Cytoskeletal dysfunction dominates in DAP12-deficient osteoclasts

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Summary

Despite evidence that DAP12 regulates osteoclasts, mice lacking the ITAM-bearing protein exhibit only mild osteopetrosis. Alternatively, *Dap12*$^{-/-}$ mice, also lacking FcRγ, are severely osteopetrotic, suggesting that FcRγ compensates for DAP12 deficiency in the bone-resorbing polykaryons. Controversy exists, however, as to whether these co-stimulatory molecules regulate differentiation of osteoclasts or the capacity of the mature cell to degrade bone. We find that *Dap12*$^{-/-}$ osteoclasts differentiate normally when generated on osteoblasts but have a dysfunctional cytoskeleton, impairing their ability to transmigrate through the osteoblast layer and resorb bone. To determine whether the FcRγ co-receptor, OSCAR mediates osteoclast function in the absence of DAP12, we overexpressed OSCAR fused to FLAG (OSCAR-FLAG), in *Dap12*$^{-/-}$ osteoclasts. OSCAR-FLAG partially rescues the abnormal cytoskeleton of *Dap12*$^{-/-}$ osteoclasts grown on bone, but not those grown on osteoblasts. Thus, cytoskeletal dysfunction, and not arrested differentiation, is the dominant consequence of DAP12 deficiency in osteoclasts. The failure of osteoblasts to normalize *Dap12*$^{-/-}$ osteoclasts indicates that functionally relevant quantities of OSCAR ligand do not reside in bone-forming cells.

Key words: Osteoclasts, Cytoskeleton, ITAM, OSCAR

Introduction

Immune receptor tyrosine activation motifs (ITAMs) are important components of many immune receptors. They are also central to osteoclastic bone resorption (Humphrey et al., 2005). In osteoclast lineage cells, the primary ITAM-containing signaling adapters are DAP12 and the FcεRIγ chain (FcRγ). Both are small proteins with very short extracellular domains containing a cysteine residue mediating homodimer formation through disulfide bonds. A single transmembrane region, containing a negatively charged amino acid, pairs DAP12 or FcRγ to their specific, associated immunoreceptors. Because the transmembrane charge bridge stabilizes the receptor, the absence of DAP12 or FcRγ obviates cell surface expression of the majority of immunoreceptors recognized by each adaptor. Both molecules also contain a single ITAM motif, within their cytoplasmic domains, whose phosphorylated tyrosines provide a high-affinity binding site for Syk family kinases.

Osteoclasts, the unique resorptive polykaryons of bone, are generated by fusion of mononuclear progenitors of the monocytes or macrophage family under the aegis of macrophage-colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL) (Teitelbaum, 2007). In the context of osteoclastic cells, DAP12 associates with the receptors TREM2 and SIRPβ1 (Colonna et al., 2007), whereas FcRγ recognizes OSCAR (Ishikawa et al., 2004; van Beek et al., 2009).

There is little question that DAP12 and FcRγ impact the osteoclast. For example, they modulate RANKL-mediated Ca^{2+} influx and NFATc1 expression, which are both important components of the resorptive process (Koga et al., 2004). Thus, DAP12 deficiency is purported to arrest osteoclast formation induced by RANKL and M-CSF, a defect rescued by co-culture with osteoblasts (Koga et al., 2004; Mocsai et al., 2004). Although absence of DAP12 yields mild osteopetrosis, added deletion of FcRγ markedly enhances the severity (Koga et al., 2004; Mocsai et al., 2004). These observations suggest that FcRγ, in association with its co-receptor, OSCAR, compensates for absence of DAP12 in the osteoclastogenic process (Koga et al., 2004; Kim et al., 2002).

Net bone resorption depends upon the rate of osteoclast recruitment and the functional competence of the individual polykaryon. Osteoclast number, in turn, reflects differentiation and proliferation of precursors and apoptosis of the committed cell. Alternatively, the capacity of the individual cell to resorb bone often reflects cytoskeletal organization (Teitelbaum, 2007). In this regard, controversy exists as to whether DAP12 and/or FcRγ exert their resorptive effects by promoting osteoclastogenesis or by enabling the osteoclast to structure its cytoskeleton. Some report failure to generate *Dap12*$^{-/-}$ osteoclasts upon exposing their precursors to RANKL and M-CSF, but *Dap12*$^{-/-}$ osteoclasts can be generated when these bone marrow cells are co-cultured with osteoblasts (Koga et al., 2004; Mocsai et al., 2004). By contrast, we show that *Dap12*$^{-/-}$ macrophages undergo osteoclastogenesis when treated with M-CSF and RANKL, but the polykaryons formed fail to organize their cytoskeleton or resorb bone (Faccio et al., 2003; Zou et al., 2008). Furthermore, although *Dap12*$^{-/-}$ osteoclasts appear in normal numbers in osteoblast co-culture, they also exhibit cytoskeletal dysfunction and, unlike the wild type (WT), fail to transmigrate through the layer of bone-forming cells. Finally, overexpressed OSCAR, the FcRγ co-receptor postulated to mediate osteoclastogenesis in the absence of DAP12 (Kim et al., 2002; Koga et al., 2004; Mocsai et al., 2004), fails to rescue the cytoskeletal defects or transmigrating capacity of osteoblast-residing *Dap12*$^{-/-}$ osteoclasts. Thus, DAP12 promotes bone resorption, principally by organizing the osteoclast cytoskeleton.

Results

Defective cytoskeletal organization of osteoblast-induced *Dap12*$^{-/-}$ osteoclasts

Osteoblasts are postulated to express a ligand for OSCAR that activates FcRγ signaling, thus compensating for arrested osteoclastogenesis in the absence of DAP12 (Kim et al., 2002). To explore this issue, we co-cultured DAP12-deficient osteoclast precursors, in the form of bone marrow macrophages, with WT calvarial osteoblasts. WT macrophages and those lacking both DAP12 and FcRγ (double knockout; DKO), served as positive and negative controls, respectively (Fig. 1A). After 7 days, osteoblasts were removed with collagenase and osteoclasts were stained for tartrate-resistant acid phosphatase (TRAP) activity. Sheets of characteristic WT osteoclasts formed, which were virtually absent in cells lacking DAP12, with or without FcRγ (Fig. 1B–D).

To further explore the failure of osteoblasts to rescue *Dap12*$^{-/-}$ osteoclastogenesis in this model, we retrovirally expressed one or the other ITAM proteins in DKO macrophages and placed them in co-culture (Fig. 2A). Osteoblasts were once again removed with collagenase after 7 days and residual cells stained for TRAP activity. DAP12 transduction yielded a population of spread osteoclasts that were indistinguishable from the WT, which numbered 358±42 cells/well (mean ± s.d.) (Fig. 2B–D). In keeping with its inability to compensate for DAP12-deficiency, DKO cells transduced with FcRγ, similar to those containing empty vector, yielded few osteoclasts. Our results suggest that DAP12, but not FcRγ, mediates osteoclast formation even in the presence of osteoblasts.

We have shown previously that the DAP12 transmembrane domain is essential for osteoclast formation and function and cannot be replaced with its FcRγ counterpart, when pure populations of macrophages are exposed to M-CSF and RANKL (Zou et al., 2008). To determine whether the same is true regarding co-culture generation of osteoclasts, we retrovirally transduced DKO macrophages with DAP12–FcRγ chimeras, containing various combinations of DAP12 (D) and FcRγ (F) extracellular, transmembrane, and cytoplasmic domains. The transduced osteoclast precursors were cultured with WT osteoblasts. Similar to our previous observations, using recombinant M-CSF and RANKL, the DAP12 transmembrane domain was required to yield normal-looking DKO osteoclasts following removal of osteoblasts.

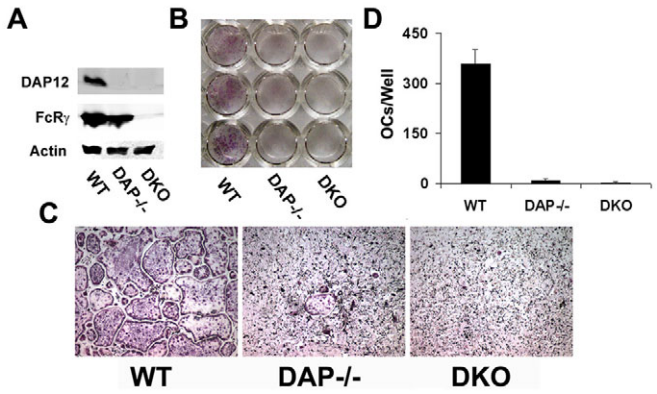

Fig. 1. DAP12-deficient macrophages in co-culture yield few osteoclasts following removal of osteoblasts. (**A**) DAP12 and FcRγ expression by WT, *Dap12*$^{-/-}$ and DKO macrophages was determined by immunoblot. Actin serves as loading control. (**B–D**) WT, *Dap12*$^{-/-}$ and DKO macrophages were co-cultured with WT osteoblasts. After 7 days, osteoblasts were removed using 0.1% collagenase and residual cells stained for TRAP activity. (B) Representative wells. (C) Histological appearance of TRAP-stained cells (40×). (D) Quantification of osteoclast number. Error bars represent s.d.

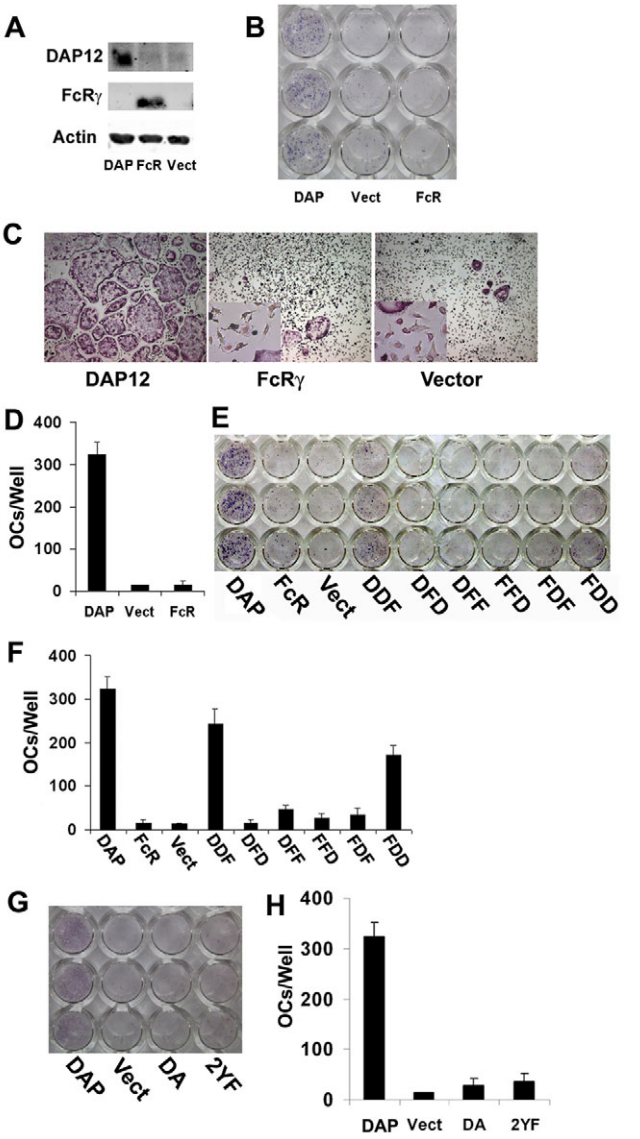

Fig. 2. DAP12, but not FcRγ rescues DKO osteoclastogenesis in co-culture. DKO macrophages were retrovirally transduced with DAP12, FcRγ or empty vector (A–D) or DAP12–FcRγ domain hybrids (E–H). The transduced cells were co-cultured with osteoblasts for 7 days. Osteoblasts were removed and cells stained for TRAP activity. (**A**) DAP12 and FcRγ expression by transduced DKO macrophages as determined by immunoblot. (**B**) Representative TRAP-stained wells following osteoblast removal. (**C**) Osteoclasts identified by TRAP staining following osteoblast removal (40×; inset 200×). (**D**) Quantification of osteoclast number in wells illustrated in B and C. (**E**) TRAP-stained wells of DKO osteoclasts transduced with DAP12, FcRγ, vector or domain hybrids of the two ITAM adaptors. F represents FcRγ and D, DAP12. First letter indicates extracellular, second letter transmembrane and third letter, cytoplasmic domain. (**F**) Quantification of osteoclast number in E. (**G**) TRAP-stained wells containing co-culture generated *Dap12*$^{-/-}$ osteoclasts transduced with WT DAP12 or its D52A or 2YF mutant. (**H**) Quantification of osteoclast number in G. Error bars represent s.d.

Specifically, the number of TRAP-expressing, plastic adherent, spread polykaryons was enhanced in mutant cells expressing FDD and DDF, but not other domain combinations (Fig. 2E,F). Although required, the DAP12 transmembrane region was not sufficient to rescue DKO osteoclasts because the FDF construct is unable to restore their numbers. However, addition of either the D extracellular or cytoplasmic domain did so. Consistent with these findings, substitution of the charged amino acid in the DAP12 transmembrane domain, which disrupts receptor association (D52A), and a nonfunctional ITAM mutation (2YF), failed to rescue *Dap12*$^{-/-}$ osteoclasts following osteoblast removal.

These data, which suggest defective osteoclastogenesis in co-culture, conflict with our previous observations that DAP12 deficiency, in M-CSF/RANKL-generated cells, impairs cytoskeletal organization and function, but not osteoclast differentiation (Zou et al., 2008). In fact, we confirmed the failure of living *Dap12*$^{-/-}$ osteoclasts to generate podosome belts when cultured on plastic (see supplementary material Movie 1). More importantly, living mutant cells failed to form actin rings or directionally move on bone (see supplementary material Movie 2). Our conclusions also differ from those of Koga and colleagues (Koga et al., 2004) and Moscai and co-workers (Mocsai et al., 2004), who claim that the impaired osteoclastogenesis of DAP12-deficient macrophages is normalized by osteoblasts.

A possible explanation for this conundrum would be failure of *Dap12*$^{-/-}$ osteoclasts to transmigrate through the osteoblasts and adhere to plastic (Saltel et al., 2006). In this circumstance, collagenase treatment would remove not only osteoblasts, but also associated osteoclasts. Consistent with this posture, TRAP-stained, non-collagenase-exposed *Dap12*$^{-/-}$ co-cultures contained osteoclasts, in numbers approximating those in the WT (Fig. 3A,B).

Exploration of the osteoclast cytoskeleton is confounded by the perception that replication of the in vivo phenotype requires residence on mineralized substrate (Saltel et al., 2008; Saltel et al., 2004). We found, however, that similarly to culture on bone (supplementary material Movie 2), WT osteoclasts, in contact with osteoblasts, formed characteristic actin rings (Fig. 3C). Unlike plasma-membrane-apposed podosome belts, which appear on non-mineralized substrate (supplementary material Movie 1), these circular structures were well within the confines of the cytoplasm, but not at the cell periphery. By contrast, *Dap12*$^{-/-}$ osteoclasts, in co-culture, failed to spread or form actin rings, but maintained isolated, individual podosomes, indicating cytoskeletal dysfunction (Fig. 3A,B,C).

To directly examine transmigration, *Dap12*$^{-/-}$ and WT macrophages transduced with GFP actin, were cultured in M-CSF and RANKL for 3 days to generate pre-fusion osteoclasts. The cells were lifted and placed on a confluent layer of osteoblast-like ST-2 cells expressing RFP-m-Cherry. Two days after culture in osteoclastogenic cytokines, the cells were examined in the *Z*-plane by confocal microscopy. Whereas WT osteoclasts transited completely through the osteoblast layer and spread on plastic, *Dap12*$^{-/-}$ cells were incapable of doing so (Fig. 3D).

DAP12 is essential for optimal M-CSF signaling in macrophage lineage cells, mediating the capacity of the cytokine to organize the osteoclast cytoskeleton (Otero et al., 2009; Zou et al., 2008). Thus, the possibility exists that the disrupted spreading of *Dap12*$^{-/-}$ polykaryons represents relative insufficiency of osteoclast cytoskeleton-regulating cytokines. However, addition of substantial quantities of M-CSF (50 ng/ml) and RANKL (100 ng/ml), alone and in combination, to WT or *Dap12*$^{-/-}$ osteoclastogenic co-cultures, altered the appearance of neither osteoclast genotype (Fig. 3E). These data indicate that the primary role of DAP12 is not osteoclast recruitment, but cytoskeletal organization and function of the mature cell.

OSCAR-FLAG activation increases osteoclast formation

OSCAR, which is selectively produced by murine osteoclasts, is a co-receptor for FcRγ, the ITAM protein proposed to partially compensate for the presumed failure of osteoclasts to form without DAP12 (Kim et al., 2002; Koga et al., 2004; Mocsai et al., 2004). Evidence suggests, but does not establish, that the OSCAR ligand(s) is probably expressed by osteoblasts, thus providing a presumptive mechanism for the generation of *Dap12*$^{-/-}$ osteoclasts in co-culture

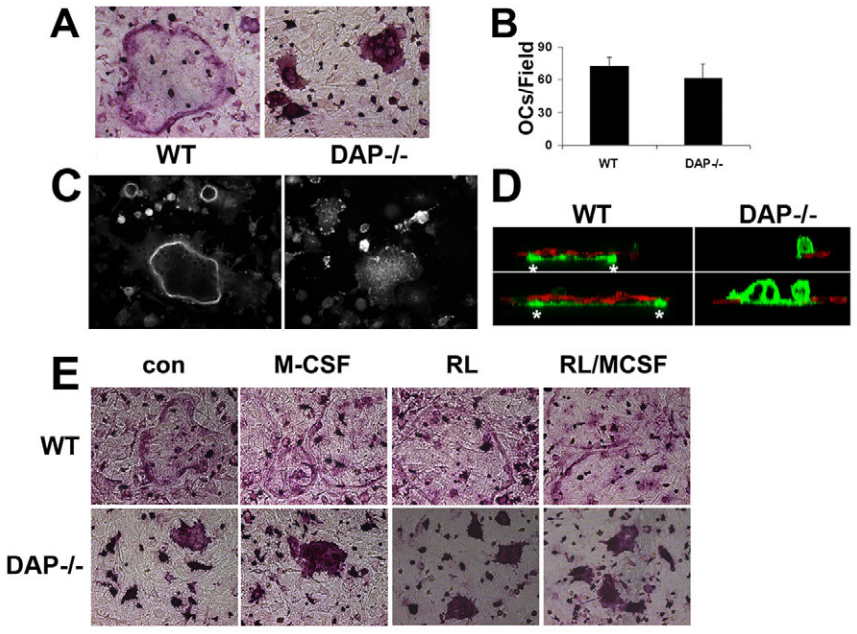

Fig. 3. Cytoskeleton-deficient DAP12 osteoclasts form in normal numbers in co-culture. WT and *Dap12*$^{-/-}$ macrophages, transduced with GFP-actin, were co-cultured with osteoblasts for 7 days. Cells were examined before osteoblast removal. (**A**) The cells were stained for TRAP activity. WT, but not *Dap12*$^{-/-}$ osteoclasts, are spread (200×). (**B**) Quantification of osteoclast number in A. Error bars represent s.d. (**C**) Fluorescent microscopy reveals actin rings in WT co-cultured osteoclasts. By contrast, those lacking DAP12, in contact with osteoblasts fail to organize podosomes (dot-like structures) into rings (200×). (**D**) WT and *Dap12*$^{-/-}$ macrophages, transduced with GFP-actin, were cultured in M-CSF and RANKL, for 3 days, to generate pre-fusion osteoclasts. The cells were lifted and placed on a confluent layer of osteoblast-like ST-2 cells expressing RFP-m-Cherry, with continued presence of the osteoclastogenic cytokines. Two days later the cells were examined, in the *Z*-plane, by confocal microscopy. (*, podosome belt; 630×). (**E**) WT and *Dap12*$^{-/-}$ macrophages were co-cultured with osteoblasts, with or without RANKL (100 ng/ml) and/or M-CSF (50 ng/ml), for 7 days. The cells were stained for TRAP activity (200×).

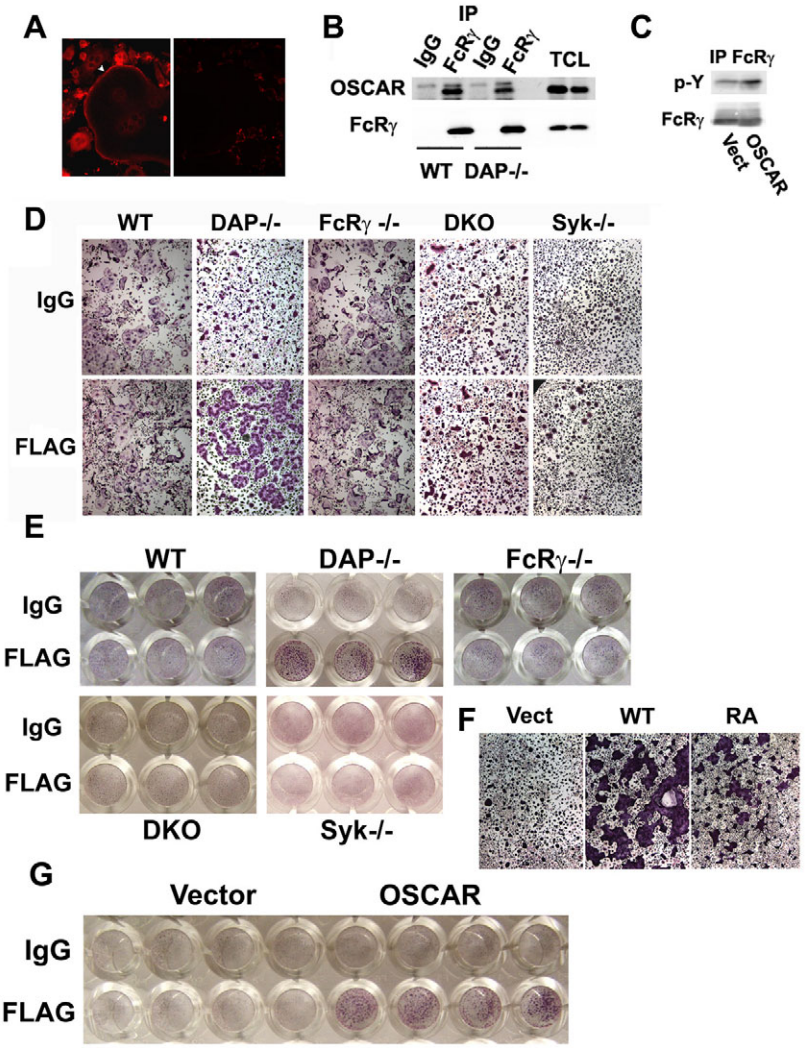

Fig. 4. OSCAR-FLAG activation induces fusion of *Dap12*$^{-/-}$ osteoclasts. (**A**) OSCAR-FLAG-transduced, cytokine-generated WT osteoclasts, were immunostained with anti-FLAG mAb (left panel). Arrowhead indicates localization of transduced protein in plasma membrane. Vector-transduced WT osteoclasts serve as negative control (right panel) (200×). (**B**) WT and *Dap12*$^{-/-}$ OSCAR-FLAG-transduced macrophages were cultured with RANKL and M-CSF for 3 days. OSCAR in FcRγ immunoprecipitates was determined by anti-FLAG immunoblot. IgG serves as negative control (TCL; total cell lysates). (**C**) OSCAR-FLAG-transduced *Dap12*$^{-/-}$ macrophages were cultured with RANKL and M-CSF for 4 days. Then cell were lifted with 0.02% EDTA and re-plated on FLAG-mAb-coated plates for 30 minutes. FcRγ immunoprecipitates were immunoblotted for phosphotyrosine (p-Y) and FcRγ. (**D**,**E**) WT, *Dap12*$^{-/-}$, FcRγ, DKO and *Syk*$^{-/-}$ macrophages, transduced with OSCAR-FLAG, were cultured, with M-CSF and RANKL, in FLAG mAb- or IgG-coated wells. After 6 days, the cells were stained for TRAP activity (200× for D). (**F**) *Dap12*$^{-/-}$ macrophages, transduced WT OSCAR-FLAG, OSCAR^{R231A}-FLAG or vector, were cultured for 6 days, with M-CSF and RANKL, on anti-FLAG mAb. The cells were stained for TRAP activity (200×). (**G**) OSCAR-FLAG or empty vector was transduced into DAP12-deficient macrophages. The cells were cultured on anti-FLAG mAb- or IgG-coated wells containing RANKL and M-CSF. After 6 days, the cells were stained for TRAP activity.

(Kim et al., 2002; Koga et al., 2004; Mocsai et al., 2004). The disrupted spreading of *Dap12*$^{-/-}$ osteoclasts, in co-culture, indicates, however, that the OSCAR–FcRγ axis, putatively activated by osteoblasts, is insufficient to rescue their abnormal cytoskeleton.

Exploration of this issue is challenged by the fact that the OSCAR ligand(s) is unknown. To surmount this problem, we used an OSCAR construct, with an extracellular domain FLAG-tag (Ishikawa et al., 2004). OSCAR-FLAG localization to the osteoclast plasma membrane was confirmed by immunofluorescence (Fig. 4A) and its association with FcRγ, by co-immunoprecipitation in both WT and *Dap12*$^{-/-}$ cells (Fig. 4B). Establishing anti-FLAG mAb activated OSCAR-FLAG, its ITAM adaptor, FcRγ, was tyrosine phosphorylated in construct-transduced, but not vector-bearing *Dap12*$^{-/-}$ osteoclasts (Fig. 4C).

To determine whether OSCAR-FLAG activation affects osteoclast formation, transduced WT, *Dap12*$^{-/-}$, FcRγ$^{-/-}$ and DKO macrophages were cultured with RANKL and M-CSF, on anti-FLAG mAb or IgG. The stimulating antibody promoted osteoclast formation in *Dap12*$^{-/-}$ cells, but not in the absence of its co-receptor, FcRγ (Fig. 4D,E). Although activated OSCAR-FLAG substantially increased the size of *Dap12*$^{-/-}$ osteoclasts, they maintained their ‘crenated’ facade. OSCAR-FLAG also did not impact the appearance of WT transductants. In contrast to WT-OSCAR bound to FLAG, a transmembrane mutant, R231A, structurally predicted to inhibit FcRγ recognition (Kim et al., 2002), only partially increased the formation of poorly spread *Dap12*$^{-/-}$ osteoclasts (Fig. 4F).

To exclude the possibility that anti-FLAG mAb enhances osteoclastogenesis independently of OSCAR, we transduced FLAG-tagged OSCAR, or empty vector, into DAP12-deficient macrophages and cultured them in FLAG-mAb- or IgG-coated wells containing RANKL and M-CSF. Osteoclast formation was increased only in OSCAR-transduced cells (Fig. 4G).

Syk interacts with phosphorylated ITAM tyrosines and is a key signaling molecule in osteoclasts (Zou et al., 2007). Hence, if OSCAR-promoted osteoclast formation is mediated by FcRγ, it should be abrogated by absence of Syk. We therefore retrovirally expressed OSCAR-FLAG in *Syk*$^{-/-}$ macrophages and maintained them for 6 days in osteoclastogenic conditions in wells coated with FLAG mAb. As in cells lacking FcRγ, absence of Syk abolished OSCAR-mediated osteoclast formation (Fig. 4D,E). Thus, Syk is an essential OSCAR effecter.

OSCAR-FLAG activation suppresses osteoclast apoptosis

Dap12$^{-/-}$ macrophages differentiate into osteoclasts, but the mutant polykaryons are dysfunctional because they fail to organize their

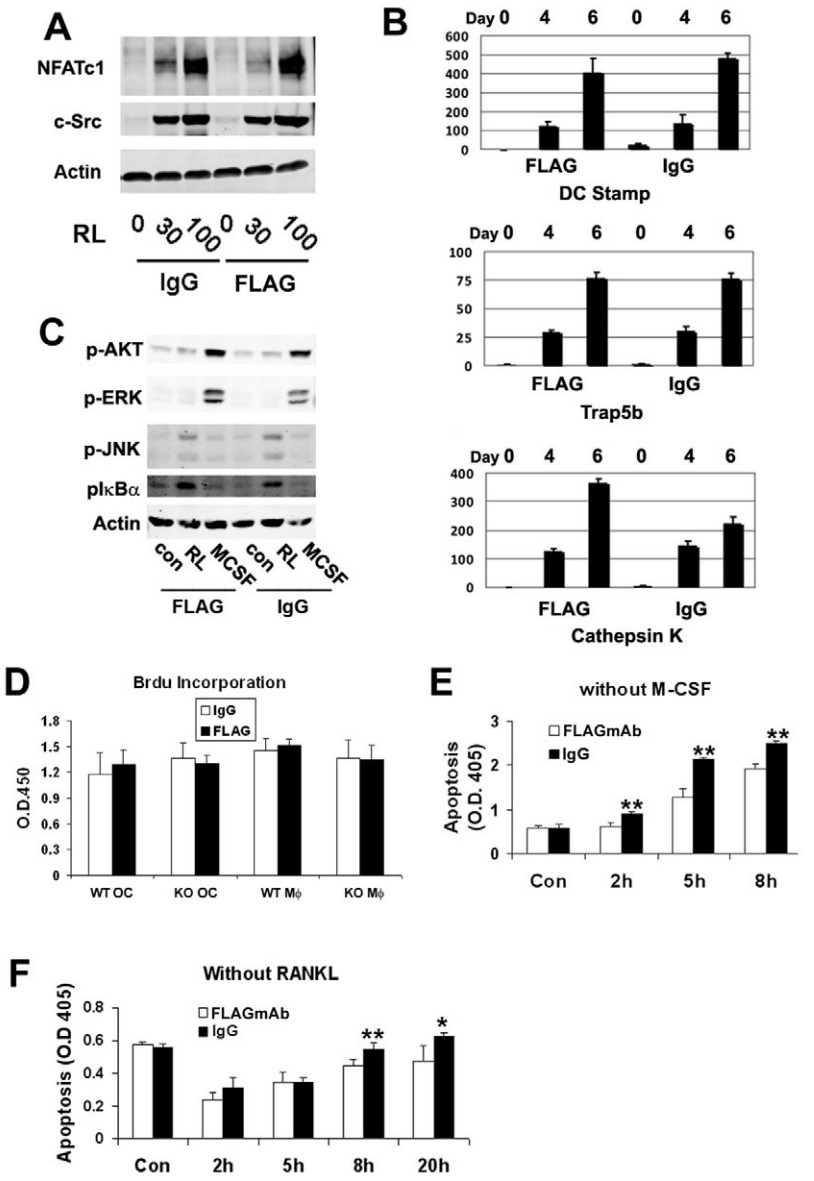

Fig. 5. OSCAR-FLAG activation does not affect osteoclast differentiation. (**A**) *Dap12*$^{-/-}$ macrophages transduced with OSCAR-FLAG or vector were cultured, for 4 days, with M-CSF and increasing amounts of RANKL (RL) in anti-FLAG mAb- or IgG-coated plates. NFATc1 and Src were determined by immunoblot. Actin serves as loading control. (**B**) OSCAR-FLAG-transduced *Dap12*$^{-/-}$ macrophages were cultured with RANKL and M-CSF for 0, 4 or 6 days in wells coated with anti-FLAG mAb or IgG. Osteoclast differentiation markers were determined by qPCR. (**C**) OSCAR-FLAG-transduced *Dap12*$^{-/-}$ macrophages, cultured on anti-FLAG mAb- or IgG-coated plates, were exposed to RANKL for 15 minutes or M-CSF for 5 minutes. MAP kinase activation was determined by immunoblot. (**D**) OSCAR-FLAG-transduced WT and *Dap12*$^{-/-}$ macrophages (KO) were cultured in M-CSF, without (Mφ) or with (OC) RANKL for 4 days on FLAG mAb- or IgG-coated plates. BrdU incorporation was assessed by ELISA. (**E**,**F**) OSCAR-FLAG-transduced *Dap12*$^{-/-}$ macrophages were cultured with RANKL and M-CSF for 4 days, on FLAG mAb- or IgG-coated plates. Then cells were deprived of either M-CSF (E) or RANKL (F) for indicated times. Apoptosis was determined by ELISA (**P*<0.05; ***P*<0.01; error bars represent s.d.).

cytoskeleton to optimally resorb bone (Zou et al., 2008). Activated OSCAR-FLAG prompted *Dap12*$^{-/-}$ osteoclast formation, but not spreading (Fig. 4D,F). NFATc1 and Src protein, which appear with osteoclastogenesis, were not increased by FLAG mAb exposure, indicating that OSCAR-FLAG-enhanced *Dap12*$^{-/-}$ osteoclast size does not reflect accelerated differentiation (Fig. 5A). The same holds regarding the abundance of mRNA encoding DC-Stamp, cathepsin K and Trap5b (Fig. 5B). Similarly, RANKL- and M-CSF-stimulated osteoclastogenic signals, including AKT, ERK, JNK and NF-κB, are unaltered by OSCAR-FLAG (Fig. 5C). These data indicate that OSCAR activation does not impact precursor differentiation into osteoclasts. Confirming that the same is true regarding proliferation of osteoclast lineage cells, WT and *Dap12*$^{-/-}$ macrophages or osteoclasts bearing OSCAR-FLAG incorporated equal amounts of BrdU whether exposed to anti-FLAG mAb or IgG (Fig. 5D). However, OSCAR-FLAG activation inhibited apoptosis of osteoclasts deprived of either M-CSF or RANKL (Fig. 5E,F).

OSCAR-FLAG activation partially rescues *Dap12*$^{-/-}$ osteoclast function

Dap12$^{-/-}$ mice develop mild osteopetrosis, whereas in DKO animals it is severe (Koga et al., 2004; Mocsai et al., 2004). This observation provides the foundation for postulating that FcRγ partially compensates for DAP12 deficiency. We addressed this issue in the context of OSCAR-FLAG activation. To this end, we generated WT and *Dap12*$^{-/-}$ osteoclasts, retrovirally transduced with OSCAR-FLAG, on anti-FLAG mAb- or IgG-coated coverslips. In both circumstances, WT cells formed classical podosome belts (Fig. 6A). Alternatively, the *Dap12*$^{-/-}$ transductants, on IgG, exhibited no evidence of cytoskeletal organization. The same cells, activated by anti-FLAG mAb, contained a few small circular actin structures at their periphery.

We next turned to the functional consequences of OSCAR-FLAG activation in the absence of DAP12 by generating transduced *Dap12*$^{-/-}$ osteoclasts on anti-FLAG mAb- or IgG-coated bone. Although vector-bearing cells exhibited no cytoskeletal

organization, atypical 'non-expanded actin rings' appeared in OSCAR-FLAG-activated osteoclasts. (Fig. 6B,C). Surprisingly, although smaller than those stimulated by anti-FLAG mAb, these abnormal actin structures were also induced in the same cells by IgG. In keeping with this observation, anti-FLAG mAb partially rescued the failed bone-resorptive capacity of *Dap12*$^{-/-}$ osteoclasts. IgG exposure also did so, but less effectively (Fig. 6D,E).

Because these experiments involved OSCAR-FLAG overexpression, the moderate rescue of osteoclast function, in the absence of anti-FLAG mAb, might represent increased quantities of OSCAR. In fact, the abundance of transduced OSCAR was approximately an order of magnitude greater than its endogenous counterpart (Fig. 6F). To exclude the possibility that failure of osteoblasts to rescue the naive *Dap12*$^{-/-}$ osteoclast cytoskeleton reflected a relative paucity of OSCAR (Fig. 3), we repeated the co-culture experiment with transduced *Dap12*$^{-/-}$ macrophages. DAP12-deficient osteoclasts, overexpressing OSCAR-FLAG, failed to spread on osteoblasts and were indistinguishable from their vector-bearing counterparts (Fig. 6G). Similarly, removal of the osteoblast layer by collagenase yielded few residual osteoclasts. These observations suggest that the quantity of OSCAR ligand present in osteoblasts is insufficient to rescue the cytoskeletal defect, regardless of receptor abundance.

To further explore this issue we turned to the DAP12-dependent, cytoskeleton-organizing canonical signaling pathway activated by the αvβ3 integrin or M-CSF (Ross and Teitelbaum, 2005; Zou et

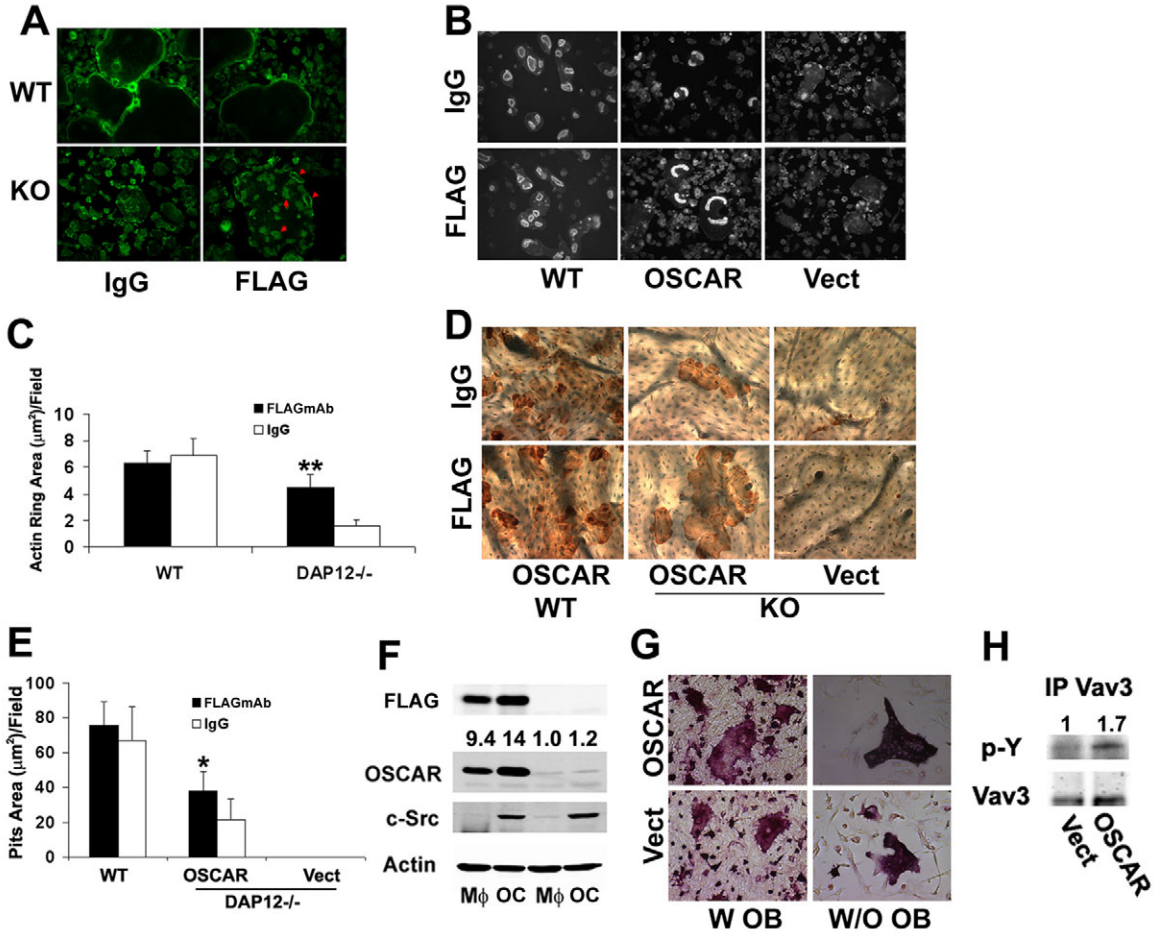

Fig. 6. OSCAR-FLAG partially rescues *Dap12*$^{-/-}$ osteoclast function on bone. (**A**) OSCAR-FLAG-transduced *Dap12*$^{-/-}$ macrophages were cultured, for 6 days, with RANKL and M-CSF on FLAG-mAb- or IgG-coated plates. The actin cytoskeleton was visualized by FITC-phalloidin staining. Arrowheads indicate small circular actin structures appearing in OSCAR-FLAG-bearing *Dap12*$^{-/-}$ cells on anti-FLAG mAb (200×). (**B**) WT or OSCAR-FLAG- or vector-transduced *Dap12*$^{-/-}$ macrophages were cultured with RANKL and M-CSF on FLAG mAb- or IgG-coated bone. The actin cytoskeleton was visualized by FITC-phalloidin staining. (**C**) Quantification of area encompassed by actin rings in WT, and ring-like structures in OSCAR-FLAG-expressing *Dap12*$^{-/-}$ cells on bone coated with anti-FLAG mAb or IgG. Vector-transduced *Dap12*$^{-/-}$ osteoclasts contain no such actin structures. (**D**) Bone resorption pits generated by cells detailed in B (200×). (**E**) Quantification of bone resorption pit area generated by cells detailed in D. No resorption pits are excavated by vector-transduced *Dap12*$^{-/-}$ osteoclasts. (**F**) OSCAR-FLAG- or vector-transduced *Dap12*$^{-/-}$ macrophages were cultured, for 4 days, in M-CSF, without (Mφ) or with (OC) RANKL. Total OSCAR expression was determined by immunoblot. OSCAR-FLAG were also analyzed by anti-FLAG immunoblot. Numbers represent densitometrically determined OSCAR:actin ratio, normalized to that endogenous in macrophages. (**G**) OSCAR-FLAG- or vector-transduced *Dap12*$^{-/-}$ macrophages were cultured with osteoblasts for 7 day. The cells were stained for TRAP activity before (W OB) and following (W/O OB) removal of osteoblasts (200×). (**H**) OSCAR-FLAG or vector transduced *Dap12*$^{-/-}$ macrophages were cultured with RANKL and M-CSF for 4 days. Then cell were lifted and re-plated on anti-FLAG mAb- or IgG-coated plates for 1 hour. Vav3 tyrosine phosphorylation was determined by immunoprecipitation and immunoblot. Numbers represent densitometric analysis of phosphorylated tyrosine:Vav3 ratio normalized to vector transductant. **P*<0.05; ***P*<0.01; error bars represent s.d.

al., 2007; Zou et al., 2008). An essential component, the guanine nucleotide exchange factor Vav3 is phosphorylated by FLAG mAb (Faccio et al., 2005), indicating that the partial rescue of *Dap12*$^{-/-}$ osteoclast function and cytoskeletal organization, extant in OSCAR-FLAG-overexpressing mutant cells, is mediated via this complex. By contrast, there is little evidence of such phosphorylation in vector-transduced *Dap12*$^{-/-}$ cells. Thus, although an abundance of activated OSCAR partially rescues the *Dap12*$^{-/-}$ osteoclast cytoskeleton by stimulating an effector signaling pathway, this is not the case with physiological amounts of the receptor.

Discussion

All forms of pathological bone loss represent enhanced resorption relative to formation. In most circumstances, accelerated skeletal degradation reflects a combination of increased osteoclast number and activity, the latter typically associated with cytoskeletal reorganization (Teitelbaum, 2007). In this circumstance, the cell polarizes to form actin rings and a ruffled border, which both participate in delivery of matrix-degrading molecules into the resorptive microenvironment between bone and the juxtaposed plasma membrane.

Although the general morphological features of osteoclast polarization have been long appreciated, insights into the relevant molecular mechanisms are recent. Matrix-derived signals, mediated via the αvβ3 integrin and M-CSF, are particularly important in organizing the resorptive cell cytoskeleton (Ross and Teitelbaum, 2005). In fact, the integrin and cytokine share many components of a canonical signaling pathway resulting in osteoclast polarization and bone degradation. This signaling complex includes Src, Syk, DAP12, Vav3, the SLP adaptor proteins and the small GTPase Rac (Faccio et al., 2005; Reeve et al., 2009; Ross and Teitelbaum, 2005; Zou et al., 2007; Zou et al., 2008). Interestingly, absence of any of these complex-residing proteins yields osteoclasts that appear 'crenated' and fail to spread.

DAP12 and FcRγ, which signal through their ITAM domains, are adaptors for transmembrane receptors that are activated by unknown ligands. These adaptors are required for normal bone homeostasis because mice lacking both develop osteopetrosis as a result of arrested resorption.

This study was prompted by discrepant observations regarding the means by which DAP12 mediates bone degradation. The authors who established the severe osteopetrosis of mice with combined deletion of the two ITAM proteins (Koga et al., 2004; Mocsai et al., 2004), conclude that DAP12 deficiency arrests osteoclastogenesis induced by RANKL and M-CSF. However, we found that DAP12-deficient osteoclasts do form when exposed to the cytokines, but fail to resorb bone because of cytoskeletal dysfunction (Faccio et al., 2003; Zou et al., 2008). Here, we confirm our conclusion in living osteoclasts. It is also of interest that this inter-laboratory discrepancy mirrors that involving generation of osteoclasts derived from cells lacking SLP adaptor proteins (Reeve et al., 2009). Furthermore, the normal appearance of osteoclast differentiation markers in DAP12-deficient M-CSF/RANKL cultures, reported by Moscai and colleagues (Mocsai et al., 2004) challenges the concept of failed osteoclastogenesis.

We have previously shown that DAP12-deficient macrophages differentiate into substrate-adherent osteoclasts in the presence of RANKL and M-CSF (Zou et al., 2008). We were therefore surprised that our initial co-culture experiments, wherein osteoblasts were removed by collagenase, failed to yield many TRAP-expressing osteoclasts. We confirmed, however, that non-collagenase-treated *Dap12*$^{-/-}$ co-cultures contain normal numbers of osteoclasts, even though they exhibited features of cytoskeletal dysfunction. Because removal of osteoblasts also eliminates the polykaryons, we suspected that the cytoskeletal abnormalities of *Dap12*$^{-/-}$ osteoclasts compromises their capacity to transmigrate through the osteoblast layer and attach to substrate, and such proves to be the case. These observations provide mechanistic insights into our previous observation that DAP12-deficient osteoclasts fail to resorb bone in vitro (Zou et al., 2008).

OSCAR, the immunoglobulin-like FcRγ co-receptor, is expressed as macrophages differentiate into mature osteoclasts (Kim et al., 2002). The receptor is apparently restricted to osteoclasts in mice, but is more widespread in humans. Indirect evidence suggests that the OSCAR ligand(s) is expressed by osteoblasts (Kim et al., 2002). This observation provided the rationale for assuming that the putative rescue of *Dap12*$^{-/-}$ osteoclastogenesis by the bone-forming cells reflects FcRγ activation. The enigmatic state of the OSCAR ligand(s), however, challenges interpretation of these data. Moreover, the significance of physiological amounts of OSCAR in the osteoclastogenic process is controversial, because it is not impacted by absence of its adaptor, FcRγ (Koga et al., 2004).

Because we found that DAP12 deficiency does not impair recruitment of osteoclasts, but does impair their function, we asked whether the FcRγ-associated receptor OSCAR rescues the compromised cell cytoskeleton. Given that the OSCAR ligand(s) is not known, we expressed the receptor as a FLAG-associated construct that binds FcRγ. When activated, the OSCAR-FLAG–FcRγ complex arrests apoptosis, thus increasing osteoclast size and number. However, the impact of mAb-stimulated OSCAR-FLAG, on the cytoskeleton of glass-residing osteoclasts, is minimal. At first sight, this failure of cytoskeletal organization by OSCAR-FLAG is surprising, because the fusion protein phosphorylates Vav3, a major component of the canonical integrin- and M-CSF-activated cytoskeleton-organizing signaling complex, in the bone-resorbing polykaryons (Faccio et al., 2005). We reasoned that this conundrum might reflect the material on which the cells are residing.

Replication of in vivo cytoskeletal structure is believed to require mineralized substrate. The actin ring, or sealing zone, for example, appears when the cell is resident on bone or similar material, but not plastic or glass, which induce podosome belts (Saltel et al., 2008; Saltel et al., 2004). Although dynamic visualization of the osteoclast cytoskeleton on artificial mineralized substrate has been achieved (Saltel et al., 2004), we have accomplished this task on authentic bone for the first time. This strategy offers the means of dynamically replicating the in vivo phenotype of the cytoskeleton of the cell and distinguishing it from the non-physiological features induced by plastic or glass.

We found, however, that podosome organization in WT osteoclasts in contact with osteoblasts mirrors that in cells resident on bone, because actin rings are generated in both circumstances. In keeping with the physical intimacy of osteoclasts and osteoblasts in vivo, and their capacity to promote the other's recruitment (Teitelbaum, 2007), these observations suggest that contact with the osteogenic cell also contributes to organizing the polykaryon cytoskeleton. However, *Dap12*$^{-/-}$ osteoclasts fail to structure their cytoskeleton regardless of the substrate.

We found that, in contrast to those on FLAG-mAb-coated glass, actin-ring-like structures, albeit abnormal, form in OSCAR-FLAG-expressing *Dap12*$^{-/-}$ osteoclasts on similarly treated bone. This observation and the fact that, unlike naive *Dap12*$^{-/-}$ cells, those

expressing OSCAR-FLAG are capable of resorption, is consistent with the capacity of the fusion protein to phosphorylate Vav3. Unexpectedly, however, these transduced cells form small, disfigured actin-ring-like structures in the absence of the mAb and exhibit approximately 50% of anti-FLAG-stimulated bone resorption. Although speculative, it is possible that, similar to its regulation of αvβ3 integrin activity (Zou et al., 2007), bone matrix contains an OSCAR-binding molecule(s), which when faced with an abundance of the receptor is sufficient to partially organize the osteoclast cytoskeleton and promote resorption.

ITAM adaptors interact with their specific receptors via their transmembrane domains. We have shown that either the FcRγ extracellular or cytoplasmic regions might substitute for those of DAP12 in rescuing the *Dap12*$^{-/-}$ cytoskeleton of cytokine-induced osteoclasts (Zou et al., 2008). We find the same results obtained in the context of osteoblast co-culture of DKO cells transduced with domain hybrids of the two ITAM proteins. By contrast, the DAP12 transmembrane component is essential for cytoskeletal rescue, in both osteoclastogenic models, and may not be replaced by that of FcRγ. These observations suggest two possible scenarios: first, endogenous FcRγ, interacting with physiological amounts of OSCAR, is incapable of affecting the osteoclast cytoskeleton; second, osteoblasts contain insufficient OSCAR ligand to activate FcRγ. In consequence, other components of the marrow environment, such as members of the immune system, deserve investigation as sources of the OSCAR ligand (Baron, 2004).

Thus, the prevalent effect of DAP12 deficiency is not arrested osteoclast development but cytoskeletal disorganization. Furthermore, although overexpressed OSCAR–FcRγ rescues the inability of *Dap12*$^{-/-}$ osteoclasts to resorb bone, there is little evidence of a physiological counterpart.

Materials and Methods

Mice

Dap12$^{-/-}$, *Syk*$^{+/-}$ (129/SV background) mice were described previously (Zou et al., 2007; Zou et al., 2008). FcRγ$^{-/-}$ mice and DAP12/FcRγ double-knockout mice were generously provided by Marco Colonna (Washington University School of Medicine, St Louis, MO). All mice used in these experiments were 6- to 8-weeks old and housed in the animal care unit of Washington University School of Medicine, where they were maintained according to guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. All animal experimentation was approved by the Animal Studies Committee of Washington University School of Medicine.

Reagents

Recombinant murine M-CSF was obtained from R&D Systems (Minneapolis, MN). Glutathione *S*-transferase (GST)-RANKL was expressed in our laboratory as described (Lam et al., 2000). The source of antibodies is as follows: rabbit anti-DAP12 polyclonal antibody was from Exalpha Biological (Watertown, MA); anti-phosphotyrosine mAb 4G10, rabbit anti-FcRγ, rabbit anti-Vav3 from Upstate (Charlottesville VA); mAb 327, directed against the Src protein were gifts from Andrey Shaw (Department of Pathology, Washington University School of Medicine, St Louis, MO); rabbit anti Src p-Y416 antibody, rabbit anti-β3-integrin antibody, from Cell Signaling (Beverly MA). Goat anti-OSCAR antibody was from R&D (Minneapolis, MN). The plasmid transfection reagent FuGENE 6 was purchased from Roche Applied Science (Indianapolis, IN). All other chemicals were obtained from Sigma.

Macrophage isolation and osteoclast culture

Primary bone marrow macrophages were obtained from WT and *Dap12*$^{-/-}$ mice and prepared as described (Faccio et al., 2003) with slight modification. Because DKO mice are osteopetrotic, splenic macrophages served as osteoclast precursors. Cells were incubated at 37°C in 6% CO_2 for 3 days and then washed with PBS and lifted with 1× trypsin-EDTA (Invitrogen, Carlsbad, CA) in PBS. A total of 5×10^3 cells were cultured in 200 μl α-MEM containing 10% heat-inactivated FBS with 100 ng/ml GST-RANKL and 30 ng/ml mouse recombinant M-CSF in 96-well tissue culture plates, some containing sterile bone slices. Cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity after 6 days in culture, using a commercial kit (Sigma 387-A). For pre-OC generation, 1.5×10^6 macrophages were plated per 10 cm tissue culture dish and cultured in 30 ng/ml M-CSF and 100 ng/ml GST-RANKL for 3 days.

Calvarial osteoblast isolation and co-culture

Primary osteoblasts were extracted from 3- to 5-day-old neonatal calvariae with collagenase. 1.2×10^4 osteoblasts and 3×10^4 macrophages were mixed and cultured in α-10 cell culture medium in 48-well plates with 1,25-dihydroxyvitamin D (10^{-8} m) for 7 days. The osteoblasts were lifted by collagenase, and the remaining cells were stained for TRAP activity. In some experiments, osteoclasts were visualized by TRAP staining before osteoblast removal.

Actin ring and bone resorptive pits stain

For staining of actin rings, cells were cultured on a bovine bone slice in the presence of M-CSF and RANKL for 6 days, at which time cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, rinsed in PBS and immunostained with Alexa Fluor 488 phalloidin (Molecule Probes). To quantify resorption lacunae, cells were removed from bone slices with mechanical agitation. Bone slices were incubated with peroxidase-conjugated wheat germ agglutinin (WGA, Sigma) for 1 hour and stained with 3,3′-diaminobenzidine (Sigma).

Proliferation assay

Macrophages (plated in 96-well dishes at a density of 5000 cells/well) were maintained for 4 days in α-10 medium with 20 ng/ml M-CSF or 20 ng/ml M-CSF and 100 ng/ml RANKL and labeled with bromodeoxyuridine (BrdU) for the last 3 hours of culture. BrdU incorporation was determined using the Biotrak ELISA system (Amersham Biosciences).

Apoptosis assay

Macrophages (plated in 96-well dishes) were cultured with 20 ng/ml M-CSF and 100 ng/ml RANKL for 4 days. Cells were deprived of either M-CSF or RANKL for indicated times. Cell death was analyzed in quadruplicate using cell death detection ELISA^{PLUS} kit (Roche Applied Science), which detects cytoplasmic histone-associated DNA fragmentation.

Plasmids and retroviral transduction

Wild-type constructs expressing mouse DAP12 and FcRγ were subcloned into the *Bam*HI and *Xho*I sites of a pMX retroviral vector in which the puromycin resistance sequence was replaced with one coding for blastocidin resistance. All DAP12-FcRγ chimeras and DAP12 ITAM, transmembrane domain mutant were described previously (Zou et al., 2008). pMX OSCAR plasmid (with CD8 signal sequence and FLAG tag) was provided by Hisahi Arase (Osaka University, Osaka, Japan). cDNA was transfected transiently into Plat-E packaging cells using FuGENE 6 Transfection Reagent (Roche). Virus was collected 48 hours after transfection. Macrophages were infected with virus for 24 hours in the presence of 100 ng/ml M-CSF and 4 μg/ml polybrene (Sigma). Cells were selected in the presence of M-CSF and 1 μg/ml blasticidin (Calbiochem) for 3 days before use as osteoclast precursors.

Western blotting and immunoprecipitation

Cultured cells were washed twice with ice-cold PBS and lysed in RIPA buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM NaF, and 1× protease inhibitor mixture (Roche). After incubation on ice for 10 minutes, cell lysates were clarified by centrifugation at 15,000 rpm for 10 minutes. Forty micrograms of total lysates were subjected to 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto PVDF membranes. Filters were blocked in 0.1% casein in PBS for 1 hour and incubated with primary antibodies at 4°C overnight followed by probing with fluorescence-labeled secondary antibodies (Jackson Lab). Proteins were detected with the Odyssey Infrared Imaging System (LI-COR Biosciences).

RNA extraction and quantitative RT-PCR

RNA from cultured cells was isolated and purified using the RNeasy RNA purification kit (Qiagen, Valencia, CA); RLT lysis buffer was supplemented with β-mercaptoethanol (1%). Purified RNA was treated with DNase I (Invitrogen) before reverse transcription (RT). RT was performed using SuperScript III (Invitrogen) and quantitative PCR (qPCR) was performed using Power SYBR green master mix and gene specific primers (Applied Biosystems, Foster City, CA). The qPCR reaction was performed on an ABI Prism 7000 (Applied Biosystems).

Microscope image acquisition

Images were acquired using the Nikon Eclipse E400 microscope with Plan Fluor lenses at room temperature. Photographs of the images were taken using the Optronics camera and displayed using MagnaFire Software (version 2.1B). Immunofluorescence images were acquired using a Leica fluorescence microscope at room temperature and analyzed using a Leica TCS SP Spectral confocal laser-scanning microscope equipped with Argon-Krypton lasers (Leica Microsystems, Heidelberg, Germany). The images were then organized in Adobe Photoshop (version 7.0.1).

Dynamic imaging of osteoclasts on bone

WT and *Dap12*$^{-/-}$ osteoclasts, transduced with GFP-Actin were maintained using standard culture conditions (37°C and 5% CO_2, 95% air atmosphere) with RANKL and M-CSF, for 5 days, in a Bioptechs (non-liquid perfused) Delta T culture system, consisting of a heated, indium-tin-oxide-coated glass dish attached to a calibrated Bioptechs micro-perfusion peristaltic pump. For dynamic studies on bone, macrophages differentiated into pre-fusion osteoclasts, by 3 days in culture with RANKL and M-CSF, were lifted and placed in the same dish containing a thin layer of pulverized bovine bone as substrate. The cells were maintained for an additional 5 days in the osteoclastogenic cytokines. All cultures were observed with the 20× objective (NA, 0.4) of an inverted automated wide-field epifluorescence DIC microscope (Leica DMIRE2, Leica Microsystems, Wetzlar, Germany). An objective lens heater was used to improve temperature homogeneity. Images (608×512 pixels spatial and 12-bit intensity resolution) were recorded with a cooled Retiga 1300 camera (Qimaging, Burnaby, BC, Canada) every 2 minutes in 2×2 binned acquisition mode, using 100–300 msecond exposures. Dynamic images were composed using ImageJ.

Transmigration and confocal microscopy

Stromal cell line ST2 cells, retrovirally transduced with pRetroQ-mCherry Vector (which express red fluorescent protein) (Clontech) were cultured to confluence in a Lab-Tek eight-well slide chamber. WT and *Dap12*$^{-/-}$ prefusion osteoclasts expressing GFP-actin were generated by culturing macrophages in RANKL and M-CSF for 3 days. These cells were lifted and placed on the layer of ST-2 cells, in the continued presence of RANKL and M-CSF, for 2 days. Immunofluorescence images were acquired using a Leica fluorescence microscope and analyzed using a Leica TCS SP Spectral confocal laser-scanning microscope equipped with argon-krypton lasers (Leica Microsystems, Heidelberg, Germany).

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