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Phospholipase Cγ1 (PLCγ1) Controls Osteoclast Numbers via Colony-stimulating Factor 1 (CSF-1)-dependent Diacylglycerol/β-Catenin/CyclinD1 Pathway*

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Phospholipases Cγ (PLCγ) 1 and 2 are a class of highly homologous enzymes modulating a variety of cellular pathways through production of inositol 1,4,5-trisphosphate and diacylglycerol (DAG). Our previous studies demonstrated the importance of PLCγ2 in osteoclast (OC) differentiation by modulating inositol 1,4,5-trisphosphate-mediated calcium oscillations and the up-regulation of the transcription factor NFATc1. Surprisingly, despite being expressed throughout osteoclastogenesis, PLCγ1 did not compensate for PLCγ2 deficiency. Because both isoforms are activated during osteoclastogenesis, it is plausible that PLCγ1 modulates OC development independently of PLCγ2. Here, we utilized PLCγ1-specific shRNAs to delete PLCγ1 in OC precursors derived from wild type (WT) mice. Differently from PLCγ2, we found that PLCγ1 shRNA significantly suppresses OC differentiation by limiting colony-stimulating factor 1 (CSF-1)-dependent proliferation and β-catenin/cyclinD1 levels. Confirming the specificity toward CSF-1 signaling, PLCγ1 is recruited to the CSF-1 receptor following exposure to the cytokine. To understand how PLCγ1 controls cell proliferation, we turned to its downstream effector, DAG. By utilizing cells lacking the DAG kinase ζ, which have increased DAG levels, we demonstrate that DAG modulates CSF-1-dependent proliferation and β-catenin/cyclinD1 levels in OC precursors. Most importantly, the proliferation and osteoclastogenesis defects observed in the absence of PLCγ1 are normalized in PLCγ1/DAG kinase ζ double null cells. Taken together, our study shows that PLCγ1 controls OC numbers via a CSF-1-dependent DAG/β-catenin/cyclinD1 pathway.

Phospholipase Cγ (PLCγ)[4] family members PLCγ1 and PLCγ2 are critical regulators of signaling pathways downstream of growth factor receptors, integrins, and immune complexes and modulate a variety of signaling pathways involved in cell differentiation, motility, and adhesion to name a few (1–3). The main function of the PLCγ family is to cleave phosphatidylinositol 4,5-bisphosphate (PIP_2) into two secondary messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3, in turn, increases intracellular calcium levels via binding to the IP_3 receptors on the endoplasmic reticulum, whereas DAG serves as an endogenous activator of protein kinase C (2, 4).

Generation of knock-out animals has identified critical and non-redundant functions of the two PLCγ enzymes. PLCγ1 is ubiquitously expressed, whereas PLCγ2 is mainly expressed in hematopoietic lineage cells (5). PLCγ1-deficient mice die soon after embryonic day 8.5, and embryos show impaired vasculogenesis and erythropoiesis (6). Interestingly, PLCγ2 is expressed in the PLCγ$1^{-/-}$ embryos but is not sufficient to prevent the early lethality (7). By contrast, global PLCγ2 knockout mice are viable but have a variety of immunological defects, including impaired inflammatory responses in models of arthritis or infections (8–10), developmental defects in the lymphatic system (11), and high bone mass due to defective osteoclastogenesis (12). Structure analysis of the two enzymes indicates over 90% homology in the catalytic domain, which mediates the conversion of PIP_2 into IP_3 and DAG, whereas 50–60% homology is observed within the SH2 and SH3 adaptive motifs (2). Interestingly, however, the different phenotype of the two null mice suggests distinct, non-overlapping specific roles for each enzyme (5).

So far, the majority of the studies have indicated that each PLCγ isoform has cell type-specific effects. For example, T cells express both PLCγ1 and PLCγ2, but only PLCγ1 modulates T cell receptor signaling and thus T cell functions (13). In B cells, deletion of PLCγ2 affects B cell differentiation (14), and there are no reports suggesting any role for PLCγ1 in this population. Similar findings were reported in dendritic cells (8), neutrophils (9), macrophages (15), platelets (16), and osteoclasts (12) where deletion of PLCγ2 impaired specific cellular functions regard-

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[4] The abbreviations used are: PLCγ, phospholipase Cγ; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; OC, osteoclast; DGKζ, DAG kinase ζ; PIP_2, phosphatidylinositol 4,5-bisphosphate; RANK, receptor activator of nuclear factor κ-B; RANKL, receptor activator of nuclear factor κ-B ligand; TRAP, tartrate-resistant acid phosphatase; LysM, lysozyme M; CSF-1, colony-stimulating factor 1; SH, Src homology; CSF-1R, CSF-1 receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; p-AKT, phospho-AKT; p-ERK, phospho-ERK; ctrl, uninfected cells; shctrl, scrambled shRNA; CA, constitutively active.

less of PLCγ1 expression. Interestingly, in platelets, enforced expression of PLCγ1 in PLCγ2$^{-/-}$ cells restores glycoprotein VI-dependent aggregation and αIIbβ3-dependent spreading defects, suggesting that reduced PLCγ1 levels might be responsible for the lack of compensation in the *in vivo* setting (16). Whether the non-overlapping effects of PLCγ1 and PLCγ2 are due to different levels of expression or non-redundant functions remains to be established.

To delve into the specificity of PLCγ signaling, we turned to the osteoclasts, which express both isoforms throughout osteoclast maturation. Osteoclasts are multinucleated giant cells derived from monocyte/macrophage lineage cells, attached to the bone surface, and responsible for bone degradation during normal bone remodeling and during pathological bone loss (17). The process of osteoclast differentiation requires activation of osteoclastogenic pathways through binding of RANKL to its receptor RANK and survival and proliferative cues activated by CSF-1 and its receptor CSF-1R (17, 18). Interestingly, both PLCγ1 and PLCγ2 are expressed and phosphorylated during the osteoclast differentiation process (12), and it is believed that both isoforms contribute to IP_3-mediated calcium fluxes and NFATc1 up-regulation in response to RANKL (12, 19). However, studies from PLCγ2$^{-/-}$ mice show a complete absence of NFATc1 expression and blockade of osteoclast differentiation despite normal expression of PLCγ1 (12, 20). The current study was designed to answer two important questions: does PLCγ1 play any role during osteoclast differentiation, and why does PLCγ1 not compensate for the lack of PLCγ2 despite the high homology? Answering these questions will aid the design of better strategies to target the PLCγ pathway in pathological bone loss and will improve our understanding of the specificity of PLCγ signaling.

Results

PLCγ1 deficient mice exhibit early embryonic lethality (6), thus limiting our ability to study the effects of PLCγ1 deletion in osteoclasts. To overcome this issue, we screened various shRNA constructs targeting PLCγ1 but not PLCγ2. We identified five PLCγ1 shRNAs showing high knockdown efficiency and specificity for PLCγ1 that did not affect PLCγ2 (Fig. 1*A*). Next, we expressed the five shRNA constructs in primary OC precursors via lentivirus infection and examined the effects of PLCγ1 deletion on osteoclastogenesis. All five shRNA-infected OC precursors exhibited a severe defect in osteoclast differentiation (Fig. 1*B*), suggesting that PLCγ1, similarly to PLCγ2, is a critical regulator of osteoclast differentiation. Interestingly, however, shPLCγ1 cultures (also referred throughout the text as PLCγ1-knocked down or PLCγ1-deficient) showed no reduction in expression of NFATc1, c-Fos, and c-Src (Fig. 1*C*), all known osteoclastogenic pathways affected by PLCγ2 deficiency (12). This finding suggested that PLCγ1 governs osteoclast differentiation via a different mechanism than PLCγ2.

Interestingly, we observed that PLCγ1-knocked down OC precursor cultures had a lower number of cells compared with control shRNA, a defect that was not observed in the context of PLCγ2 deletion. To confirm these findings, we performed an MTT assay to compare the number of uninfected cells (ctrl) and PLCγ1-deficient cells cultured in CSF-1-containing medium

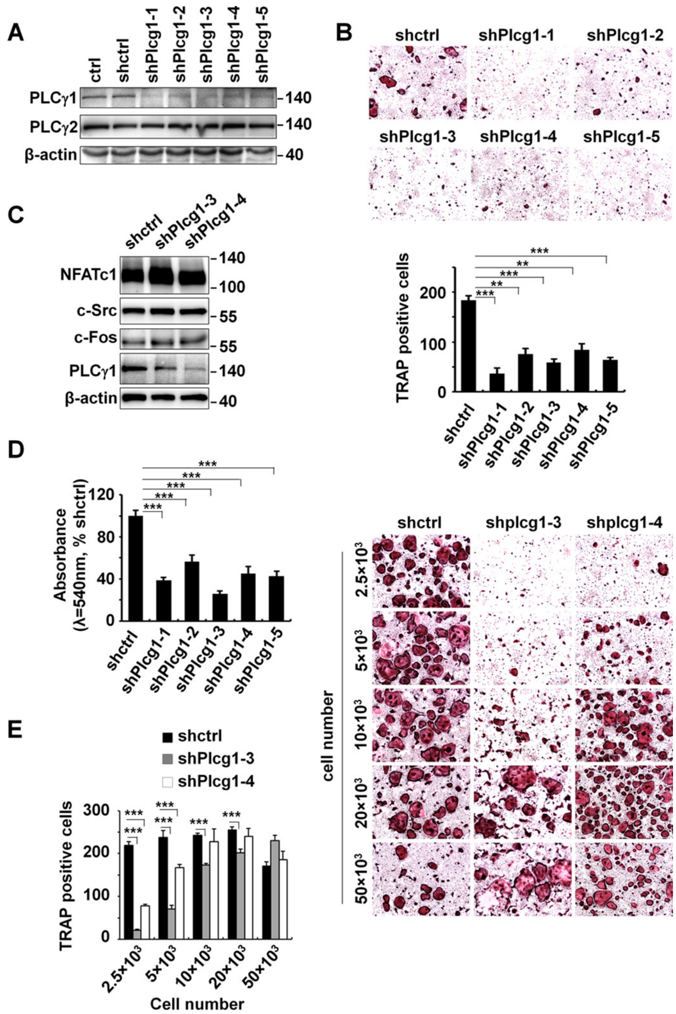

FIGURE 1. **PLCγ1 deficiency blocks osteoclastogenesis by limiting the number of OC precursors.** *A*, knockdown efficiency of five different shRNAs targeting PLCγ1 in OC precursors was compared with ctrl and shctrl. Levels of PLCγ2 and actin are used as loading controls. *B*, OC precursors infected with the indicated shPLCγ1 constructs were cultured in osteoclastogenic medium for 5 days. Representative images (*top*) and quantification data (*bottom*) show that PLCγ1 deficiency leads to a severe osteoclastogenesis defect. *C*, infected OC precursors were subjected to Western blotting analysis for NFATc1, c-Src, c-Fos, and PLCγ1. *D*, infected OC precursors cultured in CSF-1-containing medium for 6 days were subjected to MTT assay to quantify the number of cells available. *E*, increasing numbers of shPLCγ1-3- and shPLCγ1-4-infected OC precursors were plated in osteoclastogenic medium, and OCs were enumerated after TRAP staining (OC quantification and representative images of TRAP-stained wells are shown). When indicated, *error bars* represent S.D., and *asterisks* represent $p < 0.01$ (**) and $p < 0.001$ (***).

for 6 days. We found a significant decrease in the number of viable cells in the absence of PLCγ1 (Fig. 1*D*), suggesting that the defect in OC differentiation might be due to reduced availability of OC precursors.

To clarify whether PLCγ1 deficiency inhibits osteoclast differentiation via limiting cell number, we plated increasing numbers of PLCγ1-deficient OC precursors in osteoclastogenic medium and analyzed their ability to undergo osteoclast differentiation. Strikingly, the osteoclastogenesis defect was rescued by plating higher numbers of shPLCγ1 cells (Fig. 1*E*).

Reduced cell number can be due to decreased cell proliferation or increased cell death (21). CSF-1 is well recognized to activate key signaling pathways promoting OC precursor

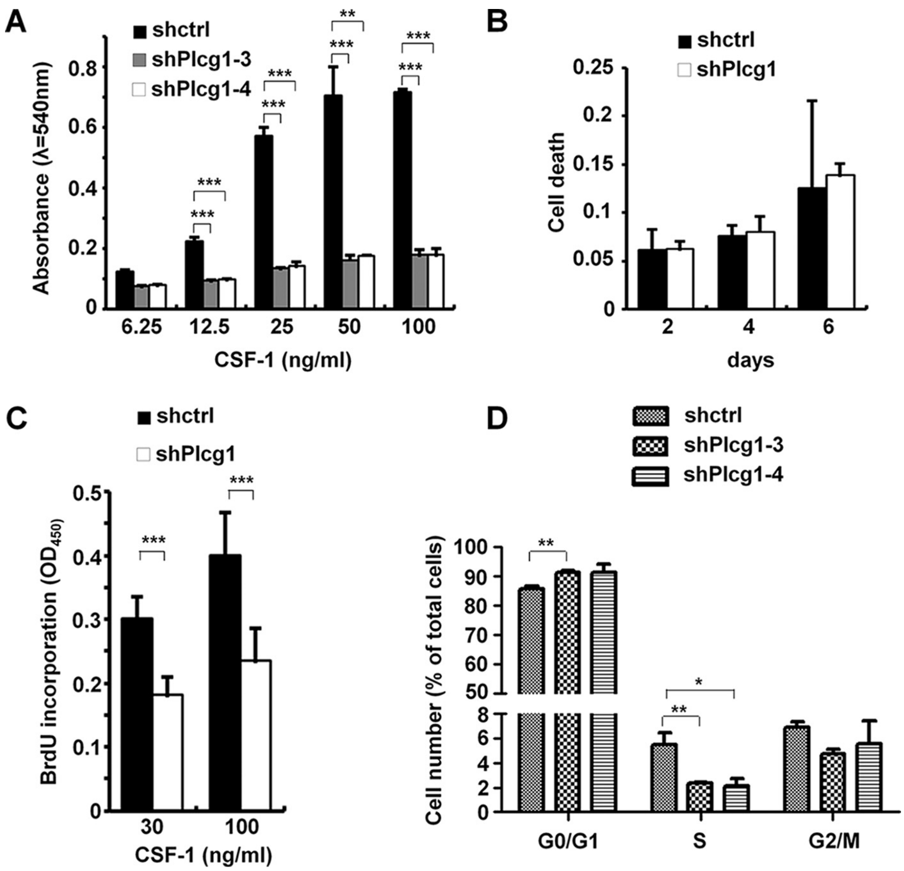

FIGURE 2. **PLCγ1 deficiency impairs CSF-1-induced proliferation.** *A*, shctrl-, shPLCγ1-3-, and shPLCγ1-4-infected OC precursors were cultured in medium containing the indicated concentrations of CSF-1 for 6 days, and cell number was assessed by MTT assay. *B*, shctrl- and shPLCγ1-infected OC precursors were cultured in the presence of 100 ng/ml CSF-1 for the indicated days, and cell death was assessed by ELISA. *C*, shctrl- and shPLCγ1-infected OC precursors were cultured in the presence of CSF-1 at the indicated concentrations for 3 days, and cells were then subjected to BrdU analysis. *D*, shctrl, shPLCγ1-3, and shPLCγ1-4 cells were serum- and cytokine-starved for 24 h and then treated with 100 ng/ml CSF-1 for an additional 24 h before cell cycle analysis. When indicated, *error bars* represent S.D., and *asterisks* represent $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

proliferation and survival (22). In previous studies, we documented that high doses of CSF-1 could rescue the osteoclastogenic defect of cells lacking the $\alpha v\beta 3$ integrin (23) or the co-stimulatory molecule DAP12 (24). However, high concentrations of CSF-1 were not sufficient to rescue cell numbers in PLCγ1-deficient cultures (Fig. 2*A*), thus suggesting that PLCγ1 is a major downstream effector of CSF-1 signaling in OC precursors.

To understand whether PLCγ1 deficiency induces apoptosis or affects cell proliferation, we examined cell death and BrdU incorporation by ELISA. As shown in Fig. 2*B*, no significant differences in cell death were observed between scrambled shRNA (shctrl) and shPLCγ1 OC precursors cultured in CSF-1-containing medium for 2, 4, or 6 days (Fig. 2*B*). By contrast, the BrdU signal was lower in shPLCγ1 cells (Fig. 2*C*) in the presence of both 30 and 100 ng/ml CSF-1. Confirming these findings, cell cycle analysis revealed that shPLCγ1 cells were arrested in G_1 phase with fewer cells entering the S phase following 24-h exposure to CSF-1 (Fig. 2*D*).

It is established that CSF-1 activates PI3K/AKT and Grb2/ERK pathways in OC precursors (22, 25). Therefore, we hypothesized that PLCγ1 modulates either AKT or ERK signaling cascades to promote cell proliferation. In contrast to our expectations, neither AKT nor ERK phosphorylation was altered by PLCγ1 deficiency in response to CSF-1 (Fig. 3*A*). CyclinD1, cyclinD2, and cyclinD3 are required for cell cycle G_1/S transition, and their expression is increased upon CSF-1 stimulation (26). Interestingly, the levels of cyclinD1 were decreased in PLCγ1-deficient cells exposed to CSF-1 (Fig. 3*B*). We observed a similar result for cyclinD3 (not shown).

CyclinD1 is a downstream target of β-catenin (27). β-Catenin has also been reported to participate in CSF-1-induced OC precursor proliferation via a mechanism that is independent of ERK/AKT phosphorylation (28, 29). Therefore, we wondered whether PLCγ1 controls β-catenin levels in OC precursors. Consistent with cyclinD1 expression, β-catenin levels were significantly reduced in cells lacking PLCγ1 (Fig. 3, *C* and *D*). The reduction in β-catenin was already observed in basal conditions and became more pronounced after short or longer exposure to CSF-1.

To clarify whether PLCγ1 mediates CSF-1-induced cyclinD1 expression via regulation of β-catenin, we used OC precursors from mice expressing a non-degradable form of β-catenin driven by the lysozyme M (Lyz2; herein indicated as LysM) promoter (LysM-Cre/*Ctnnb1*$^{\mathrm{WT/floxEx3}}$; herein referred to as β-catenin-CA) (30). To avoid confounding variables due to activity of LysM-Cre in various myeloid populations, we isolated β-catenin-CA OC precursors and infected them *ex vivo* with PLCγ1 or ctrl shRNAs to measure cyclinD1 levels follow-

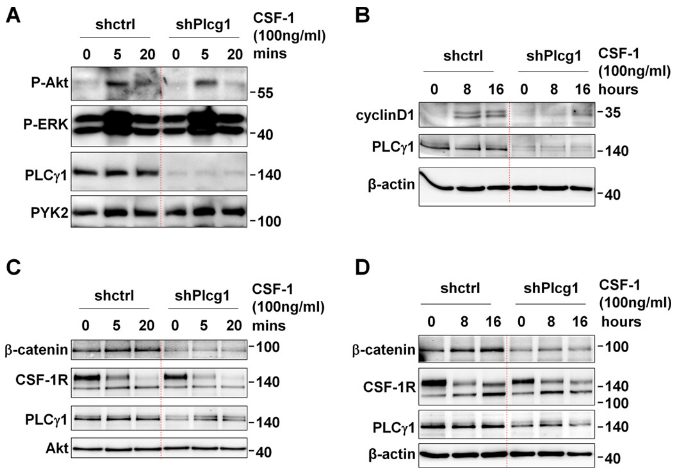

FIGURE 3. **PLCγ1 deficiency down-regulates β-catenin and cyclinD1 levels in response to CSF-1.** *A*, shctrl- or shPLCγ1-infected OC precursors were stimulated with 100 ng/ml CSF-1 for 0, 5, and 20 min and subjected to Western blotting analysis for p-AKT and p-ERK. PYK2 was used as loading control. *B* and *D*, shctrl-, shPLCγ1-3-, and shPLCγ1-4-infected OC precursors were stimulated with 100 ng/ml CSF-1 for 0, 8, and 16 h and blotted for cyclinD1, β-catenin, and CSF-1R. β-Actin and PLCγ1 are used as controls. *C*, same cells treated as in *A* blotted for β-catenin and CSF-1R.

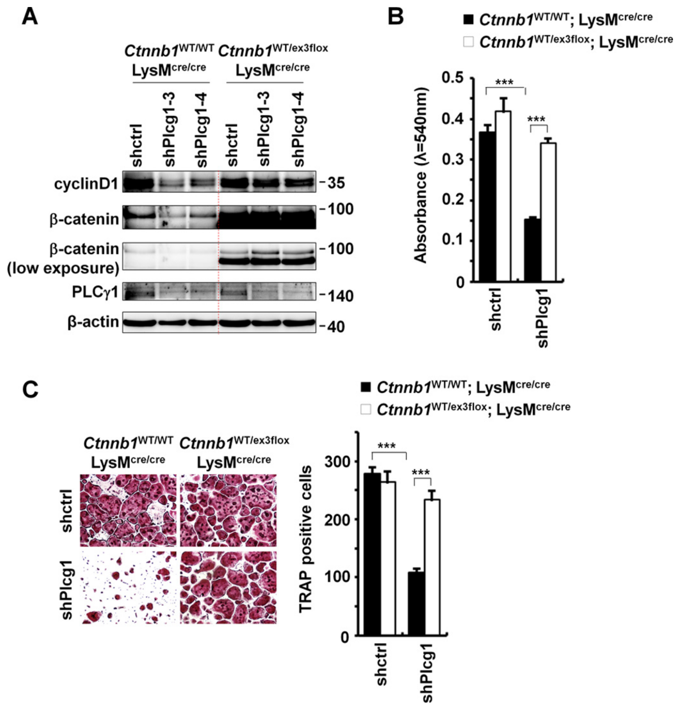

FIGURE 4. **Defects caused by PLCγ1 deficiency can be rescued in β-catenin-CA cells.** *A*, shctrl-, shPLCγ1-3-, and shPLCγ1-4-infected control (*Ctnnb1*$^{WT/WT}$; LysM$^{cre/cre}$) and β-catenin-CA (*Ctnnb1*$^{WT/ex3flox}$; LysM$^{cre/cre}$) OC precursors were stimulated with 100 ng/ml CSF-1 for 8 h, and cyclinD1 levels were analyzed by Western blotting. β-Catenin, β-actin, and PLCγ1 are used as controls. *B*, shctrl- and shPLCγ1-infected controls and β-catenin-CA OC precursors were plated at a concentration of 1×10^4/well and cultured in α-10 medium containing 100 ng/ml CSF-1 for 8 days. Cell number was assessed by MTT assay. *C*, shctrl- and shPLCγ1-infected controls and β-catenin-CA OC precursors were cultured in osteoclastogenic medium for 7 days, and differentiated OCs were analyzed via TRAP staining. Representative images (*left*) and quantification data (*right*) show that expression of β-catenin-CA can restore the osteoclastogenesis defect caused by PLCγ1 deficiency. When indicated, *error bars* represent S.D., and *asterisks* represent $p < 0.001$ (***).

ing 8-h exposure to CSF-1. As expected, cyclinD1 was down-regulated in PLCγ1-deficient cells, whereas its levels were increased in shPLCγ1/β-catenin-CA cells (Fig. 4*A*). Consistent with this result, cell number, assessed by MTT assay, was significantly increased in shPLCγ1/β-catenin-CA cultures *versus* shPLCγ1 (Fig. 4*B*). To finally determine whether higher β-catenin levels could rescue shPLCγ1 defective osteoclastogenesis, shPLCγ1/β-catenin-CA or shPLCγ1 cells were cultured in osteoclastogenic medium for 7 days. Strikingly, shPLCγ1/β-catenin-CA cells formed significantly more OCs of normal appearance than PLCγ1-deficient cells (Fig. 4*C*). Taken together, these results suggest that PLCγ1 modulates the osteoclastogenic pathway via activation of the β-catenin/cyclinD1 signaling cascade downstream of CSF-1.

PLCγ1 enzymatic activity leads to increased calcium levels and DAG production (2). Because calcium is known to modulate NFATc1 and shPLCγ1 cells have normal NFATc1 levels, we hypothesized that reduced DAG production may be responsible for impaired β-catenin/cyclinD1 expression in PLCγ1-deficient cells. To test this hypothesis, we turned to diacylglycerol kinase ζ (DGKζ)-deficient OC precursors. DGKζ modulates DAG levels in OC precursors by converting DAG into phosphatidic acid, and thus DGKζ deficiency leads to DAG accumulation (31, 32). We have recently documented that DGKζ deficiency increases osteoclast numbers (32), leading to the hypothesis that DAG accumulation could potentiate OC precursor proliferation. Indeed, MTT (Fig. 5*A*) and BrdU (Fig. 5*B*) incorporation assays revealed higher numbers of DGK$\zeta^{-/-}$ OC precursors compared with WT. This increase in cell number was observed in the presence of various concentrations of CSF-1 (Fig. 5, *A* and *B*). In line with this result, β-catenin and cyclinD1 expression was significantly higher in DGK$\zeta^{-/-}$ OC precursors compared with WT cells (Fig. 5*C*).

To further determine whether impaired cyclinD1 expression in PLCγ1-deficient OC precursors was due to decreased DAG production, we compared cell proliferation in DGK$\zeta^{+/+}$ and DGK$\zeta^{-/-}$ OC precursors infected with PLCγ1 shRNA constructs. As shown in Fig. 5*D*, shPLCγ1/DGK$\zeta^{-/-}$ cultures showed higher cell numbers when compared with shPLCγ1/DGK$\zeta^{+/+}$ cells. Consistently, the lower expression of cyclinD1 in shPLCγ1 cultures was rescued in shPLCγ1/DGK$\zeta^{-/-}$ cells (Fig. 5*E*). All together, these results demonstrate that PLCγ1 utilizes DAG signaling to promote OC precursor proliferation in response to CSF-1.

Finally, to determine whether increased DAG levels could rescue the OC differentiation defect observed in PLCγ1-deficient cultures, we cultured shPLCγ1/DGK$\zeta^{-/-}$ OC precursors in the presence of osteoclastogenic medium. Consistent with our previous report (32), DGK$\zeta^{-/-}$ OC precursors formed more and larger osteoclasts than WT, whereas fewer and smaller OCs were observed in the absence of PLCγ1 (Fig. 5*F*). Notably, OC differentiation was normalized in shPLCγ1/DGK$\zeta^{-/-}$ OC cultures. Taken together, these results demonstrate that CSF-1 activates the PLCγ1/DAG/β-catenin/cyclinD1 pathway to support OC precursor proliferation, which in turn benefits osteoclastogenesis.

We previously reported that PLCγ2 modulates OC differentiation by regulating NFATc1 activation downstream of RANKL signaling (12). PLCγ2 has also been implicated in cytoskeletal reorganization in response to CSF-1 stimulation and

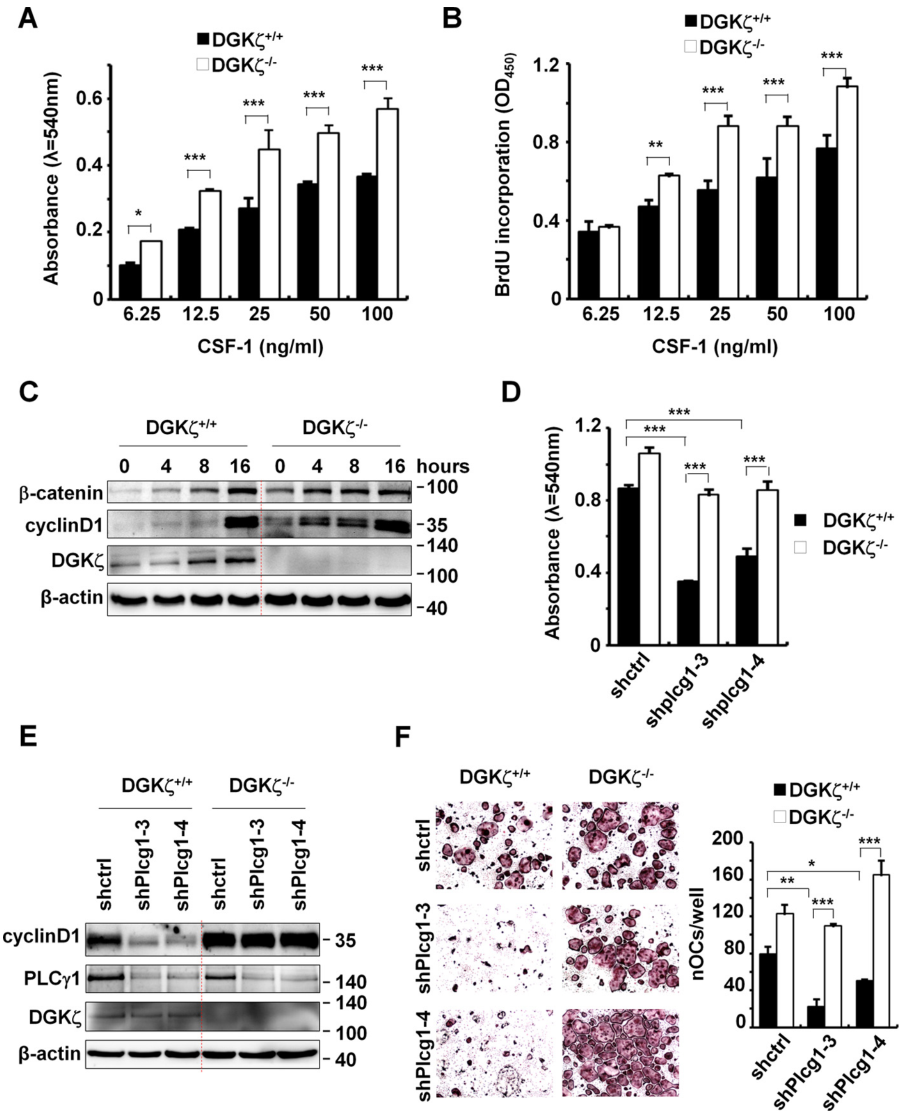

FIGURE 5. **DAG accumulation promotes proliferation and normalizes PLCγ1 deficiency-induced osteoclastogenesis defect.** *A*, DGKζ$^{+/+}$ or DGKζ$^{-/-}$ OC precursors were treated with the indicated concentrations of CSF-1 for 6 days. Cell number was assessed by MTT assay. *B*, DGKζ$^{+/+}$ or DGKζ$^{-/-}$ OC precursors were cultured as in *A* for 3 days and then subjected to BrdU analysis. *C*, DGKζ$^{+/+}$ or DGKζ$^{-/-}$ OC precursors were stimulated with 100 ng/ml CSF-1 for 0, 4, 8, and 16 h and subjected to Western blotting analysis for β-catenin, cyclinD1, DGKζ, and β-actin. *D*, DGKζ$^{+/+}$ or DGKζ$^{-/-}$ OC precursors were infected with shctrl, shPLCγ1-3, and shPLCγ1-4; cultured in the presence of 100 ng/ml of CSF-1 for 6 days; and subjected to MTT assay. *E*, DGKζ$^{+/+}$ or DGKζ$^{-/-}$ OC precursors were infected with shctrl, shPLCγ1-3, and shPLCγ1-4 and exposed to 100 ng/ml CSF-1 stimulation for 8 h. The samples were subjected to Western blotting analysis for cyclinD1, PLCγ1, DGKζ, and β-actin. *F*, DGKζ$^{+/+}$ or DGKζ$^{-/-}$ OC precursors infected with shctrl, shPLCγ1-3, and shPLCγ1-4 were cultured in osteoclastogenic medium for 5 days followed by TRAP staining. Representative TRAP images (*left*) and quantification of number of mature OCs/well (*right*) are shown. When indicated, *error bars* represent S.D., and *asterisks* represent $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

integrin engagement (33). Thus, we next asked whether PLCγ2 could also modulate CSF-1-dependent proliferative signals. To this end, we compared cyclinD1 expression in WT and PLCγ2$^{-/-}$ OC precursors exposed to CSF-1 and found no differences (Fig. 6*A*). Consistent with this finding, no differences were noted in the proliferation rate between WT and PLCγ2$^{-/-}$ cells (not shown). Furthermore, expression of shPLCγ1 in WT, PLCγ2$^{+/-}$, and PLCγ2$^{-/-}$ cells leads to a similar decrease in cell numbers, further suggesting that PLCγ2 is not sufficient to compensate for the loss of PLCγ1 (Fig. 6*B*).

The different response between PLCγ1 and PLCγ2 to CSF-1 stimulation made us hypothesize that PLCγ1 is the main modulator of CSF-1 signals required for OC precursor proliferation. To elucidate this hypothesis, we examined whether CSF-1R could associate with either PLCγ1 or PLCγ2. As shown in Fig. 6*C*, CSF-1R interacted with both PLCγ1 and PLCγ2 in basal conditions. Interestingly, the association between PLCγ1 and CSF-1R was further increased by CSF-1 stimulation, whereas CSF-1R binding to PLCγ2 was decreased. Confirming activation of the receptor, we found a time-dependent increase in CSF-1R tyrosine phosphorylation (Fig. 6*C*). In a reverse immunoprecipitation assay using anti-PLCγ1 to pull down the complex, we confirmed PLCγ1 binding to CSF-1R in basal conditions and following CSF-1 stimulation (Fig. 6*D*). By contrast, when we used anti-PLCγ2 to immunoprecipitate the complex, we did not detect PLCγ2/CSF-1R binding in response to CSF-1 stimulation (Fig. 6*E*). Taken together,

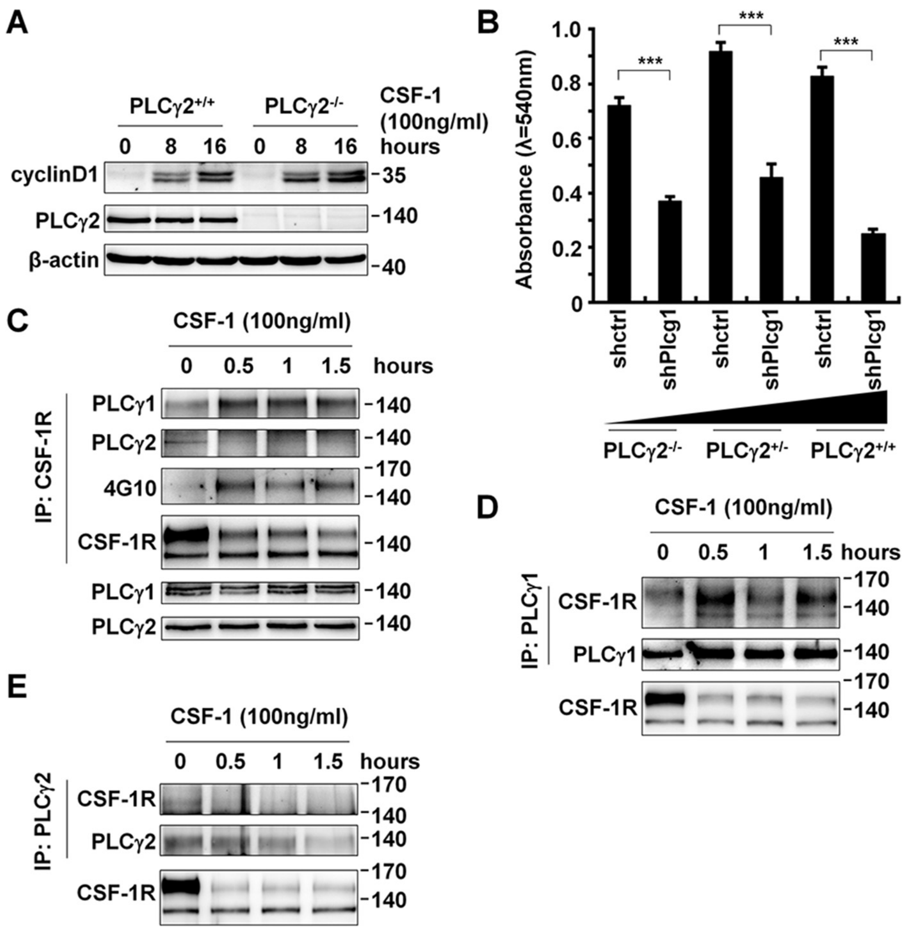

FIGURE 6. **PLCγ1 but not PLCγ2 is recruited to CSF-1R after CSF-1 stimulation.** *A*, WT and PLCγ2$^{-/-}$ OC precursors were stimulated with 100 ng/ml CSF-1 for 0, 8, and 16 h and subjected to Western blotting analysis for cyclinD1, PLCγ2, and β-actin. *B*, PLCγ2$^{+/+}$, PLCγ2$^{+/-}$, and PLCγ2$^{-/-}$ OC precursors were infected with shctrl and shPLCγ1; cultured in the presence of 100 ng/ml CSF-1 for 6 days; and subjected to MTT assay. *C–E*, WT OC precursors were stimulated with 100 ng/ml CSF-1 for 0, 0.5, 1, and 1.5 h; lysed; and subjected to immunoprecipitation (*IP*) using the following antibodies CSF-1R (*C*), PLCγ1 (*D*), and PLCγ2 (*E*) followed by Western blotting for the indicated proteins. When indicated, *error bars* represent S.D., and *asterisks* represent $p < 0.001$ (***).

these results demonstrate that CSF-1R recruits PLCγ1 but not PLCγ2 in response to CSF-1 stimulation, and this could explain why PLCγ1 but not PLCγ2 modulates CSF-1-induced OC precursor proliferation.

Discussion

PLCγ1 and PLCγ2 are two highly homologous enzymes with cell-specific, non-redundant biological functions (5). Although both enzymes can be expressed in the same cell, often one appears to play a dominant role over the other. Various hypotheses have been proposed for the non-overlapping effects of these two enzymes, ranging from different levels of expression or inability to be recruited to the same molecular complexes (3, 5). Interestingly, in the osteoclasts, both PLCγ1 and PLCγ2 are required for osteoclastogenesis but do not compensate for each other. Although our previous work demonstrated that PLCγ2 is activated downstream of RANK and regulates expression of OC markers, we now find that PLCγ1 is activated in response to CSF-1 and modulates OC precursor proliferation. To our knowledge, this is the first known cell type where deletion of either enzyme has major consequences on the cell differentiation by acting on two different signaling pathways.

CSF-1 is an important cytokine modulating critical pathways required for OC precursor proliferation, survival, and cytoskeletal reorganization (17, 34), and mice lacking CSF-1 develop osteopetrosis due to lack of osteoclasts (35). The two major signaling pathways activated by CSF-1 are ERK and AKT, which control cell proliferation and survival via the activation and stabilization of D-type cyclins. Intriguingly, however, although PLCγ1 deficiency suppresses OC precursor proliferation, it does not alter CSF-1-induced ERK or AKT phosphorylation. By contrast, PLCγ1 modulates cyclinD1 expression, a protein known to modulate cell proliferation downstream of β-catenin (36). Although the role of β-catenin in cell proliferation is well established (28, 37), contradiction exists regarding the role of β-catenin in regulating osteoclast differentiation. *In vitro* studies with cells from β-catenin conditional knock-out or β-catenin constitutively active mice using peroxisome proliferator-activated receptor γ promoter driven Cre expression show defective OC differentiation (29). By contrast, deletion of β-catenin using LysM promoter-driven Cre expression led to osteopenia due to increased number of OCs (38, 39). These data suggest that balanced β-catenin signals in osteoclast precursors may be required for proper osteoclastogenesis. In addition, treatment with Wnt3a, which induces β-catenin accumulation, did not affect RANKL-induced osteoclastogenesis (40–42), further implicating that how β-catenin is activated might also play a critical role in the OC differentiation process. We show that expression of β-catenin-CA in cells lacking PLCγ1 is sufficient to rescue cyclinD1 levels and cell proliferation, which in turn normalizes the number of mature OCs. Our finding places β-catenin downstream of CSF-1/PLCγ1 signaling and is consistent with the role of the DAP12/β-catenin axis in promoting macrophage proliferation independently of AKT/ERK activation (28).

PLCγ1 is known for converting PIP_2 to IP_3 and DAG (2, 4). DAG is a second messenger that transduces signals modulating a variety of cellular functions, including proliferation, motility, and angiogenesis to name a few. We now demonstrate that DAG production is required for PLCγ1 effects on macrophage proliferation. Because DAG is unstable and deletion of a second messenger cannot be achieved, we decided to use DGKζ knock-out mice, which lack the kinase converting DAG into phosphatidic acid. We previously reported that DGKζ knock-out mice exhibit lower bone mass and enhanced osteoclastogenesis compared with WT mice (32); however, little is known about the role of DAG in OC precursor proliferation. We now find that DGKζ-deficient OC precursors have an increased proliferation rate and higher cyclinD1 levels compared with WT mice. Most importantly, deletion of the DGKζ in PLCγ1-deficient cells rescues CSF-1-induced proliferation and osteoclastogenesis. Interestingly, we recently reported that DAG accumulation in DGKζ-null OC precursors leads to higher c-Fos expression (32), a mechanism conferring augmented osteoclast numbers. However, c-Fos levels appear to be normal in PLCγ1-deficient OC cultures. This result would suggest that DAG controls OC formation by modulating c-Fos expression and affects OC precursor proliferation via the β-catenin/cyclinD1 axis.

In conclusion, this study demonstrates that PLCγ1 works as a novel and critical signal for the promotion of OC precursor proliferation and thus osteoclastogenesis downstream of CSF-1. PLCγ1 effects on the OCs are different compared with PLCγ2, which is activated in response to RANKL. Clarifying the exact roles of PLCγ1 and PLCγ2 in the regulation of osteoclastogenesis has important implications that go beyond their effects on the OCs but also demonstrate how two highly homologous molecules can specifically activate distinct pathways in the same cell type.

Experimental Procedures

Primary Cell Cultures and Mice—OC precursors were isolated and cultured as described previously (43). Briefly, bone marrow was harvested from femora and tibiae of 6–8-week-old C57/BL6 mice. Cells were then cultured in α-minimum essential medium (M0894, Sigma) containing 10% heat-inactivated fetal bovine serum (26140, Gibco), 100 IU/ml penicillin plus 100 μg/ml streptomycin (15140, Gibco), 2 mM glutamine (25-005-Cl, Corning) (referred to as α-10 medium), and 10% CMG 14-12 cell-conditioned medium (44) containing the equivalent of 100 ng/ml CSF-1 for 3 days in Petri dishes. The attached cells represent OC precursors ready to use. In some experiments, purified CSF-1 (576404, Biolegend, San Diego, CA) was used to stimulate OC precursors. To generate mature osteoclasts, OC precursors were plated in a 96-well plate at a concentration of 5×10^3 cells/well in the presence of 100 ng/ml RANKL and 1% CMG 14-12 (containing the equivalent of 10 ng/ml CSF-1) for 6 days changing medium every day. The differentiated cells were then fixed in 4% paraformaldehyde and subjected to TRAP staining using a commercial kit (387A, Sigma). All experiments were approved by the Washington University School of Medicine animal care and use committee. Male and female WT, DGK$\zeta^{-/-}$, LysM-Cre/*Ctnnb1*$^{WT/WT}$ and LysM-Cre/*Ctnnb1*$^{WT/floxEx3}$ mice (C57BL/6 background), 6–8 weeks of age, were used in the study. Mice were housed in cages and fed with food and water *ad libitum* with a 12-h light and 12-h dark cycle.

Lentivirus Generation and Infection—HEK293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin plus 100 μg/ml streptomycin, and 1 mM sodium pyruvate (25-000-Cl, Corning). Five PLCγ1 lentiviral shRNAs constructs in PLKO.1 vector containing a puromycin resistance cassette were purchased from Washington University RNAi core (St. Louis, MO). shPLCγ1-1 (targeting GCCAGCTTGTAGCACTCAATT), shPLCγ1-2 (targeting CCCGTGAATCATGAGTGGTAT), shPLCγ1-3 (targeting CCAACTTTCAAGTGTGCAGTA), shPLCγ1-4 (targeting CCAGATCAGTAACCCAGAGTT), shPLCγ1-5 (targeting GCCAGATCAATCACACTGCTT), and shctrl were co-transfected with a packaging plasmid (Delta8.2) and envelope plasmid (VSVg) into HEK293T cells using Polyjet transfection reagent (SL100688, SignaGen Laboratories, Gaithersburg, MD). Supernatants containing the lentivirus were harvested 36–48 h after transfection, filtered, and used to infect OC precursors. 50% lentivirus supernatant and 10% CMG 14-12 in α-10 medium with additional 8 μg/ml Polybrene were added to the cells for 24 h. Cells were then selected in α-10 medium containing 10% CMG 14-12 and 2 μg/ml puromycin for 24 h prior to being used for the indicated experiments.

MTT Assay—shPLCγ1 or shctrl OC precursors were plated in a 96-well plate at a concentration of 2.5×10^3 cells/well and cultured for few days in α-10 medium containing either 10% CMG 14-12 or purified CSF-1 at the indicated concentrations. Cells were then incubated with 0.5 mg/ml MTT (M2128, Sigma) for 2.5 h. The crystalline formation was dissolved in 150 μl of DMSO (D5879, Sigma) followed by a spectrophotometric reading at A_{540} (EL-800, Bio-Tek, Winooski, VT).

BrdU Assay—The BrdU assay was performed using a cell proliferation ELISA kit (11647229001, Roche Applied Science). shctrl- and shPLCγ1-infected cells or WT (DGK$\zeta^{+/+}$) and DGK$\zeta^{-/-}$ OC precursors were plated in 96-well plates at a concentration of 5×10^3/well and cultured in α-10 medium containing purified CSF-1 at the indicated concentrations for 3 days. Cells were then incubated with 0.1% BrdU for 4 h at 37 °C, fixed for 30 min at room temperature, and incubated with blocking reagent for another 30 min. Peroxidase-labeled anti-BrdU antibody was then added for 90 min, and BrdU signal was measured at A_{450} following reaction with the appropriate substrate as indicated in the manufacturer's instructions.

Cell Death ELISA—Cell death was examined by using a commercial kit (11774425001, Roche Applied Science). Briefly, shctrl- and shPLCγ1-infected OC precursors were cultured in α-10 medium containing 10% CMG 14-12 for 2, 4, or 6 days.

Cells were then resuspended in 200 μl of lysis buffer and incubated for 30 min, and lysates were centrifuged at 200 × *g* for 10 min. 20 μl of supernatant was transferred into a streptavidin-coated microplate strip with an additional 80 μl of immunoreagent (72 μl of incubation buffer, 4 μl of anti-histone-biotin, and 4 μl of anti-DNA-peroxidase). The mixture was then incubated on a microplate shaker under gentle shaking (300 rpm) for 2 h. After washing with incubation buffer, the immunocomplex was incubated with 100 μl of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid solution for 20 min, and the reaction was stopped by adding 100 μl of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid stop solution. A_{405} and A_{490} (reference) were recorded, and cell death was assessed by calculating ($A_{405} - A_{490}$).

Cell Cycle Analysis—3 × 10^5 shctrl-, shPLCγ1-3-, and shPLCγ1-4-infected cells were seeded into 6-cm Petri dishes in α-10 medium containing 10% CMG 14-12. Cells were synchronized by 24-h starvation, stimulated with 100 ng/ml purified CSF-1 for 24 h, and fixed in 70% ethanol at −20 °C overnight. Samples were stained with 100 μg/ml propidium iodide (R4170, Sigma) in the presence of 100 μg/ml RNase (R6513, Sigma) for 30 min at 37 °C and analyzed by flow cytometry (BD FACSCalibur, BD Biosciences). Results were assessed using FlowJo software (Treestar, Inc., San Carlos, CA).

Western Blotting—For β-catenin/cyclinD1 analysis and AKT/ERK signaling, 2 × 10^5 OC precursors were plated in 6-well plates in the presence of α-10 medium containing 10% CMG 14-12. Adherent cells were then starved from serum and cytokines for 24 h followed by stimulation with 100 ng/ml CSF-1 for indicated time. Cells were directly lysed in 1× SDS loading buffer and subjected to Western blotting analysis. The following antibodies were used: cyclinD1 (2978, Cell Signaling Technology, Danvers, MA; 1:1000), β-catenin (9587, Cell Signaling Technology; 1:1000), PLCγ1 (2822, Cell Signaling Technology; 1:1000), PLCγ2 (3872, Cell Signaling Technology; 1:1000), DGKζ (sc-8722, Santa Cruz Biotechnology; 1:500), β-actin (A5441, Sigma; 1:5000), p-AKT (9271, Cell Signaling Technology; 1:1000), p-ERK (4377, Cell Signaling Technology; 1:1000), and PYK2 (3292, Cell Signaling Technology; 1:1000). For Western Blotting analysis in mature osteoclasts, 3 × 10^4 infected OC precursors were plated in 12-well plates in osteoclastogenic medium for 5 days, and the medium was changed every day. The differentiated cells were lysed in TNE buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 10% (v/v) glycerol) and subjected to Western blotting using specific antibodies for NFATc1 (sc-7294, Santa Cruz Biotechnology; 1:500), c-Src (sc-18, Santa Cruz Biotechnology; 1:500), c-Fos (2250, Cell Signaling Technology; 1:1000), PLCγ1 (1:1000), and β-actin (1:5000).

Co-immunoprecipitation—OC precursors were cultured in 10-cm Petri dishes until confluence, and cells were starved overnight in serum- and cytokine-free medium. Starved cells were then stimulated with 100 ng/ml CSF-1 for 0.5, 1, and 1.5 h after which they were lysed in TNE buffer containing protease inhibitors (78442, Thermo Fisher, Rockford, IL). 250-μg protein samples were incubated with 1 μg of anti-CSF-1R (sc-692, Santa Cruz Biotechnology), anti-PLCγ1 or anti-PLCγ2 antibody at 4 °C overnight followed by protein A/G bead incubation at 4 °C for an additional 3 h. The immunocomplex was washed with PBS three times and subjected to Western blotting using specific antibodies for PLCγ1 (1:1000), PLCγ2 (1:1000), 4G10 (05-321, Upstate Biotechnology, Lake Placid, NY; 1:2000), and CSF-1R (1:500).

Statistical Analysis—Data are represented as mean ± S.D. for absolute values as indicated in the *vertical axis* of the figures. Two-tailed one-type Student's *t* test was performed to analyze *p* values between experimental and control groups. All experiments with multiple parameters (groups, days, or concentrations) were analyzed by using a two-way analysis of variance followed by Bonferroni post-tests. $p < 0.05$ was considered to be statistically significant. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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Phospholipase Cγ1 (PLCγ1) Controls Osteoclast Numbers via Colony-stimulating Factor 1 (CSF-1)-dependent Diacylglycerol/ β-Catenin/CyclinD1 Pathway

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