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TREM2, a DAP12-Associated Receptor, Regulates Osteoclast Differentiation and Function

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ABSTRACT: Deficiency of the signaling adapter protein DAP12 or its associated receptor TREM2 is associated with abnormal OC development in humans. Here we examine the role of TREM2 in mouse OC development and function, including migration and resorption in vitro. These results provide new evidence that TREM2 regulates OC function independent of its effects on multinucleated OC differentiation.

Introduction: TREM2 (triggering receptor expressed in myeloid cells-2) associates with the signaling adapter DAP12 in osteoclasts (OCs). Genetic mutation or deletion of either the *TYROBP* (DAP12) or *TREM2* gene is associated with the human disorder of brain and bone, Nasu-Hakola disease. We and others recently showed the critical requirement for immunoreceptor tyrosine-based activation motif (ITAM) signals through DAP12 and the Fc Receptor γ chain (FcRγ) during OC development. Here, we further define the role of TREM2 in OC differentiation and describe a role for TREM2 in OC migration and bone resorption.
Materials and Methods: We generated monoclonal anti-mouse TREM2 antibodies (mAb), analyzed pre-osteoclasts and mature OCs for TREM2 surface expression, and determined the effect of antibody ligation on in vitro OC differentiation, resorption, and migration. TREM2 RNA interference (RNAi) was used to disrupt expression of TREM2 in pre-osteoclasts.
Results: Using flow cytometry, our studies reveal that TREM2 is weakly expressed on C57BL/6 bone marrow macrophages (BMMs) and is upregulated during culture with RANKL and macrophage-colony stimulating factor (M-CSF). The expression of TREM2 is unaltered in DAP12-deficient OCs. Using C57BL/6 BMMs or RAW264.7 precursors, anti-TREM2 mAb treatment with RANKL and M-CSF enhances the formation of multinuclear TRACP$^+$ OCs compared with control mAb treatment. In contrast, these agents have no effect on DAP12-deficient precursors. Monoclonal Ab blockade of TREM2 on OCs generated from C57BL/6 BMMs results in decreased resorption of artificial calcium-phosphate substrate and dentine. Reduction of TREM2 expression in RAW264.7 cells by RNAi results in loss of OC formation in response to RANKL and M-CSF. Anti-TREM2 cross-linking enhances migration of C57BL/6 OCs and RAW246.7 OCs in response to M-CSF.
Conclusions: Our studies indicate that the TREM2 receptor regulates OC multinucleation as well as resorption and migration of mature OCs. Thus, TREM2-DAP12 signals regulate both OC formation and function.
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Key words: rodent, monocytes/macrophages, osteoclast, DAP12, resorption

INTRODUCTION

Osteoclasts (OCs) are unique bone-resorbing cells that differentiate from myeloid lineage cells on activation with RANK and colony-stimulating factor-1 receptor by their corresponding ligands RANKL and macrophage-colony stimulating factor (M-CSF).[1] Recent studies have shown that OC development and function in vivo and in vitro have a critical requirement for immunoreceptor tyrosine-based activation motif (ITAM) adapter proteins that are associated with immunomodulatory receptors.[2–7] Mice deficient in both of the ITAM-signaling adapter proteins DAP12 and Fc receptor γ-chain (FcRγ) are severely osteopetrotic because of impaired formation and function of OCs,[4,6] whereas mice deficient in DAP12 only are mildly osteosclerotic with OCs that are normal in number but are impaired in function.[3,5] OCs doubly deficient in the ITAM-adapter proteins DAP12 and FcRγ are mononuclear and are unable to resorb dentine in vitro.[4,6]

The ITAM-adapter proteins DAP12 and FcRγ are homodimeric transmembrane proteins that lack extracellular ligand binding domains. Both DAP12 and FcRγ associate

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with surface receptors through charged residues in their transmembrane domains. Activation of the adapter-associated receptors leads to phosphorylation of the tyrosines within the ITAM, probably by Src-family kinases, which in turn recruit and activate SYK kinase. *SYK*-deficient OCs fail to develop and function in vitro, similar to DAP12/FcRγ doubly deficient OCs.[4] DAP12-associated receptors present in OCs include triggering receptor expressed in myeloid cells-2 and -3 (TREM2, 3), signaling regulatory protein β1 (SIRP β1), and myeloid DAP12 associated lectin-1 (MDL-1).[3,6]

Interestingly, deletions or loss of function mutations in either the *TYROBP* (DAP12) or *TREM2* genes are associated with the same rare human disorder, Nasu-Hakola disease, also called polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL).[8–10] This represents a rare example in which mutations in either partner in a heterodimeric signaling complex lead to the same phenotypic disease. In PLOSL, large cystic lesions develop in the cancellous bones, leading to arthritis and pathologic fractures before the third decade. These findings strongly suggest that the TREM2-DAP12 signaling complex is functionally important in bone remodeling and homeostasis in those boney regions. Here, we further show the role of TREM2/DAP12 signaling in OC differentiation and function by direct stimulation or blockade of TREM2. The results indicate that TREM2 is necessary for OC formation in vitro, and it regulates both OC resorption and migration.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Simonsen (Gilroy, CA, USA). Homozygous DAP12-deficient mice, backcrossed to C57BL/6 mice for nine generations, were previously described.[11] Mice were maintained under pathogen-free conditions in the animal facility of the San Francisco Veterans Affairs (VA) Medical Center. All experiments were performed in accordance with American Association for the Accreditation of Laboratory Animals (AAALC) guidelines and were approved by the VA Animal Care Committee. In vitro studies used cells isolated from mice at 6–20 weeks of age.

Cells

RAW264.7 cells (ATCC) were maintained in RPMI-1640 medium (Mediatech) supplemented with 10% FBS (Atlantic Biologics, Atlanta, GA, USA) and penicillin-streptomycin-glutamine (Mediatech). TREM2B cDNA with a FLAG epitope added to the N terminus was inserted into the retroviral vector, pMXpie, upstream of an IRES, followed by an eGFP cDNA sequence.[12] Cells were transfected with pMXpie-FLAG TREM2B, using Fugene 6 (Roche) according to the manufacturer's protocol. Cells were selected in puromycin (Sigma) at 2 μg/ml. Stable puromycin-resistant clones were screened for anti-FLAG M2 mAb (Sigma) binding by using flow cytometry and then subcloned and maintained in puromycin selection media.

Monoclonal antibody generation

Mouse TREM2-Fc fusion protein emulsified in Titermax adjuvant (Sigma) was injected into a hind footpad of Fisher rats on days 1 and 4, followed by two immunizations on days 8 and 11 with Chinese hamster ovary (CHO) cells expressing TREM2. Popliteal lymph nodes were harvested on day 12 and were fused with rat YB-2/0 plasmacytoma cells (ATCC). Hybridoma supernatants were screened for binding to CHO cells engineered to express mouse TREM2. Monoclonal Ab TREM2 78 is rat isotype IgG_1 and does not cross-react with mouse TREM1 or TREM3. Purified mAb was digested with pepsin (Sigma) into $F(ab')_2$ fragments, and intact antibody was removed by absorption to Protein G.

Osteoclast differentiation

Bone marrow monocyte/macrophage (BMM) precursor cells were isolated and were differentiated into OCs as previously described using RANKL and M-CSF (Peprotech).[3,4] RAW264.7 or transfectants were seeded in 96-well plates with 3000 cells/well in α-MEM supplemented with 10% FBS, penicillin-streptomycin-glutamine, 50 ng/ml RANKL, and 20 ng/ml M-CSF. Medium was changed every 3 days. TRACP in cultured OCs was identified using a commercial kit (product 387-A; Sigma). Multinucleated (at least three nuclei) $TRACP^+$ cells were counted and scored by light microscopy. To determine complexity and size differences, OCs were counted by number of nuclei (>10 or 3–10 nuclei). The surface area of OCs was measured by using Image J software (NIH).

Antibody stimulation/blockade assays of multinucleation

For stimulation of TREM2, plates were coated with goat $F(ab')_2$ anti-rat IgG (ICN Pharmaceuticals) at 10 μg/ml, and anti-TREM2 mAb or an isotype-matched control mAb, R334, or A110 (BD Biosciences), was added at a final concentration of 20 μg/ml. The antibodies were present throughout the duration of culture. For blockade studies, $F(ab')_2$ fragments of TREM2 mAb or control mAb (3.2.3) were added to the differentiation media at 10–20 μg/ml as indicated.

Migration assay

Migration assays were performed as previously described,[13,14] with the following modifications. Day 5 OCs were generated as above, removed from plastic culture dishes with 0.25% trypsin/0.02% versene (UCSF Cell Culture), washed, and resuspended in migration buffer (α-MEM supplemented with 1% FBS and 0.5% BSA; Sigma). For stimulation of TREM2, OCs were treated with anti-TREM2 mAb at 20 μg/ml on ice for 20 minutes, followed by goat $F(ab')_2$ anti-rat IgG at 10 μg/ml for 10 minutes. Cells (1×10^5) were placed in the top chamber of a 0.5-μm pore Transwell plate (Corning) in migration buffer with or without 100 ng/ml M-CSF (Peprotech) in the bottom chamber. Cells were allowed to migrate overnight, and the transwell membrane was fixed and stained with crystal violet. Cells on top of the membrane were removed with a cotton

swab. Crystal violet stain was eluted from the adherent migrated cells on the membrane with 100% ethanol and was quantified by determining the OD 595 of the eluate. All experiments were performed at least twice.

Resorption assay

Day 5 cultured OCs were removed from plastic dishes after 15-minute treatment with 0.25% trypsin/0.02% versene, washed, and resuspended in α-MEM supplemented with 10% FBS, penicillin-streptomycin-glutamine, RANKL 50 ng/ml, and M-CSF 20 ng/ml. Cells (5×10^4) were treated with no antibody or with either intact anti-TREM2 mAb with or without cross-linker or with $F(ab')_2$ fragments of anti-TREM2 mAb or control mAb (3.2.3) added to the medium at 20 μg/ml. Antibody treatment of cells included anti-TREM2 mAb (or control mAb) at 20 μg/ml, on ice for 20 minutes, plus or minus cross-linking antibody [goat $F(ab')_2$ anti-rat IgG at 10 μg/ml] for 10 minutes before plating on dentine discs (IDS, Tyne and Wear, UK) or BD BioCoat Osteologic Discs MultiTest Slides (BD Biosciences). Resorption area was determined by measuring the surface area of individual pits (~150/treatment) using Image J software (NIH) or as previously described.[4]

Lentivirus RNA interference

We used a protocol similar to the method described by Rubinson et al.,[15] with the following modifications. Cells (4×10^6 293T; ATCC) were plated for 24 h in 10-cm tissue culture dishes in DME-H16 medium supplemented with 10% fetal bovine calf serum (FBCS) and penicillin-streptomycin-glutamine. Before transfection, 70–80% confluent 293T cells were incubated in DME-H16 medium without antibiotics for 5 h. In tubes (Falcon 2059) 1 ml of OptiMEM medium (Gibco) was combined with 90 μl Fugene and the following plasmids: 15 μg of lentivirus RNAi vectors (pLLpuro, pLLpuro Trem2b RNAi 1 or 2), 5 μg pREV, 5 μg pVSVG, and 5 μg of pMDL g/p RRE, for a total of 30 μg DNA per transfection. After 45 minutes at room temperature, the transfection mixture was added to the 293T cells for 5 h at 37°C, and 10 ml of DME-H16 medium without antibiotics was added. At 72 h after transfection, viral supernatants were harvested and spun for 5 minutes at 500*g* to remove cell debris. The spun supernatants were filtered though a 0.45-μm syringe filter, combined, and transferred to a 50-ml Centricon tube and spun at 3000*g* for 120 minutes at 4°C. An inverted spin of 5 minutes at 1500*g* yielded concentrated viral supernatant. RAW264.7 cells were placed in 24-well plates at $1–2 \times 10^5$ cells/well in 0.25 ml of enriched α-MEM containing 20% FBS, penicillin-streptomycin-glutamine, and 4 mM HEPES (pH 7.4). Lentiviral supernatant (0.75 ml) containing 4 μg/ml polybrene (Sigma) was added to each well, and plates were centrifuged for 90 minutes at 1141*g* at room temperature. Two hours after addition of the lentivirus, the supernatant was removed and replaced with 1 ml of α-MEM containing 10% FBS and penicillin-streptomycin-glutamine. This procedure was repeated the next day, after which cells were cultured in supplemented α-MEM overnight. Cells were selected with puromycin at 2 μg/ml.

Flow cytometry

Pre-osteoclasts and OCs were removed from tissue culture plates by treatment with 0.25% trypsin/0.02% versene (UCSF Cell Culture). Nonspecific staining was blocked with rat anti-mouse FcR mAb 2.4G2 (BD Pharmingen). TREM2 surface staining was measured by using a FACS Calibur (BD Biosciences) with biotinylated TREM2 mAb followed by APC-conjugated streptavidin. Biotinylated isotype matched control antibody was R3-34 (BD Biosciences).

RESULTS

Combination of RANKL and M-CSF upregulates TREM2 during in vitro osteoclastogenesis

C57BL/6 BMMs were isolated and treated with M-CSF alone or with M-CSF and RANKL. After 1 day of culture with M-CSF alone, TREM2 was present on <10% of cells (Fig. 1A). Culture of BMMs in both M-CSF and RANKL increased the fraction of cells expressing TREM2 to 37%, and the level of expression of TREM2 on these cells was notably higher (mean fluorescence intensity, 142.89; Fig. 1B). When culture in M-CSF and RANKL was extended to 5 days, the fraction of cells expressing TREM2 rose to 57%, but the level of TREM2 expression fell (mean fluorescence intensity, 110.77; Fig. 1C).

DAP12 associates with a variety of activating receptors through paired charged residues in the transmembrane region of each protein, and this interaction is critical for stabilizing its expression at the cell surface in natural killer (NK) cells and myeloid cells. In the absence of DAP12, several DAP12-associated receptors (DARs), including TREM1 in myeloid cells and Ly49H and Ly49D in NK cells, exhibit significantly decreased surface expression.[7,11] Therefore, we analyzed $DAP12^{-/-}$ BMMs for expression of TREM2 and found that TREM2 was expressed on the cell surface of BMMs from DAP12-deficient mice maintained in M-CSF for 2 days (Fig. 1D). $DAP12^{-/-}$ OC precursors increased TREM2 surface expression in a manner similar to $DAP12^{+/+}$ precursors when stimulated with RANKL and M-CSF for 2 or 4 days (Figs. 1E and 1F).

RAW264.7 cells are dependent on TREM2 surface expression for in vitro osteoclastogenesis

RAW264.7 cells are a monocyte/macrophage leukemia line with OC potential. When treated with RANKL and M-CSF, RAW264.7 cells differentiate into large, $TRACP^+$ osteoclast-like cells (OCLs; Fig. 2A, T2-HIGH). We found, however, that over time in culture, some RAW264.7 lines lose OC differentiation potential and form fewer, small $TRACP^+$ OCLs with few nuclei (Figs. 2A and 2B, T2-LOW). We analyzed several RAW264.7 sublines that varied in OC differentiation potential for TREM2 surface expression as assessed by flow cytometry. Several cell lines that formed large OCs had bright TREM2 staining (e.g., Fig. 2C, T2-HIGH), whereas two cell lines with poor OC potential had weak TREM2 surface expression (e.g., Fig. 2C, T2-LOW). Thus, TREM2 surface expression correlated with the OC potential of RAW264.7. To test whether this

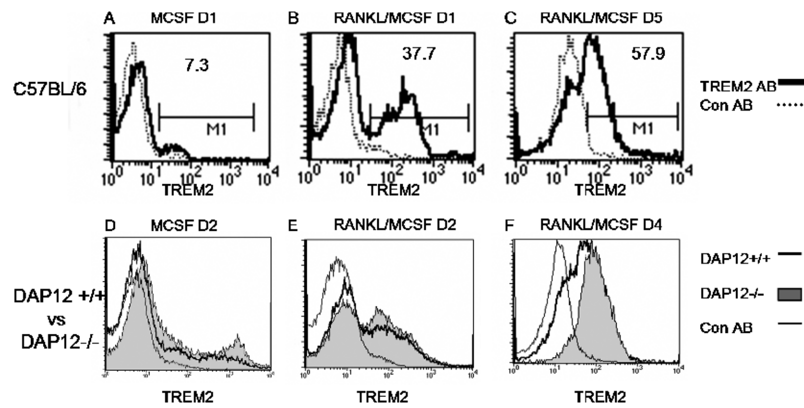

FIG. 1. TREM2 surface expression increases during in vitro OC differentiation. BMMs isolated from C57BL/6 mice were maintained for 2 days in media supplemented with M-CSF, and nonadherent cells were transferred to OC differentiation media containing 50 ng/ml RANKL and 10 ng/ml M-CSF. C57BL/6 BMMs were analyzed by flow cytometry for surface expression of TREM2 over time: (A) M-CSF day 1, (B) RANKL/M-CSF day 1, (C) RANKL/M-CSF day 5. The percentage of cells expressing TREM2 (M1 bars) is shown in the top right corner of each histogram. Data are representative of three experiments. $DAP12^{-/-}$ BMMs cultured for 2 days in M-CSF (D, gray histogram) also express TREM2. (E and F) $DAP12^{-/-}$ pre-osteoclasts upregulate TREM2 after RANKL and M-CSF stimulation and develop a homogenous $TREM2^+$ population similar to $DAP12^{+/+}$ C57BL/6 OCs.

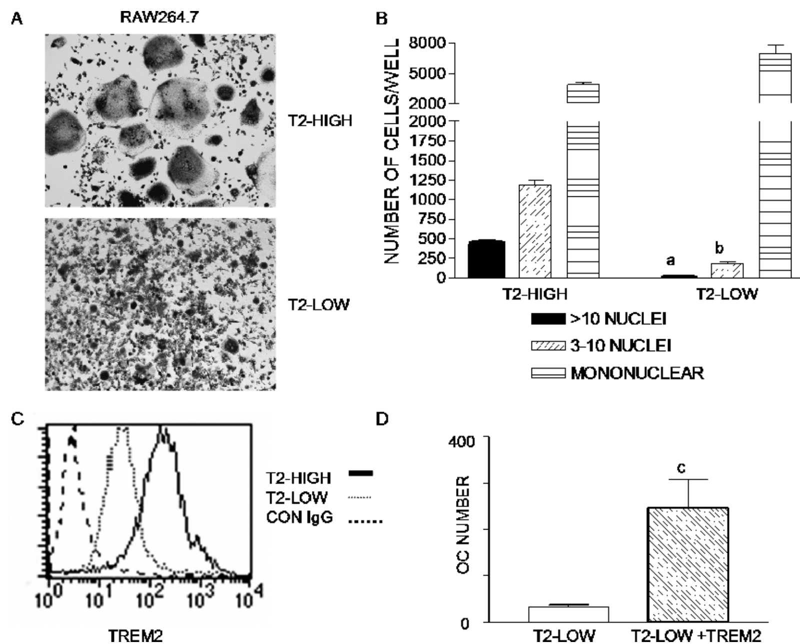

FIG. 2. TREM2 expression correlates with the osteoclastogenic potential of RAW264.7. (A) TRACP staining of OCL cells differentiated from RAW264.7 cells with a high osteoclastogenic phenotype (T2-HIGH) compared with a poorly osteoclastogenic subline (T2-LOW). (B) OCLs formed from T2-HIGH RAW264.7 were more complex compared with those formed from T2-LOW RAW264.7 as measured by nuclei per OC. (C) TREM2 surface expression is elevated in the highly osteoclastogenic line (thick line, T2-HIGH) and decreased in the poorly osteoclastogenic line (dotted line, T2-LOW; isotype anti-rat IgG Ab, dashed line). (D) Transfection of TREM2 into the T2-LOW RAW264.7 subline restores OCL cell formation in response to RANKL and M-CSF. Data are representative of two or more experiments. Paired *t*-test analysis: $^a p < 0.03$ T2 HIGH vs. T2 LOW (>10 nuclei); $^b p < 0.01$ T2 HIGH vs. T2 LOW (3–9 nuclei); $^c p < 0.05$.

correlation reflects a role for TREM2 in OC formation, we selected a line with low TREM2 and low OC potential and restored TREM2 by transfection. Re-expression of TREM2 was documented by flow cytometry (data not shown) and was accompanied by restoration of OCL formation in response to RANKL (Fig. 2D).

As a second approach to define the role of TREM2 expression in OC formation, we used lentivirus-mediated RNAi to suppress endogenous TREM2 in a highly osteoclastogenic RAW264.7 subline. Both surface expression (Fig. 3A) and total TREM2 protein (Fig. 3B) decreased after transduction with TREM2 RNAi lentivirus, but not with control virus. After suppression of TREM2 expression by RNAi, the number of $TRACP^+$ OCL cells formed in response to RANKL was significantly lower compared with control virus-treated cells (Fig. 3C). The few TREM2 RNAi OCLs that formed were notably smaller, with fewer nuclei (none with >10 nuclei), and the majority of cells remained mononuclear (>5000; Figs. 3D and 3E).

TREM2 stimulation enhances the formation of multinucleated $TRACP^+$ OCs

To directly examine the effect of TREM2 stimulation on osteoclastogenesis, we engaged TREM2 on OC precursors during stimulation with RANKL and M-CSF using anti-TREM2 mAb cross-linked with plate-bound $F(ab')_2$ anti-rat IgG. C57BL/6 BMMs formed 2-fold more OCs when

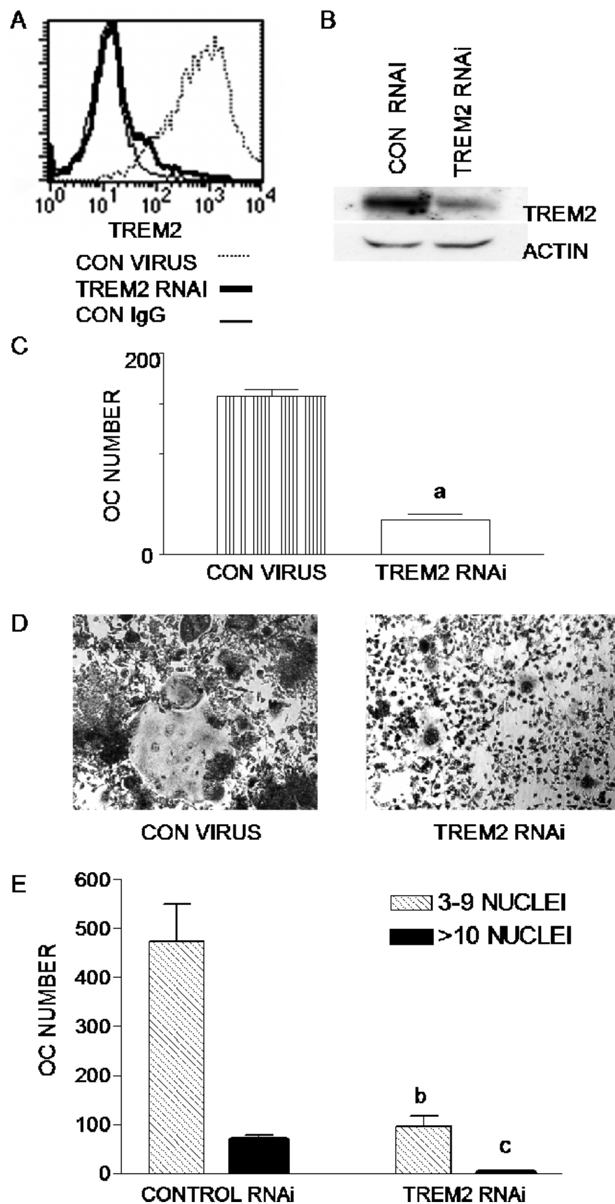

FIG. 3. Lentiviral transduction of TREM2 RNAi suppresses TREM2 expression and OC formation. (A) RAW264.7 cells were transduced with control (dotted line) or TREM2 RNAi lentivirus (bold solid line) and analyzed for TREM2 surface expression using flow cytometry (thin solid line is isotype control anti-rat IgG). (B) Western blotting of TREM2 protein and actin in whole cell lysates of RAW264.7 cells transduced with control or TREM2 RNAi lentivirus. (C) Stably transduced TREM2 RNAi RAW264.7 cells formed fewer OCs compared with control virus transduced cells after differentiation with RANKL and M-CSF. (D) Representative TRACP$^+$ OCs in control and TREM2 RNAi lentivirus-infected cells lines treated with RANKL and M-CSF. (E) OCLs formed from control virus RAW246.7 were more complex than OCLs formed from TREM2 RNAi as measured by the number of nuclei per OC. Data are representative of more than three experiments. Student's *t*-test analysis: $^a p < 0.0001$; $^b p < 0.02$ compared 3–9 nuclei control RNAi vs. TREM2 RNAi; $^c p < 0.01$ compared >10 nuclei control RNAi vs. TREM2 RNAi.

anti-TREM2 mAb was cross-linked compared with use of an isotype-matched control mAb (Fig. 4A). RAW264.7 cells stimulated with anti-TREM2 mAb formed 3-fold more OCL cells (Fig. 4B). TREM2 cross-linking also led to increased size and complexity of OCs formed compared with control mAb treatment (Figs. 4C and 4D). TREM2 cross-linking through soluble antibody failed to enhance OC formation.

To confirm the requirement for functional TREM2-DAP12 signaling in OC differentiation, we generated OCs from *DAP12*$^{-/-}$ BMMs in the presence of TREM2 cross-linking. The *DAP12*$^{-/-}$ OCs remained mononuclear and failed to generate multinuclear OCs (Fig. 4E). The finding that *DAP12*$^{-/-}$ pre-osteoclasts do not respond to anti-TREM2 mAb cross-linking indicates that, despite TREM2 expression at the cell membrane, TREM2 is nonfunctional in the absence of the signaling chain DAP12.

Blockade of TREM2 prevents OC formation

Having shown that antibody stimulation of TREM2 augments OC formation, we next considered the hypothesis that ligation of TREM2 by endogenous ligands contributes to OC formation. To this end, we tested $F(ab')_2$ anti-TREM2 mAb for the capacity to block OC formation in response to RANKL and M-CSF. As shown in Figs. 4F and 4G, blockade of TREM2 on C57BL/6 pre-osteoclasts inhibited the formation of multinuclear TRACP$^+$ OC in a dose-dependent manner compared with control $F(ab')_2$ antibody treatment.

Blockade of TREM2 inhibits bone resorption by mature OCs

To assess the role of TREM2 in resorption of bone by mature OCs, we generated day 5 OCs with RANKL and M-CSF, lifted them from plastic, and blocked TREM2 with $F(ab')_2$ anti-TREM2. C57BL/6 OCs treated with anti-TREM2 $F(ab')_2$ (T2 FAB) resorbed significantly less artificial calcium-phosphate substrate (Figs. 5A and 5B) and formed fewer resorption pits on dentine (TREM2 FAB) (Figs. 5C and 5D). Similar numbers of TRACP$^+$ OCs were present at the completion of the resorption assays independent of antibody treatment (data not shown).

Anti-TREM2 mAb alone (T2 AB) or cross-linked with 2nd step $F(ab')_2$ anti-rat IgG (T2 X) also significantly inhibited resorption of artificial calcium-phosphate substrate by C57BL/6 OCs compared with control antibody (Figs. 5A and 5B). Similar inhibition of resorption occurred in RAW264.7 OCLs treated with anti-TREM2 mAb or $F(ab')_2$ anti-TREM2 (data not shown). Thus, in contrast to OC formation, which is impaired by blockade of TREM2 but is enhanced when TREM2 is cross-linked, bone resorption by mature OCs is inhibited by anti-TREM2 even if it is cross-linked.

Chemotaxis of OCs is enhanced by stimulating TREM2

To further define the regulatory role of TREM2 in the function of mature OCs, we generated day 5 OCs with RANKL and M-CSF. The adherent OCs were removed from the plates and were used in overnight migration assays with or without cross-linking of TREM2. Crosslinking of

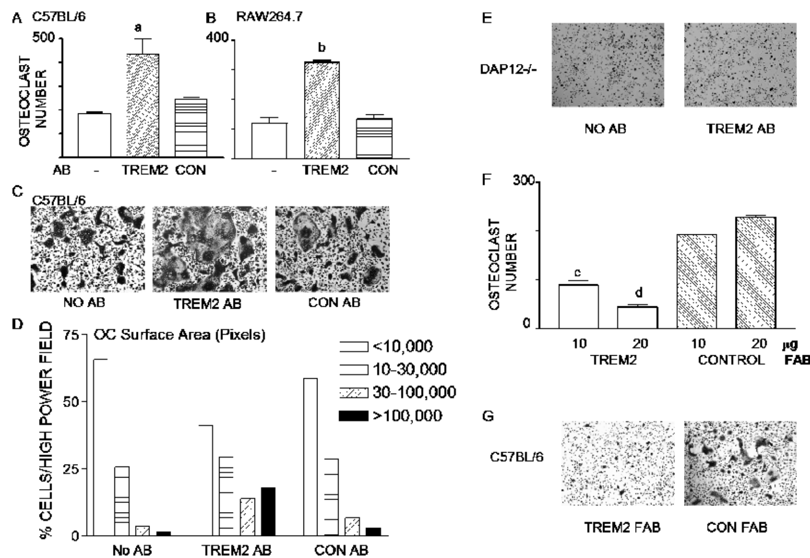

FIG. 4. Anti-TREM2 mAb stimulation enhances multinucleated OC formation in vitro. (A) OCs, generated from BMMs from C57BL/6 mice, were stimulated with plate bound anti-TREM2 mAb or control mAb in the presence of RANKL and M-CSF. The number of TRACP$^+$ cells with more than two nuclei OCs were counted at 5 days. (B) RAW264.7 cells were treated with RANKL, M-CSF, and either anti-TREM2 mAb or control mAb. After 7 days, the TRACP$^+$ cells with more than two nuclei OCs were counted. (C) Representative TRACP staining of C57BL/6 OCs generated in the presence of anti-TREM2 mAb or control mAb. (D) OCs generated in the presence of TREM2 mAb were larger, as measured by pixel area per OC. (E) OCs generated from *DAP12*$^{-/-}$ mice remained mononuclear despite anti-TREM2 mAb cross-linking. (F) Blockade of TREM2 with anti-TREM2 mAb F(ab′)$_2$ fragments inhibited the formation of OC differentiation in vitro. C57BL/6 preosteoclasts were treated with 10 or 20 μg/ml of anti-TREM2 F(ab')$_2$ or control F(ab′)$_2$ fragments in the presence of RANKL and M-CSF for 5 days, and the TRACP$^+$ cells with more than two nuclei OCs were counted. (G) Representative TRACP staining of OC generated in the presence of anti-TREM2 or control F(ab′)$_2$ fragments. All data are representative of more than three experiments. One-way ANOVA statistical analysis: $^a p < 0.01$ compared with NO AB or CON AB; $^b p < 0.001$ compared with NO AB or CON AB; $^c p < 0.001$ compared with CON 10; $^d p < 0.001$ compared with TREM2 10 or CON 10.

TREM2 on C57BL/6 OCs caused a significant increase in migration toward M-CSF (Fig. 6A). TREM2 cross-linking did not enhance migration in the absence of M-CSF. Treatment of mature OCs with F(ab′)$_2$ anti-TREM2 failed to inhibit or enhance migration (data not shown). Similar enhancement of migration by TREM2 cross-linking occurred in OCLs formed by RAW264.7 cells (Fig. 6B), even though the baseline migration by RAW264.7 cells is greater than migration of C57BL/6 OCs, perhaps reflecting the more uniform phenotype of OCs formed from RAW264.7 cells. Thus, although cross-linking of TREM2 impairs bone resorption by mature OCs, the same treatment increases their capacity for chemotaxis.

DISCUSSION

We previously showed that murine osteoclasts are dependent on DAP12 for normal development and function in vitro.[3] Here we examined the role of the DAR TREM2 in the regulation of murine OC differentiation and function in vitro. We generated anti-TREM2 mAb and showed that TREM2 surface expression increases during osteoclastogenesis by bone marrow–derived OC precursors. We also found that the expression of TREM2 on sublines of RAW264.7 cells correlates with their capacity for osteoclastogenesis, suggesting a functional role for TREM2 in OC formation. In support of this hypothesis, TREM2 cross-linking enhances OC differentiation from both primary bone marrow–derived OC cultures and RAW264.7 cells. Inhibition of TREM2 expression through RNA silencing suppresses osteoclastogenesis in RAW264.7 cells. These data suggest that TREM2 expression is a requirement for in vitro OC differentiation from RAW264.7 cells. In studies of mature OCs, we found that blockade of TREM2 inhibits resorption, whereas stimulation of TREM2 enhanced chemotaxis. These new findings indicate that TREM2-DAP12 plays a positive role in OC function as well as in differentiation.

In this study, we examined the role of the TREM2 receptor in the differentiation of OCs. TREM2 and its ITAM-bearing signaling adapter DAP12 are expressed in OCs, dendritic cells, and macrophages. ITAM-bearing receptor complexes have been extensively studied in lymphocytes, where ITAM signals are required for T- and B-cell differentiation and are also involved in the activation of mature T-cell functions, including cytokine production and cytolytic activity.[16] The role of ITAMs in OCs, however, has only recently been discovered.[2,3,6,7] In dendritic cells, TREM2-DAP12 signaling leads to prolonged survival and to partial dendritic cell maturation, with upregulation of CD40, CD86, and MHC class II, and it has been postulated to amplify other maturation signals important for the enhancement of antigen presentation.[17] Recent studies have shown that ITAM signals are critical for the activation of SYK tyrosine kinase and of phospholipase C (PLC)γ, for the activation of Ca^{2+} oscillations, and for NFATc1 produc-

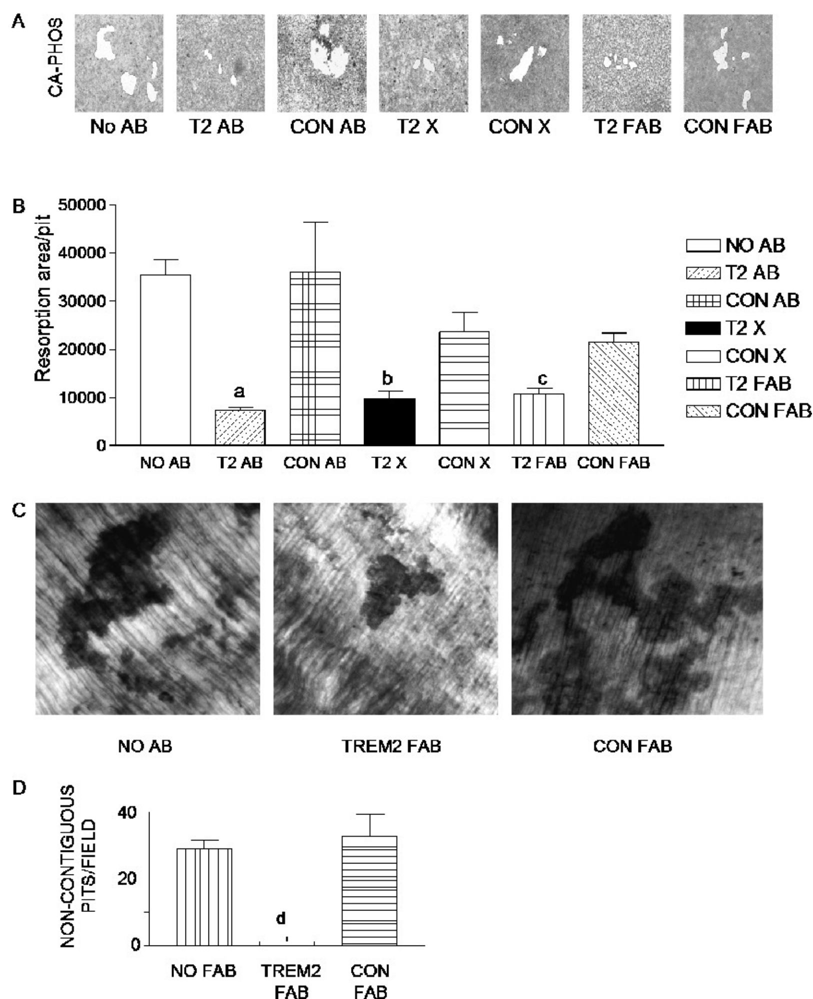

FIG. 5. Anti-TREM2 mAb inhibits resorption. Day 5 C57BL/6 OC were generated and treated with anti-TREM2 mAb or control mAb with or without additional cross-linking antibody, or with anti-TREM2 $F(ab')_2$ or control $F(ab')_2$ before placement on artificial calcium-phosphate discs or dentine. After 48 h, resorption was determined. (A) C57BL/6 OCs treated with intact TREM2 mAb (T2 AB), cross-linked TREM2 mAb (T2 X), or anti-TREM2 $F(ab')_2$ (T2 FAB) formed fewer resorption pits on calcium-phosphate discs compared with control Ab treatments. (B) Resorption of calcium-phosphate substrate quantified by measuring resorption area per pit (~100 pits/group) shows that resorption is inhibited by anti-TREM2 $F(ab')_2$ or intact TREM2 mAb compared with control $F(ab')_2$ antibody or control mAb. (C) Anti-TREM2 $F(ab')_2$ inhibited the formation of resorption pits on dentine by C57BL/6 OCs. (D) Significantly fewer resorption pits were detected on dentine when OCs were treated with TREM2 $F(ab')_2$. Data are representative of more than three experiments. One-way ANOVA statistical analysis: $^{a}p < 0.001$ compared with NO AB or CON AB; $^{b}p < 0.001$ compared with NO AB or CON X; $^{c}p < 0.001$ compared with NO AB or CON FAB; $^{d}p < 0.001$ compared with NO AB or CON FAB.

tion in RANKL-induced osteoclastogenesis.[4,6] Consistent with these findings, our study shows that cross-linking of the TREM2 receptor during in vitro osteoclastogenesis is an important stimulatory signal in OC differentiation.

OCs are the only cells known to resorb bone, and OC resorptive capacity is thought to correlate with the number of OC nuclei present in bones.[18] To explore TREM2-DAP12–mediated regulation of OC resorption, we generated mature OCs using RANKL and M-CSF, and we subsequently examined the effect of TREM2 blockade on bone resorption. TREM2 mAb treatment inhibited resorption by OC in vitro. Anti-TREM2 $F(ab')_2$ fragments also inhibited resorption, indicating that inhibition of resorption was not caused by activation of OCs by Fc receptors. These results suggest that the anti-TREM2 mAb blocks a potential TREM2 receptor–ligand interaction that is critical for activation of OC resorption in vitro. The low density of OCs in the culture makes it unlikely that TREM2 activation requires interaction with other cells. We have previously shown that TREM2 recognizes several different anionic ligands, functioning like a pattern recognition receptor.[19] Similarly, it is possible that the negatively charged inorganic bone matrix may be sufficient to trigger TREM2 signaling. Thus, antibody blockade may prevent the interaction of TREM2 with the inorganic bone matrix, thereby reducing bone resorption. TREM2 could also interact in *cis* with a ligand on the same cell or with a secreted, soluble ligand. Alternatively, it remains possible that antibody binding to TREM2 may disrupt complete osteoclast adhesion to bone or bone matrix that is required for functional resorption, which might occur with antibody used in both stimulatory and blocking conditions. Given these results, determining the physiological role of TREM2 blockade on OC activation in vivo is of significant interest.

To further explore TREM2-DAP12 in OC function, we examined the effects of TREM2 cross-linking on chemotaxis of mature OCs toward M-CSF. TREM2 stimulation enhanced migration of mature OCs generated from either C57BL/6 BMMs or from RAW264.7 cells. Prior studies have shown that *DAP12*$^{-/-}$ pre-osteoclasts show reduced in vitro migration in response to M-CSF and osteopontin, an $\alpha v\beta 3$ integrin ligand.[20] These results suggest that TREM2-DAP12 signaling either increases the sensitivity of OCs to M-CSF or that it regulates the cytoskeletal rearrangements required for cellular migration. In human dendritic cells, TREM2 signaling upregulates the expression of chemokine receptor 7 (CCR7) and enhances their chemotaxis toward the CCR7 chemokines CCL19 and CCL21.[17] ITAM-

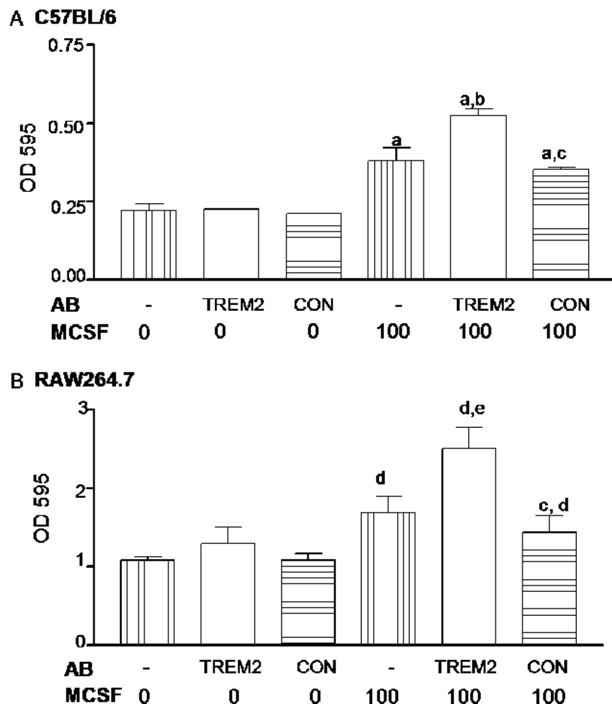

FIG. 6. Anti-TREM2 mAb stimulates migration of OCs toward M-CSF. Mature OCs were generated with RANKL and M-CSF for 5 days. Cells were treated with anti-TREM2 mAb or control mAb before placement in Transwell chambers. Bottom chambers contained media with or without 100 ng/ml M-CSF. The cells that had migrated to the undersurface of the Transwell membrane were fixed and stained with crystal violet. Membranes were removed, and the crystal violet concentration was determined by OD 595. All conditions were performed in duplicate. (A) Migration of C57BL/6 OCs. (B) Migration of RAW264.7 OCLs. Results are representative of more than three experiments. One-way ANOVA analysis: $^{a}p < 0.01$ compared with migration in the absence of M-CSF; $^{b}p < 0.001$ compared with M-CSF + no AB or Control AB; ^{c}no statistical difference between M-CSF + NO AB and M-CSF + CON AB; $^{d}p < 0.01$ compared with NO M-CSF groups; $^{e}p < 0.05$ compared with M-CSF + NO AB or M-CSF + CON AB.

deficient DAP12 knock-in mice, with nonfunctional DAP12, have an accumulation of dendritic cells in the mucocutaneous epithelia, and these dendritic cells fail to migrate to draining lymph nodes in response to a hapten skin challenge.[21] Thus, TREM2-DAP12 signaling may contribute to chemotaxis in both dendritic cells and OCs. Further studies are needed to elucidate the mechanisms of these effects, but TREM2 stimulation of OCs leading to enhanced migration of OC precursors would be of particular importance in the setting of pro-inflammatory signals generated in areas of inflammation, such as in rheumatoid arthritis erosions.

Previous human and mouse studies have clearly implicated a role for TREM2 and DAP12 in OC differentiation. Our data further show that TREM2 is required for normal bone resorption by mature OCs during in vitro conditions. Our finding that TREM2 blockade can prevent the functional resorption and regulate migration of OCs enforces the contention that TREM2 may be an attractive target for the pharmacologic modulation of bone remodeling in pathologic conditions.

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