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Phospholipase Cγ2 (PLCγ2) Is Key Component in Dectin-2 Signaling Pathway, Mediating Anti-fungal Innate Immune Responses*[S]

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Background: Dectin-2 is involved in anti-fungal immunity. However, its signaling pathway is not fully characterized.
Results: We showed that PLCγ2 deficiency impairs Dectin-2-induced NF-κB and MAPK activation in response to fungal infection.
Conclusion: PLCγ2 is a key component in Dectin-2 signaling pathway, mediating immune responses against fungal infection.
Significance: These studies reveal a potential therapeutic target for fungal infection.

C-type lectin receptors (CLRs) such as Dectin-2 function as pattern recognition receptors to sense fungal infection. However, the signaling pathways induced by these receptors remain largely unknown. Previous studies suggest that the CLR-induced signaling pathway may utilize similar signaling components as the B cell receptor-induced signaling pathway. Phospholipase Cγ2 (PLCγ2) is a key component in B cell receptor signaling, but its role in other signaling pathways has not been fully characterized. Here, we show that PLCγ2 functions downstream of Dectin-2 in response to the stimulation by the hyphal form of *Candida albicans*, an opportunistic pathogenic fungus. Using PLCγ2- and PLCγ1-deficient macrophages, we found that the lack of PLCγ2, but not PLCγ1, impairs cytokine production in response to infection with *C. albicans*. PLCγ2 deficiency results in the defective activation of NF-κB and MAPK and a significantly reduced production of reactive oxygen species following fungal challenge. In addition, PLCγ2-deficient mice are defective in clearing *C. albicans* infection *in vivo*. Together, these findings demonstrate that PLCγ2 plays a critical role in CLR-induced signaling pathways, governing antifungal innate immune responses.

Invasive *Candida albicans* infection remains a serious clinical complication in patients with compromised immune systems. *C. albicans* is a dimorphic fungus, and its pathogenicity is dependent on both its yeast and filament-shaped hyphal forms, because mutants in either form are less virulent than wild-type strains (1, 2). The host defense against systemic *C. albicans* infection primarily depends on innate immune cells, especially macrophages and neutrophils (3). To initiate an antifungal defense, the innate immune cells have to contact the fungal cell wall, which is primarily composed of carbohydrates including β-glucans, mannans, and chitin (3). Recognition of the yeast *versus* the hyphal form of *C. albicans* may be mediated through the engagement of different receptors on macrophages (4, 5).

Previous studies suggest that C-type lectin receptors, including Dectin-1 and Dectin-2, function as the pattern recognition receptors for sensing *C. albicans* infection (5–11). In response to *C. albicans* infection, Dectin-1 recognizes the β-glucan in the cell wall of the yeast form of *C. albicans* (4, 12), whereas Dectin-2 has been suggested to interact with the mannoprotein coat in the cell wall of the hyphal form of *C. albicans* (5, 6). It has been shown that the β-glucan layer in the *C. albicans* cell wall is usually buried by the mannoprotein coat in the yeast form but exposed when they transform into the hyphae under infection conditions (13), which may interact with Dectin-1 and induce a strong immune response during an infection (14). However, recent studies on Dectin-1-deficient mice suggest that there may be other redundant receptors mediating *C. albicans* infection-induced immune responses (15, 16). In contrast, studies on Dectin-2-deficient mice suggest that Dectin-2 may play a more important role for the innate immune response against *C. albicans* infection (16). Therefore, the signal transduction pathway induced by Dectin-2 is not fully characterized, and it remains to be determined whether Dectin-1 and Dectin-2 receptors share the same signaling pathway.

Although the signaling pathways induced by Dectin-2 following *C. albicans* infection are not fully defined, previous studies indicate that stimulation of Dectin-2 as well as Dectin-1 by the cell wall components of *C. albicans* can activate the spleen tyrosine kinase (Syk) (10, 17, 18). The activation of Syk leads to

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activation of multiple signaling cascades (9, 11), which induces NF-κB activation through a CARD9-dependent pathway (17, 19). Syk is a key component in the B cell receptor signaling pathway. However, whether other components in B cell receptor signaling pathway are also involved in Dectin-2 signaling pathway remains to be determined.

PLCγ hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate (20). In hematopoietic cells, two isoforms of PLCγ, PLCγ1 and PLCγ2, are expressed. PLCγ2 is a key component in B cell receptor signaling pathway (21), whereas PLCγ1 is the main effector in T cell receptor signaling (22). In dendritic cells, both PLCγ1 and PLCγ2 can be activated by the stimulation of β-glucan, zymosan, and curdlan, extracted from the cell wall of yeast (23, 24). Previous studies suggest that PLCγ1 functions downstream of Syk in the FcεR signaling pathway (25), whereas Syk controls PLCγ2 in the B cell receptor signaling pathway (21). Recent studies suggest that PLCγ2 is also involved in Dectin-1 signaling (23, 24). However, whether PLCγ1 and PLCγ2 are involved in Dectin-2 signaling pathways in response to *C. albicans* infection remains unknown. In this study, we demonstrated that PLCγ2 functions downstream of Dectin-2 receptor in response to fungal infection. Using PLCγ1- and PLCγ2-deficient mice, we show that PLCγ2 but not PLCγ1 plays an essential role in the immune responses to *C. albicans* infection by inducing expression of cytokines and generating reactive oxygen species.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Antibodies against phospho-p38, p38, phospho-ERK, phospho-IKKα/β (Ser-176/180) (2697), phospho-AKT(473), phospho-Syk, Syk, PLCγ2, and phospho-PLCγ2 (Tyr-759) were purchased from Cell Signaling Technology; antibodies against ERK (sc-154), IKKβ, Bcl10, and IκBα were from Santa Cruz Biotechnology; and CARD9 antibody was described previously (26). Bapta-AM was purchased from Calbiochem. GF109203X was purchased from Sigma-Aldrich. Fluorescence-conjugated monoclonal antibodies CD11b and F4/80 were purchased from BD Pharmingen or eBioscience. TNF-α, IL-10, IL-6, and IL-12p40 ELISA Ready-SET-GO kits were purchased from eBioscience.

Mice—PLCg2$^{-/-}$, *CARD9*$^{-/-}$, and *PLCg1*-floxed allele mice were generated as described previously (21, 22, 26). All mice have been backcrossed to C57BL6 background. PLCγ2- and PLCγ1-deficient mice were maintained in the Biological Resource Center at the Medical College of Wisconsin. CARD9-deficient mice were housed under specific pathogen-free conditions at the M. D. Anderson Cancer Center animal facility and were used at 8–16 weeks of age. Bone marrow cells from *Myd88*$^{-/-}$ and *Dectin-1*$^{-/-}$ mice were kindly provided by Dr. Shizuo Akira (Department of Host Defense, Research Institute for Microbial Diseases, Osaka University) and Dr. David Underhill (University of California at Los Angeles), respectively. All of the animal experiments were performed in compliance with the institutional guidelines and according to the protocol approved by the institutional animal use and care committees of the Medical College of Wisconsin and M. D. Anderson Cancer Center. To generate *PLCg1*$^{f/f}$/Mx1Cre chimera mice, *PLCg1*-Floxed (*PLCg1*$^{f/f}$) mice were bred with Mx1Cre mice (Jackson Laboratory stock 005673). For experiments, *PLCg1*$^{+/+}$Mx1Cre mice and *PLCg1*$^{f/f}$Mx1Cre mice received intraperitoneal injections of 300 μg of poly(I·C) on days 1 and 3. The mice were sacrificed 14 days after the first injection. Bone marrow cells from these mice were harvested and injected into C57B6 wild-type mice irradiated with 11 gray (Gy). For *PLCg2*$^{-/-}$ chimera mice, similar procedures were performed. Two months after bone marrow transplantation, chimera mice were used for yeast infection experiments.

Bone Marrow-derived Macrophage Preparation—Primary cultures of bone marrow-derived macrophages (BMDMs)[2] were prepared as described previously (26–28). Briefly, bone marrow cells were harvested from the femurs and tibias of mice. Erythrocytes were removed from cells samples by subjecting the samples to hypotonic solution. The cells were cultured for 7 days in DMEM containing 20% FBS, 55 μM β-mercaptoethanol, streptomycin (100 μg/ml), penicillin (100 units/ml), and 30% conditioned medium from L929 cells expressing macrophage colony-stimulating factor. Nonadherent cells were removed, and cells were passed every 3 days. After 1 week of cultures, flow cytometry analysis indicated that the harvested cell population contained 86–95% CD11b^{+} F4/80^{+} cells as assessed.

Lentivirus-encoded shRNA Knockdown of Dectin-2 in BMDMs—Lentiviral particles used for infection were prepared as described previously (28) using shRNA for mouse Dectin-2 (Sigma-Aldrich) or a nontargeting sequence. Mouse bone marrow cells were infected with virus on days 1 and 3 and selected with 2 μg/ml puromycin on day 5 of culture.

C. albicans Preparation—C. albicans (strain SC5314) was kindly provided by Dr. Michael C. Lorenz (Department of Microbiology and Molecular Genetics, University of Texas Medical School at Houston). A single colony of *C. albicans* was grown overnight at 30 °C in yeast peptone dextrose medium. The cells were washed three times with PBS and then used as live yeast. For hyphae, the washed yeast cells were resuspended in RPMI with 10% FCS, grown at 37 °C for 3 h, and washed in PBS. The hyphae were then used for live stimulations. For heat-inactivated yeast, yeast cells were heated at 65 °C for 1 h.

In Vivo C. albicans Infection—For *in vivo C. albicans* infection, male mice aged at 8–14 weeks were injected with 2×10^5 or 1×10^6 live *C. albicans* in 0.3 ml of PBS (pH 7.4). Candidal burden was determined by killing the mice 42 h after infection and harvesting and homogenizing their kidneys, lungs, livers, and spleens. Fungal colony formation units were quantified by plating serial dilutions of the homogenized organs on yeast extract peptone dextrose agar. For histological analysis, the tissue sections were cut and stained for glycogen using periodic acid-Schiff or hematoxylin and eosin.

Reactive Oxygen Species (ROS) Production Assay—For ROS production evaluation, 1×10^5 BMDMs were washed with Hanks' balanced salt solution without phenol red. Then Hanks' balanced salt solution containing 100 μM luminol and 5 units of horseradish peroxidase (Sigma) were added to the cells, and the

[2] The abbreviations used are: BMDM, bone marrow-derived macrophage; ROS, reactive oxygen species; MOI, multiplicity of infection; BAPTA-AM, 1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester).

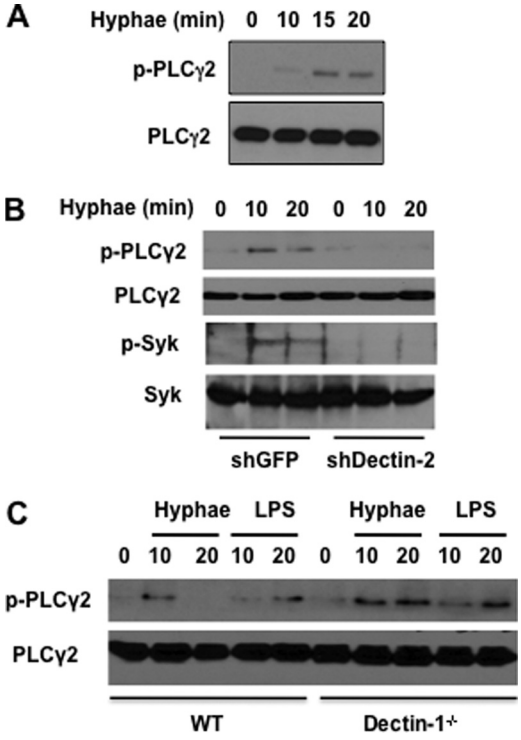

FIGURE 1. **Dectin-2 is required for *C. albicans*-induced PLCγ2 activation.** *A*, wild-type macrophages were stimulated with *C. albicans* hyphae (MOI = 1), and the phosphorylation of PLCγ2 was examined by Western blotting. *B*, BMDMs were infected with lentivirus encoding Dectin-2 shRNA or GFP shRNA, then selected with puromycin, and stimulated on day 9 with *C. albicans* hyphae (MOI = 1) at different times. Cell lysates were subjected to Western blotting analysis using indicated antibodies. *C*, WT and Dectin-1$^{-/-}$ BMDMs were stimulated with *C. albicans* hyphae (MOI = 1) or LPS (100 ng/ml) for the indicated times. Cell lysates were subjected to Western blotting analysis using the indicated antibodies.

cells were incubated for 10 min at 37 °C. The cells were then stimulated with either hyphae (multiplicity of infection (MOI) = 2) or phorbol myristate acetate. ROS production was measured every 3 min using a luminometer.

EMSA—After BMDMs were stimulated with hyphae or yeast, their nuclear extracts were prepared. The resulted nuclear protein (5 μg) was incubated with ^{32}P-labeled NF-κB or Oct-1 probe (Promega) for 15 min at 25 °C and then subjected to PAGE and exposed to x-ray films.

RESULTS

PLCγ2 Is Involved in Dectin-2-induced Signaling Pathway—To determine whether *C. albicans* can activate PLCγ2 through Dectin-2 receptor in macrophages, we examined PLCγ2 phosphorylation in BMDMs after infecting them with *C. albicans* hyphae. Stimulation of BMDMs with *C. albicans* led to an inducible phosphorylation of PLCγ2 (Fig. 1*A*), indicating that PLCγ2 is activated in response to *C. albicans* infection. Because both Dectin-1 and Dectin-2 have been shown to be involved in antifungal innate immune response, we determined whether Dectin-1 and Dectin-2 are required for *C. albicans* hyphae-induced PLCγ2 activation using BMDMs treated with a specific shRNA to Dectin-2 or BMDMs from Dectin-1 knock-out mice. Consistent with previous findings that Dectin-2 but not Dectin-1 is involved in *C. albicans* hyphae-induced signal transduction, we found that PLCγ2 activation was dependent on Dectin-2 but not Dectin-1 (Fig. 1, *B* and *C*). These results indicate that although *C. albicans* hyphae may activate multiple receptors on macrophages, only Dectin-2 leads to activation of PLCγ2.

PLCγ2 Is Required for C. albicans Infection-induced NF-κB Activation—We next sought to identify the PLCγ2-dependent signaling pathways in macrophages and determine whether these signaling pathways are redundant in response to the hyphal and yeast forms of *C. albicans*. Previous studies indicate that heat-inactivated yeast of *C. albicans* can activate Dectin-1 signaling pathway, whereas hyphae of *C. albicans* induces Dectin-2 pathway. Therefore, we examined the contribution of PLCγ2 in NF-κB activation induced by these signaling pathways and found that NF-κB activation induced by hyphae was defective in PLCγ2-deficient macrophages (Fig. 2*A*), indicating that PLCγ2 is a key component in Dectin-2 signaling pathway. Consistent with recent studies (23, 24), PLCγ2-deficient macrophages were also defective in NF-κB activation induced by the stimulation of heat-inactivated yeast (Fig. 2*B*) that activates Dectin-1 signaling pathway. Together, these data indicate that PLCγ2 functions downstream of both Dectin-1 and Dectin-2 receptors and mediates NF-κB activation induced by these receptors.

Earlier studies suggest that TLR2 functionally cooperates with Dectin-1 to regulate cytokine production following fungal infection (29, 30). Because TLR2 can potently activate NF-κB through a MyD88-dependent pathway, we examined whether the MyD88-dependent pathway might also be involved in fungal infection-induced NF-κB activation. However, we have found that, unlike PLCγ2 deficiency, MyD88 deficiency does not affect *C. albicans* hyphae- and yeast-induced NF-κB activation (Fig. 2, *C* and *D*). Therefore, our data suggest that Dectin-1- and Dectin-2-induced NF-κB activation are mediated through a PLCγ2-dependent but TLR/MyD88-independent pathway.

The regulation of the IKK complex in response to hyphae is likely mediated through at least two signaling events, in which a Syk-dependent pathway regulates the phosphorylation of IKKα/β, whereas the adaptor protein CARD9 regulates the ubiquitination of IKKγ in a Syk-independent manner (28). We next examined whether PLCγ2 regulates the phosphorylation of IKKα/β and found that the signal-induced phosphorylation of IKKα/β was defective in PLCγ2$^{-/-}$ but not in Card9$^{-/-}$ macrophages in response to hyphae stimulation (Fig. 2*E*).

Because CARD9 forms a complex with Bcl10 following hyphae stimulation, we investigated whether the formation of this complex would be affected in the absence of PLCγ2. CARD9 was immunoprecipitated from WT and PLCγ2$^{-/-}$ macrophages that were stimulated with or without hyphae, and then the association of CARD9 with Bcl10 was examined. Interestingly, we found that PLCγ2 deficiency did not significantly affect the formation of the CARD9-Bcl10 complex in response to the stimulation of *C. albicans* hyphae (Fig. 2*F*). These results suggest that PLCγ2 is a critical enzyme downstream of Syk and regulates the IKK complex in a CARD9-independent manner.

MAPK Signaling Is Defective in PLCγ2-deficient Macrophages—Previous studies show that stimulation of bone marrow-derived dendritic cells by *C. albicans* induces tyrosine phosphorylation of MAPKs (17). Therefore, we examined

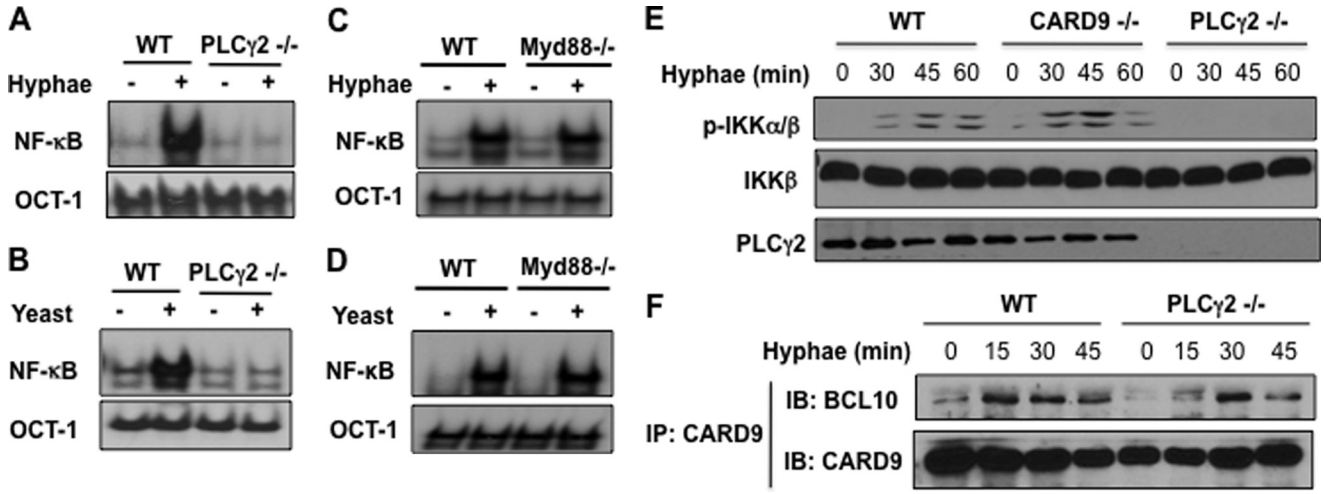

FIGURE 2. **NF-κB activation induced by *C. albicans* hyphae and yeast is PLCγ2-dependent.** *A* and *B*, WT and PLCγ2-deficient BMDMs were either untreated or treated with *C. albicans* hyphae (MOI = 1) (*A*) or heat-inactivated yeast (MOI = 5) (*B*) for 90 min. The nuclear extracts were prepared from these cells and then subjected to the electrophoretic mobility shift assay using ^{32}P-labeled NF-κB or OCT-1 probe. *C* and *D*, WT and Myd88$^{-/-}$ BMDMs were stimulated with *C. albicans* hyphae (MOI = 1) (*C*) or heat-inactivated yeast (MOI = 5) (*D*) for 90 min. The nuclear extracts were subjected to EMSA using ^{32}P-labeled Oct-1 or NF-κB probe. *E*, WT, *CARD9*$^{-/-}$, and *PLCg2*$^{-/-}$ BMDMs were stimulated with *C. albicans* hyphae for the indicated time points, and IKKα/β phosphorylation was examined using anti-phospho-IKKα (Ser-176)/IKKβ (Ser-180) antibody. *F*, WT and PLCγ2-deficient BMDMs were stimulated with *C. albicans* hyphae (MOI: 1) for the indicated time points, and the cell lysates were immunoprecipitated with CARD9 antibody-conjugated agarose. The immunoprecipitates were probed with BCL10 and CARD9 antibodies. *IP*, immunoprecipitation; *IB*, immunoblot.

whether MAPK activation is dependent on PLCγ2 following fungal infection. We found that the activation of ERK and JNK was completely defective in PLCγ2$^{-/-}$ BMDMs following the stimulation by hyphae (Fig. 3*A*), whereas the activation of p38 and AKT in WT and PLCγ2$^{-/-}$ BMDMs was comparable (Fig. 3*A*). Although *C. albicans* yeast could also induce ERK and JNK activation in WT cells, ERK activation was only partially defective in PLCγ2$^{-/-}$ BMDMs, whereas JNK activation was significantly decreased (Fig. 3*B*). These results indicate that the activation of ERK and JNK in response to *C. albicans* hyphae is solely dependent on PLCγ2, whereas *C. albicans* yeast may induce ERK activation through two pathways, one of which is dependent on PLCγ2, whereas the other is PLCγ2-independent.

Because PLCγ1, a homolog of PLCγ2, is highly expressed in macrophages, we would like to investigate whether PLCγ1 plays a role in antifungal innate immunity. To determine whether PLCγ1 is also involved in this fungal infection-induced signaling pathway, we obtained BMDMs from PLCγ1-conditional knock-out (PLCγ1$^{F/F}$/MxCre) or its control (PLCγ1$^{F/+}$/MxCre) mice and examined the MAPK activation following stimulation with *C. albicans*. Unlike PLCγ2-deficient cells, PLCγ1-deficient cells are not defective in *C. albicans* hyphae-induced ERK and IKK activation (Fig. 3*C*). These data indicate that PLCγ1 is not involved in Dectin-2 signaling pathway to induce anti-fungal innate immune responses.

PLCγ2 but Not PLCγ1 Is Required for C. albicans-induced Cytokine Production—To determine the functional requirement of PLCγ2 in antifungal innate immune responses, we compared the levels of cytokine production in PLCγ2$^{-/-}$ macrophages with those in WT macrophages in response to the hyphal (Fig. 4, *A–D*) or yeast (Fig. 4, *E–H*) forms of *C. albicans*. We found that the production of TNFα, IL-10, IL-6, and IL-12p40 in PLCγ2-deficient BMDMs was significantly lower than that in WT BMDMs (Fig. 4). Because PLCγ1 is also expressed in macrophages and is activated in DCs following the stimulation by zymosan, the extract of yeast cell walls (10), we decided to examine the role of PLCγ1 in cytokine production in BMDMs in response to the stimulation with *C. albicans*. We found that PLCγ1 deficiency did not affect cytokine production in BMDMs following the stimulation with *C. albicans* (Fig. 5). Together, these data indicate that PLCγ2 but not PLCγ1 plays an essential role in antifungal immune response in macrophages.

PLCγ2 Mediates ROS Production in Macrophages in Response to C. albicans Hyphae—ROS production in phagocytes in response to *C. albicans* is important in antifungal host responses. In macrophages, the production of ROS in response to zymosan has been shown to be Syk-dependent (18). Aside from Syk, the signaling components that are crucial to NADPH oxidase assembly in response to *C. albicans* stimulation remain to be identified. Because PLCγ enzymes were shown to link integrin-mediated adhesion to NADPH oxidase activation in neutrophils (31), we decided to examine whether PLCγ2 is involved in ROS production in response to *C. albicans* infection. The hyphal form of *C. albicans* induced robust ROS production in WT BMDMs but not in PLCγ2$^{-/-}$ BMDMs (Fig. 6*A*), whereas phorbol myristate acetate could effectively induce ROS production in both WT and PLCγ2$^{-/-}$ cells (Fig. 6*B*). In contrast to PLCγ2$^{-/-}$ macrophages, WT and PLCγ1$^{-/-}$ BMDMs showed no significant difference in ROS production following hyphae stimulation (Fig. 6*C*). These data indicate that PLCγ2 but not PLCγ1 is an essential signaling component for ROS production in response to *C. albicans* hyphae and that loss of PLCγ2, therefore, impairs the initial step of fungal killing in macrophages and increases host susceptibility to *C. albicans* infection.

Because activation of PLCγ2 induces diacylglycerol and inositol 1,4,5-trisphosphate, which activate PKC and increase the level of intracellular calcium, we next examined

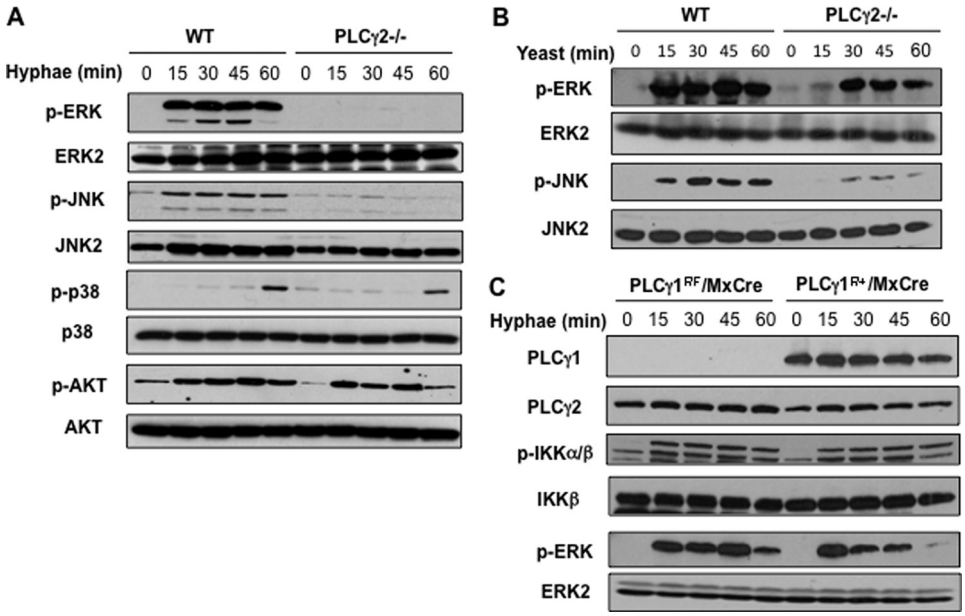

FIGURE 3. **PLCγ2-deficient BMDMs display defective MAPK activation.** *A* and *B*, wild-type and PLCγ2-deficient (PLCγ2$^{-/-}$) BMDMs were stimulated with *C. albicans* hyphae (MOI = 1) (*A*) or yeast (MOI = 5) (*B*) for the indicated time points. The cell lysates were prepared from these cells and then subjected to immunoblotting analysis using the indicated antibodies. *C*, BMDMs from MxCre/*PLCg1*$^{fl/+}$ and MxCre/*PLCg1*$^{fl/fl}$ mice were stimulated with *C. albicans* hyphae for the indicated time points. Cell lysates were prepared from these cells and then subjected to Western blotting. The activation of ERK and IKK was examined by using the indicated antibodies. The data shown are a representative of three independent experiments.

how PLCγ2 is involved in the generation of ROS. Using the PKC inhibitor GF109203X and the calcium chelator Bapta-AM, we found that hyphae-induced ROS generation primarily depends on PKC activation, because treatment with PKC inhibitor significantly blocked ROS generation, whereas Bapta-AM only caused a modest decrease in ROS generation (supplemental Fig. S1).

PLCγ2 Controls Antifungal Immunity in Vivo—To evaluate the role of PLCγ2 in anti-fungal immunity *in vivo*, we sought to determine whether PLCγ2-deficient mice were highly susceptible to fungal infection. To avoid the influence of PLCγ2 deficiency in other cell types, we generated PLCγ2-deficient bone marrow chimera (PLCγ2-deficient chimera) by reconstituting γ-irradiated wild-type mice with the bone marrow from PLCγ2-deficient mice and control mice with the bone marrow from WT mice. We then intravenously injected *C. albicans* into control or PLCγ2-deficient chimera mice. High dose (1×10^6) injection of *C. albicans* resulted in the death of PLCγ2-deficient mice but not WT control mice within 24 h (data not shown). Given a low dose (2×10^5) injection of *C. albicans*, PLCγ2-deficient chimera mice were dead within 48 h, but WT mice survived for more than 5 days (Fig. 7*A*). Cytokine (IL-6) levels in the sera from PLCγ2-deficient mice were significantly decreased in response to *C. albicans* infection (Fig. 7*B*). To provide the quantitative assessment of *C. albicans* burdens, we sacrificed some of these mice 42 h after infection. The kidneys, lungs, livers, and spleens from these mice were collected. Some of these organs were homogenized, and the serial dilutions of the homogenized organs were plated on yeast extract peptone dextrose agar, and fungal colonies grown on these plates were counted and plotted (Fig. 7*C*). To visualize *C. albicans* in these organs, the tissue sections from some collected organs were stained with the periodic acid-Schiff. Compared with WT mice, PLCγ2-deficient mice had substantially higher levels of germinating hyphal *C. albicans* in the kidneys, lung, spleen, and liver (supplemental Fig. S2). Together, these results demonstrate that PLCγ2 plays an essential role in antifungal innate immune response *in vivo*.

DISCUSSION

Recent work has highlighted the role of C-type lectin receptors, Dectin-1 and Dectin-2, as pattern recognition receptors for fungal infections. So far, Syk and CARD9 are the few known signaling components downstream of Dectin-1 and Dectin-2 receptors, which initiate signal transduction cascades in response to fungal infection. In this study, we demonstrate that PLCγ2 but not PLCγ1 is an essential signaling component in the Dectin-2 signaling pathway and mediates NF-κB and MAPK activation, leading to antifungal innate immune responses. Therefore, PLCγ2-deficient mice are highly susceptible to *C. albicans* infection.

Previous studies show that TLR signaling may collaborate with Dectin-1 signaling to induce the inflammatory response (29, 30), in which they have found that cytokine expression induced by zymosan, the β-glucan component extracted from yeast cell wall, is dependent on TLR2 and MyD88 (29, 30). These studies suggest that TLR signaling may be also involved in anti-fungal inflammation. However, in this study, we have found that the stimulation by both *C. albicans* hyphae and heat-inactivated yeast can effectively activate NF-κB in MyD88-deficient cells but is defective in PLCγ2-deficient cells, indicating that NF-κB activation induced by both Dectin-1 and Dectin-2 receptors is TLR/MyD88-independent but PLCγ2-dependent. Therefore, our data indicate that previous results obtained by using zymosan are signifi-

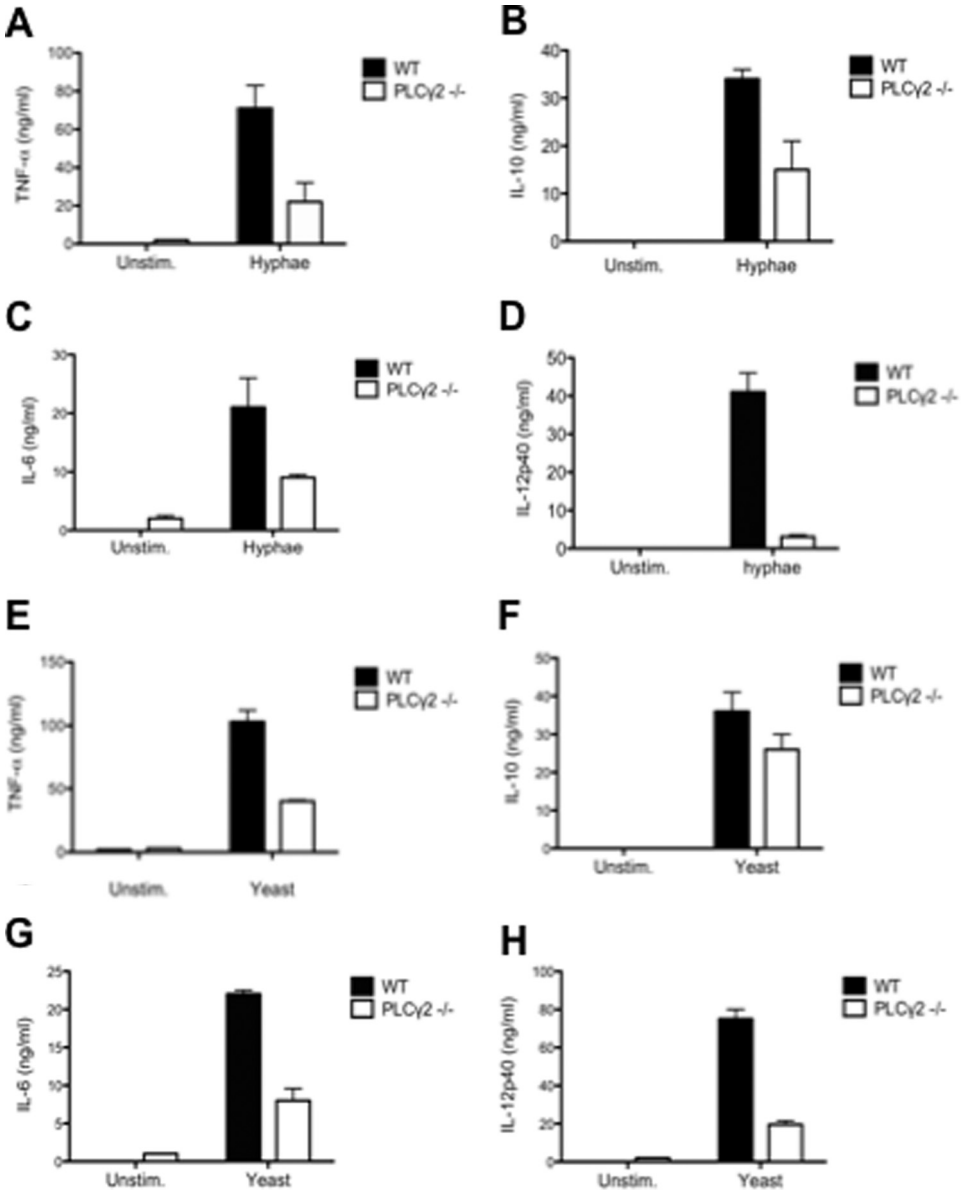

FIGURE 4. **PLCγ2 contributes to cytokine induction by *C. albicans* stimulation.** BMDMs from WT (*black bars*) and PLCγ2-deficient (PLCγ2$^{-/-}$, *white bars*) mice were stimulated overnight with *C. albicans* hyphae (MOI = 1) (*A–D*) or heat-killed yeast (MOI = 5) (*E–H*). ELISA was used to measure the level of cytokines in these cultured media. The data are the means ± S.D. of triplicate wells and are representative of three independent experiments.

cantly different from the results obtained by using the fungal organism itself.

In the classical NF-κB pathway, the activation of IKK complex requires the signal-induced phosphorylation of IKKα/β subunits, in addition to the K63-linked ubiquitination of the regulatory subunit IKKγ/NEMO for the degradation of the inhibitory IκBα proteins and the subsequent translocation of NF-κB into the nucleus (32, 33). Recent work from our lab demonstrated that Syk and the adaptor protein CARD9 cooperate in activating the IKK complex with CARD9 mediating IKK ubiquitination, whereas Syk is necessary for the phosphorylation of IKKα/β subunits (28). In the current study, we find that PLCγ2$^{-/-}$ macrophages are defective in the activation of IKKα/β, whereas Card9$^{-/-}$ macrophages are not. In addition, PLCγ2 deficiency did not alter the formation of the CARD9-BCL10 complex. These findings suggest that although PLCγ2 and CARD9 are both required for NF-κB activation in response to *C. albicans* (34), they mediate their signaling in an independent manner. However, it remains to be determined which signaling components are required for linking CARD9 to the upstream signaling cascade.

The mechanism that couples ERK activation to upstream signaling in *Candida*-induced responses is not well understood. We find that ERK activation in response to hyphae is completely abrogated, whereas ERK activation in response to yeast is partially defective in PLCγ2-deficient cells. This result suggests that the ERK activation induced by hyphal form of *C. albicans* is through a PLCγ2-dependent pathway, whereas yeast form of *C. abicans* may stimulate both PLCγ2-dependent and -independent pathways leading to ERK activation. Because PLCγ2 is downstream of Syk, our results are

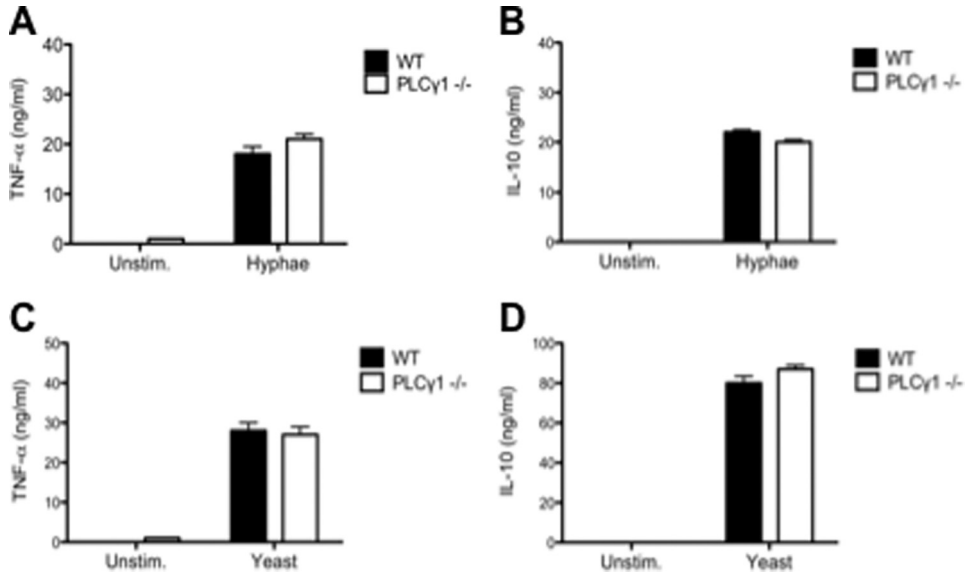

FIGURE 5. **PLCγ1 is not required for cytokine production by *C. albicans* yeast and hyphae.** BMDMs from MxCre/*PLCg1*$^{fl/+}$ mice (WT, *black bars*) and MxCre/*PLCg1*$^{fl/fl}$ mice (PLCγ1$^{-/-}$, *white bars*) were stimulated overnight with hyphae (MOI = 1) (*A* and *B*) or heat-killed *C. albicans* yeast (MOI = 5) (*C* and *D*). ELISA was used to measure the level of cytokines in these cultured media. The data are the means ± S.D. of triplicate wells and are representative of three independent experiments.

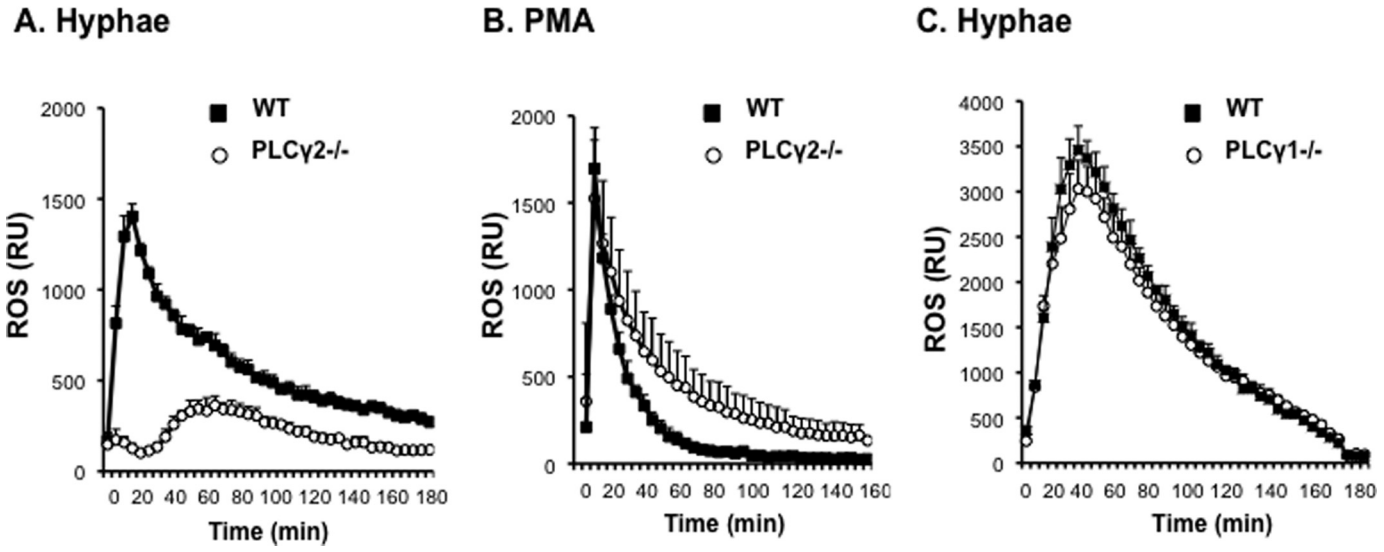

FIGURE 6. **PLCγ2 is required for ROS production in response to *C. albicans* hyphae stimulation.** *A* and *B*, ROS production in WT (*black squares*) or PLCγ2-deficient (*white circles*) BMDMs was measured by using a luminometer following the stimulation with *C. albicans* hyphae (*A*, MOI = 2) or phorbol myristate acetate (*B*, 100 μM). *C*, ROS production in MxCre/*PLCg1*$^{+/+}$ (WT, *black squares*) and MxCre/*PLCg1*$^{fl/fl}$ (PLCγ1$^{-/-}$, *white circles*) BMDMs was measured following the stimulation with hyphae (MOI = 2). The data are the means ± S.D. and are representative of at least three independent experiments. *RU*, relative units.

consistent with an earlier observation that zymosan-induced ERK activation is dependent on Syk in macrophages and DCs (34). However, a recent study using human macrophages suggests that zymosan-induced ERK activation is mediated through a PLCγ2-independent, but a Syk-CaMK-Pyk2-dependent pathway (35). These different results may be due to differences between using zymosan *versus* the fungal organism or to species-specific differences between human and mouse macrophages.

It is well established that the Grb2-SOS-Ras-Raf1 signaling cascade leads to a potent ERK activation. Upon receptor engagement, SOS, a Ras guanine nucleotide exchange factor, is recruited to the membrane by the adaptor protein Grb2 leading to Ras activation (36, 37). Activated Ras can then activate the Raf-1 kinase, which will ultimately activate ERK1 and ERK2 kinases (38, 39). Thus, it is possible that *C. albicans* yeast, but not hyphae, may also activate the Grb2/SOS/Ras/Raf1 signaling cascade, which may explain the partial defect of ERK activation. We will test this hypothesis in our future studies.

In contrast to ERK activation, JNK activation is significantly reduced in PLCγ2-deficient cells following the stimulation by both yeast and hyphae, indicating that JNK is activated through the same PLCγ2-dependent pathway by the stimulation of yeast and hyphae. Because our preliminary studies found that calcium chelator Bapta-AM and PKC inhibitor could not block

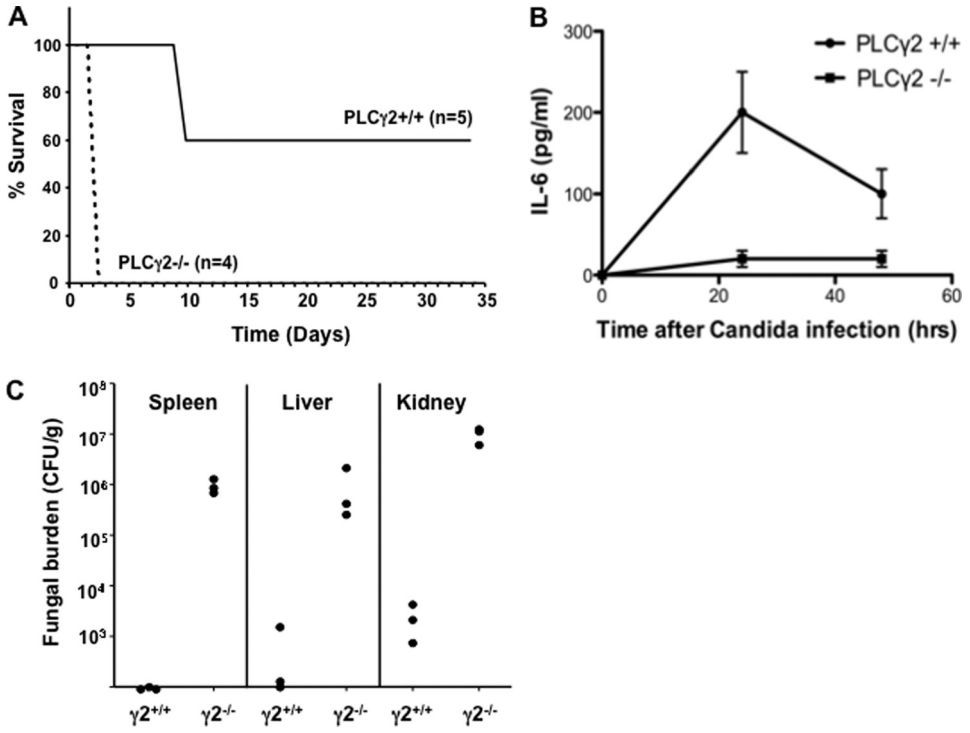

FIGURE 7. **PLCγ2-deficient mice show higher susceptibility to *C. albicans* infection than WT mice.** *A*, WT and PLCγ2-deficient mice were challenged with *C. albicans* (2×10^5) intravenously and monitored every day for lethality. *B*, WT and PLCγ2-deficient mice ($n = 3$) were challenged with *C. albicans* (2×10^5) intravenously, and serum samples from each mouse were tested for IL-6 levels by ELISA. The data are the means ± S.D. from triplicate samples. *C*, 42 h after intravenous infection with *C. albicans* (2×10^5 cells), some of the infected mice were sacrificed. The liver, kidney, lung, and spleens from WT and PLCγ2-deficient mice ($n = 3$) were homogenized, and serial dilutions of the homogenized organs were plated on yeast extract peptone dextrose agar plates to determine the yeast colony formation unit (*CFU*). The data are colony formation unit/organ weight. The data are represented as CFU/organ weight. Similar results were obtained in two repeated experiments.

the JNK activation in response to fungal infection (data not shown), it suggests that JNK is activated through an unknown pathway that is independent of PKC and calcium. Future studies are needed to reveal this signaling cascade following fungal infection.

The phagocytosis of β-glucans in macrophages can activate the production of ROS (4). Although Syk is not involved in controlling phagocytosis, it is required for ROS production following fungal infection (18). However, CARD9, another known component in the C-type lectin pathway, is only partially required for fungal infection-induced ROS production (27). Therefore, the mechanism by which fungal infection triggers ROS production remains largely unknown. In the current study, we provide evidence that PLCγ2 but not PLCγ1 is necessary for ROS production in macrophages in response to stimulation with *C. albicans*. Although it remains to be determined how PLCγ2 links to ROS production, our data suggest that PLCγ2-dependent PKC activation is a critical link, because PKC has been shown to regulate ROS production, and inhibition of PKC significantly blocks ROS production following fungal infection (supplemental Fig. S1). Future studies will determine which isoform(s) of PKC are involved in fungal infection-induced ROS production. In addition, ROS production in DCs has been shown to be required for *C. albicans*-induced inflammasome activation (40). Therefore, our findings suggest a possible role for PLCγ2 in Nlrp3 inflammasome activation. This hypothesis will be tested in the future studies. In conclusion, we demonstrate that PLCγ2 is a critical component of Dectin-2 signaling and mediates anti-fungal innate immune responses.

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