 8 days in α-MEM supplemented with 10% FBS. Cytokines were replenished every 3 days. On day 9, cells were fixed and stained for TRAP using an acid phosphatase leukocyte diagnostic kit (Sigma-Aldrich) as recommended by the manufacturer. Multinucleated (more than three nuclei) TRAP+ osteoclasts were counted in triplicate wells. For detection of actin ring formation, cells were fixed, permeabilized, and incubated with FITC-conjugated phalloidin (Sigma-Aldrich) on ice for 30 min. After washing with PBS, cells were imaged using a Zeiss Axioplan microscope with an attached Leica DC 200 digital camera.

Immunoblotting and immunoprecipitation

Whole-cell extracts were prepared by lysis in buffer containing 20 mM HEPES (pH 7.0), 300 mM NaCl, 10 mM KCl, 1 mM MgCl2, 0.1% Triton X-100, 0.5 mM DTT, 20% glycerol, and 1× protease inhibitor cocktail (Roche). Nuclear extracts were isolated as described previously (33). The protein concentration of extracts was quantitated using the Bradford assay (Bio-Rad). For immunoblotting, 10 μg of cell lysates was fractionated on 7.5% or 10% polyacrylamide gels using SDS-PAGE, transferred to polyvinylidene fluoride membranes (Millipore), incubated with specific Abs, and ECL was used for detection. Phospho-CaMKII (catalog no. 3361), p-Erk (no. 9101), p-p38 (no. 9215), p-CREB (no. 9198), I-κB (no. 4812), and c-jun (no. 6096) Abs were from Cell Signaling Technology. TBP and TBP (catalog no. sc-535) Abs were from Santa Cruz Biotechnology, Fos Ab was from BD Pharmingen, TREM-2 (no. AF-1828) Ab was from R&D Systems, and RANK (no. ALX 804) Ab was from Axonora.

Gene expression analysis

For real-time PCR, total RNA was extracted using an RNeasy Mini kit and 1 μg of total RNA was reverse transcribed using a First Strand cDNA Synthesis kit (Fermentas). Real-time, quantitative PCR was performed using iQ SYBR Green Supermix and an iCycler iQ thermal cycler (Bio-Rad) following the manufacturer’s protocols. Triplicate reactions were run for
levels were normalized relative to the expression of GAPDH, and results are shown as means ± SD from triplicate wells. B, Cells were fixed and stained using FITC-phalloidin to detect actin ring formation. Results are shown as means ± SD from at least three independent experiments.

IL-10 (100 ng/ml) was added at the same time as M-CSF at the initiation of cultures. Data are shown as means ± SD from triplicate wells. B, Cells were fixed and stained using FITC-phalloidin to detect actin ring formation. Results are shown as means ± SD from at least three independent experiments.

Intracellular calcium measurements

Cells that had been plated on 22-mm² glass coverslips were loaded at 37°C for 30 min with the membrane-permeant form of the Ca²⁺-indicator dye fluo-2 (10 μM; Molecular Probes), washed, and then incubated for 10 min in HEPES-buffered NaCl (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM CaCl₂, and 1 mM MgCl₂, pH 7.4). The dye-loaded cells were imaged in real time in a flow-through superfusion chamber that maintained 37°C and was attached to an inverted epifluorescence microscope (Nikon Eclipse TE-2000), as described previously (37). Quantitative image pairs at 340- and 380-nm excitation with emission at 510 nm were obtained every 15 s. The fluorescence excitation was shuttled off except during the short intervals required to record image pairs. Calibration of the emitted fluorescence signal from each cell in the field was performed at the end of each experiment using the Ca²⁺ ionophore ionomycin (10 μM) in a HEPES buffer containing either 2.0 mM CaCl₂ or 10 mM EGTA, titrated to pH 7.4, as previously described (38). Intracellular calcium levels were calculated as described by Grynkiewicz et al. (39). The imaging workstation was controlled with the MetaFluor software package (Universal Imaging).

Results

IL-10 inhibits human osteoclastogenesis

We tested the direct effects of IL-10 on human osteoclastogenesis in a standard, validated culture system (40). In this system primary human monocytes are initially cultured for 1–2 days with M-CSF to induce RANK expression (thereby generating RANKL-responsive pOCs) and are subsequently treated with RANKL to induce formation of multinucleated TRAP⁺ osteoclast-like cells that are capable of resorbing calcified matrix, which we confirmed in our system (data not shown). Control cells cultured with M-CSF and RANKL differentiated into multinuclear (more than three nuclei per cell) TRAP⁺ osteoclasts that formed large actin rings that represent a marker of the later stages of osteoclast differentiation (Fig. 1A and B). Eighty percent of mature osteoclasts in control conditions contained ≥10 nuclei (data not shown). The addition of IL-10 resulted in a dramatic near complete suppression of RANKL-induced osteoclastogenesis, as evidenced by diminished formation of TRAP⁺ multinucleated cells (Fig. 1A). This inhibitory effect of IL-10 was observed in a dose dependent manner.
Substantial inhibition of osteoclastogenesis by IL-10 was observed at IL-10 concentrations as low as 1.25 ng/ml, and inhibition was nearly complete at 5 ng/ml IL-10. Additionally, in IL-10-treated cells, staining for actin expression revealed diffuse staining in small mostly mononuclear cells, in sharp contrast to the discrete peripheral actin rings observed in large multinucleated polykaryons observed in control cells (Fig. 1B). Thus, IL-10 disrupted the formation of actin rings. Quantitation of the strong suppression of osteoclastogenesis by IL-10 (90% inhibition) in a representative experiment is shown in Fig. 1C. Cell viability was assessed by MTT assay and was not affected by addition of IL-10 (supplemental Fig. S1B). Additionally, IL-10 strongly suppressed RANKL-induced expression of the osteoclast-related genes cathepsin K, calcitonin receptor, TRAP, and matrix metallopeptidase 9 (MMP9) (Fig. 1D). Collectively, the results demonstrate that IL-10 strongly inhibits human RANKL-induced osteoclastogenesis and acts directly on monocytes and myeloid precursor cells.

**IL-10 preferentially inhibits RANKL-induced ERK MAPK signaling that is important for osteoclastogenesis**

Signaling induced by M-CSF, RANK, and ITAM-containing co-stimulatory immunoreceptors is essential for osteoclastogenesis, and thus we investigated the effects of IL-10 on these signaling pathways. IL-10 did not inhibit activation of MAPKs or Akt by M-CSF and actually modestly prolonged the kinetics of activation (Fig. 2A), suggesting that IL-10 did not inhibit osteoclastogenesis by suppressing M-CSF signaling. We next investigated the effects of IL-10 on RANK signal transduction in human osteoclast precursors. RANKL induced rapid and transient activation of ERK (2–30 min post-RANKL stimulation) in pOCs, and ERK activation was essentially completely abrogated by IL-10 (Fig. 2B); this inhibitory effect was consistently observed in more than 10 independent experiments with different blood donors. IL-10-mediated inhibition of ERK activation was specific to RANKL, as IL-10 did not block ERK activation by immune complexes that utilize the ITAM-containing FcR (Fig. 2B), although modest suppressive effects were observed with some blood donors (data not shown). Baseline JNK phosphorylation was elevated and not further induced by RANKL (supplemental Fig. S2C). Overall, the results show that IL-10 selectively inhibited RANK-mediated activation of the ERK MAPK pathway.

To further explore the functional consequences of inhibition of ERK activation by IL-10, we tested whether IL-10 suppressed...
FIGURE 3. IL-10 inhibits calcium-dependent signaling downstream of RANK. A and B, Human CD14+ monocytes were cultured with 10 ng/ml M-CSF and treated with 40 μM BAPTA (A) and 40 μM W-7 (B) for 1 h before stimulation with 100 ng/ml RANKL, and whole-cell lysates were analyzed by immunoblotting. C, Human CD14+ monocytes were cultured with 10 ng/ml M-CSF in the presence or absence of 100 ng/ml IL-10. Cells were then stimulated with 100 ng/ml RANKL for the indicated times. Whole-cell lysates were subjected to immunoblotting with anti-phospho-CaMKII, phospho-CREB, and p38 Abs. D, Human CD14+ monocytes were cultured with 10 ng/ml M-CSF and treated with 40 μM KN-92 or KN-93 for 1 h before stimulation with 100 ng/ml RANKL. Whole-cell lysates were analyzed by immunoblotting. E and F, Human CD14+ monocytes were cultured with 20 ng/ml M-CSF in the presence or absence of 100 ng/ml IL-10 for 24 h. In E, cells were then stimulated with 100 ng/ml RANKL for 24 h and intracellular calcium was monitored for 500 s. In F, cells were cultured with 40 ng/ml RANKL for 48 h. Whole-cell lysates were immunoblotted with anti-NFATc1 Abs. Data are representative of at least three independent experiments.

IL-10 preferentially inhibits a RANKL-induced calcium pathway that activates ERK MAPKs

ERK-dependent gene induction and sought the role of the ERK MAPK pathway in osteoclastogenesis. IL-10 activates an NFAT-mediated program of gene expression that becomes apparent a day after RANKL addition, but little is known about early gene responses to RANKL stimulation. First, we tested the effects of IL-10 on expression of c-Jun, which is important for osteoclastogenesis (41), and whose expression is dependent on ERKs in other systems. IL-10 inhibited RANKL-induced augmentation of c-Jun expression and osteoclastogenesis in human pOCs (supplemental Fig. 2D). Thus, inhibition of ERK activation by IL-10 is translated into inhibition of gene activation responses downstream of ERK. ERKs have been previously implicated in osteoclastogenesis in murine systems (42) and we wanted to link the ERK MAPK pathway to osteoclastogenesis in our system. Inhibition of the ERK MAPK pathway suppressed RANKL-induced expression of NFATc1 (Fig. 2F) and concomitantly suppressed RANKL-induced osteoclastogenesis (Fig. 2G). Thus, ERK activation in response to RANKL was necessary for osteoclastogenesis. Collectively, the results show that IL-10 preferentially suppresses the activation of ERKs downstream of the RANK receptor and suggest that IL-10 inhibits osteoclastogenesis in part by inhibiting the MEK ERK pathway.

ERK has been reported to be downstream of calcium/calcmodulin pathways in other systems (43). We confirmed this in human osteoclast precursors, finding that the calcium chelator BAPTA and the calmodulin inhibitor W-7 strongly suppressed RANKL activation of ERKs (Fig. 3, A and B). These results show that ERK activation by RANKL is dependent on calcium signaling and suggest that IL-10 inhibits calcium-dependent RANK signals. We next wanted to more directly examine the effects of IL-10 on calcium signaling. An acute RANKL-induced calcium flux has been difficult to detect (4, 18, 44), a finding that we confirmed in human cells (data not shown). However, tonic signaling by ITAM-coupled costimulatory receptors that supports calcium-dependent signaling has been observed in osteoclast precursors (7, 16, 45). Additionally, a role for calcium signaling in acute RANKL responses is suggested by rapid RANKL-induced activation of phospholipase C (PLC)γ2 that has been observed in serum starved murine pOCs; PLC-γ2 plays a key role in calcium-mediated responses and osteoclastogenesis in murine systems (6, 17, 46). Consistent with previous findings in mouse models, the PLC-γ inhibitor U73122 suppressed RANKL-induced NFATc1 expression and osteoclastogenesis in human pOCs (supplemental Fig. S3, A and B). However, in contrast to murine cells, RANKL did not detectably increase PLC-γ2 phosphorylation above the baseline; baseline PLC-γ2 phosphorylation was observed even in serum-starved human pOCs (supplemental Fig. S3D and data not shown). Moreover, IL-10 had no effect on PLC-γ2 activity (supplemental Fig. S3C). Collectively, these results confirmed a role for PLC-γ in human osteoclastogenesis, but suggested that RANKL also engages a parallel calcium signaling pathway that is targeted by IL-10. Therefore, we further investigated tonic and RANKL-inducible calcium signaling by measuring the phosphorylation of CaMKII, a calcium-dependent kinase that is phosphorylated and active at ambient calcium concentrations generated by tonic signaling, and whose phosphorylation increases in response to transient calcium fluxes (47). Consistent with previous reports suggesting both tonic and RANKL-inducible calcium
FIGURE 4. Regulation of TREM-2 expression. A. Human CD14+ monocytes were cultured with 10 ng/ml M-CSF in the presence or absence of 100 ng/ml IL-10. “Fresh” represents freshly isolated monocytes. mRNA was measured using real-time PCR. Representative results from at least three independent experiments are shown; suppression of TREM-2 expression by IL-10 was observed in >10 independent experiments. B. Whole-cell lysates from pOCs were subjected to SDS-PAGE and immunoblotted with either RANK, TREM-2, or STAT3 Abs. C. Human CD14+ monocytes were cultured with 10 ng/ml M-CSF in the presence or absence of 100 ng/ml IL-10. mRNA was isolated at the indicated times and was measured using real-time PCR. The values were normalized relative to the expression of GAPDH. D, CD14+ macrophages were purified from synovial fluids of patients with inflammatory arthritis (Synovial CD14+, n = 7) or blood monocytes from disease-free donors (ctrl, n = 4). mRNA expression was analyzed by real-time PCR. Each data point represents data corresponding to an individual donor, and the horizontal line depicts the mean; control data points are overlapping and thus individual data points are not apparent. E. Mouse bone marrow cells were cultured with 20 ng/ml murine M-CSF and harvested at the indicated times. mRNA was measured using real-time PCR. Representative results from at least two independent experiments are shown.

signaling in pOCs (4, 6, 17, 18, 44), we observed basal phosphorylation of CaMKII in human pOCs; the level of basal phosphorylation varied with different blood donors and was diminished after serum starvation (Fig. 3C and supplemental Fig. S4). RANKL stimulation rapidly increased CaMKII phosphorylation, and both basal and RANKL-induced CaMKII phosphorylation were strongly suppressed by IL-10 (Fig. 3C and supplemental Fig. S4). IL-10 also suppressed RANKL-induced phosphorylation of CREB, which occurs downstream of CaMKII in other systems (Fig. 3C and supplemental Fig. S4, A and B). These results suggest a role for CaMKII in the early phase of RANKL-induced calcium signaling and support the notion that IL-10 suppresses calcium-dependent signaling pathways downstream of RANK.

We used the specific CaMK inhibitor KN-93 to test the role of CaMKs in rapid RANK signaling. KN-93, but not the control compound KN-92, suppressed RANKL-induced activation of ERK, and also of the MEK kinase that lies upstream of ERK and phosphorylates ERK (Fig. 3D), thus placing MEK and ERK downstream of CaMK in calcium-dependent RANK signaling. In contrast, KN-93 had a minimal effect on RANKL-induced CREB phosphorylation, suggesting that rapid activation of CREB for the most part occurs independently of CaMK in our system. KN-93 did not prevent RANKL-induced I-κBα degradation (supplemental Fig. S5). Consistent with CaMK-mediated modulation of the NF-κB pathway, KN-93 down-regulated basal I-κBα expression, likely secondary to diminished NF-κB p65 serine 536 phosphorylation (supplemental Fig. S5). Overall, these results define a rapidly activated calcium-dependent CaMK-MEK-ERK pathway downstream of RANK that is inhibited by IL-10.

RANKL induces calcium oscillations and activation of calmodulin at delayed time points (24 h) after RANKL stimulation (18, 48), and these calcium oscillations have been suggested to be dependent on ITAM signaling and important for the activation of NFATc1. Strikingly, IL-10 also inhibited delayed RANKL-induced calcium oscillations (Fig. 3E). IL-10 also inhibited RANKL-induced NFATc1 expression (Fig. 3F). These results confirm the previously described inhibition of NFAT expression by IL-10 in murine cells (30), and they reveal an IL-10-induced defect in proximal and delayed RANKL-induced calcium signaling.

TREM-2 expression is induced during human pOC formation and suppressed by IL-10

Current models suggest that calcium signaling downstream of RANK is mediated by ITAM-dependent signals. Thus, we tested the hypothesis that IL-10 suppresses RANKL-induced calcium signaling by affecting the expression or function of ITAM-containing adaptors or associated costimulatory receptors. RANK and immunoreceptor expression was analyzed during culture of primary human monocytes with M-CSF for 1–2 days to induce osteoclast precursors in the absence or presence of IL-10. As expected, freshly isolated monocytes expressed low levels of RANK mRNA and protein that increased during culture with M-CSF (Fig. 4, A and B). Surprisingly, TREM-2 mRNA and protein expression were low in monocytes and strongly increased in parallel with RANK expression (Fig. 4, A and B). TREM-2 expression increased in a time-dependent manner, with the earliest increases apparent after 6–12 h of culture (Fig. 4C). In contrast, the expression of the other costimulatory receptors OSCAR, ILT7 (the human ortholog of PIR-A), and SIRPβ1, and of DAP12 and FcRγ, did not change during generation of human osteoclast precursors (Fig. 4A and data not shown). These data suggest that, in addition to RANK expression, induction of TREM-2 expression is an important component of the formation of human pOCs. This notion was supported by the
parallel increase in RANK and TREM-2 expression in joint macrophages in rheumatoid arthritis, an inflammatory disease characterized by increased osteoclast formation and bone resorption (Fig. 4D).

Interestingly, the increase in TREM-2 mRNA and protein expression that occurred during generation of human pOCs was strongly suppressed by IL-10 (Fig. 4, A and B). IL-10 suppressed increases in TREM-2 expression beginning at the earliest time points in culture (Fig. 4C). IL-10 had a small to modest suppressive effect on RANK expression in cultures with cells from multiple blood donors and had no detectable effect on expression of OSCAR, ILT7, SIRPβ1, DAP12, and FcγR (Fig. 4, A and B, and data not shown). In contrast to the human system, during murine osteoclast differentiation cultures the kinetics of increased TREM-2 mRNA expression were delayed until the later stages of culture after RANKL had been added (Fig. 4E), which is consistent with the previously reported delayed increase in TREM-2 protein (49). In the murine system, IL-10 inhibited the late phase of TREM-2 expression, but had minimal effects at earlier time points (Fig. 4E). Delayed increases of TREM-2 expression that occur at time points when expression of other immunoreceptors such as OSCAR is elevated provide a plausible explanation for a more redundant role for TREM-2 in murine osteoclastogenesis.

TREM-2 is important for human osteoclastogenesis, as patients with TREM-2 mutations develop defects in bone remodeling that manifest as Nasu-Hakola disease (9–11). We confirmed an overall positive role for TREM-2 in human osteoclastogenesis using RNAi to knock down TREM-2 expression in primary human pOCs. Decreased TREM-2 expression resulted in a significant decrease of osteoclastogenesis (Fig. 5, left panel; \( p < 0.01 \), Student’s t test), although the degree of inhibition varied with different blood donors. The striking decrease in osteoclastogenesis observed in some donors when TREM-2 expression was diminished is shown in a representative photomicrograph in Fig. 5, right panels. Overall, these results suggest that IL-10 suppresses the generation of human pOCs at least in part by inhibiting TREM-2 expression.

TREM-2 regulates RANK signaling at an early point in human osteoclastogenesis

We took several approaches to substantiate a role for inhibition of TREM-2 expression by IL-10 in mediating the effects of IL-10 on RANK signaling and osteoclastogenesis. TREM-2 expression decreased markedly after stimulation of human pOCs with RANKL, and IL-10 had minimal further effects on this low level of TREM-2 expression (Fig. 6A). This decrease of TREM-2 expression likely...
represents a shift from the use of TREM-2 toward other costimulatory molecules, such as $\alpha_v\beta_3$ integrins, whose expression is induced in preosteoclasts after RANKL stimulation (8). Interestingly, addition of IL-10 after RANKL stimulation, at a time when TREM-2 expression was low, no longer suppressed osteoclastogenesis (Fig. 6B). Furthermore, addition of IL-10 after RANKL stimulation did not inhibit RANKL-induced expression of NFATc1 (Fig. 6C) or osteoclast-related genes (Fig. 6A). These results show that IL-10 works at an early stage of osteoclast differentiation by affecting myeloid precursor cells, and they also show a loss of IL-10 inhibitory activity in cells that do not express significant levels of TREM-2.

Next, we determined whether suppression of TREM-2 expression or function would have similar effects on RANK signaling as did treatment with IL-10. RNAi-mediated knockdown of TREM-2 expression resulted in diminished RANKL-induced phosphorylation of ERK (Fig. 7A). RANKL-induced ERK activation was also inhibited by the Syk kinase inhibitor piceatannol that inhibits proximal TREM-2 signaling (Fig. 7B). Consistent with the role of TREM-2 in osteoclastogenesis (Fig. 5) and with the dependence of NFATc1 expression on ERKs (Fig. 2F), suppression of TREM-2 expression inhibited RANKL-mediated induction of NFATc1 (Fig. 7C). We then tested whether forced expression of TREM-2 was sufficient to overcome the inhibitory effects of IL-10 on RANKL-induced ERK activation and downstream NFATc1 expression. Forced expression of TREM-2 in primary human pOCs using nucleofection of a TREM-2 expression vector abolished the capacity of IL-10 to inhibit RANKL-induced phosphorylation of ERK (Fig. 7D). Additionally, lentiviral expression of TREM-2 reversed IL-10-mediated inhibition of NFATc1 expression (Fig. 7E). The levels of TREM-2 expression in both nucleofected and transduced cells were comparable to physiologic levels (1–1.5-fold, data not shown). Collectively, these results show that TREM-2 mediates rapid calcium signaling, ERK activation, and NFATc1 expression downstream of RANK, and that IL-10 inhibits this signaling pathway by down-regulating TREM-2 expression. However, forced expression of TREM-2 was not sufficient to reverse the inhibitory effects of IL-10 on osteoclastogenesis (Fig. 7F), suggesting that IL-10 inhibits additional aspects of the osteoclastogenesis program, possibly the activation or function of NFATc1.

**IL-10 inhibits TREM-2 transcription**

Analysis of gene expression during osteoclastogenesis has predominantly focused on the period after RANKL addition (5). Little is known about regulation of TREM-2 expression (45) or generally about gene expression during generation of pOCs beyond the basic observation that RANK expression is increased by M-CSF (5, 40). We wanted to investigate mechanisms that regulate TREM-2 expression in human pOCs. First, we measured TREM-2 transcription using primary transcript analysis. Expression of primary transcripts reflects rates of transcription, rather than the balance between transcription and mRNA decay that determines levels of
steady-state mRNA, and primary transcript analysis has gained broad acceptance as a method to measure transcription rates (50, 51). TREM-2 primary transcripts increased during osteoclastogenesis cultures (Fig. 8A). IL-10 effectively suppressed the increase in TREM-2 primary transcripts (Fig. 8A), indicating that IL-10 inhibited TREM-2 gene transcription. Consistent with this result, IL-10 suppressed methylation of histone H3 on lysine 4, a mark of “poised” and transcriptionally active promoters, at the TREM-2 locus (Fig. 8B). Thus, IL-10 regulates TREM-2 at the level of gene expression.

RANK expression is dependent on the transcription factor PU.1 (52) and on M-CSF (2, 40). RNA interference experiments demonstrated that TREM-2 expression was dependent on PU.1, similar to RANK expression (Fig. 8C). However, in contrast to RANK, the increase in TREM-2 expression during generation of pOCs was not dependent on M-CSF (Fig. 8D). These results suggest that, in addition to the well-described M-CSF-dependent program of pOC development that increases RANK expression, there is a parallel M-CSF-independent program that increases expression of the costimulatory receptor TREM-2 and is thus required for effective human osteoclastogenesis. The M-CSF-independent expression of TREM-2 was broadly suppressed by well-known inhibitors of osteoclastogenesis, as IFN-γ and IFN-α also suppressed TREM-2 expression (Fig. 8E). In contrast, RANK expression was not inhibited by IL-10 or IFN-α (data not shown). These results indicate that TREM-2 is a common target for inhibitors of osteoclastogenesis, and they suggest that TREM-2 and RANK expression are induced by different mechanisms during pOC generation.

Discussion

In this report, we have delineated a new rapidly activated calcium-mediated costimulatory pathway in human osteoclast precursors that is dependent on TREM-2 and leads to ERK activation and NFATc1 expression and thus promotes RANKL-induced osteoclastogenesis. IL-10 suppressed human osteoclastogenesis in part by down-regulating TREM-2 expression and thereby inhibiting costimulation of RANK signaling by attenuating calcium pathways. IL-10 and other homeostatic cytokines inhibited an early step in human osteoclastogenesis, the generation of RANK/TREM-2 osteoclast precursors, by inhibiting TREM-2 transcription. These results reveal a new mechanism of inhibition of osteoclastogenesis that is particularly relevant for human cells that are highly dependent on TREM-2 (9–11). The results also yield insights into mechanisms that mediate crosstalk between ITAM-coupled receptors and heterologous receptors such as RANK, and they identify a new mechanism by which IL-10 can suppress cellular responses to receptors of the TNFR family.

An important role for calcium-mediated signaling and NFATc1 activation in RANK-induced osteoclastogenesis is well established, as is the dependence of calcium signals on the ITAM-containing adaptors DAP12 and FcRγ (4, 6, 18). Induction of delayed calmodulin activity and calcium oscillations that activate NFATc1 after RANKL stimulation has been reported, but it has been difficult to detect an acute RANKL-induced calcium flux (18, 48, 53). A plausible explanation for this difficulty in observing an acute calcium flux is high background due to tonic calcium signaling by...
DAP12 and FcRγ that are basally phosphorylated and active (7, 16, 45). A high baseline makes it difficult to detect additional RANKL-induced calcium signals; indeed, serum starvation to diminish background was required to detect RANK-induced activation of PLCγ that typically leads to a calcium flux (6, 17). We monitored tonic and inducible calcium signaling by measuring the calcium-dependent phosphorylation of CaMKII. This approach showed baseline CaMKII phosphorylation consistent with tonic calcium signaling, and it was able to detect increased phosphorylation that occurred rapidly after RANKL stimulation. These results provide clear evidence for rapidly induced RANK-mediated calcium-dependent signaling, and they provide support for a model whereby tonic ITAM signaling (7, 16, 45) and an additional RANKL-induced signal that is also dependent on ITAM-containing adaptors (6, 17) are integrated downstream of RANK. Additionally, we found that rapid RANKL-induced phosphorylation of MEK and ERK was calcium- and CaMK-dependent, and ERK activation was required for osteoclastogenesis. These results delineate a new CaMK-MEK-ERK calcium-dependent signaling pathway downstream of RANK, in addition to the previously described delayed calcium oscillations and NFATc1 activation. Inhibition of the various phases of RANKL-induced calcium signaling by IL-10 contributes to its suppressive effects on human osteoclastogenesis.

IL-10 is a potent inhibitor of myeloid cell responses to many inflammatory factors, including LPS, TNF, and IL-1 (54), which induce IL-10 production as a feedback mechanism. Mechanisms underlying the suppressive effects of IL-10 have been under intense investigation but have proven difficult to elucidate (55). The suppressive effects are mediated by IL-10-induced genes, but the identity of these genes and their targets for inhibition are not known, and it appears that IL-10 induces multiple inhibitory genes that act via different mechanisms. Inhibitory effects of IL-10 on inflammatory signal transduction have not been consistently observed, although the IL-10-induced protein ABIN-3 has been recently shown to inhibit NF-κB activation in human but not murine cells, secondary to species differences in the ABIN homology domain (29). Consistent with previous work, we found that IL-10 did not directly inhibit signaling when an ITAM-containing adaptor was activated by crosslinking (supplemental Fig. S2A). Instead, IL-10 indirectly affected RANK signaling by suppressing expression of its costimulatory receptor TREM-2 in human cells. These results identify a new mechanism by which IL-10 suppresses myeloid cell responses to cytokines and suggest that analysis of the effects of IL-10 on the expression of coreceptors or costimulatory receptors may be a fruitful new line of investigation into mechanisms of IL-10 function in other systems.

Forced expression of TREM-2 only partially reversed the inhibitory effects of IL-10 and did not restore osteoclastogenesis. Thus, IL-10 activates inhibitory mechanism(s) in addition to down-regulation of TREM-2. Our results yield two insights into additional mechanisms by which IL-10 can regulate myeloid cell responses to environmental factors. First, IL-10 inhibited NFAT activation by inhibiting calcium oscillations and possibly suppressed NFATc1 function (Figs. 3 and 7 and K.-H.P.-M., unpublished observations). Additionally, IL-10 suppressed the expression of genes important for the generation and function of osteoclasts even in the absence of RANKL stimulation. For example, expression of OSCAR, ATP6v0d2, TRAP, and MMP9 increased during generation of human pOCs, and it was further superinduced in response to RANKL stimulation. IL-10 inhibited basal and RANKL-induced expression of these genes in both osteoclasts and macrophages (K.-H.P.-M., unpublished observations). In combination with previous reports, these data imply that IL-10 regulates myeloid cell fate decisions by suppressing differentiation toward osteoclasts and instead promotes differentiation into M2 macrophages (9–11). pOCs are contained within the pool of monocytes that migrate into sites of inflammation, where they are exposed to IL-10 and also osteoclastogenic cytokines such as RANKL and TNF. At early stages after their entry into inflammatory sites, IL-10 would suppress pOC differentiation down the osteoclast pathway; the extent of osteoclastogenesis would be determined by the balance between IL-10 and osteoclastogenic factors present in the inflammatory microenvironment. However, at later stages of differentiation after monocytes/pOCs have matured in response to RANKL or other cytokines found at inflammatory sites, they would become refractory to the inhibitory effects of IL-10 and the osteoclastogenesis pathway would predominate.

TREM-2 is a DAP12-associated receptor that plays a nonredundant role in the differentiation of human osteoclasts and in bone resorption in vivo in humans (11). We found that TREM-2 expression was rapidly induced during the generation of human pOCs and played a key role in costimulation of RANK signaling. We showed that TREM-2 had a direct role in human osteoclastogenesis, but the effects of TREM-2 varied with donors. In our system, the Fcγ-associated OSCAR and ILT7 are not engaged and expression of SIRPβ1 did not change during culture. A recent study showed that MDL-1 is coupled with DAP-12 and DAP-10 and plays an important role in osteoclastogenesis (56). Thus, under these culture conditions, which are clearly relevant for human osteoclastogenesis in vivo (57, 58), TREM-2 played a dominant costimulatory role early in osteoclast differentiation, at the stage of pOC generation and initial RANK signaling. Later during osteoclast differentiation, after addition of RANKL, the relative expression of various costimulatory receptors changes (8, 57). For example, expression of OSCAR and αβ integrins that also signal via DAP12 (8) increases and expression of TREM-2 in human cells decreases after RANKL stimulation. Thus, it is likely that the relative importance of individual costimulatory receptors varies with time, expression of ligands, and physiological context.

IL-10 has been previously shown to inhibit murine osteoclastogenesis (30, 31). In one study, IL-10 was shown to inhibit RANKL-induced expression and nuclear localization of NFATc1 in the RAW267.4 murine cell line (30). This study suggested that IL-10 suppressed calcium signaling but did not address underlying mechanisms. A second study (31) also showed that IL-10 inhibited NFATc1 expression in RAW267.4 cells and primary murine pOCs by a mechanism that involved decreased activation of JNK and diminished expression of Fos; these findings suggest inhibition of signaling downstream of RANK and TRAF6, rather than inhibition of costimulation. In contrast, our study shows that in human pOCs, IL-10 inhibits RANK responses by abrogating TREM-2-mediated costimulation, thereby identifying a new inhibitory mechanism. The different findings in human and murine systems are most likely explained by differences in the kinetics of TREM-2 expression, and its regulation by IL-10, between human and murine pOCs. In human cells, TREM-2 expression increases early in parallel with increased RANK expression and contributes to initial RANK signaling. In murine cells TREM-2 expression increases only modestly during generation of pOCs, and lower expression of TREM-2 likely results in a smaller contribution to initial RANK signaling (49). TREM-2 may provide more effective costimulation for RANK signaling at later time points in murine osteoclast differentiation cultures when it is highly expressed, or secondary to constitutive high level expression in murine RAW267 subclones (1). Thus, the less prominent role of TREM-2 in murine osteoclastogenesis likely arises from differences in the kinetics and magnitude of expression relative to other costimulatory receptors, rather
than differences in TREM-2 function. Overall, our findings help to clarify reasons underlying the differences between the role of TREM-2 in human and murine osteoclast differentiation and bone resorption.

Investigation of the regulation of osteoclastogenesis has predominantly focused on the RANKL-induced differentiation program, and thus on later events, after the generation of osteoclast precursors (1). Our results reveal an unexpected complexity in the regulation of human pOCs that goes beyond M-CSF-induced expression of RANK and includes regulation of the expression of costimulatory receptors such as TREM-2, and possibly other molecules important in osteoclastogenesis. Regulation of TREM-2 occurred at the level of transcription and was targeted by several inhibitors of osteoclastogenesis such as IFNs and IL-10. Regulation of the transcription of RANKL-induced genes has been studied extensively, with important roles defined for constitutive PU.1 and Mist1 that collaborate with RANKL-induced NF-κB, AP-1, and NFATc1. In contrast, little is known about transcriptional regulation during generation of osteoclast precursors, especially the newly described M-CSF-independent component that drives TREM-2 expression. Our findings highlight the functional importance of transcriptional regulation during pOC development and open this new area for future investigation.

In conclusion, we have found that IL-10 inhibits the development of human osteoclast precursors by suppressing the transcription of the costimulatory receptor TREM-2. Low expression of TREM-2 results in selective defects in RANK calcium-dependent signaling and human osteoclast differentiation. These findings identify a mechanism for the physiologically important suppressive effects of IL-10, and they suggest that diminished calcium-dependent RANK signaling represents the molecular basis for defective osteoclast differentiation in patients with Nasu-Hakola disease who harbor mutations in TREM-2.

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Disclosures
The authors have no financial conflicts of interest.

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