Signal adaptor DAP10 associates with MDL-1 and triggers osteoclastogenesis in cooperation with DAP12

Masanori Inui[a,1], Yuki Kikuchi[a,1], Naoko Aoki[b], Shota Endo[a], Tsutomu Maeda[a], Akiko Sugahara-Tobinai[a], Shion Fujimura[a], Akira Nakamura[a], Atsushi Kumanogoh[c], Marco Colonna[d], and Toshiyuki Takai[a,2]

[a]Department of Experimental Immunology, Institute of Development, Aging and Cancer, Tohoku University, Seiryo 4-1, Sendai 980-8575, Japan; [b]Department of Pathology, Asahikawa Medical College, Higashi 2-1-1, Midorigaoka, Asahikawa 078-8510, Japan; [c]Department of Immunopathology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan; and [d]Department of Pathology and Immunology, Washington University School of Medicine, 660 South Euclid, St. Louis, MO 63110

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Osteoclasts, cells of myeloid lineage, play a unique role in bone resorption, maintaining skeletal homeostasis in concert with bone-producing osteoblasts. Osteoclast development and maturation (osteoclastogenesis) is driven by receptor activator of NF-κB ligand and macrophage-colony stimulating factor and invariably requires a signal initiated by immunoreceptor tyrosine-based activation motif (ITAM)-harboring Fc receptor common γ chain or DNAX-activating protein (DAP)12 (also referred to as KARAP or TYROBP) that associates with the cognate immunoreceptors. Here, we show that a third adaptor, YINM costimulatory motif-harboring DAP10, triggers osteoclastogenesis and bone remodeling. DAP10-deficient (*DAP10*$^{-/-}$) mice become osteopetrotic with age, concomitant with a reduction in osteoclasts. The DAP10-associating receptor was identified as myeloid DAP12-associating lectin-1 (MDL-1), whose physiologic function has not been found. MDL-1-mediated stimulation of osteoclast precursor cells resulted in augmented osteoclastogenesis in vitro. MDL-1 associates with both DAP12 and DAP10 in osteoclasts and bone marrow-derived macrophages, where DAP10 association depends almost entirely on DAP12, suggesting a formation of MDL-1–DAP12/DAP10 trimolecular complexes harboring ITAM/YINM stimulatory/costimulatory motifs within a complex that could be a novel therapeutic target for skeletal and inflammatory diseases.

immunoreceptor tyrosine-based activation motif-harboring adaptor | osteoclast development | synergistic signal

Homeostasis of bone tissues is maintained by 2 types of cells, osteoblasts and osteoclasts of mesenchymal and myeloid-cell lineages, respectively. Although osteoblasts are bone-generating cells, osteoclasts are present on bone erosive surfaces in the marrow and play a unique role in bone resorption, maintaining the mass and structure of bone tissues in collaboration with osteoblasts. Osteoclast development and maturation (osteoclastogenesis) depends on receptor activator of NF-κB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) (1, 2). M-CSF interaction with its receptor c-fms, a receptor tyrosine kinase expressed on osteoclasts, supports the survival of osteoclast precursor cells (3, 4). However, binding of RANKL to its receptor RANK on the osteoclast precursor cell surface induces TNF receptor-associated factor 6 (TRAF6) and NF-κB activation, leading to transcription of a master transcription factor for osteoclastogenesis, nuclear factor of activated T cells (NFAT)c1 (5, 6). Full activation and induction of NFATc1 for this intracellular signal initiated by RANKL, however, invariably require the tyrosine phosphorylation-based stimulating signal initiated by the Fc receptor common γ chain (FcRγ) or DNAX-activating protein (DAP)12 (also known as KARAP or TYROBP) (7–14).

FcRγ and DAP12 are immunoreceptor tyrosine-based activation motif (ITAM)-harboring, membrane-bound signal adaptors expressed in leukocytes that associate with and support the cell-surface expression of various activating-type immunoreceptors of the Ig superfamily or C-type lectin family. They primarily mediate a Src-family kinase-initiated activation signal, leading to a series of events including Syk kinase recruitment, triggering of calcium signaling mediated by phospholipase Cγ (PLCγ), and activation of the MAPK cascade, culminating in cell proliferation and cytokine production as the output of cellular responses. In osteoclast precursor cells, FcRγ and DAP12 associate with several cognate cell-surface immunoreceptors (9) such as osteoclast-associated receptor (OSCAR) (15) and paired Ig-like receptor-A (PIR-A) (16, 17) or triggering receptor expressed on myeloid cells-2 (TREM-2) (18) and signal regulatory protein β1 (SIRPβ1) (19), respectively. Mice deficient in both FcRγ and DAP12 exhibit a markedly excessive bone mass (osteopetrosis) caused by a severe defect in the development of osteoclasts (9, 10). In vitro osteoclastogenesis was greatly impaired when bone marrow cells from DAP12-deficient (*DAP12*$^{-/-}$) mice or monocytes from *DAP12*$^{-/-}$ humans were cultured with RANKL and M-CSF (12–14) or such cells from FcRγ/DAP12 double-deficient (*FcRγ*$^{-/-}$*DAP12*$^{-/-}$) mice were cocultured with osteoblasts (9, 10). Moreover, we demonstrated that RANKL stimulation of osteoclast precursor cells led to tyrosine phosphoryation of DAP12 and FcRγ and activation of PLCγ (9). These results indicate the indispensable roles of these ITAM-harboring signal adaptors and associated receptors in osteoclastogenesis. This notion is further supported by the fact that the bone marrow cells in mice lacking Syk or PLCγ also fail to differentiate into mature osteoclasts (10, 20). Btk and Tec kinases were identified very recently as the link between RANK activation and ITAM phosphorylation of FcRγ and DAP12 (21).

Although FcRγ and DAP12 have been identified as indispensable stimulators for osteoclastogenesis (9, 10), a markedly reduced, but small number of osteoclasts was still found in *FcRγ*$^{-/-}$*DAP12*$^{-/-}$ mice (9), suggesting the existence of other activating adaptors or pathways that promote or compensate for osteoclastogenesis in vivo. A candidate molecule that might operate in the absence of FcRγ and DAP12 could be DAP10, which is also a membrane-associated signal adaptor harboring a costimulatory YINM motif and is cognate to DAP12 in the immune system (22, 23), but it remains unknown whether DAP10 is involved in the skeletal system. DAP10 is expressed broadly on hematopoietic cells, such as monocytes and macrophages (22, 23), NK cells, and CD8$^+$ T cells (23), and functions through its association with NKG2D, the only known DAP10 partner receptor that recognizes

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[1]M.I. and Y.K. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: tostakai@idac.tohoku.ac.jp.

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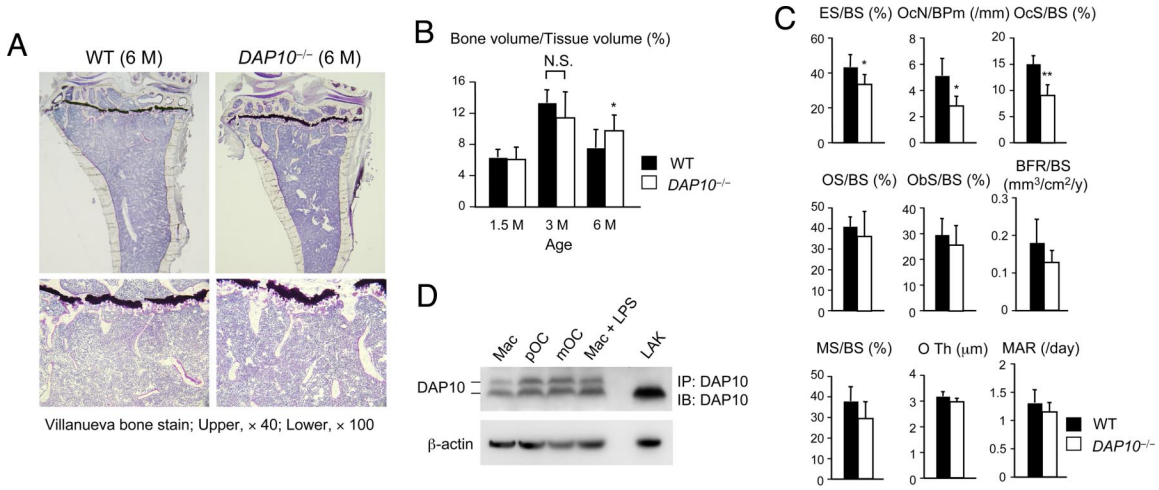

Fig. 1. Bone histological analysis reveals osteopetrosis in *DAP10*$^{-/-}$ mice developing with age. (*A*) Villanueva bone staining of tibial sections from female 6-month-old WT and *DAP10*$^{-/-}$ mice. Trabecular bones were increased, particularly in the metaphysis of *DAP10*$^{-/-}$ mice. (*B*) Bone morphometric analysis reveals the bone volume was increased in *DAP10*$^{-/-}$ mice at 6 months of age but not at 1.5 and 3 months of age, indicating osteopetrosis developing with age in the mice. Data are expressed as means ± SD ($n = 9$, female). *, $P < 0.05$. N.S., not significant. (*C*) Analysis of bone morphometric parameters in 6-month-old WT and *DAP10*$^{-/-}$ mice reveals reduced osteoclast parameters in *DAP10*$^{-/-}$ animals but comparable osteoblast parameters. BS, bone surface; ES, erosive surface; OcN, osteoclast number; BPm, bone perimeter; OcS, osteoclast surface; OS, osteoid surface; ObS, osteoblast surface; BFR, bone formation rate; MS, mineral surface; O Th, osteoid thickness; MAR, mineral apposition rate. Data are expressed as means ± SD ($n = 9$, female). *, $P < 0.05$; **, $P < 0.01$. (*D*) DAP10 protein expression was examined by immunoprecipitation and immunoblot analysis of cell extracts prepared from bone marrow-derived macrophages (Mac), pOCs, mOCs, macrophages stimulated with 0.1 μg/ml LPS for 24 h, and splenic DX5^{+} NK cells stimulated with 1,000 units/mL of IL-2 for 4 days. As a loading control, a β-actin blot is shown.

stress-induced MHC class I-like ligands (22, 24). DAP10 is unique in that it does not have an ITAM in its cytoplasmic domain but instead contains a different tyrosine-based motif, YINM, that is similar to those found in costimulatory receptors such as CD28 and ICOS and binds to the p85 subunit of PI3K (22) and Grb2 (23), leading to the triggering of calcium signaling and cytotoxicity in NK cells.

We attempted to clarify whether DAP10 could be a hidden signal adaptor molecule that promotes osteoclastogenesis. Here, we demonstrate the physiological function of myeloid DAP12-associating lectin-1 (MDL-1) (25) and the significance of its novel amplifying role in osteoclastogenesis through the cooperation of DAP10 with DAP12.

Results

DAP10-Deficient Mice Exhibit Mild Osteopetrosis Caused by a Reduction in the Osteoclast Number in Bone Tissues. To clarify whether DAP10 could be an activating adaptor for osteoclast development, we first performed bone morphometric analyses of DAP10-deficient (*DAP10*$^{-/-}$) mice (see *SI Text*). Tibial bone sections from 6-month-old *DAP10*$^{-/-}$ mice were found to exhibit slight increases in the metaphyses and trabecular bones, indicating mild osteopetrosis (Fig. 1*A*). Osteopetrosis was not evident in mice at 1.5 and 3 months of age, but it was significant in 6-month-old *DAP10*$^{-/-}$ mice (Fig. 1*B*). This observation was in sharp contrast to that for *DAP12*$^{-/-}$ mice, in which mild osteopetrosis was evident even in 1.5-month-old animals and was significant afterward at least up to 12 months of age (12). This difference is probably because the contribution of DAP10 to osteoclast development depends on DAP12 (see below). A small number of tartrate-resistant acid phosphatase (TRAP)$^{+}$ osteoclasts was seen in bone tissues from FcRγ/DAP12/DAP10 triple knockout (*FcRγ*$^{-/-}$*DAP12*$^{-/-}$*DAP10*$^{-/-}$) mice and *FcRγ*$^{-/-}$*DAP12*$^{-/-}$ mice (Fig. S1), supporting the above notion.

Bone morphometric analyses of the tibia of these mice at 6 months of age revealed significant reductions in osteoclast-related parameters (Fig. 1*C Top*), indicating reductions in the number and function of osteoclasts in *DAP10*$^{-/-}$ mice, while keeping comparable values for osteoblastic parameters (Fig. 1*C Middle* and *Bottom*). These parameters for 1.5- and 3- month-old *DAP10*$^{-/-}$ mice were comparable with those for WT mice (Fig. S2). These results indicate that the increased bone mass in *DAP10*$^{-/-}$ mice was caused by attenuated osteoclastogenesis, although the overall magnitude of the defect was apparently smaller than that in *DAP12*$^{-/-}$ mice, as judged from the occurrence of osteopetrosis in *DAP12*$^{-/-}$ animals at as early as 1.5 months of age (12). We found that DAP10 protein is expressed in immature, prefusion osteoclasts (pOCs), mature, multinucleated osteoclasts (mOCs), and bone marrow-derived macrophages in vitro (Fig. 1*D Upper*), but not in osteoblasts (Fig. S3) on immunoblot analysis with DAP10-specific antibodies (Fig. S4), suggesting a role of DAP10 in the development of osteoclasts.

In Vitro Osteoclastogenesis Was Impaired in the Absence of DAP10. With the RANKL/M-CSF system, culturing of bone marrow-derived monocyte/macrophage lineage cells (BMMs) as osteoclast precursor cells from *DAP10*$^{-/-}$ mice resulted in a significant reduction in the number of TRAP^{+} mOCs, especially with suboptimal concentrations of RANKL and M-CSF (5–20 and 1–10 ng/mL, respectively) (Fig. 2*A* and *B*). Concomitant with the reduction in mOCs, the resorptive pit area formed by *DAP10*$^{-/-}$ mOCs was also reduced (Fig. 2*C*). In the presence of suboptimal concentrations of RANKL, an excess concentration (100 ng/mL) of M-CSF did not rescue the reduced osteoclastogenesis (Fig. 2*D*). Reduced osteoclastogenesis was also observed with the osteoblast coculture system (Fig. 2*E*). Flow cytometric analyses (Fig. S5) verified that the cell-surface expression levels of the M-CSF receptor c-fms and the RANKL receptor RANK on pOCs were comparable among B6, *DAP10*$^{-/-}$, *DAP12*$^{-/-}$, and *DAP12*$^{-/-}$*DAP10*$^{-/-}$ (Fig. S6) cells, indicating that the reduced development of *DAP10*$^{-/-}$ osteoclasts is not caused by reduced expression levels of c-fms and RANK but a deficit in DAP10. These observations suggest that the RANKL/M-CSF-mediated signal is insufficient in *DAP10*$^{-/-}$ osteoclast precursor cells.

Identification of MDL-1 as a Distinct Cell-Surface Receptor That Associates with DAP10 in Osteoclasts. How does DAP10 participate in osteoclastogenesis? Although DAP10 physiologically associates with the only known partner, NKG2D, in immune cells (22), we failed to detect a significant amount of NKG2D mRNA in osteoclast lineage cells after GeneChip (9) and RT-PCR analyses (Fig. S7).

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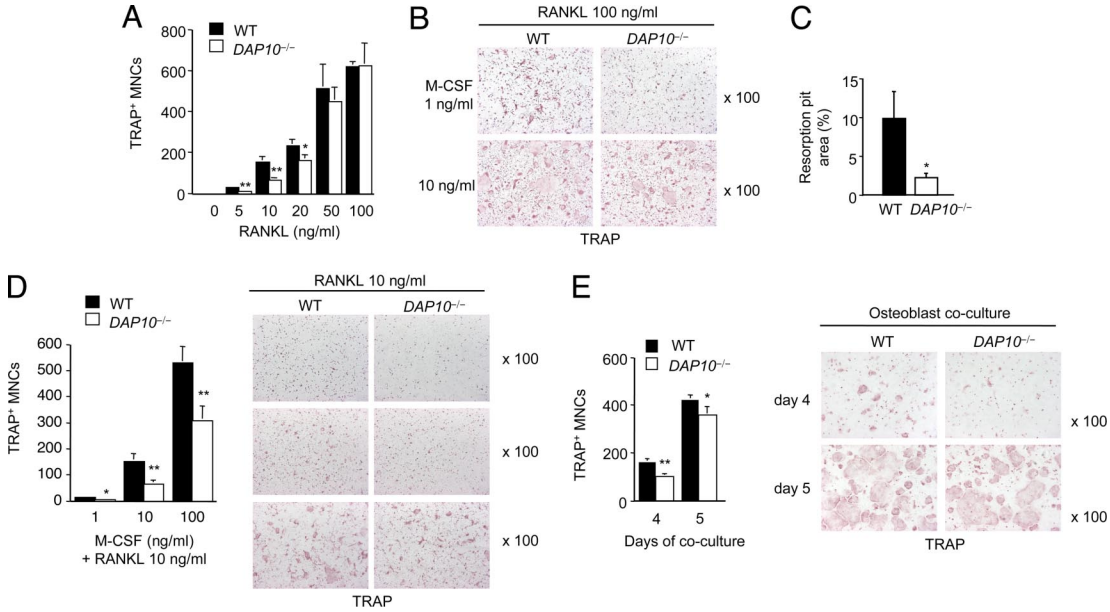

Fig. 2. Osteoclast development and function are impaired in an in vitro culture. (*A*) Reduced osteoclastogenesis in *DAP10*$^{-/-}$ bone marrow-derived monocyte/macrophage lineage cells (BMMs) stimulated with suboptimal concentrations of RANKL in the presence of 20 ng/mL M-CSF. TRAP^{+} multinucleated cells (MNCs) were counted. (*B*) Reduced osteoclastogenesis in *DAP10*$^{-/-}$ BMMs with suboptimal concentrations of M-CSF (1 or 10 ng/mL) in the presence of sufficient RANKL (100 ng/mL). (*C*) Reduced pit formation of *DAP10*$^{-/-}$ osteoclasts obtained in culture in the presence of 100 ng/mL RANKL and 20 ng/mL M-CSF. (*D*) Reduced osteoclastogenesis in *DAP10*$^{-/-}$ BMMs stimulated with 10 ng/mL RANKL and 1, 10, or 100 ng/mL M-CSF. (*E*) Reduced osteoclastogenesis in *DAP10*$^{-/-}$ BMMs cocultured with osteoblasts. Data are representative of 3 separate experiments with similar results and are expressed as means ± SD for triplicate cultures. *, $P < 0.05$; **, $P < 0.01$.

Therefore, the formation of a functional NKG2D–DAP10 complex in osteoclasts is unlikely. To find a candidate receptor that associates with DAP10, we prepared macrophages, pOCs, and mOCs from B6 and *DAP10*$^{-/-}$ mice, which were surface-biotinylated, either glycosidase-treated or left untreated, and then immunoprecipitated with anti-DAP10 antibodies. Comparison of the profiles for surface-biotinylated proteins of DAP10-sufficient and -deficient cells enabled us to detect a discrete band of ≈27-kDa glycosylated protein species, whose deglycosylated form was ≈18 kDa (Fig. S8*A*). We excised a gel strip containing this protein and subjected it to TOF/MS analysis (see *SI Text*). Sixty-six tryptic peptide sequences obtained were searched for in the NCBInr database (www.protein.sdu.dk/gpmaw/GPMAW/Databases/NCBInr/ncbinr.html), and 6 murine proteins were shown to be significant hits, among which C-type lectin domain family 5 member A (i.e., MDL-1) (25) was the only notable one in the context of our investigation (Fig. S8*B*). The expression of mRNA for MDL-1 was confirmed by RT-PCR analysis of samples from pOCs, mOCs, and macrophages as described (26) (Fig. S8*C*). MDL-1 was identified originally (25) as a type II transmembrane glycoprotein of the C-type lectin superfamily (Fig. S8*D*), expressed as a DAP12-dependent manner as both long and short forms possibly arising from alternative splicing, but neither the physiological role of nor the ligand for MDL-1 has been elucidated. Based on the knowledge obtained in these preceding studies, our present observations indicate that MDL-1 could associate with DAP10 in addition to DAP12 in macrophages and osteoclasts and might function as an activating receptor in osteoclasts. Although C-type lectin superfamily molecules are often expressed on the cell surface as a disulfide-linked homodimer like NKG2D or Ly49C (22, 27), or a heterodimer such as CD94–NKG2A (28), the results from our immunoblot analyses indicated that the majority of the MDL-1 short and long forms exist as monomers (*SI Text* and Fig. S8*E*).

DAP10 Forms an MDL-1–DAP12/DAP10 Trimolecular Complex Depending Almost Solely on DAP12. To determine whether MDL-1 expression solely depends on DAP12 or not, we performed flow cytometry of MDL-1 expression on macrophages and pOCs from mice deficient in either DAP12, DAP10, DAP12 and DAP10, or FcRγ (Fig. 3*A*). The MDL-1 expression levels on macrophages and pOCs were not changed by FcRγ deletion, but were reduced markedly in the absence of DAP10 and decreased to a minimum with either DAP12 or DAP12 and DAP10 double deletion. We verified that the MDL-1 mRNA levels in pOCs from these mutant mice were not reduced when compared with that in pOCs from B6 mice (Fig. S9). Thus, MDL-1 cell-surface expression primarily depends on DAP12, and partially, but substantially also depends on DAP10.

Given that the DAP12-associated receptor TREM-2 (18) has been implicated in osteoclastogenesis (29–31), it would be interesting to determine whether the expression of TREM-2 is also diminished in *DAP10*$^{-/-}$ and *DAP12*$^{-/-}$*DAP10*$^{-/-}$ osteoclasts like that of MDL-1. Our flow cytometric analysis (Fig. 3*B*) revealed that TREM-2 expression on pOCs did not change with the deficiency of DAP10 or DAP10 and DAP12, and in the deficiency of DAP12, as reported (29), indicating that the TREM-2 expression itself does not depend on DAP10 or DAP12, which is in sharp contrast to the case of MDL-1. However, this observation does not exclude the possibility that DAP10 could enhance TREM-2–DAP12-mediated signaling.

To confirm the DAP12- and DAP10-dependent expression of MDL-1, lysates of macrophages, pOCs, and mOCs derived from B6, *DAP10*$^{-/-}$, and *DAP12*$^{-/-}$ mice were immunoprecipitated with anti-MDL-1 polyclonal antibodies and then subjected to immunoblotting with antibodies to MDL-1 or DAP10 and DAP12. As shown in Fig. 4*A*, top 2 panels), immunoprecipitation/immunoblotting analyses with MDL-1 antibodies and with or without *N*-glycosidase treatment revealed that macrophages, pOCs and mOCs from B6 mice (lanes 1–3) and macrophage-like RAW cells (lane 11) exhibited high expression of a diffuse, glycosylated 38- to 45-kDa protein species corresponding to the highly glycosylated, monomeric long form of MDL-1, and several discrete and a diffuse protein species ≈20–28 kDa corresponding to the differently glycosylated, monomeric short form of MDL-1. These protein species were confirmed to migrate at the positions consistent with the predicted core sizes for MDL-1 long and short forms after

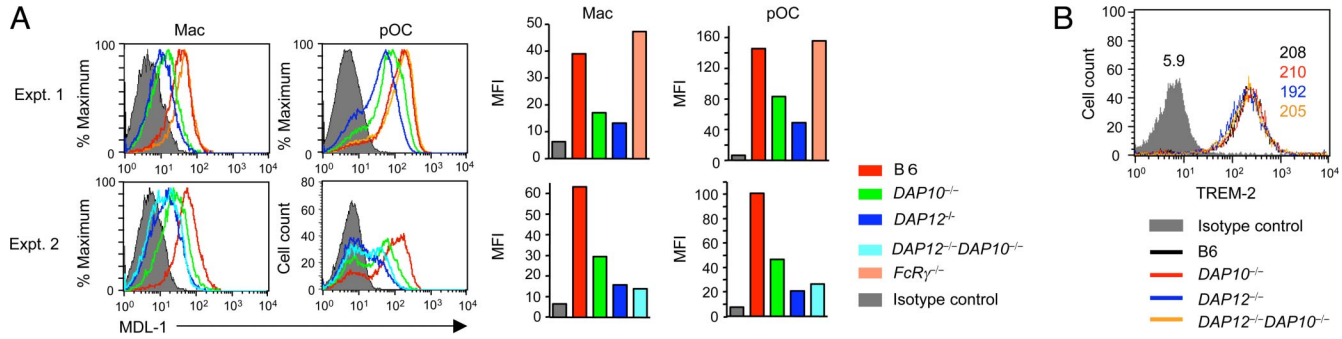

Fig. 3. Cell surface expression of MDL-1 depends on DAP12 and is enhanced by DAP10. (*A*) The results or flow cytometric analysis of surface MDL-1 expression in bone marrow-derived macrophages (Mac) and pOCs are shown as histograms and as graphs for mean fluorescence intensity (MFI) of each histogram profile. Macrophages and pOCs obtained from B6 (red), *DAP10*$^{-/-}$ (green), *DAP12*$^{-/-}$ (blue), and *FcRγ*$^{-/-}$ (orange) (experiment 1) or *DAP12*$^{-/-}$*DAP10*$^{-/-}$ (light blue) (experiment 2) mice were stained with phycoerythrin-conjugated anti-MDL-1 mAb or an isotype control, and then subjected to flow cytometry. (*B*) Flow cytometric analysis of TREM-2 expression on pOCs obtained from B6 (black), *DAP10*$^{-/-}$ (red), *DAP12*$^{-/-}$ (blue), and *DAP12*$^{-/-}$*DAP10*$^{-/-}$ (orange) mice. MFI value of each histogram is shown.

treatment with the combination of *N*- and *O*-glycosidases and neuraminidase (Fig. S10; see also Fig. S8*E*). We observed less abundant, long and short form MDL-1 bands for samples of macrophages, pOCs and mOCs from *DAP10*$^{-/-}$ and *DAP12*$^{-/-}$ mice (Fig. 4*A*, lanes 4–9).

As shown in Fig. 4*A* (bottom 2 panels), we observed DAP12 and DAP10 coimmunoprecipitation with MDL-1 for all 3 cell populations from WT B6 mice (lanes 1–3) and in RAW cells (lane 11). For *DAP10*$^{-/-}$ cells, we observed DAP12 coprecipitation with MDL-1 (lanes 4–6) as expected from the results of flow cytometric analysis. Rather curiously, however, we did not detect DAP10–MDL-1 coprecipitation for *DAP12*$^{-/-}$ cells (lanes 7–9). To check the overall expression levels of DAP10 and DAP12, and their possible interaction, we performed immunoprecipitation and immunoblot analyses of these 2 adaptors in macrophage lysates from B6, *DAP10*$^{-/-}$ and *DAP12*$^{-/-}$ mice (Fig. 4*B*). It was evident that both DAP10 and DAP12 were expressed in total macrophage lysates (Fig. 4*B Upper*, lanes 7 and 8 and Fig. 4*B Lower*, lanes 1 and 2). Interestingly, DAP10 expression was markedly reduced in the absence of DAP12 (Fig. 4*B Upper*, *lane 9* and Fig. 4*B Lower*, lane 3) but importantly not lost completely (Fig. 4*B Upper*, lane 6), suggesting that the DAP10 protein is unstable in the absence of DAP12. This finding is in sharp contrast to in the case of IL-2-activated killer cells (LAK) in which DAP10 is abundantly expressed in the absence of DAP12 (Fig. 4*B Upper*, lane 10 and Fig. 4*B Lower*, lane 5) and is functional because DAP10-dependent NKG2D expression is normal in the absence of DAP12 (Fig. S11). Also, it was verified that DAP10 was coimmunoprecipitated with DAP12 (Fig. 4*B Upper*, lanes 2 and 4), suggesting that DAP10 is a component of one or more DAP12-receptor complexes in macrophages and RAW cells. To examine directly the association among MDL-1, DAP10, and DAP12, we performed immunoprecipitation/immunoblot analysis of RAW cell extracts with polyclonal antibodies specific to each molecule (Fig. 4*C*). We found that DAP10 and DAP12 immunoprecipitation resulted in coprecipitation of DAP12 and DAP10, respectively, in addition to MDL-1. To examine the presence of DAP12–DAP10 heterodimers associating with MDL-1, total lysates of pOCs were immunoprecipitated with anti-MDL-1 mAb, separated by SDS/PAGE under nonreducing conditions, and then immunoblotted with anti-DAP12 or DAP10 antibodies (Fig. 4*D*). We found the 16-kDa protein species in B6 pOCs that was reactive with both DAP12 and DAP10 antibodies (Fig. 4*D*, lanes 1 and 3) and was lost in the DAP10 deficiency (Fig. 4*D*, lane 2), suggestive of the DAP12–DAP10 heterodimer. Collectively, these results suggest that the DAP12–MDL-1 complex is a prototype structure for the surface expression of MDL-1, and that DAP10 association with MDL-1 almost solely depends on the presence of DAP12–MDL-1, forming an MDL-1–DAP12/DAP10 trimolecular complex.

MDL-1 Functions as a Positive Regulator of Osteoclastogenesis. To determine how MDL-1 functions in osteoclastogenesis, we stimulated BMMs from B6 mice with a plate-bound anti-MDL-1 mAb and found an increase in RANKL-induced mOCs (Fig. 5*A*). To further confirm the activating function of MDL-1, we examined the effect of suppression of mRNA for MDL-1 by the RNA silencing technique. Transfection of MDL-1 siRNA, but not nontargeting siRNA, reduced the level of MDL-1 mRNA (Fig. S12) and led to the reduced number of mOCs (Fig. 5*B*). These results led to our conclusion that MDL-1 functions as a positive regulator of osteoclastogenesis.

Discussion

Murine NKG2D can be expressed as a NKG2D–DAP12 complex or as a NKG2D–DAP10 complex, both of which are capable of initiating a signal (32, 33). In addition, recent stoichiometric analysis revealed that the human NKG2D homodimer can associate with 2 homodimeric forms of DAP10, resulting in the formation of a hexameric structure (34). Thus, binding of a single ligand can result in the phosphorylation of 4 DAP10 chains, which may be relevant as to the sensitivity of the receptor, in particular in the situation of low ligand density (34). It has been pointed out that a hexameric NKG2D complex is assembled in activated murine NK cells, which incorporates 1 DAP10 and 1 DAP12 homodimer. The formation of such a trimolecular hexamer could be functionally relevant, because the 2 homodimers initiate distinct signaling cascades: DAP12 activates the Syk/ZAP70 pathway, and DAP10 signals through the PI3K pathway (24, 35, 36). SIRPβ1 (19) is also capable of associating with either the DAP12 or DAP10 homodimer or the DAP12/DAP10 heterodimer in the RBL-2H3 leukemic mast cell line (37).

In sharp contrast to NKG2D, MDL-1 expression solely depends on the association with DAP12 and is accelerated by the additional association with DAP10, possibly through, most simply, the ultimate formation of an MDL-1–DAP12/DAP10 trimolecular complex, although we do not exclude a possibility of any unidentified molecule intervening MDL-1–DAP12 and DAP10, or a possible sharing of DAP10 between MDL-1–DAP12 and any unidentified receptor–DAP10: the unidentified molecule or receptor could also be an MDL-1 itself. In any case, MDL-1 cannot be expressed robustly on the cell surface as an MDL-1–DAP10 complex. It has not ever been clarified whether MDL-1 itself is expressed as a monomeric form or a homodimer, or both (25, 38). Our immunoblot analysis (Fig. 4*A* and Fig. S8*E*) indicates that the majority of MDL-1 is expressed as a monomeric form on macrophages, pOCs, and mOCs, while it also did not exclude the possibility of the presence of minor populations containing homodimeric or long form–short form heterodimeric complexes or heterooligomeric complexes with other membrane proteins such as CD94 (28). In an MDL-1–DAP12/DAP10 trimeric complex, monomeric MDL-1

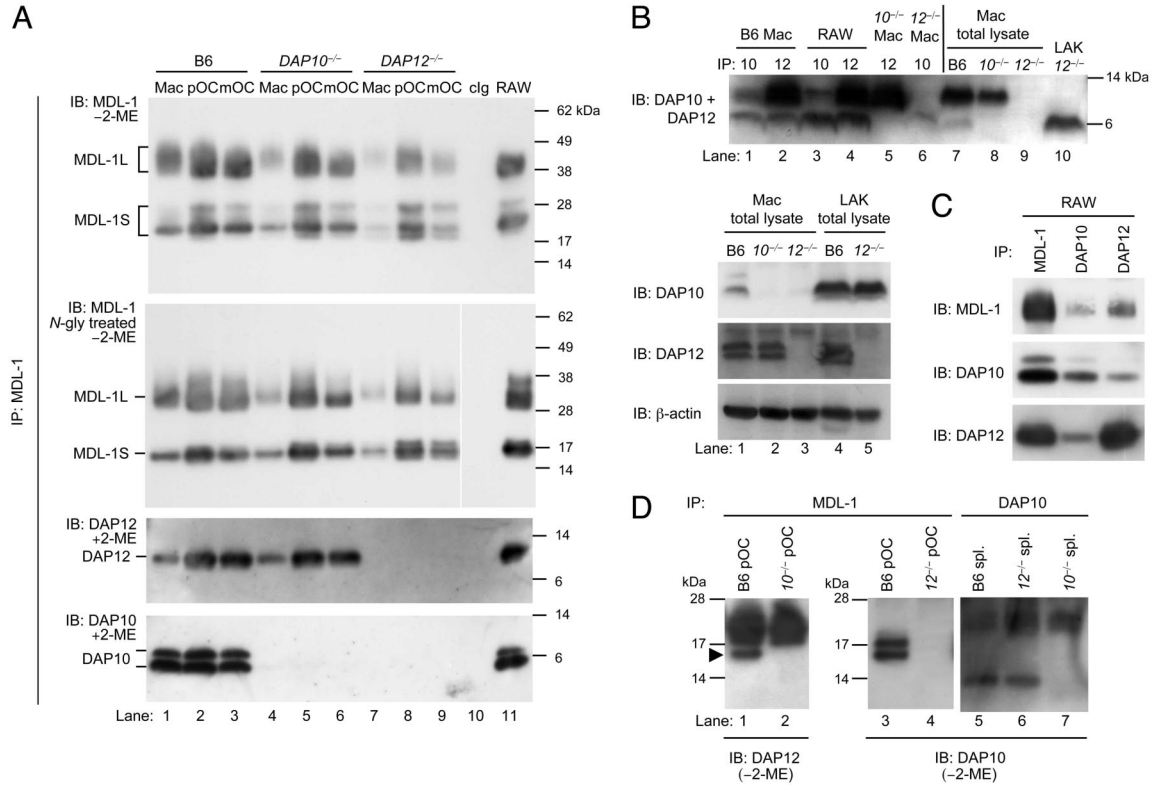

Fig. 4. DAP10 forms an MDL-1–DAP12/DAP10 trimolecular complex depending almost solely on DAP12. (*A*) Immunoprecipitation and immunoblot analysis of MDL-1 (top 2 panels) and its DAP10/DAP12 association (bottom 2 panels). Cell lysates of bone marrow-derived macrophages (Mac), pOCs, and mOCs from B6, *DAP10*$^{-/-}$ and *DAP12*$^{-/-}$ mice were immunoprecipitated with anti-MDL-1 antibodies, treated with *N*-glycosidase or left untreated, separated by SDS/PAGE under nonreducing (−2-ME) or reducing conditions (+2-ME), and immunoblotted with anti-MDL-1, anti-DAP12, or anti-DAP10 antibodies. Lysates of RAW264.7 cells immunoprecipitated with anti-MDL-1 or control Ig (cIg) were used as positive and negative controls, respectively. Long-form and short-form MDL-1 are denoted. (*B*) Immunoprecipitation and immunoblot analysis of DAP10 and DAP12 in macrophages. Cell lysates of macrophages (Mac) cultured from bone marrow cells of B6, *DAP10*$^{-/-}$ and *DAP12*$^{-/-}$ mice, and of RAW, as a positive control, were immunoprecipitated with anti-DAP10 or DAP12 antibodies, and immunoblotted with anti-DAP10 and anti-DAP12 antibodies (*Upper*, lanes 1–6). Total lysates of macrophages from B6, *DAP10*$^{-/-}$, and *DAP12*$^{-/-}$ mice, and of IL-2-activated splenic NK cells (LAK) from *DAP12*$^{-/-}$ mice are simultaneously blotted (*Upper*, lanes 7–10). Note that DAP10 band becomes weak in the absence of DAP12 for macrophages (*Upper*, lane 9) but not for LAK (*Upper*, lane 10) and that DAP12 immunoprecipitation yields DAP10 coprecipitation (*Upper*, lanes 2 and 4). The disappearance of the DAP10 band in the absence of DAP12 for macrophages but not for LAK is reproduced (*Lower*, lanes 3 and 5). (*C*) DAP10 coprecipitates with DAP12 and vice versa together with MDL-1. Lysates of RAW cells were immunoprecipitated with either anti-MDL-1, anti-DAP10, or anti-DAP12 anbitodies, separated by SDS/PAGE under reducing conditions, and then immunoblotted with each antibody. (*D*) DAP10 possibly forms an MDL-1–DAP12–DAP10 triplex. Total cell lysates of pOCs and splenocytes (spl.) from B6, *DAP10*$^{-/-}$, and *DAP12*$^{-/-}$ mice were immunoprecipitated with anti-MDL-1 mAb or anti-DAP10 antibodies, separated by SDS/PAGE under nonreducing conditions, and immunoblotted with anti-DAP12 or DAP10 antibodies. The robust and seemingly triplet bands migrating at 18–25 kDa detected with anti-DAP12 antibodies in B6 pOCs (lane 1) are assigned to DAP12–DAP12 homodimers with different glycosylation, because these protein species do not react with anti-DAP10 antibodies (lane 3) and are present even in the absence of DAP10 (lane 2). Immunoprecipitation of splenocyte samples with anti-DAP10 antibodies followed by detection with anti-DAP10 revealed a ≈13-kDa band for the less-glycosylated or nonglycosylated DAP10–DAP10 homodimer that presumably associates with NKG2D on NK cells in splenocytes (lanes 5 and 6; see also Figs. S4, S6, and S11). This form of homodimeric DAP10–DAP10 was not detected in B6 pOC samples (lane 3). The less-glycosylated or nonglycosylated monomeric 6-kDa DAP10 is truly a component of the MDL-1 complex (see *A*, lane 2), suggesting the pairing with a different molecule. The 16-kDa band (lane 1, arrowhead), detected with anti-DAP12 antibodies in B6 pOCs, is attributable to a heterodimer of DAP12 paired with less-glycosylated or nonglycosylated DAP10, because it was lost in the DAP10 deficiency (lane 2). Consistent with this notion, the 16-kDa band was detectable also with anti-DAP10 antibodies in B6 pOCs (lane 3). The 18-kDa band, reactive with anti-DAP10 antibodies (lane 3), is probably also a DAP12–DAP10 heterodimer in a different glycosylation state, whose supposed disappearance in DAP10 deletion might be masked by the DAP12 homodimer bands (lane 2).

would associate with a disulfide-bonded DAP12–DAP10 heterodimer, thereby allowing the full cascade of events including Syk and PLCγ1 activation via ITAM-harboring DAP12 (9, 10), and PI3K and Grb2–Vav1 recruitment via YINM-harboring DAP10 located just proximal to DAP12. By being positioned very close to each other in a trimeric complex, the 2 adaptor molecules could collaborate intimately, enabling them to signal much more efficiently than as singlets.

We showed (9) that TREM-2 (18) and SIRPβ1 could accelerate osteoclastogenesis via the DAP12 signal. Then, is it pertinent to suppose that MDL-1–DAP12/DAP10 is as equally important as TREM-2–DAP12 and SIRPβ1–DAP12? In a normal situation, a restricted number of DAP12 molecules might be competitively recruited by at least 3 different receptors, MDL-1, TREM-2, and SIRPβ1, which evokes a presence of any complex mechanism for functional allotment among them. If, for example, DAP12 can preferentially associate with TREM-2 than others, it indirectly reduces the chance of MDL-1 associating with DAP12, and thus lowers the expression level of MDL-1–DAP12/DAP10. We speculate that TREM-2– and SIRPβ1–DAP12 have to cooperate with MDL-1–DAP12/DAP10 for full-blown osteoclastogenesis. Relative significance of the individual receptors in osteoclastogenesis awaits experimental verifications such as examinations in MDL-1-deficient mice.

A defect in the DAP12–TREM-2 function leads to the development of heritable Nasu–Hakola disease in humans (11, 14). Involvement of MDL-1–DAP12/DAP10 in osteoclastogenesis suggests that Nasu–Hakola disease by DAP12 deficiency may also

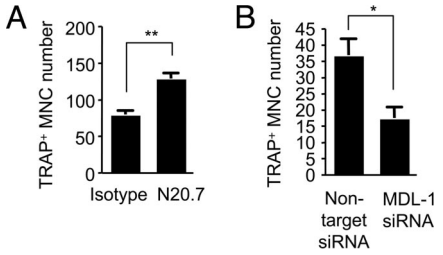

Fig. 5. MDL-1 functions as a positive regulator of osteoclastogenesis. (*A*) Stimulation of pOCs with plate-bound MDL-1 mAb leads to enhanced osteoclastogenesis. Bone marrow-derived monocyte/macrophage lineage cells (BMMs) prepared from B6 mice were stimulated with plate-bound N20.7 mAb or an isotype control antibody in the presence of RANKL/M-CSF. The number of $TRAP^+$ cells (>3 nuclei) was determined. (*B*) Reduced osteoclastogenesis by MDL-1 siRNA. BMMs transfected with MDL-1 or nontargeting siRNA were cultured for 4 days in the presence of M-CSF, and then further cultured with RANKL for 4 days. The number of $TRAP^+$ cells (>3 nuclei) was determined. Data are expressed as means ± SD of triplicate culture. *, $P < 0.05$; **, $P < 0.01$.

include a defect in the MDL-1 function, albeit it has not been tested yet. The *MDL-1* gene is located on human chromosome 7q33 and murine chromosome 6B2 as a single copy gene (official symbol, *Clec5a*), where no disease relation has been mapped to date. MDL-1 is a member of a large complex of genes termed the C-type lectin domain (CLEC) family (39), although both the murine and human MDL-1 genes are apart from the CLEC locus present in murine 6F3 and human chromosome 12. The CLEC family comprises 13 structurally-related molecules such as Dectin-1 and Dcir encoded by genes at the 6F3 region, which is adjacent to the locus for the NKG2 family and the Ly49 family. A recent gene targeting study has revealed the significance of Dcir in autoimmune diseases (40). Our present study suggests that an altered function of MDL-1 could also be related to osteopetrosis, osteoporosis, rheumatoid arthritis, and other inflammatory diseases, where osteoclast/macrophage development and function is perturbed or dysregulated.

During preparation of our article, we found out that DAP10 has been coimmunoprecipitated with Ly49H and Ly49D from IL-2-cultured mouse NK cells (41), and that DAP10 and DAP12 supports Ly49H surface expression on NK cells, and the Ly49H–DAP10 receptor complex is functional (M. Orr and L. L. Lanier, personal communication). Therefore, DAP10 could be a more versatile adaptor in terms of the receptor expression/function than we previously thought. Our current identification of MDL-1 as the newly recognized receptor that associates with DAP10 may open up an additional avenue for dissecting the full spectrum of DAP10- and DAP12-mediated activation cascade of various cells not only in terms of osteoclastogenesis but also in other cellular development and function and diseases. After submission of this article, it was reported that MDL-1 can bind the dengue virion and initiate proinflammatory cytokine release (42). It is of no doubt that identification of physiological MDL-1 ligands will facilitate our understanding of the roles of MDL-1–DAP12/DAP10 in health and disease.

Materials and Methods

All statistics were generated with Student's *t* test. $P < 0.05$ was considered significant. Other methods are detailed in *SI Text* and refs. 9 and 33).

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