

## **DAP12/TREM2 Deficiency Results in Impaired Osteoclast Differentiation and Osteoporotic Features**

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### **Abstract**

Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOS), Nasu-Hakola disease, is a globally distributed recessively inherited disease. PLOS is characterized by cystic bone lesions, osteoporotic features, and loss of white matter in the brain leading to spontaneous bone fractures and profound presenile dementia. We have earlier characterized the molecular genetic background of PLOS by identifying mutations in two genes, *DAP12* and *TREM2*. *DAP12* is a transmembrane adaptor protein that associates with the cell surface receptor *TREM2*. The *DAP12*–*TREM2* complex is involved in the maturation of dendritic cells. To test a hypothesis that osteoclasts would be the cell type responsible for the bone pathogenesis in PLOS, we analyzed the differentiation of peripheral blood mononuclear cells isolated from *DAP12*- and *TREM2*-deficient PLOS patients into osteoclasts. Here we show that loss of function mutations in *DAP12* and *TREM2* result in an inefficient and delayed differentiation of osteoclasts with a remarkably reduced bone resorption capability in vitro. These results indicate an important role for *DAP12*–*TREM2* signaling complex in the differentiation and function of osteoclasts.

Key words: bone diseases • central nervous system diseases • osteoporosis • monocytes • dementia

### **Introduction**

Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOS), Nasu-Hakola disease, is a genetically heterogeneous, recessively inherited disease. The histological hallmarks of PLOS are cystic bone lesions, osteoporotic features, and loss of white matter in the brain. The pathological changes lead to bone fractures after minimal trauma, severe dementia, and premature death (1–3). We have recently identified mutations in all PLOS patients either in *DAP12* or *TREM2* (4, 5). *DAP12* is a transmembrane adaptor molecule that forms a complex with several cell surface receptors depending on the cell

type, and is implicated in the activation of myeloid and NK cells (6, 7). On the cell membrane of monocyte-derived dendritic cells, *DAP12* is expressed as a complex with *TREM2* (8, 9). The interaction between *TREM2* and an unidentified ligand results in the phosphorylation of an immunoreceptor tyrosine-based activation motif in the cytoplasmic domain of *DAP12*. Phosphorylated *DAP12* binds the cytoplasmic protein tyrosine kinases SYK and ZAP70. This interaction results in the activation of downstream signal transduction pathways (6, 7, 10).

Although the primary cause of PLOS has now been characterized, the pathogenic mechanisms behind the lesions in the bone and brain have remained unknown. We have earlier proposed that the cystic bone lesions and loss of trabecular bone in PLOS could be caused by dysfunction of osteoclasts, the cells responsible for resorption and

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remodeling of bone (4). To test this hypothesis, we studied the differentiation and bone resorption capability of osteoclasts derived from the PBMC isolated from four Finnish and one German PLOSL patient with homozygous loss of function mutations in *DAP12* (*TYROBP* or *KARAP*) or *TREM2* (4, 5).

## Materials and Methods

**Patients.** The ethical committee of National Public Health Institute, Helsinki, Finland has approved this study. An informed consent was obtained from all subjects. The mutation analyses were performed as previously described (5). RT-PCR analyses of *TREM2* of the German patient were performed using the following primer pairs (sense and antisense): (full-length coding sequence) ATGGAGCCTCTCCGGCTGCT and TCACGTGTCTCTCAGCCCTG, (5' half of the coding sequence) TCACGTGTCTCTCAGCCCTG and ATCCAGGGGGTCTGCCAGCA, and (3' half of the coding sequence) TACAACCCCATGATGCGGGTC and TCACGTGTCTCTCAGCCCTG.

**Induction of Osteoclasts.** PBMCs were isolated from buffy coat cells over Ficoll-Paque (Amersham Biosciences). The cells were resuspended in  $\alpha$ -MEM (GIBCO BRL), FCS, and antibiotics. Samples of  $5 \times 10^6$  cells were allowed to adhere for 1 h at 37°C in a round cell culture dish 36 mm in diameter containing four round glass coverslips 13 mm in diameter. Adherent cells were stimulated for 1, 3, 7, 14, and 21 d with 25 ng/ml M-CSF (R&D Systems) and 40 ng/ml RANKL (Qbiogene). The media with cytokines was replaced twice a week.

**Histochemistry and Immunofluorescence Stainings.** Staining for tartrate-resistant acid phosphatase (TRAP) was performed using TRAP staining kit (Sigma-Aldrich). The cytoskeletal actin was stained using Alexa Fluor 633 phalloidin reagent (Molecular Probes). The nuclei were visualized using DAPI reagent (Sigma-Aldrich). The staining for cathepsin K was performed using polyclonal anti-human cathepsin K antibody (Santa Cruz Biotechnology, Inc.).

**Quantitative RT-PCR Analysis.** Monocytes were stimulated with M-CSF and RANKL for 1, 3, 7, and 21 d as described above. Total RNA was isolated using TRIzol reagent (GIBCO BRL). Quantitative RT-PCR experiments were performed using LightCycler PCR machine (Roche) as previously described (5, 11). Serial dilutions of human *DAP12* (sequence data are available from GenBank/EMBL/DDBJ under accession no. AA481924), *TREM2* (sequence data are available from GenBank/EMBL/DDBJ under accession no. BF343916), *TRAP* (sequence data are available from GenBank/EMBL/DDBJ under accession no. J04430), and calcitonin receptor (*CALCR*; sequence data are available from GenBank/EMBL/DDBJ under accession no. NM\_001742) cDNA cloned in a plasmid vector were used to determine the copy number of the amplicon per housekeeping gene cDNA copies (porphobilinogen deaminase [*PBGD*]; sequence data are available from GenBank/EMBL/DDBJ under accession no. M95623). Probes and human genomic DNA were used to determine the copy number of *Cathepsin K*, *PBGD*, and receptor activator of nuclear factor  $\kappa$ B (*RANK*). The sequences for the PCR primers (sense and antisense, respectively) and probes are: (*CALCR*) TCTCAGGAGTCAAAGCATTGCACATA and AATGCTATGACCGAATGCAGCAGTTA; (*DAP12*) ATGGGGGGACTTGAACCC and TCATTTGTAATACGGCCTCTGTG; (*TRAP*) CACACAGCTGTC-

CTGGCTCAAGAA and CAGGTAGGCAGTGACCCCGTATGT; (*TREM2*) ATGGAGCCTCTCCGGCTGCT and TCACGTGTCTCTCAGCCCTG; (*Cathepsin K*) CAGTGAAAGGTGGTTTCCAGA and AGAGTCTGGGGCTCTACCTT, (*Cathepsin K* probe) TCCCGCAGTAATGACACCCCTT; (*PBGD*) GGGAAACCTCAACACCCGGCT and ACCCGGTGTGTGCCAGCCCAT, (*PBGD* probe) ATCCTGGCAA-CAGTGGCCTGCA; and (*RANK*) GCAAGACCGAGATAGAGGAAGACAGCT and CAGGCTCAGTGAGGAACAGTAACCTGGT, (*RANK* probe) TGCCATGTATTTCATCTCTGTGGGCATCT.

**In Vitro Analysis of Bone Resorption.** In vitro bone resorption analysis was performed by first stimulating PBMCs from three *DAP12*-deficient and four healthy individuals with M-CSF and RANKL for 7 or 21 d.  $4 \times 10^5$  cells were then transferred to a well 7.5 mm in diameter containing a dentin slice 5 mm in diameter (Immunodiagnostic Systems). Cells were incubated on dentin in the presence of media with cytokines (replaced twice a week) for 7 d, fixed, and stained for TRAP. The number of multinucleated osteoclasts and nuclei per cell was calculated using light microscope. The cells were then brushed away and the dentin slices were stained with toluidine blue to visualize the resorption pits. The surface area and depth of the resorption pits were determined using ANALYSIS 3.2 software (Soft Imaging System) and a confocal microscope, respectively.

**Statistical Analysis.** Statistical analyses were performed using GraphPad Prism 3.0 software (GraphPad Software).

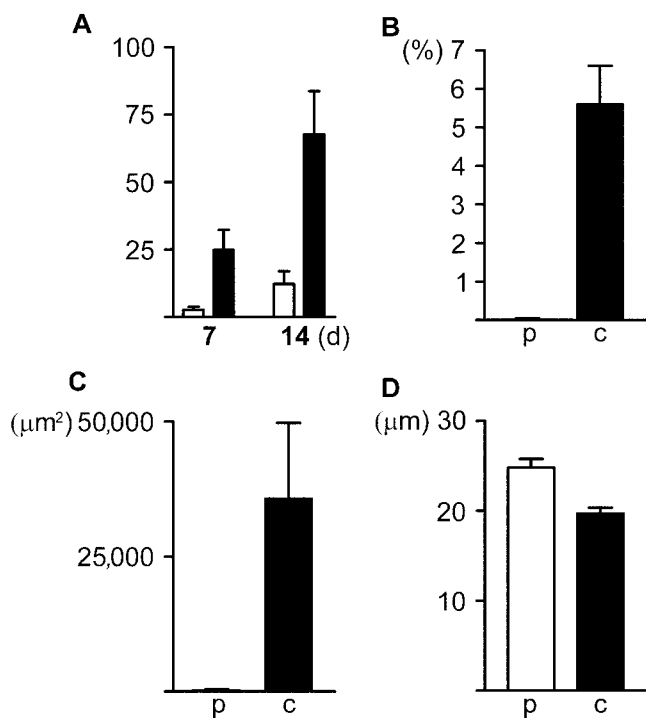
## Results and Discussion

***DAP12*- and *TREM2*-deficient Monocytes Show a Delayed Differentiation into Osteoclasts.** Multinucleated osteoclasts in humans are formed by the fusion of mononuclear hematopoietic precursor cells circulating in the monocyte fraction (12–14). To generate osteoclasts, we stimulated the PBMCs of four patients with *DAP12* mutations, one with a *TREM2* mutation and four healthy individuals using a cytokine combination consisting of M-CSF and receptor activator of nuclear factor  $\kappa$ B ligand (RANKL), known to induce cells with all morphological and functional characteristics of osteoclasts (14, 15). All Finnish patients carried a homozygous 5.3 kb deletion encompassing exons 1–4 of the five exons of *DAP12*, resulting in a total loss of expression of *DAP12* transcripts or polypeptide (4). The German patient was homozygous for a conversion of nucleotide G 40 to T at the last position of exon 1 of *TREM2*, resulting in the creation of a premature translation termination codon. We could not detect *TREM2* transcripts in the stimulated PBMCs of the German patient in contrast to the control cells, implying the knockout character of this mutation as well (not depicted).

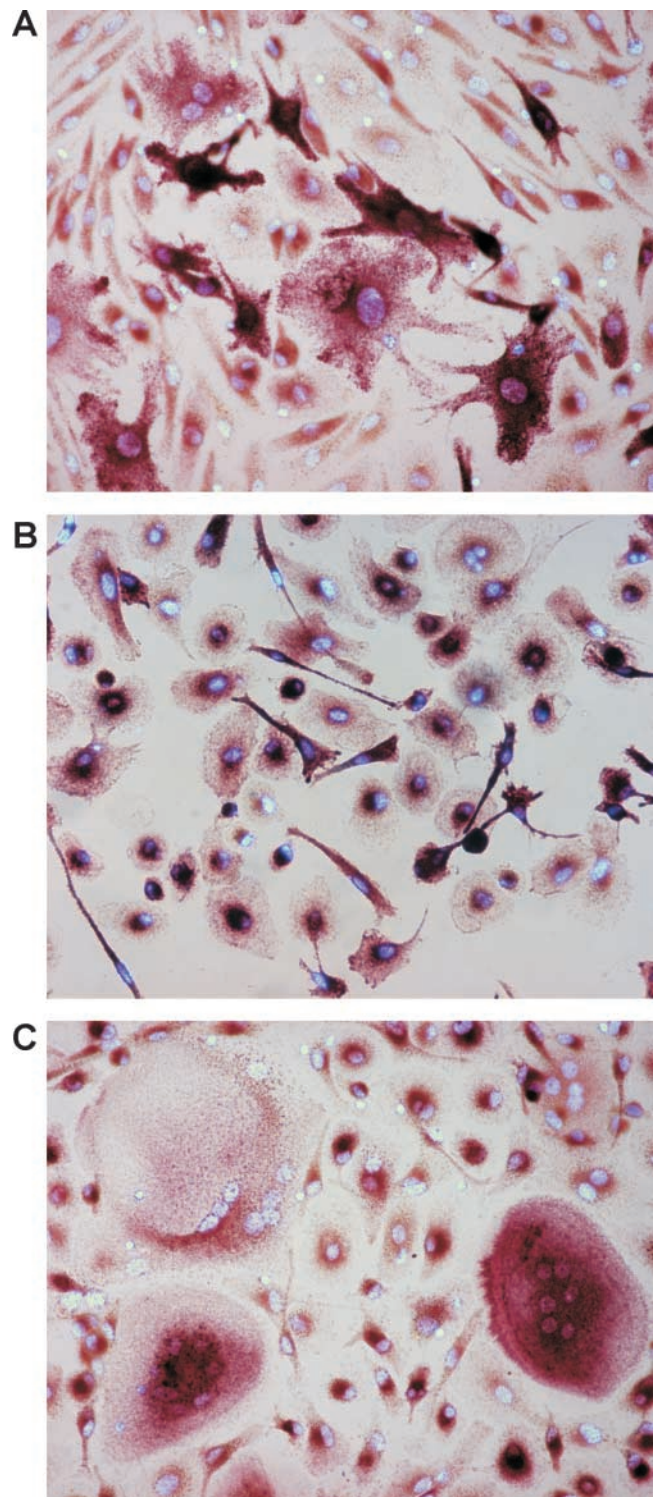
Induction with M-CSF and RANKL generated TRAP<sup>+</sup> and cathepsin K<sup>+</sup> osteoclasts with 10–20 nuclei in 3–7 d from the PBMCs of healthy individuals. Remarkably, the differentiation of *DAP12*- and *TREM2*-deficient PBMCs into multinucleated giant cells was seriously impaired. After 7 d stimulation the number of multinucleated (three or more nuclei) cells generated from *DAP12*- and *TREM2*-deficient PBMCs was only 10% of that of control cells ( $P < 0.01$ ). A vast majority of these genetically deficient

multinucleated cells had three or four nuclei. The proportion of cells with five or more nuclei was only 6% of the controls ( $P = 0.01$ ). After stimulation for 14 d the number of genetically deficient multinucleated cells had increased to 18% ( $P < 0.01$ ) and the proportion of cells with five or more nuclei increased to 20% of that of controls ( $P < 0.05$ ; Fig. 1 A). The multinucleation process of DAP12- and TREM2-deficient cells was comparable to each other. DAP12- and TREM2-deficient multinucleated cells were cathepsin K<sup>+</sup> and TRAP<sup>+</sup> (Fig. 2). The calculated cell density of cultured DAP12- and TREM2-deficient PBMCs was similar to the controls at all time points, indicating that the genetically deficient cells were able to proliferate in a similar manner as the controls. Taken together, the DAP12-TREM2 complex mediates the differentiation of osteoclasts. Further, in the absence of DAP12/TREM2 signaling, the fusion is very inefficient, but proceeds in the course of time.

*DAP12- and TREM2-deficient Osteoclastic Cells Show an Aberrant Morphology.* To characterize the morphology of the induced osteoclastic cells in more detail, we stained the



**Figure 1.** Impaired multinucleation and bone resorption capability of genetically deficient osteoclasts. (A) The number of multinucleated (three or more nuclei) DAP12- or TREM2-deficient (open bars) osteoclastic cells per  $10^6$  adherent PBMCs calculated at day 1 was lower than that of controls (solid bars;  $P < 0.01$ ) after stimulation with M-CSF and RANKL for 7 and 14 d. (B) The total surface area per dentine slice resorbed by DAP12-deficient osteoclasts (p) after incubation on dentine for 7 d was significantly reduced compared with the controls (c;  $P < 0.01$ ). (C) The resorbed surface area per DAP12-deficient osteoclast (p) was markedly smaller than that of the controls (c;  $P < 0.05$ ). (D) The resorption pits generated by DAP12-deficient osteoclasts were significantly deeper compared with the controls ( $P < 0.0001$ ). The error bars indicate the standard error of the mean.



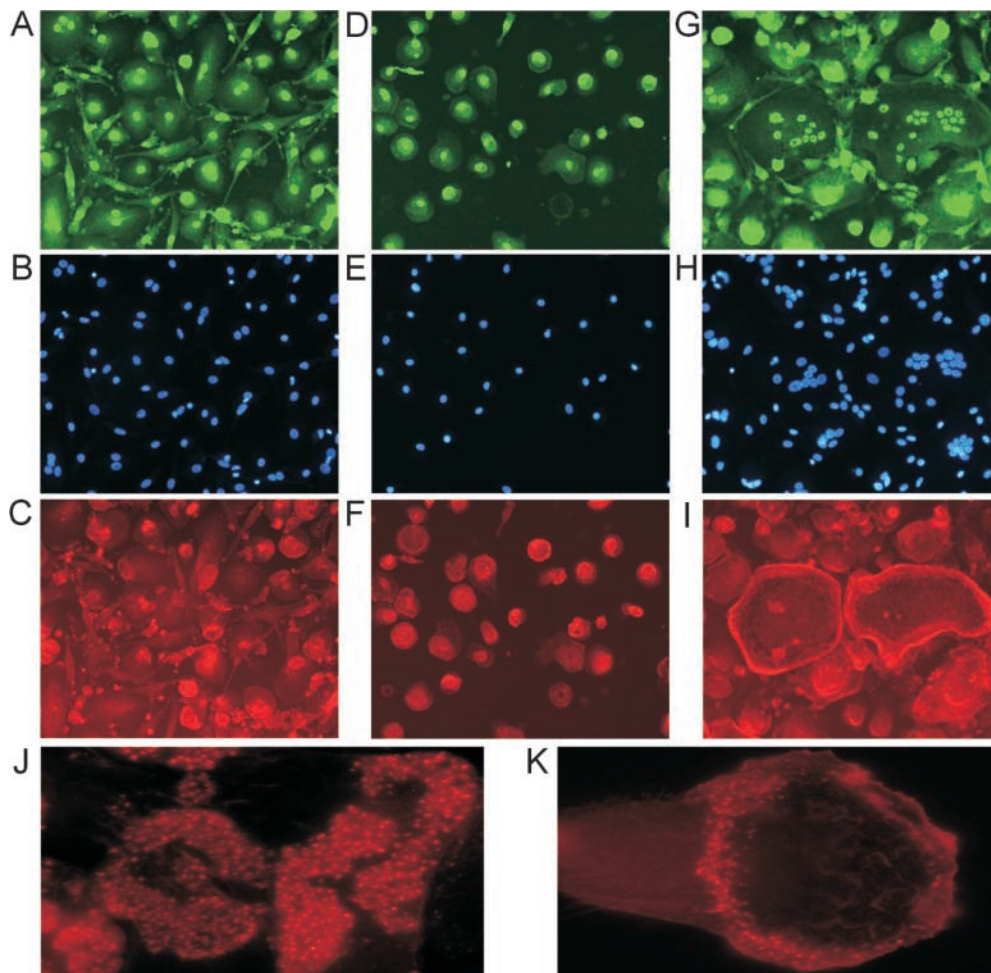
**Figure 2.** TRAP staining of DAP12-, TREM2-deficient, and control osteoclastic cells differentiated from PBMCs. DAP12- (A) and TREM2-deficient (B) osteoclastic cells are intensely TRAP<sup>+</sup> and much smaller than the control osteoclasts (C) after stimulation for 7 d. Only occasional genetically deficient cells contain two to three nuclei. Note the numerous processes in DAP12-deficient osteoclastic cells.



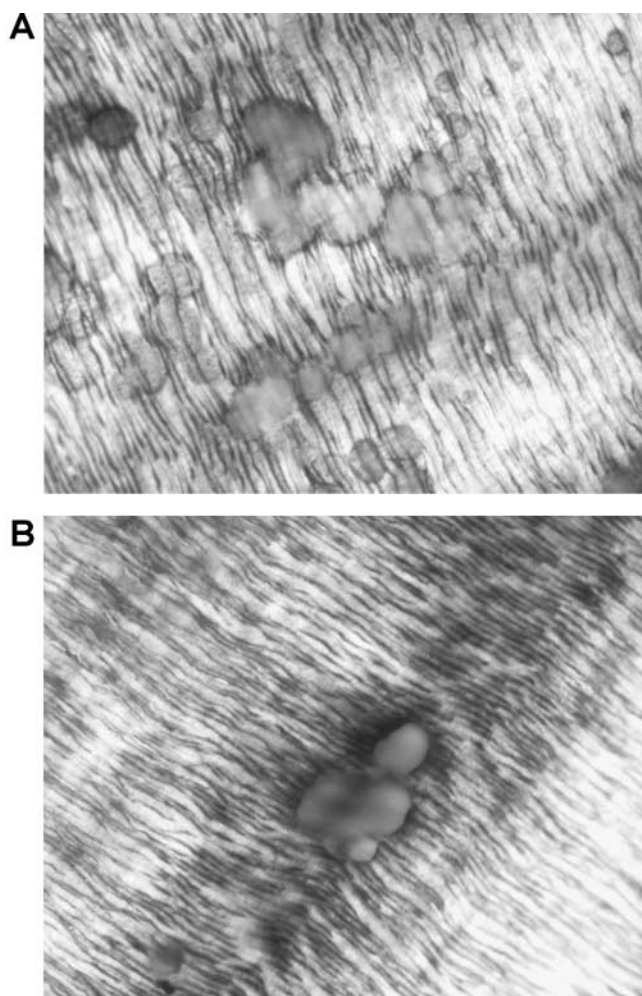
cells for cytoskeletal actin. An actin ring, consisting mostly of F-actin filaments, is a functional characteristic of resorbing osteoclasts and delineates the bone resorption area between an osteoclast and bone (16–18). After 7 d the F-actin filaments in the control osteoclasts formed the typical single large actin ring, whereas even after stimulation for 14 d the multinucleated DAP12-deficient osteoclastic cells had several small, unorganized granular actin clusters. The small TREM2-deficient osteoclastic cells showed only one granular, ring-like actin staining pattern after 14 d (Fig. 3). The transmembrane adaptor molecule DAP12 forms a complex with several different activating receptors depending on the cell type (7). In DAP12-deficient cells, all DAP12-associated cell surface receptors are likely to be inactive. The slightly different morphology of actin rings in DAP12- and TREM2-deficient osteoclastic cells could be explained by the potential costimulatory function of other DAP12-associated cell surface receptors in TREM2-deficient cells.

**DAP12-deficient Osteoclastic Cells Resorb Bone.** To assess the bone resorption capability of DAP12-deficient multinucleated cells, we performed an *in vitro* bone resorption assay. We first induced DAP12-deficient and control PBMCs with M-CSF and RANKL for 7 or 21 d and then transferred the cells on dentin slices for 7 d. Multinu-

cleated DAP12-deficient cells stimulated for 7 d before transferring on dentine slices were capable of bone resorption (Fig. 4). Although the same number of DAP12-deficient and control cells were transferred per well, the total surface area per dentine slice resorbed by DAP12-deficient osteoclasts was reduced, being only 0.03% of that of control osteoclasts ( $P < 0.01$ ; Fig. 1 B). Similarly, we observed a significant difference in the average resorbed surface area per multinucleated osteoclast between DAP12-deficient and control osteoclasts ( $36 \mu\text{m}^2$  and  $35,700 \mu\text{m}^2$ , respectively,  $P < 0.05$ ; Fig. 1 C). Surprisingly, the surface area per resorption pit generated by DAP12-deficient and control osteoclasts showed no difference. However, the resorption pits generated by DAP12-deficient osteoclasts were 25% deeper compared with the controls ( $25 \mu\text{m}$  and  $20 \mu\text{m}$ , respectively,  $P < 0.0001$ ; Fig. 1 D). Neither DAP12-deficient nor control osteoclasts were capable of bone resorption in resorption assays initiated after stimulation for 21 d. Taken together, DAP12-deficient multinucleated cells can resorb mineralized bone and they fulfill the criteria for osteoclasts. The lower resorption rate of these osteoclasts could either be due to an impaired actin ring formation or to a loss of controlled cyclic changes in the actin cytoskeleton required for osteoclast movement (17).



**Figure 3.** Morphological characteristics of DAP12- and TREM2-deficient osteoclastic cells and control osteoclasts after stimulation for 14 d. (A–C, same visual field) DAP12-deficient osteoclastic cells are small and cathepsin  $\text{K}^+$  (A). Staining for DAPI demonstrates that only occasional cells have a few nuclei (B). Phalloidin staining for actin demonstrates numerous small unorganized actin clusters in DAP12-deficient osteoclastic cells (C). (D–F, same visual field) TREM2-deficient osteoclastic cells are small and cathepsin  $\text{K}^+$  (D). Majority of the cells are mononuclear (E) and show a single granular ring-like actin staining pattern (F). (G–I, same visual field) The control cells are large and cathepsin  $\text{K}^+$  (G), contain multiple nuclei (H), and a single large actin ring (I). (J) A high magnification of two DAP12-deficient mononuclear osteoclastic cells demonstrates several granular, unorganized actin clusters. (K) A high magnification of a mononuclear TREM2-deficient cell shows a single granular ring-like actin staining pattern.



**Figure 4.** DAPI2-deficient osteoclasts are capable of bone resorption in vitro. (A) Control osteoclasts generated long continuous resorption pits. (B) The average surface area per resorption pit generated by DAPI2-deficient osteoclasts is similar to controls. The resorption pits generated by genetically deficient osteoclasts are deeper compared with controls (toluidine blue staining).

Abnormal function of the actin ring or cytoskeleton in the genetically defective osteoclasts could potentially lead to a decrease in resorbed surface area, prolonged resorption process, and abnormally deep resorption pits.

**Quantitative RT-PCR Analysis.** To study the molecular basis for resorption capability and osteoclastic character, we performed a time course study of mRNA of the stimulated cells for cathepsin K, *RANK* (19), *TRAP*, and *CALCR* (20) using quantitative RT-PCR (Table I). *Cathepsin K* transcripts were expressed at a very low level at days 1–3, but became strongly up-regulated after stimulation for 7–21 d ( $P < 0.001$ ). There was no difference between DAPI2/TREM2-deficient and control cells. Transcripts of the gene encoding RANK, the receptor for RANKL, were expressed in both genetically deficient and control cells after stimulation for 1 d. No significant difference was observed between the patient and control cells. *TRAP* transcript level was initially low but became up-regulated after

stimulation for 3–7 d. No significant differences between the patients and controls were observed. Both DAPI2- and TREM2-deficient cells differentiating along the osteoclastic lineage expressed *CALCR*, the receptor regulating the resorption by osteoclasts. Reliable quantitation could not be performed due to a low transcript level. However, there were no obvious differences between genetically deficient and control cells during the stimulation for 1–21 d. The transcript levels of *DAP12* and *TREM2* in control cells showed a progressive increase during the stimulation for up to 21 d. A fivefold increase in *DAP12* ( $P < 0.01$ ) and a 39-fold increase in *TREM2* transcript level ( $P < 0.001$ ) was observed between stimulations for 1 and 21 d. Taken together, the delayed differentiation and impaired resorption function of genetically deficient osteoclasts is not due to loss of *RANK*, *CALCR*, or *TRAP* expression. There were no significant differences between DAPI2/TREM2-deficient and control cells, suggesting that the steady-state expression levels of the genes studied are not dependent on DAPI2 and TREM2. TREM2 polypeptide has been shown to be undetectable in macrophages derived by stimulation of monocytes for up to 14 d with M-CSF alone (9). Thus, osteoclasts are likely to express *DAP12* and *TREM2* because the expression of *DAP12* and *TREM2* in PBMCs differentiating along the osteoclastic lineage became progressively up-regulated parallel with the increasing number of multinucleated osteoclasts.

**DAPI2- and TREM2-deficient PBMCs Migrate Efficiently.** To determine if the fusion of genetically deficient osteoclast precursor cells is delayed due to reduced motility we performed a motility assay. We plated  $10 \times 10^6$  DAPI2-, TREM2-deficient, or control PBMCs per well 36 mm in diameter, each well containing four round coverslips 13 mm in diameter. A cylindrical silicone object 5 mm in diameter attached to the center of each coverslip before plating was removed after 24 h stimulation. After 7 d, the genetically deficient cells effectively migrated to the center of the coverslip, and multinucleated osteoclasts were found at the center. A notable difference was observed in the control cell density between the center and surrounding areas ( $P < 0.01$ ), whereas there was no significant difference in the density of genetically deficient cells between these areas ( $P > 0.05$ ). This implies that DAPI2- and TREM2-deficient PBMCs migrate efficiently and that the delayed fusion of prefusion osteoclasts is not caused by impaired motility.

The receptors and signals required for the fusion of osteoclast precursor cells during osteoclast maturation are not fully understood. Our results demonstrate that DAPI2/TREM2 signaling mediates the differentiation of osteoclasts and that DAPI2/TREM2-deficiency results in an aberrant osteoclast morphology and a severely delayed maturation process in vitro, as indicated by the inefficient fusion and impaired actin ring formation of the immature osteoclasts.

Kaifu et al. (21) have recently reported a defect in osteoclast differentiation in DAPI2-deficient mice. Interestingly, their mouse model presents a mild osteopetrosis and no cystic bone lesions. This observation is in contradiction

**Table I.** Quantitative RT-PCR Analysis of RANKL/M-CSF-stimulated PBMCs of DAP12- and TREM2-deficient and Healthy Individuals

Gene	Time of stimulation			
	1 d	3 d	7 d	21 d
DAP12 (controls) <sup>a</sup>	260 ± 132	365 ± 95	450 ± 161	1,304 ± 254
TREM2 (controls) <sup>a</sup>	14 ± 6	65 ± 8	76 ± 25	541 ± 125
TRAP (controls) <sup>b</sup>	9 ± 4	63 ± 27	63 ± 11	76 ± 43
TRAP (patients)	5 ± 1	36 ± 8	59 ± 16	16 ± 4
Cathepsin K (controls) <sup>c</sup>	0.3 ± 0.2	1 ± 0.9	374 ± 360	324 ± 183
Cathepsin K (patients)	16 ± 16	15 ± 13	963 ± 710	533 ± 155
RANK (controls) <sup>b</sup>	8 ± 3	33 ± 17	5 ± 3	25 ± 15
RANK (patients)	71 ± 29	71 ± 13	8 ± 3	54 ± 11

The numbers indicate the transcript copy number per housekeeping gene copies (PBGD) ± SEM. Note: DAP12, TREM2, TRAP, and cathepsin K copy number is presented as (the absolute copy number)/(PBGD copy number), and RANK expression as 1,000 × (the absolute copy number)/(PBGD copy number).

*n* (controls) = 4, *n* (patients) = 5.

<sup>a</sup>DAP12 and TREM2 expression increased significantly in stimulated PBMCs (*P* < 0.01; Bonferroni's multiple comparison test).

<sup>b</sup>No difference between controls and patients (*P* > 0.05).

<sup>c</sup>A significant up-regulation in patients and controls (*P* < 0.001; Kruskal-Wallis test), but no difference between patients and controls.

with human PLOSL where the pathological hallmarks are cystic bone cavities and osteoporosis.

The symptoms of PLOSL appear approximately at age 20 as skeletal pain. Spontaneous fractures in the bones of the extremities occur a few years later due to bone cavities filled with membranous lipid material (3, 22). The lesions are found in all limb bones, especially in the bones of the wrists, hands, ankles and feet. In the long tubular bones, the lesions are typically located in the distal end of the bones. In addition, the bones show osteoporotic features, i.e., severe loss of trabecular bone. The development of the bone lesions is slowly progressive and the age at which the lesions begin to develop is unknown (23). The normal average height and the macroscopic structure of the bones in PLOSL patients, apart from the lesion cavities and trabecular bone loss, indicate that the resorptive phase of bone development and growth is not severely affected. As expected, multinucleated, TRAP<sup>+</sup> and CD68<sup>+</sup> osteoclasts were found in the bones of DAP12-deficient PLOSL patients. The size of osteoclasts appeared normal (not depicted). Although the bone resorption capability of DAP12-deficient osteoclasts was impaired in vitro, the bones of PLOSL patients are osteoporotic rather than osteopetrotic, as is seen in severe osteoclastic failure (24). Our finding of an impaired resorption capability of genetically deficient osteoclasts in vitro is somewhat contradictory with the local osteolytic process in PLOSL patients. Current knowledge of DAP12/TREM2 signaling does not provide self-evident explanations for this paradox. The locally increased bone loss in vivo could be explained by systemic or local factors (endocrine, paracrine, etc.) affecting the differentiation or activation of osteoclasts in situ.

The reason why the bone lesions in DAP12- and TREM2-deficient PLOSL patients are found only in the limb bones, and especially in the distal segment of the bones, remains unclear. Bones develop either by endochondral or intramembranous ossification. In endochondral ossification a cartilage model serves as the precursor of bone, whereas intramembranous ossification occurs without an intervening cartilage precursor (25). Interestingly, all bones affected by PLOSL are formed by endochondral ossification. No lesions have been found in the bones developed by intramembranous ossification (e.g., the skull, clavicle, and mandible; 23).

So far, the function of DAP12 and TREM2 has been thought to be limited to transmitting activating signals to cells of the immune system. Our findings provide, for the first time, direct evidence that DAP12 and TREM2 in humans also play a key role in the normal functions of cells not directly involved in immune responses. Further characterization of the role of DAP12/TREM2 signaling in common disorders of bone, such as osteoporosis, should be stimulated by our findings. Finally, despite the characterization of a defective osteoclast maturation and function caused by DAP12/TREM2 deficiency, understanding the role of osteoclasts in the bone pathogenesis of PLOSL in detail still requires additional studies in vitro and in vivo.

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