Phospholipase Cγ2 Mediates RANKL-stimulated Lymph Node Organogenesis and Osteoclastogenesis*

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Phospholipase Cγ2 (PLCγ2) is an important signaling effector of multiple receptors in the immune system. Here we show that PLCγ2-deficient mice displayed impaired lymph node organogenesis but normal splenic structure and Peyer’s patches. Receptor activator of NF-κB ligand (RANKL) is a tumor necrosis factor family cytokine and is essential for lymph node organogenesis. Importantly, PLCγ2 deficiency severely impaired RANKL signaling, resulting in marked reduction of RANKL-induced activation of MAPKs, p38 and JNK, but not ERK. The lack of PLCγ2 markedly diminished RANKL-induced activation of NF-κB, AP-1, and NFATc1. Moreover, PLCγ2 deficiency impaired RANKL-mediated biological function, leading to failure of the PLCγ2-deficient bone marrow macrophage precursors to differentiate into osteoclasts after RANKL stimulation. Re-introduction of PLCγ2 but not PLCγ1 restores RANKL-mediated osteoclast differentiation of PLCγ2-deficient bone marrow-derived monocyte/macrophage. Taken together, PLCγ2 is essential for RANK signaling, and its deficiency leads to defective lymph node organogenesis and osteoclast differentiation.

PLCγ2 is a lipid enzyme, and activation of PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol and inositol 1,4,5-trisphosphate (1, 2). Both diacylglycerol and inositol 1,4,5-trisphosphate are important second signaling messengers for diverse cellular responses (1, 2). Diacylglycerol activates protein kinase C (PKC), whereas inositol 1,4,5-trisphosphate mediates the mobilization of Ca2+ from internal stores, resulting in a transient intracellular Ca2+ flux (1). Activated PKC, via a three component complex composed of CARMA1 (CARD, membrane-associated guanylate kinase, MAGUK, protein 1), Bcl10 (B-cell lymphoma protein 10), and MALAT1 (mucosa-associated lymphoid tissue lymphoma translocation protein 1), leads to the activation of IκB kinase (3–5). Activated IκB kinase then phosphorylates a family of cytoplasmic inhibitory proteins IκB, triggering its ubiquitination and proteolysis by the proteasome complex (5). Ultimately, the degradation of IκB releases sequestration of transcription factors of the NF-κB family in the cytoplasm, leading to its nuclear localization and activation of its target genes (6, 7). Meanwhile, the elevated intracellular Ca2+ binds to calmodulin, activating the serine/threonine phosphatase calcineurin. Activation of calcineurin leads to dephosphorylation of the transcription factor NFAT, resulting in its translocation from the cytoplasm to the nucleus and ultimate activation of its target genes (8). Moreover, the PLCγ/Ca2+/PKC pathway has been shown to participate in the activation of all types of MAP kinases (ERKs, JNKs, and p38 MAPks) (9–13) even though PKC-independent Grb2/SOS/Raf1 pathway plays a primary role in the activation of MAPks (10, 14, 15). Activated PKC can promote activation of ERK-1 and ERK-2 and is required for the maximum activation of p38 MAPK (12, 13, 16). In addition, calcium and PKC are involved in JNK activation (12, 13). Ultimately, the activation of the three MAP kinase leads to the activation of transcription factors, including AP-1 (17–19).

PLCγ2 is primarily expressed in hematopoietic cell lineages (1). Targeted gene disruption studies have revealed a critical role of PLCγ2 in multiple receptor-mediated biological functions. PLCγ2 is essential for pre-B cell receptor (BCR)- and BCR-mediated B cell development and functions, and its deficiency affects early B cell development and severely impairs B cell maturation and responses to antigen challenges (20–23). PLCγ2 is also a critical component of FcγR chain-containing collagen receptor signaling pathways, and deficiency of PLCγ2 results in platelet dysfunction and fetal hemorrhage (20). In addition, PLCγ2 participates in FcR signaling, and its deficiency impairs FcγR-induced degranulation and cytokine lating factor; α-MEM, α-minimal essential medium; RT, reverse transcription; GFP, green fluorescent protein; LT, lymphotixin; TRAP, tartrate resistant acid phosphatase; CTR, calcitonin receptor; CATH K, cathepsin K.
secretion in mast cells (24). Last, PLCγ2 correlates with defective FcγR-mediated ADCC (antibody-dependent cell-mediated cytotoxicity) activity in NK cells (20) and is involved in signaling of the major activating receptor, NKG2D, of the NK cells (25, 26), wherein PLCγ2 deficiency disrupts NKG2D-mediated NK cell maturation and function (27, 28).

Receptor activator of NF-κB ligand (RANKL) is a tumor necrosis factor family cytokine (29, 30). RANKL is essential for early lymphocyte development and lymph node organogenesis (31, 32). RANKL also mediates the final differentiation of bone marrow derived monocyte/macrophage precursors (BMMs) into osteoclasts (29, 30, 32), whereas macrophage-colony stimulating factor (M-CSF) controls the survival and proliferation of these precursors (33). RANKL deficiency impairs the early development of both T and B cells and blocks the formation of lymph nodes, resulting in immunodeficiency disease (32).

Upon binding to its receptor RANK, RANKL initiates the recruitment of the immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor proteins, DAP12 and the γ chain of Fc receptor (FcRγ), and tumor necrosis factor receptor associated factor 6 (TRAF6), an important signaling molecule, to the receptor complex (34–38). Then, RANKL activates multiple pathways, including the mitogen-activated protein kinase (MAPK), such as ERK, JNK, and p38, and Ca2+ dependent pathways (39–42). Ultimately, RANKL leads to activation of transcription factors, including NFATc1, NF-κB, and AP-1 (42–45). Gene disruption studies have demonstrated the important role of DAP12, FcRγ (42–45). Gene disruption studies have demonstrated the important role of DAP12, FcRγ, TRAF6, NFATc1, NF-κB, and c-Fos, a component of AP-1, in RANKL-mediated biological functions (34–37, 42, 46, 47). Although Ca2+-dependent NFATc1 and PKC-dependent NF-κB pathways are indispensable for RANKL signaling, the PLC isosforms responsible for activation of these pathways is not known.

Here we demonstrate that PLCγ2 is activated upon RANKL stimulation. Importantly, deficiency of PLCγ2 severely impairs RANKL signaling and results in the lack of lymph node organogenesis in mice.

**EXPERIMENTAL PROCEDURES**

**Mice**—PLCγ2−/− mice were as previously described (20). Bone morphology assessment was performed with age- and gender-matched PLCγ2−/− and wild-type control littermates. All animal usage followed the guideline of the Institutional Animal Care and Use Committees at the University of Alabama at Birmingham and the Blood Research Institution at the Blood Center of Wisconsin.

**Antibodies and Reagents**—Rabbit polyclonal anti-ERK (sc-93), anti-p38 (sc-535), and anti-JNK2 (sc-572) antibodies and mouse monoclonal anti-PLCγ1 (sc-7290), anti-PLCγ2 (sc-5283), anti-phospho-ERK (pThr202/pTyr204, sc-7383), and anti-NFATc1 (sc-7294) antibodies were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-phospho-p38 (pThr180/pTyr182, #9216) and mouse monoclonal anti-phospho-JNK (pThr183/pTyr185, #9255) antibodies were purchased from Cell Signaling Technology. Mouse monoclonal anti-γ-glutamyl-3-phosphate dehydrogenase antibodies (RDITRKS4G-6C5) were purchased from Research Diagnostics Inc. Mouse monoclonal anti-actin antibodies (MAB1501R) were purchased from CHEMICON. Fluorescein isothiocyanate-conjugated anti-mouse CD11 (11-041), phosphatidylethanolamine-conjugated anti-mouse Mac-1 (12-0112), PE-conjugated anti-mouse CD11b (c-Fms) (12-1152), PE-conjugated anti-mouse RANK (12-6612) fluorescein isothiocyanate-conjugated Rat IgG2a isotype control (11-4321), and PE-conjugated Rat IgG2b isotype control (12-4031) were purchased from eBioscience. Murine M-CSF was purchased from R&D Systems. Glutathione S-transferase-RANKL was purified as previously described (48).

**Deriving BMMs and RANKL-mediated Differentiation of BMMs in Vitro**—In vitro osteoclastogenesis was performed as previously described (49, 50). Briefly, bone marrow (BM) cells were isolated from long bones of 8–12-week-old PLCγ2−/− and wild-type control littermates. The cells were grown in complete α-MEM (Invitrogen) with murine M-CSF (10 ng/ml) for 72 h. Then the nonadherent BMMs were cultured with glutathione S-transferase-RANKL (100 ng/ml) and M-CSF (10 ng/ml). Cell differentiation was examined by TRAP staining according to the manufacturer’s instruction (Sigma).

The osteoclast-specific gene expression was detected as previously described (51). Briefly, total RNA was isolated from the indicated cells with TRizol reagents, reverse-transcribed, and amplified with primers for mouse TRAP, CTR, Cath K, and RANK. Expression of glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. RT-PCR and quantitative real-time PCR was performed and analyzed using SYBR Green I Supermix (Bio-Rad) in a Bio-Rad i-Cycler. Primers and conditions for PCR assays were as following: RANK, 5′-TTTGGTGGGATTTGGGTCATGAT-3′ and 5′-ACCTCGTGACCCAGTTGTGAA-3′; TRAP, 5′-GACAGTGCCGCTGACTTCA-3′ and 5′-GGGCTTGGAGAGATCTTAGAT-3′; Cath K, 5′-ACGAGGCTAGCTGACTGAA-3′ and 5′-GATGCCAAGCTTGCGTTCAT-3′; CTR, 5′-GACACACTGTCGTAGTCT-3′ and 5′-GAAGCATTAGATAGTCCGCA-3′. Standard curves were generated for all PCR assays. PCR conditions were 95 °C for 7 min followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, 68 °C for 30 s, and then 68 °C for 7 min.

**Retroviral Transduction of BMMs of PLCγ1 and PLCγ2**—The retroviral transduction was as previously described (22). Briefly, rat PLCγ1 or rat PLCγ2 gene has been cloned into a vector with bicistronic retrovirus murine stem cell virus promoter-internal ribosome entry site (IRES)-GFP to generate GFP-IRES-PLCγ1 and GFP-IRES-PLCγ2 vectors. Conditioned media containing high titer, amphotropic retrovirus particles derived from 293T cells were filtered and used for transduction. Wild-type and PLCγ2−/− BMMs were exposed to filtered conditioned media that contained PLCγ1 or PLCγ2 retrovirus for 2 days, and subsequently RANKL (100 ng/ml) and M-CSF (10 ng/ml) were added. retrovirus with GFP alone served as the control. Media were changed every other day. The effects on osteoclastogenesis were determined by TRAP staining and normalized by measuring infection efficiency assessed by GFP expression.

**Western Blot Analysis**—Cells were lysed, and cell lysates were subjected to SDS-PAGE and Western blot analysis as previously described (52). For measuring activation of MAPKs, nonadherent BM cells were cultured in complete α-MEM with 10% fetal bovine serum and murine M-CSF (10 ng/ml) for 3 days. The cells were then starved in serum-free media for 6 h fol-
PLCγ2 Deficiency Impairs Lymph Node Organogenesis—We examined the effect of PLCγ2 deficiency on lymph node organogenesis. Anatomical analysis of secondary lymphoid organs demonstrated that PLCγ2−/− mice displayed dramatically impaired mesenteric, cervical, mandibular, inguinal, axillary, para-aortic, and popliteal lymph nodes compared with wild-type mice (Fig. 1A and data not shown). Thus, PLCγ2 deficiency severely impairs lymph node organogenesis, similar to either the lack of lymphotxin pathway or RANKL pathway.

Previous studies have linked the formation of lymph node with that of Peyer’s patch and splenic architecture in the lymphotxin pathway but not in the RANKL pathway. Lymphotxin-deficient mice lack lymph nodes and also display defects in the formation of Peyer’s patch and exhibit disorganized splenic architecture (55–58). Interestingly, despite the severe defects of all lymph nodes in PLCγ2−/− mice, the mutant mice had normal Peyer’s patches compared with wild-type mice (Fig. 1B). Moreover, PLCγ2−/− mice exhibited intact splenic architecture, including normal distribution of red and white pulp and normal primary follicle structure, including T- and B-cell areas and marginal zones (Fig. 1C and data not shown). Taken together, these data demonstrate that PLCγ2 is essential for lymph node but not Peyer’s patch or splenic architecture formation, which is consistent with the role of RANKL.

RESULTS

PLCγ2 Deficiency Impairs Lymph Node Organogenesis—We measured up-regulation of NFATc1, nonadherent BM cells were cultured in complete α-MEM with 10% fetal bovine serum and murine M-CSF (10 ng/ml) for 3 days. The cells were cultured in serum-free medium with or without RANKL (100 ng/ml) or M-CSF (10 ng/ml) for 16 h. Nuclear extracts were prepared for gel mobility shift assays using [32P]-labeled probes containing AP-1 binding (purchased from Promega) or Oct-1 binding (purchased from Santa Cruz Biotechnology) sequences. For NF-κB, nonadherent BM cells were cultured in complete α-MEM with 10% fetal bovine serum and murine M-CSF (10 ng/ml) for 3 days. The cells were starved in serum-free medium for 18 h and then stimulated with RANKL (100 ng/ml) for the indicated times. Nuclear extracts were prepared for gel mobility shift assays using [32P]-labeled probes containing NF-κB (purchased from Promega) or Oct-1 binding sequences.

Bone Histomorphometry Analysis—Femurs were removed from the indicated mice and fixed in 10% formalin, decalcified in EDTA, and embedded in paraffin. Longitudinal sections (5 μm thick) were stained with hematoxylin and eosin. Bone histomorphometry analysis was performed as previously described (53) with Bioquant image Analysis Software (R & M Biometrics). Various bone parameters, including bone thickness, osteoid surface, and osteoblast and osteoclast numbers were determined. Goldner’s Trichrome staining was performed as previously described (53).

Statistical Analysis—Statistical analysis was performed as previously described (54). The differences between two groups were identified by Student’s t tests. For multiple groups, one-way analysis of variance and Student-Newman-Keuls tests were used to identify differences. Significance was defined as p < 0.05.

FIGURE 1. Lymph node organogenesis in PLCγ2-deficient mice. A, the absence of mesenteric lymph nodes in PLCγ2-deficient mice. Shown is a macroscopic view of mesenteric lymph nodes from wild-type (PLCγ2+/+) and PLCγ2-deficient (PLCγ2−/−) mice. Lymph nodes are indicated by the arrowheads. B, presence of Peyer’s patches in PLCγ2-deficient mice. Small intestines derived from wild-type and PLCγ2-deficient mice were sectioned and stained with hematoxylin and eosin to visualize morphology of Peyer’s patches. C, intact splenic architecture in PLCγ2−/− mice. Spleens derived from wild-type and PLCγ2-deficient mice were sectioned and stained with hematoxylin and eosin to visualize their architecture.
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PLCγ2 Deficiency Severely Impairs RANKL Signaling—To further determine whether PLCγ2 plays an important role in RANKL signaling, we examined the effect of PLCγ2 deficiency on RANKL signaling. BMMs were derived from wild-type and PLCγ2−/− mice. Both BMMs expressed comparable levels of myeloid cell lineage markers CD14 and Mac-1 (Fig. 2C). PLCγ2−/− BMMs also displayed normal levels of M-CSF receptor c-Fms relative to wild-type cells (Fig. 2C). Importantly, PLCγ2−/− BMMs exhibited normal levels of RANK relative to wild-type cells (Fig. 2C). Therefore, lack of PLCγ2 does not affect M-CSF-mediated BMM development or reduce RANK expression on these precursors.

Then we examined RANKL-mediated MAPK, such as ERK, p38, and JNK, activation in PLCγ2-deficient BMMs. Activation of ERK1 and ERK2 was evaluated by immunoblotting with antibodies that detect phosphorylation of pThr180/pTyr182 within p38 (65), was markedly reduced in PLCγ2−/− relative to wild-type BMMs (Fig. 3B). Furthermore, RANKL-mediated activation of JNK, which was measured by immunoblotting with antibodies detecting phosphorylation of pThr183/pTyr185 within JNK1/2 (66), was reduced in PLCγ2−/− relative to wild-type BMMs (Fig. 3C). Therefore, PLCγ2 deficiency specifically impairs RANKL-mediated activation of p38 and JNK but not ERK.

RANKL signaling eventually results in activation of transcription factors, including NFAT, NF-κB, and AP-1, which are important for its function (42–45). Thus, we examined the effect of PLCγ2 deficiency on RANKL-induced activation of NFAT, NF-κB, and AP-1. As expected, RANKL induced up-regulation of NFATc1 expression in wild-type BMMs (Fig. 3D), consistent with a previous study (42). In contrast, PLCγ2 deficiency impaired NFATc1 expression in PLCγ2−/− BMMs (Fig. 3E). Taken together, PLCγ2 deficiency impairs RANKL-mediated activation of NFATc1, NF-κB, and AP-1.

PLCγ2 Deficiency Severely Impairs RANKL-mediated Osteoclast Formation—Last, we examined the effect of PLCγ2 deficiency on RANKL-mediated biological function. In the presence of M-CSF, RANKL induced wild-type BMMs to differentiate into giant multinucleated and TRAP-positive osteoclasts, whereas PLCγ2−/− BMMs failed to become multinucleated osteoclasts, although some of them became TRAP-positive (Fig. 4A). Moreover, semiquantitative RT-PCR demonstrated that RANKL-induced expression of TRAP and other osteoclast-associated genes, such as CathK and CTR, was markedly impaired in PLCγ2−/− compared with wild-type BMMs (Fig. 4B). Of note, both wild-type and PLCγ2−/− BMMs expressed comparable levels of RANK before and after RANKL stimulation (Fig. 4B), consistent with the results from fluorescence-activated cell sorter analysis shown in Fig. 2C. Furthermore, the marked impairment of RANKL-induced expression of pThr180/pTyr182 within p38 (65), was markedly reduced in PLCγ2−/− relative to wild-type BMMs (Fig. 3B).
**PLCγ2 Mediates Lymph Node Organogenesis and Osteoclastogenesis**

![Diagram](image)

**FIGURE 3.** RANKL-mediated activation of MAPK family members ERK, p38, and JNK and transcription factors NFATc1, NF-κB, and AP-1 in PLCγ2-deficient BMMs. (A) RANKL-mediated activation of ERK1 and ERK2 in PLCγ2-deficient BMMs. BMMs were infected with a retrovirus, MSCV-PLCγ2-1-IRES-GFP, encoding PLCγ2. As controls, PLCγ2-/- BMMs transduced with a retrovirus, MSCV-IRES-GFP, encoding GFP alone served as a negative control. (B) RANKL-mediated activation of NF-κB in PLCγ2-deficient BMMs. (C) RANKL-mediated activation of JNK in PLCγ2-deficient BMMs. (D) RANKL-mediated activation of NFATc1 in PLCγ2-deficient BMMs. (E) RANKL-mediated activation of AP-1 in PLCγ2-deficient BMMs. (F) RANKL-mediated activation of M-CSF in PLCγ2-deficient BMMs.

**PLCγ2 but Not PLCγ1 Restores RANKL-mediated Biological Functions of PLCγ2-/- BMMs**—The impaired RANKL-mediated biological functions by PLCγ2 deficiency could be due to other genes that were affected during the targeted disruption of the PLCγ2 locus. To exclude this possibility, we assessed the ability of PLCγ2 to restore RANKL-induced differentiation of PLCγ2-/- BMMs. We employed a retrovirus-mediated gene transfer strategy (68, 69). PLCγ2-/- BMMs were infected *in vitro* with a retrovirus, MSCV-PLCγ2-1-IRES-GFP, encoding PLCγ2. As controls, PLCγ2-/- BMMs transduced with a retrovirus, MSCV-IRES-GFP, encoding GFP alone served as a negative control, whereas wild-type BMMs infected with MSCV-IRES-GFP served as a positive control. Subsequently, the retrovirally transduced BMMs were treated with RANKL in the absence of M-CSF. As expected, upon RANKL stimulation, GFP-transduced wild-type BMMs differentiated into osteoclasts, whereas GFP-transduced PLCγ2-/- BMMs failed to differentiate into osteoclasts (Fig. 5A). Importantly, PLCγ2-transduced PLCγ2-/- BMMs were able to differentiate into osteoclasts after RANKL treatment (Fig. 5A). As expected, PLCγ2 expression was restored in PLCγ2-transduced PLCγ2-/- BMMs (Fig. 5B). Thus, a lack of PLCγ2 directly disrupts RANKL-mediated biological functions.

**PLCγ1**, the other family member of PLCγ, might play a similar role as PLCγ2 in RANKL signaling. Although PLCγ1 is expressed in BMMs and its expression is not affected by PLCγ2 deficiency (Fig. 2A), it is possible that overexpression of PLCγ1 might compensate for PLCγ2 deficiency in RANKL-mediated osteoclastogenesis. To test this hypothesis, we assessed the ability of PLCγ1 to restore RANKL-mediated biological functions in PLCγ2-/- BMMs. PLCγ2-/- BMMs were infected *in vitro* with a retrovirus, MSCV-PLCγ1-1-IRES-GFP, encoding PLCγ1. PLCγ1-transduced PLCγ2-/- BMMs failed to differentiate into osteoclasts upon RANKL treatment (Fig. 5). Of note, the level of PLCγ1 expression was markedly increased in PLCγ1-deficient BMMs relative to GFP- or PLCγ2-transduced PLCγ2-/- BMMs (Fig. 5B). Thus, PLCγ2 plays an important and unique role which cannot be replaced by the highly homologous PLCγ1 in RANKL-mediated biological functions.

**DISCUSSION**

RANKL is an important cytokine for early lymphocyte development and lymph node organogenesis (29–32). The Ca^{2+}-dependent activation of NFAT and the PKC-dependent activation of NF-κB are indispensable for RANKL-mediated biological...
functions (42, 46). RANKL is a tumor necrosis factor family cytokine (29, 30), and thus, the mechanism by which RANKL activates the Ca\(^{2+}\)-dependent pathway has been a puzzle (42). The recruitment of the immunoreceptor tyrosine-based activation motif-containing adaptor proteins, DAP12 and FcR\(\gamma\), to the RANK receptor complex has been shown to play a role in RANKL-induced Ca\(^{2+}\)-flux (34–36). PLC\(\gamma\) is involved in signaling of the FcR\(\gamma\)-chain-containing collagen receptor (20) and the DAP12-associated NKG2D receptor (27, 28). Here we report that PLC\(\gamma\) plays a critical role in RANKL-induced activation of p38, JNK, AP-1, NF-\(\kappa\)B, and NFAT and in RANKL-mediated lymph node organogenesis and osteoclastogenesis. It is highly possible that immunoreceptor tyrosine-based activation motif-containing DAP12 and FcR\(\gamma\) may play a role in RANKL-induced activation of PLC\(\gamma\). Consistent with this notion, deficiency of DAP12 or FcR\(\gamma\) disrupts RANKL-induced signaling and RANKL-mediated biological functions such as osteoclastogenesis, a defect very similar to that observed in PLC\(\gamma\) mice (34–36, 70).

Although PLC\(\gamma\) deficiency severely impairs RANKL signaling and RANKL-mediated cell differentiation, PLC\(\gamma\) mouse exhibit markedly reduced osteoclasts that appear normal.

**Figure 4.** PLC\(\gamma\) deficiency severely impairs RANKL-mediated cell differentiation. A, RANKL-induced cell differentiation of PLC\(\gamma\)-deficient BMMs in vitro. BMMs derived from wild-type and PLC\(\gamma\)-deficient mice were cultured with RANKL and M-CSF for 6 days. The cells were stained with TRAP staining and visualized by light microscopy. B, RT-PCR analyses of RANKL-induced expression of differentiation associated genes in PLC\(\gamma\)-deficient BMMs. RANKL-stimulated BMMs from A were subjected to RT-PCR analyses of expression of the indicated genes. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. C, real-time RT-PCR analyses of RANKL-induced expression of differentiation associated genes in PLC\(\gamma\)-deficient BMMs. RANKL-stimulated BMMs from A were subjected to quantitative real-time RT-PCR analyses of expression of the indicated genes. The expression data of each gene were normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase expression. D, an increased bone mineral density of femurs derived from PLC\(\gamma\)-deficient mice. Femurs derived from wild-type (PLC\(\gamma\)) and PLC\(\gamma\)-deficient (PLC\(\gamma\)) mice were subjected to dual-energy x-ray absorptiometry analysis of bone mineral density. E, histology of the femurs from PLC\(\gamma\)-deficient mice by Goldner’s Trichrome and microcomputed tomography evaluation. Femurs derived from wild-type and PLC\(\gamma\)-deficient mice were subjected to Goldner’s Trichrome (upper) and microcomputed tomography evaluation (lower). The figures shown A, D, and E are representative of six pairs of age- and gender-matched wild-type and PLC\(\gamma\)-deficient littermates. The figures shown in B and C are representative of three independent analyses.
Organogenesis of lymph node or other secondary lymphoid organs, such as spleen and Peyer’s patch, is highly regulated by chemokines and cytokines (74, 75). For instance, the chemokine CXCL13 is required for secondary lymphoid organ development and deficiency of CXCL13, or its receptor CXCR5 severely impairs development of lymph node and Peyer’s patch and organization of splenic microarchitecture (76, 77). The tumor necrosis factor family of cytokines, such as lymphotakin (LTα) and LTβ, are also essential for secondary lymphoid organ development, and mice deficient in LTα or LTβ lack lymph node and Peyer’s patch have and have disorganized splenic microarchitecture (55, 56, 78, 79). Activation of transcription factor NF-κB seems to be a common pathway that controls organogenesis of lymph nodes, spleens, and Peyer’s patches. Mice deficient in 1κB kinase α or NF-κB-inducing kinase (NIK), important kinases in NF-κB activation, have severe defects in lymph nodes and Peyer’s patches (80, 81). However, organogenesis of lymph node also has its distinct requirement. For example, mice deficient in RANKL or and its receptor RANK lack lymph nodes but have normal Peyer’s patches and organization of splenic microarchitecture (32, 82). Interestingly, our current study has found that PLCγ2 plays an essential role in RANKL/RANK signaling, and its deficiency specifically blocks organogenesis of lymph node but not Peyer’s patch or spleen. Of note, LT-induced up-regulation of VCAM (vascular cell adhesion molecule), a NF-κB-dependent event (83), is normal in the absence of PLCγ2 (data not shown). Thus, RANKL/RANK mediates lymph node organogenesis through signaling molecule PLCγ2, whereas LTα/LTβ does not depend on PLCγ2 in regulating organogenesis of peripheral lymphoid organs.

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REFERENCES
1. Rhee, S. G., and Bae, Y. S. (1997) J. Biol. Chem. 272, 15045–15048
2. Rhee, S. G., and Choi, K. D. (1992) J. Biol. Chem. 267, 12393–12396
3. Lin, X., and Wang, D. (2004) Semin. Immunol. 16, 429–435
4. Thome, M. (2004) Nat. Rev. Immunol. 4, 348–359
5. Karin, M., and Ben-Neriah, Y. (2000) Annu. Rev. Immunol. 18, 621–663
6. Ghosh, S., May, M. J., and Kopp, E. B. (1998) Annu. Rev. Immunol. 16, 225–260
7. Baldwin, A. S., Jr. (1996) Annu. Rev. Immunol. 14, 649–683
8. Rao, A., Luo, C., and Hogan, P. G. (1997) Annu. Rev. Immunol. 15, 707–747
9. Chao, T. S., Foster, D. A., Rapp, U. R., and Rosner, M. R. (1994) Annu. Rev. Immunol. 12, 707–747
10. Hirasawa, N., Santini, F., and Beaven, M. A. (1995) J. Immunol. 154, 5391–5402
11. Roa, M., Paumet, F., Le Mao, J., David, B., and Blank, U. (1997) J. Immunol. 159, 2815–2823
12. Jiang, A., Craxton, A., Kuroasaki, T., and Clark, E. A. (1998) J. Exp. Med. 188, 1297–1306
13. Hashimoto, O., Okada, H., Jiang, A., Kuroasaki, M., Greenberg, S., Clark, E. A., and Kuroasaki, T. (1998) J. Exp. Med. 188, 1287–1295
14. Zhang, C., Hirasawa, N., and Beaven, M. A. (1997) J. Immunol. 158, 4968–4975
15. Kawakami, Y., Hartman, S. E., Holland, P. M., Cooper, J. A., and...
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70. Mao, D., Eppe, H., Uthgenannt, B., Novack, D. V., and Faccio, R. (2006) *J. Clin. Investig.* 116, 2869–2879
71. Ji, Q. S., Winnier, G. E., Niswender, K. D., Horstman, D., Wisdom, R., Magnuson, M. A., and Carpenter, G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 2999–3003
72. Matsuo, K., Galson, D. L., Zhao, C., Peng, L., Laplace, C., Wang, K. Z., Bachler, M. A., Amano, H., Aburatani, H., Ishikawa, H., and Wagner, E. F. (2004) *J. Biol. Chem.* 279, 26475–26480
73. Matsumoto, M., Kogawa, M., Wada, S., Takayanagi, H., Tsujimoto, M., Katayama, S., Hisatake, K., and Nogi, Y. (2004) *J. Biol. Chem.* 279, 45969–45979
74. Muller, G., and Lipp, M. (2003) *Curr. Opin. Immunol.* 15, 217–224
75. Forster, R., Mattis, A. E., Kremmer, E., Wolf, E., Brem, G., and Lipp, M. (1996) *Cell* 87, 1037–1047
76. Ansel, K. M., Ngo, V. N., Hyman, P. L., Luther, S. A., Forster, R., Sedgwick, J. D., Browning, J. L., Lipp, M., and Cyster, J. G. (2000) *Nature* 406, 309–314
77. Banks, T. A., Rouse, B. T., Kerley, M. K., Blair, P. J., Godfrey, V. L., Kuklin, N. A., Bouley, D. M., Thomas, J., Kanangat, S., and Mucenski, M. L. (1995) *J. Immunol.* 155, 1685–1693
78. Koni, P. A., Sacca, R., Lawton, P., Browning, J. L., Ruddle, N. H., and Flavell, R. A. (1997) *Immunol. 6*, 491–500
79. Miyawaki, S., Nakamura, Y., Suzuki, H., Koba, M., Yasumizu, R., Ikehara, S., and Shibata, Y. (1994) *Eur. J. Immunol.* 24, 429–434
80. Dougall, W. C., Glaccum, M., Charrier, K., Rohrbach, K., Brasel, K., De Smedt, T., Daro, E., Smith, J., Tometsko, M. E., Maliszewski, C. R., Armstrong, A., Shen, V., Bain, S., Cosman, D., Anderson, D., Morrisey, P. J., Peschon, J. J., and Schuh, J. (1999) *Genes Dev.* 13, 2412–2424
81. Dejardin, E., Droin, N. M., Delhase, M., Haas, E., Cao, Y., Makris, C., Li, Z. W., Karin, M., Ware, C. F., and Green, D. R. (2002) *Immunity* 17, 525–535
82. Dejardin, E., Droin, N. M., Delhase, M., Haas, E., Cao, Y., Makris, C., Li, Z. W., Karin, M., Ware, C. F., and Green, D. R. (2002) *Immunity* 17, 525–535