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Phosphoinositide 3-Kinase δ Regulates Dectin-2 Signaling and the Generation of Th2 and Th17 Immunity

Min Jung Lee,*,† Eri Yoshimoto,*,† Shinobu Saijo,‡ Yoichiro Iwakura,§ Xin Lin,¶ Howard R. Katz,*,† Yoshihide Kanaoka,*,† and Nora A. Barrett*,†

The C-type lectin receptor Dectin-2 can trigger the leukotriene C_4 synthase–dependent generation of cysteinyl leukotrienes and the caspase-associated recruitment domain 9– and NF-κB–dependent generation of cytokines, such as IL-23, IL-6, and TNF-α, to promote Th2 and Th17 immunity, respectively. Dectin-2 activation also elicits the type 2 cytokine IL-33, but the mechanism by which Dectin-2 induces these diverse innate mediators is poorly understood. In this study, we identify a common upstream requirement for PI3Kδ activity for the generation of each Dectin-2–dependent mediator elicited by the house dust mite species, *Dermatophagoides farinae*, using both pharmacologic inhibition and small interfering RNA knockdown of PI3Kδ in bone marrow–derived dendritic cells. PI3Kδ activity depends on spleen tyrosine kinase (Syk) and regulates the activity of protein kinase Cδ, indicating that PI3Kδ is a proximal Syk-dependent signaling intermediate. Inhibition of PI3Kδ also reduces cysteinyl leukotrienes and cytokines elicited by Dectin-2 cross-linking, confirming the importance of this molecule in Dectin-2 signaling. Using an adoptive transfer model, we demonstrate that inhibition of PI3Kδ profoundly reduces the capacity of bone marrow–derived dendritic cells to sensitize recipient mice for Th2 and Th17 pulmonary inflammation in response to *D. farinae*. Furthermore, administration of a PI3Kδ inhibitor during the sensitization of wild-type mice prevents the generation of *D. farinae*–induced pulmonary inflammation. These results demonstrate that PI3Kδ regulates Dectin-2 signaling and its dendritic cell function. *The Journal of Immunology*, 2016, 197: 278–287.

Dectin-2 is a myeloid C-type lectin receptor (CLR) with well-described roles in antifungal immunity (1, 2). Upon activation with hyphae from *Candida albicans*, Dectin-2 triggers the generation of NF-κB–dependent inflammatory cytokines IL-6, IL-10, IL-23, and TNF-α and the development of Th17 immunity (3). Dectin-2 has a short cytoplasmic tail, but it pairs with the ITAM-bearing FcRγ-chain to initiate Src homology region 2 domain–containing phosphatase 2– and spleen tyrosine kinase (Syk)–dependent signaling (3–5). The downstream activation and nuclear translocation of NF-κB by Dectin-2, and by several other CLRs, requires the protein kinase Cδ (PKCδ)–dependent phosphorylation of the adaptor protein caspase-associated recruitment domain (CARD)9 (6) and its assembly with B cell lymphoma/leukemia 10 and MALT lymphoma translocation protein 1 (7). Inhibition of MALT lymphoma translocation protein 1 abrogates Th17 immunity to *Candida* (8), which is in keeping with the importance of the Dectin-2/CARD9 pathway in the generation of Th17 immunity.

Dectin-2 activation can also elicit Th2-dependent pulmonary inflammation through the immediate generation of proinflammatory lipid mediators, cysteinyl leukotrienes (cys-LTs). Cys-LTs are derived from membrane arachidonate through the serial actions of cytosolic phospholipase A_2, 5-lipoxygenase in the presence of the 5-lipoxygenase activating protein, and LTC_4 synthase (LTC_4S) (9–11). Dectin-2-dependent cys-LT generation is elicited by glycans from common aeroallergens, including *Aspergillus fumigatus* and the house dust mite (HDM) species *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* (12). Dectin-2–dependent arachidonate metabolism in macrophages can also be triggered by *C. albicans* (13), suggesting it is a central feature of Dectin-2 signaling. Whereas Dectin-2 signaling is required for the generation of both Th2 and Th17 allergic pulmonary inflammation elicited by HDM (14–16), the Dectin-2/LTC_4S pathway in dendritic cells (DCs) is required only for Th2 immunity to HDM through the autocrine actions of cys-LTs at the type 1 cys-LT receptor (14).

HDM-elicited allergic pulmonary inflammation also requires the type 2 cytokine IL-33, which promotes sensitization to HDM through the ST2-dependent upregulation of OX40L on lung DCs (17, 18). Notably, HDM can elicit IL-33 generation in bone marrow–derived DCs (BMDCs) through a Dectin-2– and PI3K-dependent pathway (19), suggesting that IL-33 production may be a second Dectin-2–dependent autocrine signal that conditions DCs for Th2 immunity. However, the mechanism by which Dectin-2 controls the generation of these diverse mediators remains poorly understood.

Class I PI3Ks catalyze the phosphorylation of phosphatidylinositol at the 3-position to generate second messengers in response

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Abbreviations used in this article: Akt, protein kinase B; BAL, bronchoalveolar lavage; BMDC, bone marrow–derived dendritic cell; CARD, caspase-associated recruitment domain; CLR, C-type lectin receptor; cys-LT, cysteinyl leukotriene; DC, dendritic cell; EGFP, enhanced GFP; HDM, house dust mite; IRF, IFN regulatory factor; LT, leukotriene; LTC_4S, leukotriene C_4 synthase; MDLN, mediastinal lymph node; PAS, periodic acid–Schiff; PKCδ, protein kinase Cδ; PLCγ, phospholipase Cγ; siRNA, small interfering RNA; Syk, spleen tyrosine kinase; WT, wild-type.

to transmembrane signaling (20). Although the upstream signaling that activates PI3Ks is incompletely understood, YXXM-bearing receptors, G protein–coupled receptors, and rat sarcoma–dependent receptors have been implicated (21). The PI3K p110δ isoform (PI3Kδ) is highly expressed in hematopoietic cells and plays a key role in lymphocyte activation through the TCR and the BCR (22). PI3Kδ is also activated by the high-affinity receptor for IgE, FcεR1, and mediates the phosphorylation of protein kinase B (Akt) and degranulation in mast cells (23, 24). As FcεR1 and Dectin-2 each uses the FcRγ-chain to initiate ITAM/Syk-dependent cys-LT generation, we sought to determine whether PI3Kδ is required for Dectin-2–dependent cys-LT generation, and whether it may be critical for Dectin-2 signaling more broadly.

In this study, we find that *D. farinae* stimulation of BMDCs elicits phosphorylation of Akt, a PI3K-dependent kinase, and inhibition of PI3K reduces *D. farinae*–elicited cys-LTs, IL-23, and IL-33, suggesting that PI3K activity regulates Dectin-2 signaling. PI3Kδ is the most abundant class I PI3K isoform in BMDCs, and both pharmacologic inhibition and small interfering RNA (siRNA) knockdown of PI3Kδ reduce *D. farinae*–elicited cys-LTs, IL-23, and IL-33. Inhibition of PI3Kδ also reduces cys-LTs and cytokines elicited by Dectin-2 cross-linking, demonstrating that PI3Kδ is an important signaling intermediate in the Dectin-2 pathway. Whereas *D. farinae*–elicited PI3Kδ activity and the generation of each inflammatory mediator depends on Syk, inhibition of PI3Kδ has no effect on Syk phosphorylation but reduces the phosphorylation of PKCδ, indicating that PI3Kδ is a proximal Syk-dependent signaling intermediate. Inhibition of PI3Kδ potently reduces DC-mediated sensitization, as wild-type (WT) mice sensitized with PI3Kδ-treated *D. farinae*–pulsed BMDCs have a dramatic attenuation in allergic pulmonary inflammation and Th2/Th17 cytokine production after *D. farinae* challenge. Furthermore, selective treatment of WT mice with a PI3Kδ inhibitor during sensitization attenuates the generation of *D. farinae*–induced pulmonary inflammation. These findings demonstrate that PI3Kδ regulates divergent Dectin-2–dependent signaling pathways to promote both Th2 and Th17 immunity. Thus, strategies to inhibit the mucosal activation of PI3Kδ may have therapeutic efficacy in allergen-induced pulmonary inflammation.

Materials and Methods

Mice

C57BL/6 WT mice were purchased from Charles River Laboratories and ROSA26–enhanced GFP (EGFP) transgenic mice [Tg(Gt(ROSA)26Sor-EGFP)I1Able)] were purchased from The Jackson Laboratory. *Ltc4s*$^{-/-}$ mice were generated and maintained in our laboratory (25). *Clec4n*$^{-/-}$ mice (2) and *Card9*$^{-/-}$ mice (26) were generated as previously described. IL-33–deficient (*Il33*$^{-/-}$) mice were provided by Pfizer Research (Cambridge, MA). All mice were on C57BL/6 background and were 8–12 wk old for in vivo experiments. All animal studies were approved and in accordance of the Animal Care and Use Committee of the Dana-Farber Cancer Institute.

Reagents

D. farinae extracts (Greer Laboratories, Lenoir, NC) were reconstituted in PBS. LPS from *Escherichia coli* 055:B5 was obtained from Sigma-Aldrich (St. Louis, MO). Curdlan (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in DMSO. Rat anti-mouse Dectin-2 IgG2a (clone D2.11E4; AbD Serotec, Raleigh, NC) and goat anti-rat IgG2a (Jackson ImmunoResearch Laboratories, West Grove, PA) were used for Dectin-2 cross-linking. Pan-PI3K inhibitor (Ly294002; EMD Millipore, Billerica, MA), PI3K p110δ inhibitors (CAL-101 and IC87114; Selleckchem, Houston, TX), PI3K p110β inhibitor (TGX-221; Selleckchem), Syk inhibitor II (Santa Cruz Biotechnology, Dallas, TX), and Syk inhibitor (R406; Selleckchem) were dissolved in DMSO.

BMDC generation and Dectin-2 activation

BMDCs were generated with GM-CSF according to Lutz et al. (27) and as previously described (28). Briefly, bone marrow was harvested from the femur, washed, and plated in petri dishes at 4×10^5 cells/ml in complete media consisting of RPMI 1640 with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 5 μM 2-ME, and 40 ng/ml recombinant mouse GM-CSF (PeproTech, Rocky Hill, NJ). This suspension was cultured at 37°C in a 5% CO_2 incubator. On day 3, 10 ml complete media was added. On day 6, 10 ml complete media was exchanged. Harvested cells on day 7 were washed and counted for stimulation. Cells were plated at 1×10^6 cells/ml and stimulated with *D. farinae* for the indicated times.

Dectin-2 cross-linking assay

Tissue culture plates were incubated with goat F(ab′)$_2$ anti-rat IgG2a at 4°C overnight. The next day, the plates were washed with PBS and incubated with rat anti-mouse Dectin-2 IgG2a dissolved in PBS with 1% BSA at indicated doses for 2 h. The plate was washed again with PBS, and BMDCs ($2.5–5 \times 10^5$ cells) were added and incubated in 37°C in a 5% CO_2 incubator for indicated times. Supernatants and cell lysates were collected for cytokine analysis.

Cys-LT and cytokine measurements

Cys-LTs in the cultured supernatants at 30 min were analyzed by ELISA according to the manufacturer's protocol (Cayman Chemical, Ann Arbor, MI). IL-23 and TNF-α in the cultured supernatants and IL-33 in the cell lysates after freeze and thaw were measured by ELISA (eBioscience, San Diego, CA) at an 8 h time point, unless otherwise indicated. Lymph node cells were cultured at 4×10^6 cells/ml, stimulated with 20 μg/ml *D. farinae* for 72 h, and IL-4, IL-5, IL-13, IL-17A, and IFN-γ were measured by ELISA (eBioscience) in the cultured supernatants.

Quantitative PCR

RNA was extracted from BMDCs with TRIzol reagent, and cDNA was generated using a first-strand kit (Thermo Scientific, Waltham, MA). Transcripts were measured using Mx3006P real-time PCR systems (Agilent Technologies, Santa Clara, CA) with PI3Kβ, PI3Kδ, IL-23, IL-33, 18S, and GAPDH primers (SABiosciences, Frederick, MD).

Western blot

After treatment with or without *D. farinae* in the presence or absence of PI3K inhibitor for the indicated times, the cells were lysed in Nonidet P-40 lysis buffer (Boston BioProducts, Ashland, MA). The total cellular protein concentrations were then quantified using a bicinchoninic acid assay (Pierce, Rockford, IL, USA). After quantification, 10 μg denatured protein was loaded onto 10 or 12% SDS-PAGE gels (Bio-Rad Laboratories, Hercules, CA). The protein was then transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories) and blocked in TBS-T containing BSA or nonfat milk (1× TBS, 5% BSA or nonfat milk, 0.05% Tween 20) depending on the primary Ab for 60 min. The blocked membranes were then incubated overnight at 4°C with Abs to phosphorylated Akt Ser^{473}, phosphorylated Syk Tyr$^{525/526}$, phosphorylated PKCδ Tyr^{311} (1:1000 dilution; Cell Signaling Technology, Beverly, MA) or PI3Kδ (1:500 dilution; Santa Cruz Biotechnology). The following day, membranes were washed three times with TBS-T wash buffer (1× TBS, 0.05% Tween 20). Membranes were incubated for 60 min with HRP-conjugated anti-rabbit secondary Ab (Santa Cruz Biotechnology; 1:10,000 dilution) and washed three times in TBS-T wash buffer. The membrane was developed using SuperSignal West Femto (Thermo Scientific). For total Akt, the same membranes were stripped with Restore Plus Western blot stripping buffer reagent (Thermo Scientific) according to the manufacturer's instructions, followed by TBS wash and blocked in TBS-T containing nonfat milk (1× PBS, 5% nonfat milk, 0.05% Tween 20) for 60 min. The blocked membrane was then incubated at 4°C overnight with Akt or GAPDH (1:1000–1:5000 dilution; Cell Signaling Technology) Ab for subsequent analysis.

PI3Kδ siRNA knockdown

Day 7 WT BMDCs were transfected with PI3Kδ or nontargeting control siRNA (GE Dharmacon, Lafayette, CO) using an Amaxa DC transfection kit reagent (Invitrogen) according to the manufacturer's protocol. After transfection, BMDCs were stimulated with 200 μg/ml *D. farinae* or 100 ng/ml LPS for 30 min for cys-LTs and 6 h for cytokines and analyzed by quantitative PCR or ELISA.

Adoptive transfer protocol

Day 7 BMDCs were stimulated with PBS or *D. farinae* in the presence or absence of CAL-101 at the concentration of 10 nM for 8 h and washed twice and resuspended in PBS. WT mice were sensitized with 10^4 cells in 20 μl PBS intranasally on day 0, challenged with 3 μg *D. farinae* on days

11 and 13, and killed on day 15 with pentobarbital overdose. Bronchoalveolar lavage (BAL) fluid and mediastinal lymph node (MDLN) cells were collected for analysis. For adoptive transfer of BMDCs from transgenic GFP mice, 1×10^6 cells in 20 μl PBS were administered intranasally into WT recipients, and MDLN cells were collected the following day for analysis.

Flow cytometry

For live cell/dead cell discrimination, Zombie Aqua dye (BD Biosciences, San Jose, CA) was used according to the manufacturer's protocol. Isolated MDLN cells were first blocked with 1% mouse IgG (Sigma-Aldrich) and 1% anti-mouse CD16/CD32 (BD Biosciences), then stained with anti-mouse MHC class II–Alexa Fluor 627 (clone M5/114.15.2; BD Biosciences) and anti-mouse CD11c-allophycocyanin-Cy7 (clone N418; BD Biosciences), according to the manufacturer's protocol. Analyses were performed on a FACSCanto flow cytometer (BD Biosciences), and data were analyzed with FlowJo 7.5.

Direct sensitization and challenge protocol

On day 0, WT mice were sensitized with 3 μg *D. farinae* intranasally. PI3Kδ inhibitor IC87114 (30 mg/kg) (29, 30) or vehicle control (PEG-400) was administered orogastrically at 1 h before and 8 h after *D. farinae* administration. Mice were then challenged intranasally with 3 μg *D. farinae* on days 11 and 13 and killed on day 15. BAL fluid was collected as described below.

BAL and lymph node isolation

BAL fluid was collected via three instillations of 0.75 ml PBS plus 1 mM EDTA, and cells were pelleted for counting (14). Cells (4×10^5) in 200 μl were centrifuged and counted after staining with Hema 3 Stain Set (Thermo Scientific). A total of 200 cells per slide were counted and discriminated based on morphological differences. Isolated mediastinal lymph node cells were filtered through 70-μm strainers, washed, and plated at 4×10^6 cells/ml. Cells were restimulated with *D. farinae* at 20 μg/ml for 72 h and supernatants were collected for cytokine analysis.

Histology

The left lung was removed and fixed in 4% paraformaldehyde solution. Histology sections were prepared as previously described (28). They were stained with H&E for general morphology and periodic acid–Schiff for mucus and goblet cell identification.

Statistical analysis

Results were expressed as means ± SEM. Unpaired Student *t* tests were used for the statistical analysis unless stated otherwise. To compare between multiple genotypes or stimuli, one-way ANOVA tests were used. To compare multiple genotypes over doses or time, two-way ANOVA tests were used with Bonferroni posttests. A p value <0.05 was considered significant.

Results

HDM stimulation elicits PI3K activity and the generation of cys-LTs, IL-23, and IL-33 in a PI3K-dependent manner

To determine whether PI3K plays a central role in Dectin-2 signaling, we first assessed BMDC activation in response to *D. farinae*. Phosphorylation of Akt, a PI3K-dependent serine-threonine kinase, was detected in WT BMDCs stimulated with *D. farinae* at 1 min (Fig. 1A). This induction was absent in *D. farinae*–stimulated BMDCs treated with the pan-PI3K inhibitor Ly294002. To determine whether PI3K activity was required for the production of each Dectin-2–dependent inflammatory mediator, we stimulated WT BMDCs with *D. farinae* in the presence of Ly294002. *D. farinae* elicited cys-LTs at 30 min, and IL-23 and IL-33 at 8 h in a dose-dependent manner, which was absent in *Clec4n*$^{-/-}$ BMDCs (Fig. 1B–D). The specificity of IL-33 protein measurement was verified by the absence of detection in *D. farinae*–stimulated *Il33*$^{-/-}$ BMDCs (data not shown). PI3K inhibition reduced production of *D. farinae*–induced cys-LTs, IL-23, and IL-33 with IC_{50} values of 0.7, 0.9, and 0.5 μM, respectively (Fig. 1E–G). PI3K inhibition had no effect on LPS-induced TNF-α generation (Fig. 1H), demonstrating the specificity of PI3K inhibition.

D. farinae elicits cys-LTs through Dectin-2, the ITAM-bearing FcRγ-chain, and the downstream activation of Syk. To determine whether PI3K activity depends on Syk, we assessed the phosphorylation of Akt in the presence of the Syk inhibitor R406. *D. farinae* stimulation of WT BMDCs triggered phosphorylation of Akt at 1 min (Fig. 2A), which was reduced by 88 ± 2% in the presence of R406. In keeping with the role of Syk as an upstream mediator of the Dectin-2 pathway, blockade of Syk with two different inhibitors, Syk II and R406, significantly reduced the production of *D. farinae*–elicited cys-LTs, IL-23, and IL-33 in a dose-dependent manner (Fig. 2B–G). LPS-induced TNF-α generation was intact in the presence of each Syk inhibitor, indicating the specificity of inhibition (Fig. 2H). These findings demonstrate that both PI3K and Syk are required for production of each Dectin-2–dependent mediator in response to *D. farinae* and that PI3K activity depends on Syk.

PI3K p110δ isoform regulates Dectin-2 signaling

Next we investigated which isoform of PI3K is required for Dectin-2 signaling elicited by *D. farinae*. WT BMDCs expressed each class I PI3K isoform, with PI3Kδ being the most abundant (Fig. 3A). There was no difference in PI3K isoform expression between WT and *Clec4n*$^{-/-}$ BMDCs. Two isoforms of class I PI3K, p110β (PI3Kβ) and p110δ (PI3Kδ), have been linked to the function of FcRγ-chain–dependent receptors in myeloid cells (24, 31). To assess their role in Dectin-2 signaling, we first used available inhibitors. Inhibition of PI3Kδ with CAL-101 reduced *D. farinae*–induced cys-LTs, IL-23, and IL-33 with IC_{50} values of 0.5, 1.63, and 1.67 nM, respectively (Fig. 3B–D). The inhibition was specific, as BMDCs treated with higher levels of inhibitor had no reduction in LPS-induced TNF-α (Fig. 3E). Similarly, use of another highly specific PI3Kδ inhibitor, IC87114, also significantly reduced *D. farinae*–induced cys-LTs, IL-23, and IL-33 (Fig. 3F–H), with intact LPS-induced TNF-α (Fig. 3I). In contrast, treatment with a PI3Kβ inhibitor at doses specific for the p110β isoform had no effect on the levels of *D. farinae*–induced cys-LTs, IL-23, or IL-33 and no effect on LPS-induced TNF-α (Supplemental Fig. 1). At 100 nM PI3Kβ inhibitor, a dose previously reported to also block PI3Kδ (32), there was a small trend to reduced cys-LTs and IL-33 that was not significant.

In keeping with our prior data suggesting that PI3K activity is downstream of Syk (Fig. 2A), PI3Kδ inhibition had no effect on Syk phosphorylation (Fig. 3J). PKCδ, another downstream Syk-dependent signaling molecule in the Dectin-2 pathway, phosphorylates CARD9 to facilitate NF-κB activation and the generation of Dectin-2–dependent cytokines elicited by *C. albicans* (2, 6). To determine whether PI3Kδ is upstream of PKCδ and CARD9 activation, we assessed the effect of PI3Kδ inhibition on *D. farinae*–elicited PKCδ phosphorylation. *D. farinae* stimulation of WT BMDCs induced phosphorylation of PKCδ, which was detected at 5 and 15 min (Fig. 3K). PI3Kδ inhibition reduced PKCδ phosphorylation by 90 ± 3% at 15 min, indicating that PI3Kδ is proximal to PKCδ and CARD9 signaling. In keeping with a downstream role for CARD9 in *D. farinae*–elicited signaling, we did find that *D. farinae*–stimulated *Card9*$^{-/-}$ BMDCs had reduced generation of both IL-23 and IL-33, but preserved cys-LT generation (Supplemental Fig. 2).

To confirm a requirement for PI3Kδ in Dectin-2 signaling, we used a knockdown approach. WT BMDCs were harvested on day 7, treated with PI3Kδ siRNA or nontargeting siRNA control (control siRNA) by nucleofection, and stimulated with *D. farinae* 24 h later. At 24 h, PI3Kδ siRNA–treated BMDCs had a significant reduction in PI3Kδ transcript (Fig. 4A), but no reduction in transcript for PI3Kα, β, or γ isoforms (Fig. 4B–D). Western blotting confirmed a reduction in PI3Kδ protein in siRNA-treated

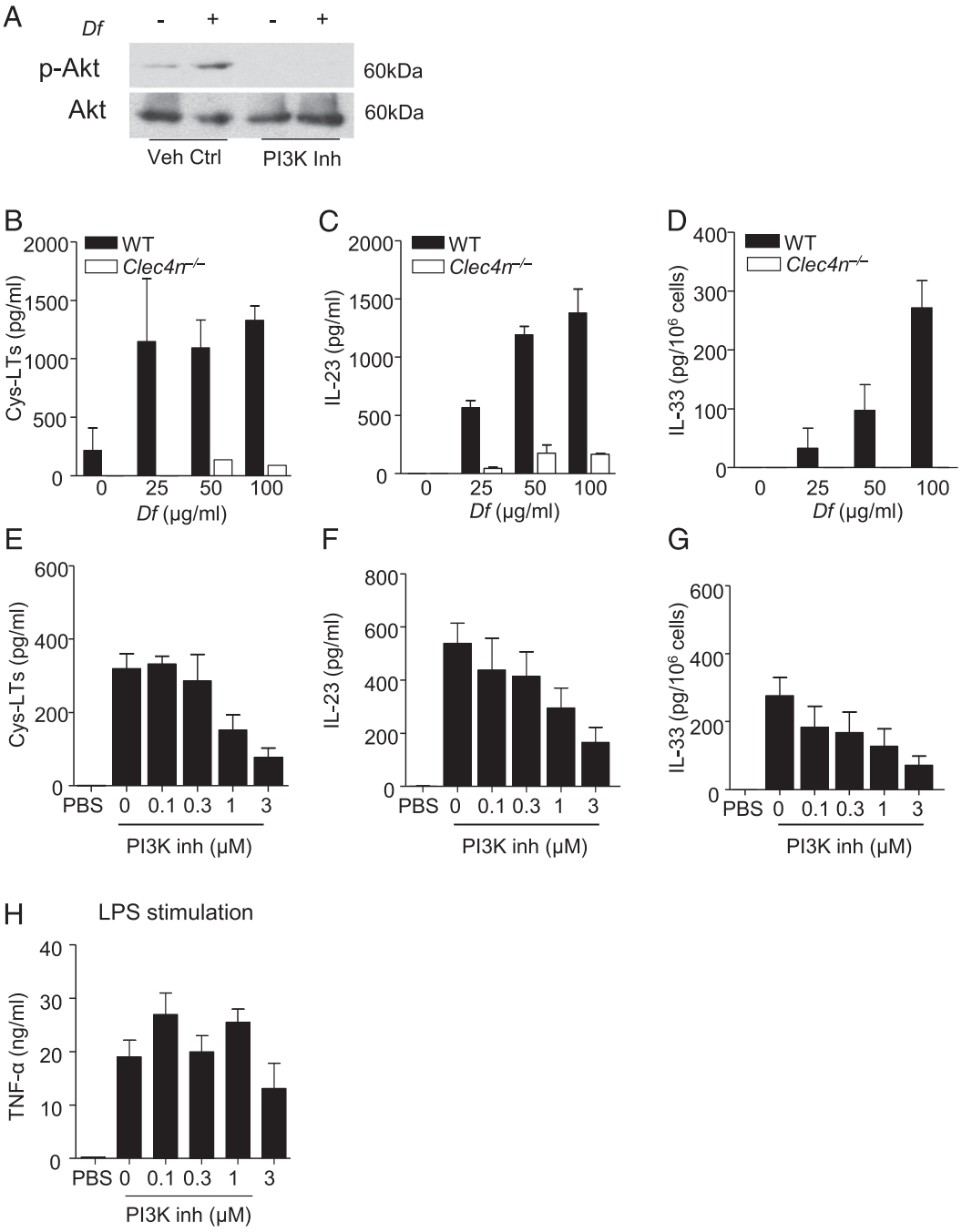

FIGURE 1. PI3K activity is required for the generation of each Dectin-2 mediator. (**A**) Representative Western blot from one of two independent experiments. Phosphorylation of Akt in WT BMDCs stimulated with 0 (−) or 100 μg/ml *D. farinae* (+) for 1 min in the presence of 3 μM Ly294002 (pan-PI3K inhibitor) or vehicle control. (**B**–**D**) Day 7 BMDCs from WT and *Clec4n*$^{-/-}$ mice were stimulated with increasing doses of *D. farinae*. Cys-LTs and IL-23 were measured in supernatants and IL-33 in the cell lysates by ELISA at 30 min, 8 h, and 8 h, respectively. $p = 0.0004$ for cys-LTs, $p < 0.0001$ for IL-23 and IL-33. (**E**–**H**) WT BMDCs were stimulated with PBS or (E–G) 100 μg/ml *D. farinae* or (H) 100 ng/ml LPS in the presence of Ly294002 at indicated doses. $p < 0.01$ for cys-LTs and IL-23, $p < 0.05$ for IL-33, and not significant for TNF-α. Results are means ± SEM pooled from three independent experiments. Significance was determined with two-way ANOVA. *Df*, *D. farinae*.

BMDCs relative to controls (Fig. 4E), and the reduction averaged 64 ± 4% at 24 h (Fig. 4F). Accordingly, *D. farinae*–induced cys-LTs, IL-23, and IL-33 were significantly reduced (Fig. 4G–I), as compared with control siRNA–treated BMDCs. The inhibition was specific, as PI3Kδ siRNA–treated BMDCs had no reduction in LPS-elicited TNF-α (Fig. 4J). These results demonstrate that PI3Kδ mediates *D. farinae*–elicited Dectin-2 signaling.

To verify that PI3Kδ activity contributes specifically to Dectin-2 signaling, BMDCs were activated with plate-bound Dectin-2 Ab overnight in the presence of 0, 10, or 100 nM PI3Kδ inhibitor. Dectin-2 cross-linking elicited cys-LTs and IL-33, each of which was absent in *Clec4n*$^{-/-}$ BMDCs (Fig. 5A, 5B). IL-23 was also detected, but high levels were elicited from plate-bound IgG controls (data not shown). TNF-α, another Dectin-2– and CARD9-dependent cytokine, was also elicited by Dectin-2 cross-linking and significantly reduced in *Clec4n*$^{-/-}$ BMDCs (Fig. 5C). Treatment with a PI3Kδ inhibitor reduced Dectin-2-elicited cys-LTs, IL-33, and TNF-α in a dose-dependent fashion (Fig. 5). These results

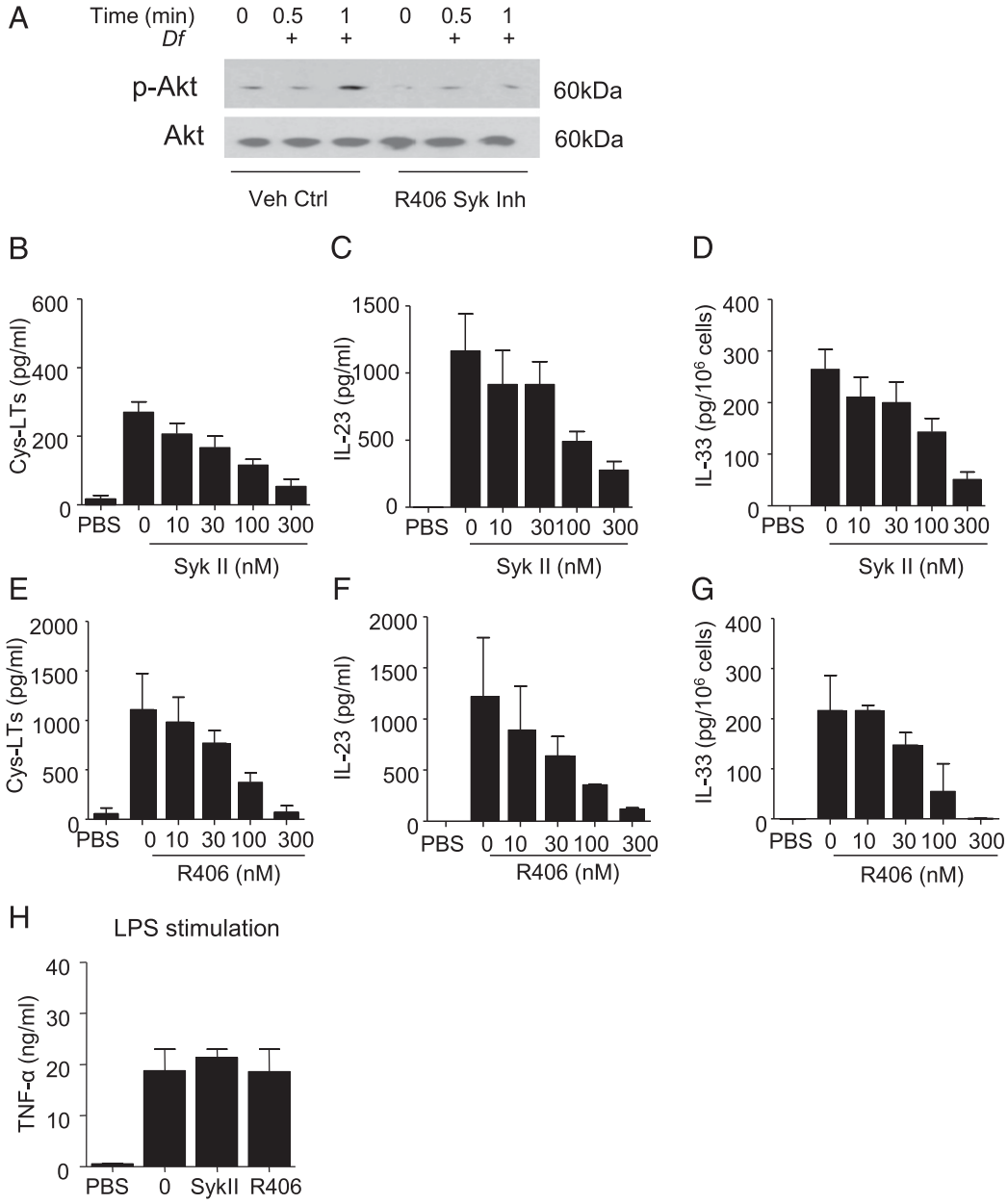

FIGURE 2. *D. farinae*–elicited PI3K activity, cys-LTs, and cytokines depend on Syk. (**A**) Phosphorylation of Akt in WT BMDCs stimulated with 100 μg/ml *D. farinae* (+) at various time points in the presence of 300 nM R406 (Syk inhibitor) or vehicle control. (**B**–**G**) WT BMDCs were stimulated with PBS or 100 μg/ml *D. farinae* or (**H**) 100 ng/ml LPS in the presence of specific Syk inhibitors, (B–D) Syk II inhibitor, $p < 0.0001$ for cys-LTs, $p < 0.05$ for IL-23 and IL-33, or (E–G) R406, $p < 0.05$ for cys-LTs, IL-23, and IL-33. Results are means ± SEM pooled from three independent experiments. Significance was determined with one-way ANOVA. *Df*, *D. farinae.*

further demonstrate that PI3Kδ is an essential component of Dectin-2 signaling and mediates the generation of both cys-LTs and cytokines.

PI3Kδ is required for Dectin-2–mediated pulmonary inflammation in vivo

To understand whether PI3Kδ activity is essential for *D. farinae*–induced pulmonary inflammation, we used an adoptive transfer model in mice (14). WT BMDCs were pulsed with either PBS (PBS-DCs) or *D. farinae* in the presence of the PI3Kδ inhibitor CAL-101 (PI3Kδ-*D. farinae*-DCs) or vehicle control (*D. farinae*-DCs) for 8 h, washed, and transferred intranasally into naive WT C57BL/6 recipient mice on day 0. All recipients were challenged intranasally with 3 μg *D. farinae* on days 11 and 13, and killed on day 15. WT mice sensitized with *D. farinae*-DCs showed inflammation in the BAL fluid after *D. farinae* challenge, as compared with mice sensitized with PBS-DCs (Fig. 6A). This included an influx of macrophages, neutrophils, and eosinophils. Mice sensitized with PI3Kδ-*D. farinae*-DCs had significant and marked reductions in total cell counts of 80%, neutrophils of 91%, and eosinophils of 96%, as compared with mice sensitized with vehicle control–treated *D. farinae*-DCs.

PI3Kδ treatment of BMDCs did not influence BMDC survival, as PI3Kδ-*D. farinae*-DCs cultured for an additional 48 h demonstrated no difference in staining with a nonpermeant amine-reactive fluorescent viability dye compared with vehicle control–treated *D. farinae*-DCs (Supplemental Fig. 3A). Furthermore, $EGFP^+$ PI3Kδ-*D. farinae*-DCs were detected in the MDLNs of recipient mice at 24 h after adoptive transfer in comparable numbers to $EGFP^+$ vehicle control–treated *D. farinae*-DCs (Supplemental Fig. 3B).

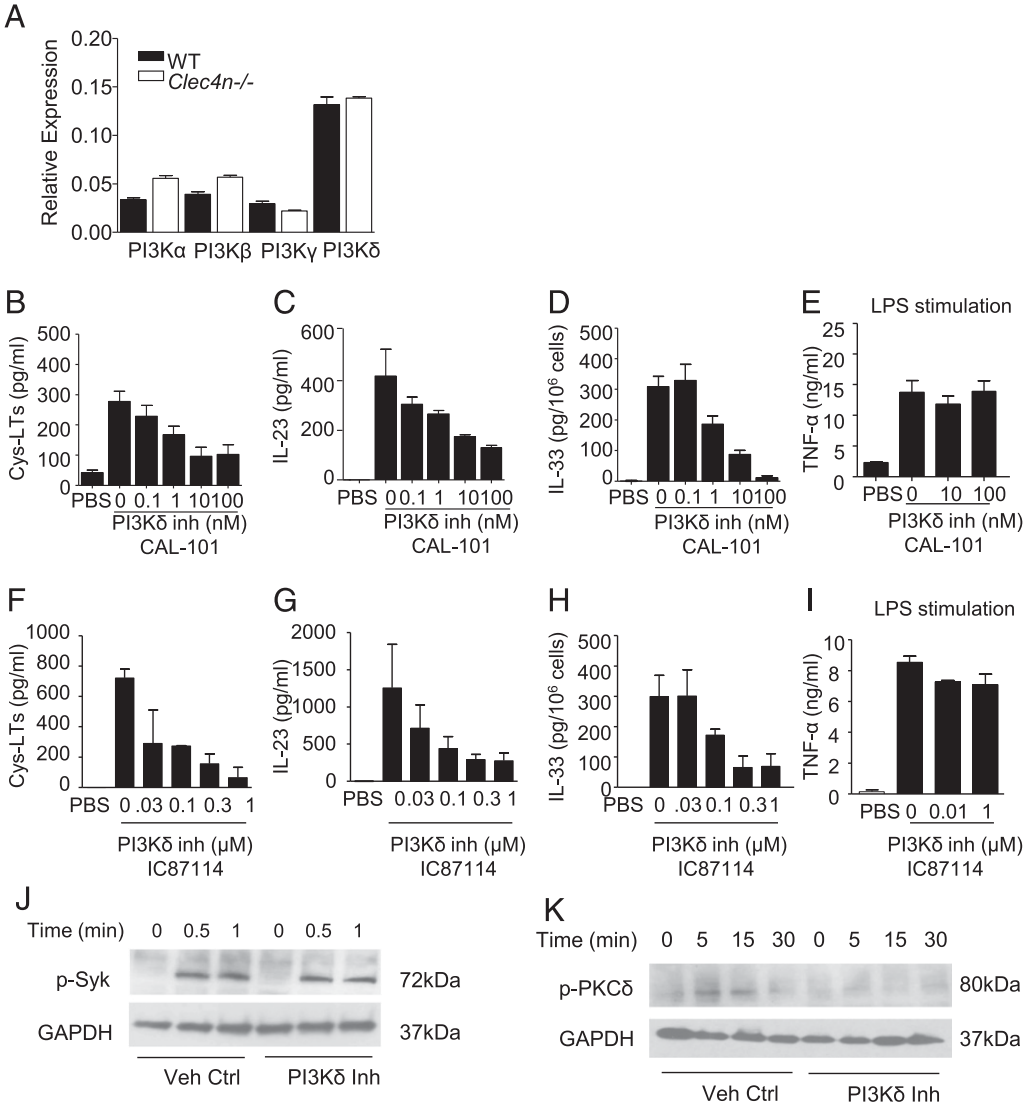

FIGURE 3. Pharmacologic inhibition suggests that PI3Kδ is required for cys-LTs, cytokines, and activation of PKCδ. (**A**) PI3Kα, PI3Kβ, PI3Kγ, and PI3Kδ transcript expression relative to GAPDH in WT and *Clec4n*$^{-/-}$ BMDCs. (**B–I**) WT BMDCs were stimulated with PBS or 100 μg/ml *D. farinae* or 100 ng/ml LPS in the presence of (B–E) CAL-101 or (F–I) IC87114. For CAL-101, $p < 0.001$ for cys-LTs and for IL-23, $p < 0.0001$ for IL-33. For IC87114, $p < 0.05$ for cys-LTs, IL-23, and IL-33. Results are means ± SEM pooled from three independent experiments. Significance was determined with one-way ANOVA. (**J**) Phosphorylation of Syk in WT BMDCs stimulated with 100 μg/ml *D. farinae* at various time points in the presence of 100 nM CAL-101 (PI3Kδ inh) or vehicle control (Veh Ctrl). (**K**) Phosphorylation of PKCδ in WT BMDCs stimulated with 100 μg/ml *D. farinae* at various time points in the presence of 100 nM CAL-101 or vehicle control. Representative Western blot from one of two independent experiments (J and K).

Histologic analysis of the lung stained with H&E showed that mice sensitized with *D. farinae*-DCs had dense cellular infiltrates around the bronchovascular bundles that were not present in mice sensitized with PBS-DCs (Fig. 6B, H&E). These infiltrates were markedly diminished in mice sensitized with PI3Kδ-*D. farinae*-DCs. Goblet cell metaplasia and mucus production, as detected by periodic acid–Schiff (PAS) staining, were also present in mice sensitized with *D. farinae*-DCs, but were absent in mice sensitized with either PBS-DCs or PI3Kδ-*D. farinae*-DCs (Fig. 6B, PAS). Thus, PI3Kδ is critical for the capacity of BMDCs to elicit *D. farinae*–induced pulmonary inflammation.

To understand whether PI3Kδ-*D. farinae*-DCs elicited an altered T cell cytokine response, we harvested the MDLN cells from each cohort at day 15, restimulated them with 20 μg/ml *D. farinae* for 72 h, and assayed for T cell cytokines in cultured supernatants (14, 33). MDLN cell cultures from WT mice sensitized with *D. farinae*-DCs generated high levels of IL-4, IL-5, IL-13, and IL-17A, as compared with those sensitized with PBS-DCs (Fig. 6C). MDLN cell cultures from WT mice sensitized with PI3Kδ-*D. farinae*-DCs generated significantly less IL-4, IL-5, IL-13, and IL-17A. MDLN cell cultures from each group generated high levels of IFN-γ, consistent with an Ag-independent response to DC adoptive transfer previously reported (14). Thus, PI3Kδ is critical for the capacity of BMDCs to generate pulmonary inflammation and Th2 and Th17 immunity to *D. farinae*.

To verify that PI3Kδ was important in priming through the mucosal route, we treated WT mice with 3 μg intranasal *D. farinae* on days 0, 10, and 12, and with the PI3Kδ inhibitor IC87114 or vehicle control by oral gavage on day 0. IC87114 was chosen based on its established use in murine models of inflammation (29, 30, 34). The BAL fluid at day 14 revealed pulmonary inflammation in WT mice sensitized and challenged with *D. farinae* that included both eosinophils and neutrophils (Fig. 6D). Mice treated with the PI3Kδ inhibitor on day 0 showed attenuated development of *D. farinae*–elicited pulmonary inflammation with significant reductions in total BAL fluid cell counts, macrophages, neutrophils,

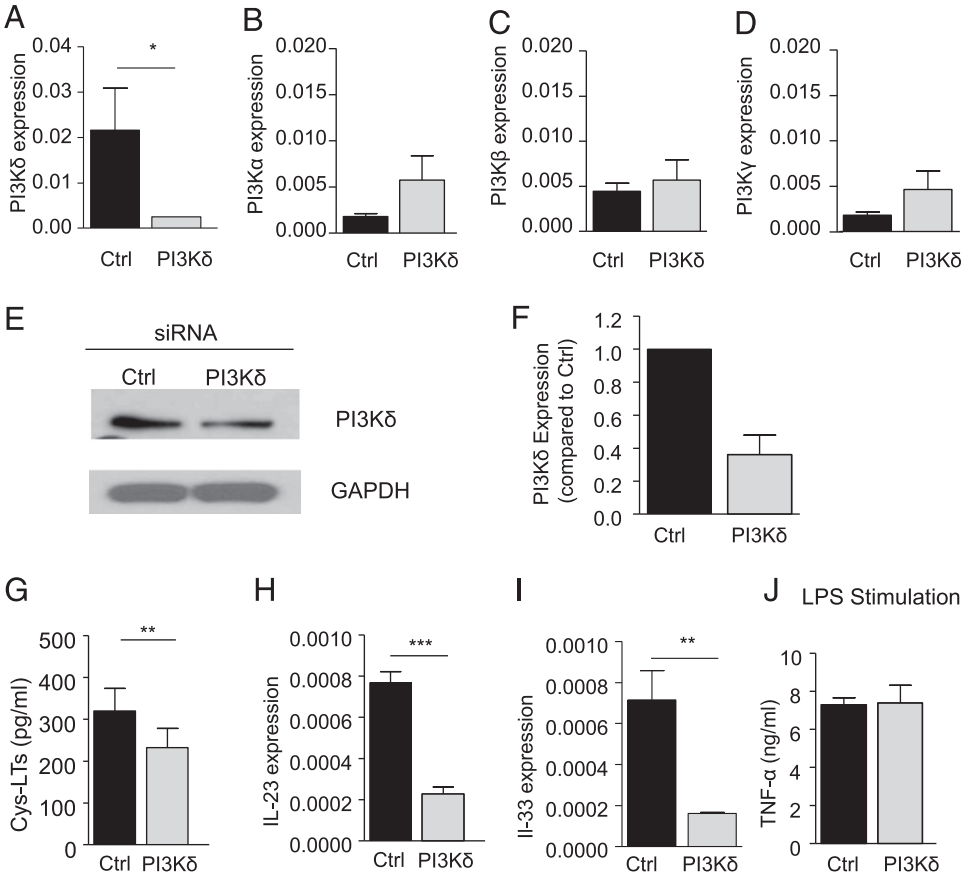

FIGURE 4. PI3Kδ knockdown confirms a role for PI3Kδ in Dectin-2 signaling. Day 7 WT BMDCs were transfected with nontargeting control siRNA or PI3Kδ siRNA. (**A**–**D**) PI3Kδ, PI3Kα, PI3Kβ, and PI3Kγ transcript expression relative to 18S in nontargeting control siRNA or PI3Kδ siRNA group. (**E**) Representative Western blots of PI3Kδ and GAPDH in BMDCs transfected with either nontargeting control siRNA or PI3Kδ siRNA. (**F**) Densitometric analysis of PI3Kδ proteins relative to GAPDH on Western blots in control (set as 1) and PI3Kδ siRNA transfected groups. (**G**–**I**) *D. farinae*–elicited cys-LTs at 30 min, and IL-23 and IL-33 expression at 6 h, as compared with control siRNA-treated BMDCs. (**J**) LPS-elicited TNF-α at 6 h, as measured by ELISA. Results are means ± SEM pooled from three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.0001$ compared with control siRNA.

and eosinophils, as compared with mice treated with vehicle control. These results further suggest that PI3Kδ plays an important role in the generation of *D. farinae*–elicited allergic pulmonary inflammation.

Discussion

Our study demonstrates that PI3Kδ is an important signaling intermediate in the DC Dectin-2 signaling pathway elicited either by *D. farinae* or by Ab-mediated cross-linking. We found that PI3Kδ is Syk-dependent and regulates PKCδ activation and the generation of both cys-LTs and cytokines elicited by *D. farinae*. Accordingly, two models of *D. farinae*–elicited Dectin-2–dependent allergic pulmonary inflammation were significantly attenuated by PI3Kδ inhibition, indicating the importance of Dectin-2 and PI3Kδ in the immune response triggered by this common allergen.

To our knowledge, this is the first study to identify PI3Kδ in CLR signaling. However, because several members of the myeloid CLR family use a shared Syk signaling pathway (1), PI3Kδ may regulate the signaling of additional receptors. Indeed, we have found that the Dectin-1 agonist, curdlan, also induces cys-LTs in a manner that partially depends on PI3Kδ (data not shown). As PI3Kδ inhibitors are in clinical trials for B cell malignancies, further identifying such roles will be important to understand the possible risks associated with systemic PI3Kδ inhibition.

PI3Kδ is an upstream mediator of several membrane-bound receptors that use ITAM/Syk-dependent signaling, including the BCR and the TCR (22). Ligation of the BCR causes tyrosine kinase–mediated phosphorylation of Igα and Igβ, and recruitment of PI3K through phosphorylation of the YXXM-bearing CD19 coreceptor and the B cell adaptor for PI3K (35, 36). TCR activation of PI3K also depends on tyrosine kinase signaling and has been variably attributed to the YXXM-bearing adaptor protein TCR interacting molecule (37) or to phosphorylation of additional non-ITAM tyrosine-containing proteins such as the linker of activated T cells and Src homology 2 domain–containing leukocyte protein of 76 kDa (38, 39). Our finding that *D. farinae*–elicited PI3K activity

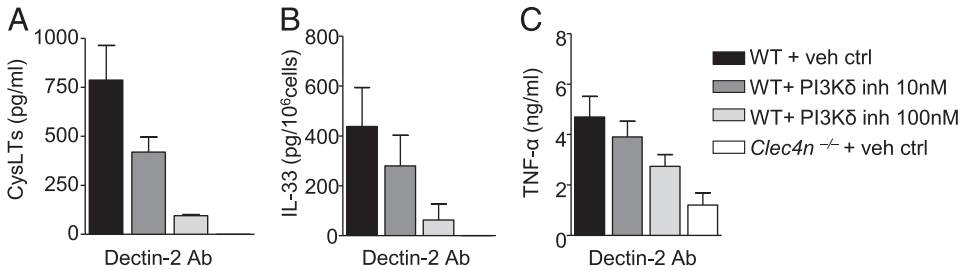

FIGURE 5. PI3Kδ inhibition reduces Dectin-2 signaling elicited by Ab-mediated cross-linking. WT and *Clec4n*$^{-/-}$ BMDCs were stimulated with plate-bound Dectin-2 Ab overnight in the presence or absence of CAL-101. (**A**–**C**) The concentrations of cys-LTs and TNF-α in the supernatants and of IL-33 in the cell lysates were measured by ELISA. Results are means ± SEM pooled from three independent experiments. $p < 0.001$ (cys-LTs), $p < 0.01$ (TNF-α), $p < 0.05$ (IL-33). Significance was determined with one-way ANOVA.

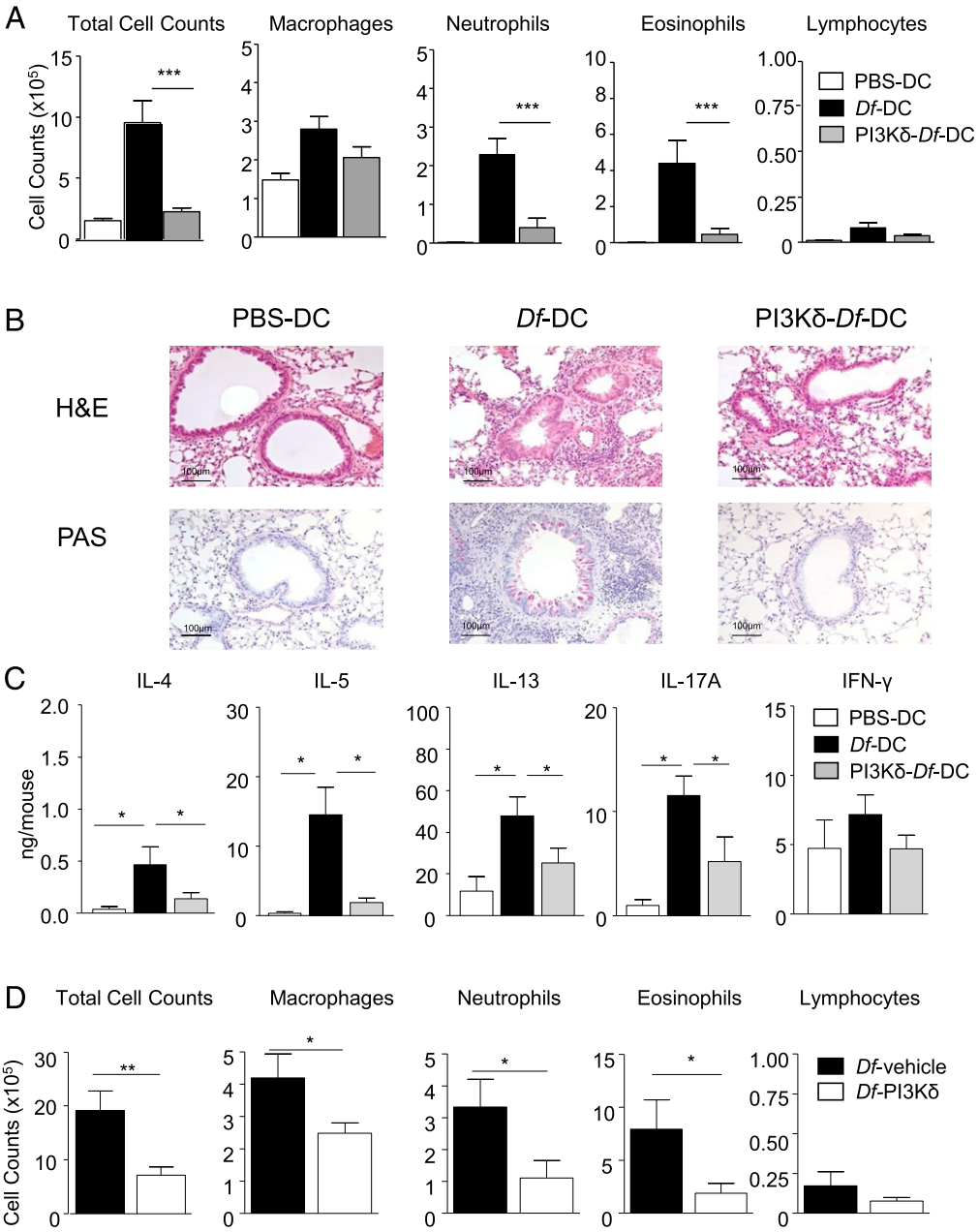

FIGURE 6. PI3Kδ is required for Dectin-2–mediated pulmonary inflammation in vivo. (**A**–**C**) WT mice were sensitized with 10^4 PBS- or *D. farinae*–pulsed BMDCs in the presence or absence of PI3Kδ inhibitor CAL-101 at 10 nM (PBS-DCs, *D. farinae*-DCs, PI3Kδ-*D. farinae*-DCs), challenged with 3 μg *D. farinae* on days 11 and 13 and killed on day 15. (A) Total and differential cell counts for BAL fluid macrophages, neutrophils, eosinophils, and lymphocytes. (B) H&E and PAS staining of lung tissue from each group. (C) MDLN cells were isolated and restimulated with 20 μg/ml *D. farinae* for 72 h. Cytokines were measured in the supernatants. Results are means ± SEM (n = 8, 13, and 15 mice for PBS-DC, *D. farinae*-DC, PI3Kδ-*D. farinae*-DC groups, respectively) from three experiments. (**D**) On day 0, WT mice were sensitized with 3 μg *D. farinae* intranasally. PI3Kδ inhibitor IC87114 (30 mg/kg) or vehicle control (PEG-400) was administered orogastrically at 1 h before and 8 h after *D. farinae* administration. Mice were then challenged intranasally with 3 μg *D. farinae* on days 11 and 13 and killed on day 15. Total and differential cell counts for BAL fluid macrophages, neutrophils, eosinophils, and lymphocytes are shown. Results are means ± SEM (n = 8 and 10 mice for *D. farinae*-vehicle– and *D. farinae*-PI3Kδ–treated groups, respectively) pooled from three independent experiments. *$p < 0.05$, **$p < 0.01$. *Df, D. farinae*.

depends on Syk is in keeping with these studies. However, neither Dectin-2, FcRγ, nor Dectin-3, which dimerizes with Dectin-2 (40), contains the tyrosine motifs that support PI3Kδ recruitment. As such, our study suggests that Dectin-2 uses additional adaptor proteins as the docking site for the Src homology 2 domain–containing p85 regulatory subunit of PI3Kδ. Notably, the tyrosine-containing adaptor protein linker for activation of B cells/non–T cell activating linker was recently reported to be phosphorylated after Dectin-2 activation (41), but whether Dectin-2 utilizes this protein or an as yet unidentified YXXM-bearing adaptor protein to support PI3Kδ activation remains to be determined.

The generation of cys-LTs is unique to CLRs among pattern recognition receptor families, likely due to the sustained calcium flux that is required to translocate cytosolic phospholipase A_2 from the cytosol to the nuclear membrane to release arachidonate from membrane phospholipids (10, 42). PI3K plays a key role in mobilizing calcium and activating PKCs in response to FcεR1 signaling or BCR signaling by activating PLCγ (24, 43, 44). Recent studies

on Dectin-1 and Dectin-2 have also demonstrated that PLCγ is required for eliciting a calcium flux and for PKCδ- and CARD9-dependent cytokine generation (6, 45, 46). Moreover, our data demonstrate that inhibition of PI3Kδ impairs PKCδ phosphorylation elicited by *D. farinae*. Taken together, these studies indicate that PI3Kδ is a proximal mediator of Dectin-2–dependent arachidonate metabolism and cytokine generation and likely acts by triggering activation of PLCγ.

We found that Dectin-2, PI3Kδ, and CARD9 were required for *D. farinae*–elicited IL-33 generation in DCs. IL-33 generation in APCs can be induced by multiple stimuli such as influenza virus (18), *Nippostrongylus brasiliensis* (47), *Histoplasma capsulatum* (48), and TLR agonists (49), and prior studies have demonstrated roles for both IFN regulatory factors (IRFs) and NF-κB in the transcriptional regulation of IL-33. IRF3 is required for TLR3 and TLR4-induced IL-33 in murine macrophages (49), and IRF4 is required for both Dectin-1– and Dectin-2–induced IL-33 in macrophages and DCs, respectively (48, 50). However, NF-κB is required for TLR5-induced IL-33 generation from murine DCs (51) and for TLR3- and TLR5-induced IL-33 from human epithelial cells (52). In keeping with these findings, our results show that *D. farinae*–induced IL-33 generation is reduced in *Card9*$^{-/-}$ BMDCs but more significantly attenuated with PI3Kδ inhibition, suggesting that PI3Kδ signaling can promote IL-33 induction through both CARD9-dependent NF-κB activation and CARD9-independent IRF4 activation.

We and others have identified a role for Dectin-2 in the generation of both Th2- and Th17-dependent allergic pulmonary inflammation to HDM (14, 15, 19, 50). In the present study, we demonstrate that PI3Kδ signaling in DCs is required for this function, as the adoptive transfer of PI3Kδ-inhibited *D. farinae*-DCs had a reduced capacity to generate Th2- and Th17-dependent allergic pulmonary inflammation. We have previously demonstrated a role for the Dectin-2/LTC_4S pathway in the type 1 cys-LT receptor–dependent conditioning of DCs to promote Th2 sensitization (14), but our findings in the present study suggest that an additional Dectin-2 mediator, IL-33, may participate in this process. IL-33 promotes Th2 immunity through several actions, including the upregulation of DC costimulatory molecules and the polarization of naive T cells to Th2 (18). Although IL-33 is widely expressed (18), it is bound and neutralized by circulating soluble ST2 (18), suggesting that anatomic and temporal control of IL-33 generation may regulate its function and that the DC-specific generation of IL-33 in response to allergen may contribute to local polarization in the lymph node.

We found that inhibition of PI3Kδ solely on the day of *D. farinae* sensitization prevented allergic pulmonary inflammation elicited by *D. farinae* challenge 2 wk later. Because of the short $t_{1/2}$ of the PI3Kδ inhibitor used (53), the results of PI3Kδ inhibition in vivo are likely due to proximal effects on DC-mediated sensitization, which occurs during the first 24–72 h (54), but additional effects on the activation of T cells or B cells cannot be excluded. Several previous studies have demonstrated a role for PI3Kδ in the effector phase of Ag-induced allergic pulmonary inflammation. In these studies, the airway administration of a PI3Kδ inhibitor during the OVA challenge of previously sensitized mice reduced BAL eosinophilia, Th2 and Th17 lung cytokine production, airway hyperresponsiveness, and OVA-specific IgE (29, 34). Furthermore, $PI3K\delta^{D910A/D910A}$ mice, which express a catalytically inactive form of PI3Kδ, were also protected from BAL eosinophilia and Th2 cytokine production in models of Th2 pulmonary inflammation using sensitization and challenge to OVA (55) or to uric acid (56). Although the present study demonstrates a key role for PI3Kδ in DC function during the sensitization phase, the same Dectin-2 pathway contributes to pulmonary inflammation in effector phase responses (33). Thus, use of a well-tolerated and highly specific PI3Kδ inhibitor such as CAL-101 (also known as idelalisib), with recently reported efficacy in lymphoma (57), may be a rational therapeutic strategy to prevent the mucosal activation of both lymphoid and myeloid cells that participate in type 2 pulmonary inflammation.

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Disclosures

The authors have no financial conflicts of interest.

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