Dectin-1 is a C-type lectin that recognizes β-glucan in the cell walls of fungi and plays an important role in anti-fungal immunity. It signals via tyrosine kinase Syk and adaptor protein Card9 to activate NF-κB leading to proinflammatory cytokine production in dendritic cells (DCs). Other than this, not much else is known of the mechanism of Dectin-1 signaling. We demonstrate here that stimulation of DCs with zymosan triggers an intracellular Ca$^{2+}$ flux that can be attenuated by a blocking anti-Dectin-1 antibody or by pre-treatment of cells with the phospholipase C (PLC) γ-inhibitor U73122, suggesting that Dectin-1 signals via a PLCγ pathway to induce Ca$^{2+}$ flux in DCs. Interestingly, treatment of DCs with particulate curdlan, which specifically engages Dectin-1, results in the phosphorylation of both PLCγ1 and PLCγ2. However, we show that PLCγ2 is the critical enzyme for Dectin-1 signaling in DCs. PLCγ2-deficient DCs have drastic impairment of Ca$^{2+}$ signaling and are defective in their secretion of interleukin 2 (IL-2), IL-6, IL-10, IL-12, IL-23, and tumor necrosis factor α. PLCγ2-deficient DCs also exhibit impaired activation of ERK and JNK MAPKs and AP-1 and NFAT transcription factors in response to Dectin-1 stimulation. In addition, PLCγ2-deficient DCs are also impaired in their activation of NF-κB upon Dectin-1 engagement due to defective assembly of the Card9-Bcl10-Malt1 complex and impaired IKKα/β activation and IkBα degradation. Thus, our data indicate that pattern recognition receptors such as Dectin-1 could elicit Ca$^{2+}$ signaling and that PLCγ2 is a critical player in the Dectin-1 signal transduction pathway.

The C-type lectin receptors (CLRs)$^3$ are a large family of proteins that possess one or more C-type lectin-like domains, which were initially characterized for their calcium-dependent carbohydrate-binding property, but recently found to have diverse functions and are now defined based on their similarity to the structural motif found in the protein-fold of the carbohydrate-recognition domain of the mannose-binding lectin (1, 2). Some CLRs have been shown to be intimately involved in innate immunity by binding pathogen-associated molecular patterns found on microbes to mediate host responses. Examples of these include the soluble CLRs such as the collectins that are found in serum and could activate complement upon binding microbes (3), and the membrane-bound CLRs such as DEC-205 and DC-SIGN that could mediate endocytosis of ligands for pathogen clearance or antigen presentation (4, 5).

Dectin-1 is a CLR that is expressed mainly on myeloid cells such as macrophages, DCs, and neutrophils and possesses a single extracellular carbohydrate-recognition domain that recognizes β(1,3)-glucans found mainly in the cell walls of fungi (6–8). It has been shown to mediate the phagocytosis of yeast and yeast-derived particles such as zymosan (9–11). Upon binding its substrates, Dectin-1 could also activate the production of inflammatory cytokines in innate cells, suggesting that it could couple pathogen-associated molecular pattern recognition to the induction of immune response genes. The importance of Dectin-1 in anti-fungal immunity was seen in recent studies that revealed that Dectin-1-deficient mice were more susceptible to systemic yeast infection by Candida albicans (12) and Pneumocystis carinii (13).

Dectin-1 belongs to the natural killer cell-receptor-like subgroup of CLRs that also include Ly49D and NKG2D (14). However, unlike these CLRs, which signal through an associated adaptor protein, DNAX activation protein 12, which bears ITAM motifs, Dectin-1 mediates its own signaling through its cytoplasmic tail (15–17). However, the cytoplasmic portion of Dectin-1 contains only an ITAM-like domain, termed as hemi-ITAM, as it is composed of only one classical YXXL (where X is any amino acid) motif. It has been demonstrated that upon binding zymosan or β-glucan, Dectin-1 is phosphorylated at its hemi-ITAM, possibly by a member of the Src family of tyrosine kinases (17). The phosphorylated ITAM-like motif of Dectin-1 could directly recruit Syk, which subsequently signals downstream to activate mitogen-activated protein kinases (MAPKs) and nuclear factor κB (NF-κB), with the latter dependent on the adaptor protein Card9 (18). Thus, Syk and Card9 play critical roles in coupling Dectin-1 engagement to the regulation of innate gene expression.

However, other than Syk and Card9, the other participants of the Dectin-1 signal transduction pathways upstream of the activation of MAPKs and NF-κB are largely unknown. The usage of Syk...
as an upstream kinase and the structural similarity between the signaling competent hemi-ITAM of Dectin-1 and the classical ITAMs found in the signaling subunits of the antigen receptor complex such as those of the BCR and TCR have led to the speculation that Dectin-1 could signal in an antigen-receptor-like manner (16, 17), and perhaps, also uses some additional components of the BCR or TCR signaling pathways in its signaling.

One of the hallmarks of BCR or TCR signaling is the elicitation of Ca\(^{2+}\) flux in lymphocytes upon the engagement of these antigen receptors (19, 20). In BCR signaling, the enzymatic activity of PLC\(_{\gamma}\)2 is required for the induction of Ca\(^{2+}\) flux (21), whereas in TCR signaling, PLC\(_{\gamma}\)1 plays the critical role (22). PLC\(_{\gamma}\)1 is ubiquitously expressed, whereas PLC\(_{\gamma}\)2 is mainly found in hematopoietic cells. It is currently not known whether Dectin-1 signaling could trigger Ca\(^{2+}\) flux in DCs and if it does, whether PLC\(_{\gamma}\)1 or PLC\(_{\gamma}\)2 plays the predominant role in this process given that DCs expressed both phospholipases.

Here we demonstrate that the engagement of Dectin-1 induces Ca\(^{2+}\) flux in DCs and PLC\(_{\gamma}\)2 is the critical phospholipase. We further show that the activation of PLC\(_{\gamma}\)2 is dependent on Syk and Src family kinases. In the absence of PLC\(_{\gamma}\)2, the activation of ERK and JNK MAPKs and the induction of transcription factors AP-1, NFAT, and NF-\(\kappa\)B as well as the secretion of cytokines are severely compromised in DCs.

**EXPERIMENTAL PROCEDURES**

**Mice and Cells**—C57BL/6 (Jackson Laboratories) and plcy\(_{\gamma}\)2\(^{-/-}\) mice were bred and maintained in our animal facilities and used with approval from the Institutional Animal Care and Use Committee and according to the guidelines issued by the National Advisory Committee on Laboratory Animal Research (NACLAR). Bone marrow-derived DCs (BMDCs) were differentiated as described (23). Briefly, BM cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin, 2 mM L-glutamine, 50 \(\mu\)M 2-mercaptoethanol, 10% heat-inactivated fetal calf serum, and 10% supernatant of granulocyte-macrophage colony-stimulating factor-transduced X-63 cells. After 6 to 7 days of culture, DCs were purified with anti-CD11c monoclonal antibody-coupled magnetic beads (Miltenyl Biotec) and the purity was routinely verified by flow cytometry to be >90%.

**Reagents**—The following reagents were purchased: Zymosan (Invitrogen), Curdlan (Wako), Src family kinase inhibitor-PP2, Syk inhibitor-Piceatannol, Btk inhibitor-LFM-A13, and PLC\(_{\gamma}\) inhibitor-U73122 (Calbiochem), and Laminarin (Sigma). Antibodies used for immunoblot analyses were as follows: from Santa Cruz: anti-Bcl10 (331.3), anti-ERK2 (C-14), anti-phospho-ERK (E-4), anti-IxK-\(\alpha\), anti-PLC\(_{\gamma}\)2 (G-20), anti-PLC\(_{\gamma}\)1 (1249), anti-p38 (N-20), anti-IKK\(\alpha/\beta\), anti-decitin-1 (26094), anti-NFATc1 (7A6), anti-NFATc2 (4G6-G5), anti-NFATc3 (M-75), anti-NFATc4 (H-74), goat anti-rabbit IgG-horseradish peroxidase, and goat anti-mouse IgG-horse radish peroxidase; from Cell Signaling: anti-phospho-Syk (Ty\(_r^{525/526}\)), anti-phospho-p38 (Thr\(_r^{180/182}\)) (3D7), anti-phospho-PLC\(_{\gamma}\)1 (Tyr\(_r^{783}\)), anti-phospho-SAPK/JNK (Thr\(_r^{183/185}\)), anti-Malt1, and anti-phospho-IKK\(\alpha\) (Ser\(_r^{180}\)) (IKK\(\beta\) (Ser\(_r^{181}\)). Anti-Card9 antibody was from Sigma. Anti phosphotyrosine horseradish peroxidase-conjugated antibody (4G10) was from Upstate.

**BMDC Stimulation Assay**—For analysis of cytokine production and cell surface marker expression, 5 \(\times\) 10\(^5\) BMDCs per well were cultured in complete Dulbecco’s modified Eagle’s medium for 4–6 h in 12-well plates in the presence of zymosan (50 \(\mu\)g/ml) or curdlan (100 \(\mu\)g/ml). Cytokines in the supernatants were measured using enzyme-linked immunosorbent assay kits from BD Pharmingen. Cell surface marker expression was examined on a FACSCalibur (BD Biosciences) and analyzed with Flowjo software (TreeStar). For biochemical analyses, 5 \(\times\) 10\(^6\) BMDCs were stimulated for various times with zymosan (100 \(\mu\)g/ml) or curdlan (500 \(\mu\)g/ml).

**Calcium Flux**—For analysis of calcium flux, BMDCs were loaded with Indo-1 AM (2 \(\mu\)M; Molecular Probes) as described previously (24). To block Dectin-1, Indo-1-loaded BMDCs were preincubated with anti-Dectin-1 blocking antibody or laminarin for 30 min at room temperature. After resting for 30 min, cells were stimulated with zymosan (100 \(\mu\)g/ml) and calcium flux was monitored on a LSR II or FACSAria flow cytometer in real time for 6–8 min. The kinetics studies of calcium flux were performed with Flowjo software.

**Immunoprecipitation, Western Blotting, and Electrophoretic Mobility Shift Assay**—After stimulation, whole cell lysates were prepared and subjected to immunoprecipitation and Western blotting as described previously (24). Electrophoretic mobility shift assay was performed using end-labeled \([\gamma-32P]ATP\), double-stranded probes containing binding sites for NF-\(\kappa\)B and AP-1. The double-stranded probes used are as follow: NF-\(\kappa\)B-binding sites, 5’-CATGGCCTGGGAAAGTCCCCTCAACT-3’ and 5’-CATGAGTGGAGGGACTTTCCCAGGC-3’ (25); AP-1-binding sites, 5’-CGCTTGATGACTCAAGCGGAA-3’ and 5’-GCGAATACTGAGTCGCGCCTT-3’ (26).

**RESULTS**

Dectin-1 Engagement Induces Ca\(^{2+}\) Flux and Activates PLC\(_{\gamma}\) in BMDCs—It has been speculated that Dectin-1 could signal in a manner analogous to the lymphocyte antigen receptor as it possesses an ITAM-like motif in its cytoplasmic tail and activates Syk when stimulated by its ligands (11, 16, 17). Because Ca\(^{2+}\) mobilization is one of the most important signaling events after BCR or TCR engagement, we first investigated if the activation of Dectin-1 could induce Ca\(^{2+}\) flux in DCs. As shown in Fig. 1, stimulation of BMDCs by zymosan, a yeast derivative that is commonly used as an agonist of Dectin-1 (8, 10, 15), led to an induction of intracellular Ca\(^{2+}\) flux in these cells. As zymosan is also thought to engage TLR2 (10, 15), we next clarified if zymosan-induced Ca\(^{2+}\) flux in BMDCs is elicited through Dectin-1 or TLR2. To address this, we treated BMDCs with Pam3Csk, which is a pure TLR2 agonist. It was readily apparent that Pam3Csk could also trigger Ca\(^{2+}\) flux in BMDCs but the transient nature of the Ca\(^{2+}\) flux elicited by Pam3Csk was quite different from the sustained pattern of Ca\(^{2+}\) flux elicited by zymosan. Although one might argue that the stimulation of TLR2 by Pam3Csk might not be equivalent to the
engagement of TLR2 or TLR2 and Dectin-1 together with zymosan, the different nature of the Ca²⁺ flux elicited by Pam3Csk and zymosan nevertheless suggested that Dectin-1 activation could possibly induce Ca²⁺ signaling in BMDCs. To confirm that the engagement of Dectin-1 could induce Ca²⁺ flux in BMDCs, we preincubated the cells with a blocking anti-Dectin-1 antibody (8) or with laminarin, a soluble β-glucan that could effectively inhibit the binding of zymosan to Dectin-1 (7), prior to stimulating them with zymosan. As revealed in Fig. 1, both the soluble β-glucan and the blocking anti-Dectin-1 antibody could dampen the Ca²⁺ flux elicited by zymosan treatment in BMDCs, implying that stimulation through Dectin-1 could indeed trigger Ca²⁺ signaling in these cells.

The Ca²⁺ flux induced by lymphocyte antigen receptors such as the BCR and TCR is mainly mediated by PLCγ2 and PLCγ1, respectively (21, 22). Because the stimulation of Dectin-1 could activate Ca²⁺ flux in BMDCs, we determined if the enzymatic activity of any PLCγ might be involved in this process. To this end, we preincubated BMDCs with the PLCγ inhibitor U73122 and showed that this prior treatment could completely abolish the Ca²⁺ flux elicited by zymosan treatment in BMDCs, implying that stimulation through Dectin-1 could indeed trigger Ca²⁺ signaling in these cells.

FIGURE 1. Engagement of Dectin-1 elicits Ca²⁺ flux in DCs. CD11c⁺ wild-type BMDCs were loaded with Indo-1 (2 µM) and stimulated with 100 µg/ml zymosan (Zym), 50 µg/ml Pam3Csk, or 100 µg/ml zymosan in the presence of 0.5 mg/ml laminarin or 0.5 µg/ml anti-Dectin-1 blocking antibody or 0.1 µM inhibitor of PLCγ-U73122. Intracellular Ca²⁺ flux was depicted as a ratio of Indo-1 violet/blue fluorescence versus time. Data shown are representative of more than five independent sets of experiments.
Role of PLCγ2 in Dectin-1 Signaling

It is known that stimulation of BMDCs by zymosan or curdlan leads to an increase in cytosolic calcium (Ca²⁺) levels. The induction of Ca²⁺ is an important step in the signaling pathway leading to the activation of Dectin-1. PLCγ2, a calcium-dependent enzyme, is activated by Syk and responsible for BCR-induced signaling in B cells and is implicated in the Dectin-1 signal transduction pathway. During BCR signaling, Src family kinases, Syk and Btk, are critical for the activation of PLCγ2. Syk and Src family kinases play roles in the phosphorylation of PLCγ2, which leads to its activation. Btk is a kinase that is not involved in the Dectin-1 signaling pathway.

To investigate the role of PLCγ2 in Dectin-1 signaling, we performed experiments where wild-type and Btk-deficient BMDCs were treated with zymosan or curdlan and examined the levels of PLCγ2 phosphorylation. As shown in Fig. 3, PLCγ2 phosphorylation appeared sustained in zymosan-stimulated wild-type BMDCs, indicating that PLCγ2 phosphorylation is required for its activation in the Dectin-1 signal transduction pathway. However, in Btk-deficient BMDCs, the residual Ca²⁺ flux seen in zymosan-stimulated PLCγ2-deficient BMDCs, it could not completely compensate for the lack of PLCγ2 in Dectin-1-induced Ca²⁺ signaling.

As control to ensure that wild-type and PLCγ2-deficient DCs were equivalently stimulated via Dectin-1, we also analyzed the cell surface expression level of Dectin-1 in wild-type and mutant BMDCs. As shown in Fig. 3D, the cell surface expression level of Dectin-1 in PLCγ2-deficient BMDCs was normal and not different from that found in wild-type BMDCs and thus could not have contributed to the defect in Ca²⁺ signaling seen in the mutant cells.

Syk and Src family kinases but not Btk are important for Dectin-1-induced PLCγ2 activation—We next explored how PLCγ2 is activated by determining which upstream kinase is required for its activation in the Dectin-1 signal transduction pathway. During BCR signaling, Src family kinases, Syk and Btk, are important for PLCγ2 activation and Ca²⁺ flux in B cells (29, 30). Recent studies demonstrated that Syk and probably Src family kinases played critical roles in Dectin-1 signaling (16, 17). To assess if these tyrosine kinases are important for Dectin-1-induced activation of PLCγ2, we pretreated wild-type BMDCs with specific inhibitors for the individual kinase, followed by stimulation with 500 μM Btk inhibitor LFM-A13, or 50 μM Syk inhibitor Piceatannol, followed by treatment with 500 μg/ml curdlan for 10 min. The activation of PLCγ2 was assayed by immunoblotting (IB) as described in the legend to Fig. 2. Data shown are representative of three independent experiments.

Syk and Src family kinases but not Btk are important for Dectin-1-induced PLCγ2 activation. Wild-type BMDCs were preincubated for 20 min with DMSO, 200 nM Src family kinase inhibitor PP2, 25 μM Btk inhibitor LFMA13, or 50 μM Syk inhibitor Piceatannol, followed by treatment with 500 μg/ml curdlan for 10 min. The activation of PLCγ2 was assayed by immunoblotting (IB) as described in the legend to Fig. 2. Data shown are representative of three independent experiments.
to their maturation and up-regulation of activation markers (27). Thus we assessed whether signal transduced via PLCγ2 was involved in this process. As shown in Fig. 5A, stimulation with either zymosan (blue histogram) or curdlan (red histogram) up-regulated the expression of CD40, CD80, and CD86 on wild-type BMDCs. By contrast, the up-regulation of these activation markers was significantly inhibited in curdlan-treated PLCγ2−/− BMDCs. Interestingly, the up-regulation of CD80 and CD86 was only slightly reduced, whereas the up-regulation of CD40 was not affected in zymosan-treated PLCγ2−/− BMDCs compared with wild-type BMDCs. This again reflects the fact that zymosan is a more complex agonist and part of its stimulatory effect is through other innate receptors such as TLR2. Nevertheless, the data derived from the study with curdlan indicate that PLCγ2 signaling is important for the up-regulation of activation markers on BMDCs upon Dectin-1 engagement.

Stimulation of Dectin-1 by β-glucan also induces the production of various cytokines in DCs (10, 27). Thus we next determined if PLCγ2 regulates Dectin-1-mediated cytokine secretion in BMDCs. As seen in Fig. 5B, the production of IL-2, IL-10, IL-23p19, tumor necrosis factor-α, IL-6, and IL-12p40 was either completely abrogated or significantly reduced in curdlan-treated PLCγ2−/− BMDCs compared with wild-type controls. Similarly, the production of IL-2 and IL-23p19 was almost completely abolished in zymosan-stimulated PLCγ2−/− BMDCs suggesting that PLCγ2 signaling critically regulates these two cytokines in anti-fungal immunity. The production of IL-6, IL-10, and tumor necrosis factor-α was also significantly decreased in zymosan-treated PLCγ2−/− BMDCs. Interestingly, the production of IL-12p40 was not perturbed in zymosan-stimulated PLCγ2−/− BMDCs. This latter finding was quite similar to the situation found in syk−/− (27) or card9−/− (18) DCs, suggesting that zymosan-induced production of IL-12p40 only partially relied on Dectin-1 signaling. Taken together, our data collectively indicate that PLCγ2 plays a critical role in regulating Ca2+ mobilization and cytokine production in response to Dectin-1 stimulation in DCs.

**Role of PLCγ2 in Dectin-1 Signaling**

**FIGURE 5.** PLCγ2 signaling is required for Dectin-1-induced maturation of DC and their production of cytokines. A, flow cytometry analyses of the expression of maturation or activation markers on zymosan- and curdlan-treated wild-type and PLCγ2-deficient BMDCs. BMDCs were either untreated (black histogram) or stimulated with 50 μg/ml zymosan (blue histogram) or 100 μg/ml curdlan (red histogram) for 6 h and examined for their cell surface expression of CD40, CD80, and CD86. B, PLCγ2-deficient DCs exhibit defective cytokine secretion upon Dectin-1 engagement. Wild-type (black columns) or PLCγ2-deficient (white columns) BMDCs were left untreated (m) or were stimulated with zymosan (Zym) or curdlan for 6 h and the culture supernatants were collected and assayed for the presence of various cytokines by enzyme-linked immunosorbent assay. Results shown are mean ± S.D. of triplicate cultures and are representative of five independent set of experiments.
increasing amounts of these three proteins were induced to form a complex upon Dectin-1 engagement in wild-type DCs. Interestingly, the interaction of Card9 and Bcl10 was not affected but the interaction of Malt1 and Bcl10 was significantly reduced by the lack of PLCγ2 signaling in the mutant BMDCs that were similarly treated with curdlan. This suggested that PLCγ2 signaling might be important to induce the association of Malt1 with Bcl10 but not Card9 with Bcl10. Although we do not know the hierarchy of the interactions of these three proteins, our data nonetheless indicate that PLCγ2 signaling is required for proper stoichiometry and stable assembly of the Card9-Bcl10-Malt1 complex in Dectin-1 signaling.

Taken together, the data presented in this section indicate that PLCγ2 plays an important role in coupling Dectin-1 recognition of fungal pathogen-associated molecular patterns to the activation of NF-κB in BMDCs via inducing the assembly of the Card9-Bcl10-Malt1 complex and the subsequent activation of IKKα/β and degradation of IκB proteins.

Defective Activation of ERK and JNK MAPKs and AP-1 and NFAT transcription factors in Dectin-1-activated PLCγ2-deficient BMDCs—Besides inducing NF-κB, Dectin-1 also activates MAPKs, which are important for signaling the production of inflammatory cytokines in BMDCs. It was demonstrated previously that the production of IL-2 and IL-10 by zymosan-stimulated BMDCs was dependent on Dectin-1-Syk but not MyD88 signaling (16) and that in curdlan-treated syk−/− cells, the activation of ERK, JNK, and p38 MAPKs was substantially impaired (27). More recently, it was shown that ERK signaling was required for zymosan-induced production of IL-2 and IL-10 in murine BMDCs (33). Because we have shown that PLCγ2 is downstream of Syk in Dectin-1 signaling (Fig. 4) and that plcyγ2−/− BMDCs were defective in cytokine secretion upon Dectin-1 activation (Fig. 5B), we further examined if the activation of MAPKs was affected in plcyγ2−/− BMDCs upon Dectin-1 stimulation. As shown in Fig. 7A, the activation of ERK and JNK was severely attenuated across all time points tested in curdlan-stimulated plcyγ2−/− BMDCs compared with similarly treated wild-type controls. However, the activation of p38 was apparently comparable in both curdlan-stimulated wild-type and mutant BMDCs. Thus, the absence of PLCγ2 specifically...
Role of PLCγ2 in Dectin-1 Signaling

NFATc3 was almost completely abrogated in plcγ2−/− BMDCs compared with wild-type controls upon Dectin-1 stimulation. Although NFATc1 could be mildly activated by curdlan treatment of wild-type BMDCs, its activation was not substantially compromised in plcγ2−/− BMDCs. Collectively, our results indicate that the activation of NFATc2 and NFATc3 are important in Dectin-1 signaling and they are impaired in the absence of PLCγ2.

DISCUSSION

Dectin-1, a NK cell-receptor-like C-type lectin, has been shown to be important for the recognition of β-glucans found in the cell wall of pathogenic fungi (8, 10, 15). Its engagement could trigger intracellular signaling that activates NF-κB and MAPKs and leads to the production of inflammatory cytokines in DCs as well as phagocytosis and the production of reactive oxygen species in macrophages (1, 14). So far, only Syk and Card9 have been identified to be involved in Dectin-1 signaling (16–18, 27). In this report, we provide definitive evidence that PLCγ2 also plays a critical role in Dectin-1 signaling. PLCγ2 is required not only for the induction of Ca2+ flux in Dectin-1-activated DCs (Fig. 3A) but also for the activation of ERK and JNK MAPKs and the induction of NF-κB, AP-1, and NFAT transcription factors (Figs. 6 and 7). In the absence of PLCγ2, BMDCs have an impaired production of IL-2, IL-6, IL-10, IL-23, and tumor necrosis factor α (Fig. 5). Thus, PLCγ2 plays a central role in coupling Dectin-1 recognition of fungal pathogen-associated molecular patterns to the mobilization of intracellular Ca2+ and activation of inflammation signaling in DCs, which leads ultimately to their secretion of inflammatory cytokines.

It is interesting to note that although DCs express both PLCγ1 and PLCγ2, and either enzyme could mobilize Ca2+ in various cell types, only PLCγ2 seems to be important for signaling Ca2+ flux during Dectin-1 or TLR2 or both engagements in DCs (Fig. 3). It is not clear why PLCγ1 could not fully compensate for the lack of PLCγ2 during Dectin-1 signaling even though PLCγ1 is clearly activated upon treatment of DCs with curdlan that engages Dectin-1 (Fig. 2). Future experiments using plcγ1−/− DCs or plcγ1−/− plcγ2−/− DCs may help to shed light on this puzzle.

Another interesting finding that arises from the current study is the discovery that stimulation of Dectin-1 by zymosan could elicit intracellular Ca2+ flux in DCs. Zymosan is also known to activate TLR2 other than Dectin-1. Interestingly, TLR2 engagement by Pam3Csk could also induce Ca2+ flux in DCs, although the pattern of Ca2+ flux is much more transient compared with the more sustained Ca2+ flux elicited by zymosan stimulation. It is possible that zymosan triggering of Dectin-1 and TLR2 together contribute to a more synergistic activation of Ca2+ flux in DCs. However, blocking experiments using laminarin and anti-Dectin-1 antibody clearly demonstrated that Dectin-1 engagement alone could contribute substantially to the induction of Ca2+ flux in these cells. Taken together, our data indicate that innate pathogen recognition receptors such as Dectin-1 and TLR (TLR2 in this case) could also elicit intracellular Ca2+ flux in DCs, thus suggesting that intracellular Ca2+ mobilization, as a mechanism of cellular sig-
naling in the immune system, is not confined to lymphocytes and their antigen receptors. Dectin-1 possesses a hemi-ITAM in its cytoplasmic tail and has been postulated to signal in a lymphocyte antigen-receptor manner (11, 16, 17). Adding to this hypothesis is the discovery that the phosphorylated hemi-ITAM of Dectin-1 could recruit Syk, a tyrosine kinase that plays a critical role in BCR signaling (30, 37). Activated Syk subsequently leads to the downstream activation of MAPKs and NF-κB (27). Here, we identified PLCγ2 as a critical component downstream of Syk in Dectin-1 signaling as the inhibition of Syk abrogated curdlan-induced phosphorylation of PLCγ2 (Fig. 2E). In further support of this, the cellular and molecular defects of Dectin-1-activated plcγ2−/− DCs are quite similar to that of syk−/− DCs. When compared with wild-type DCs, both mutant DCs have defective up-regulation of activation markers and secretion of inflammatory cytokines upon curdlan stimulation. With regard to the molecular defects, both plcγ2−/− and syk−/− DCs (27) have impaired activation of ERK and JNK MAPKs and NF-κB transcription factor when stimulated with curdlan. The only signaling difference appears to be the normal activation of p38 MAPK in plcγ2−/− DCs (our current study) but compromised activation of this MAPK in syk−/− DCs. The reason for this difference is not apparent but it could be that other signaling pathways downstream of Syk but independent of PLCγ2 control the activation of p38 MAPK in response to Dectin-1 engagement. Nevertheless, our data largely supported the idea that PLCγ2 acts downstream and transduces most of the signals emanated from the Syk pathway during Dectin-1 signaling.

The defective responses of plcγ2−/− DCs to Dectin-1 stimulation are also reminiscent of the defects found in card9−/− DCs treated in a similar manner (18). Card9 has been shown to be important for NF-κB activation in certain ITAM-bearing receptors such as the FcγR and was thought to be the functional homologue of Card11 in NF-κB activation induced by the cross-linking of lymphocyte antigen receptors (18, 38, 39). A dectin-1-Syk-Card9 signaling cascade has been delineated based on the studies of mutant DCs deficient for the individual molecule (27). However, it has not been established how Dectin-1/Syk signals trigger Card9 and consequently activate NF-κB. In our current study, we found that PLCγ2 is important in signaling NF-κB activation in response to Dectin-1 engagement and this occurs via the induction of the assembly of a Card9-Bcl10-Malt1 complex (Fig. 4). Together with the finding that Syk is crucial for PLCγ2 activation upon Dectin-1 stimulation (Fig. 5), a more detailed signaling cascade emerges: Dectin-1 engagement triggers Syk that further activates PLCγ2, leading to the formation of the Card9-Bcl10-Malt1 complex that mediates the activation of NF-κB. However, it is currently still not known how Dectin-1/Syk/PLCγ2 signals induce the three proteins to form a complex and what the other intermediate signaling events and players are involved. Moreover, our data show that PLCγ2 signaling seems to be critical for the recruitment of Malt1 to the protein complex as in its absence, the interaction of Malt1 with Bcl10 was compromised, whereas the interaction of Card9 with Bcl10 appears normal, suggesting that there might be some other signaling pathways contributing to the formation of this multiprotein complex. Nevertheless, our data clearly indicate that PLCγ2 is required for at least inducing the association of Bcl10 with Malt1, and therefore it plays an essential role in mediating Dectin-1/Syk signals to the activation of NF-κB.

Last but not least, it is increasingly being appreciated that C-type lectins might play crucial roles in initiating and sustaining immune responses against various pathogens. For example, Dectin-1 is important for host recognition of fungi and Mycobacterium tuberculosis (1, 14, 40, 41), whereas DC-SIGN and CLEC5A are known to bind HIV and Dengue virus (42, 43), respectively. Therefore the elucidation of the signaling of these CLR might be important for our understanding of host defense and microbial spread in the organism. Our current study demonstrating a role for PLCγ2 in Dectin-1 signal transduction and possibly the signaling of other CLR could provide new targets for therapeutic intervention to enhance or suppress the host response.

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