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Protein Kinase D1 Is Essential for the Proinflammatory Response Induced by Hypersensitivity Pneumonitis-Causing Thermophilic Actinomycetes Saccharopolyspora rectivirgula

Young-In Kim,*‡,†, Jeoung-Eun Park,*‡,†, David D. Brand,*‡,.§,* Elizabeth A. Fitzpatrick,* and Ae-Kyung Yi*‡,§,¶

Hypersensitivity pneumonitis is an interstitial lung disease that results from repeated pulmonary exposure to various organic Ags, including Saccharopolyspora rectivirgula, the causative agent of farmer’s lung disease. Although the contributions of proinflammatory mediators to the disease pathogenesis are relatively well documented, the mechanism(s) involved in the initiation of proinflammatory responses against the causative microorganisms and the contribution of signaling molecules involved in the host immune defense have not been fully elucidated. In the current study, we found that S. rectivirgula induces the activation of protein kinase D (PKD1) in lung cells in vitro and in vivo. Activation of PKD1 by S. rectivirgula was dependent on MyD88. Inhibition of PKD by pharmacological PKD inhibitor G66976 and silencing of PKD1 expression by small interfering RNA revealed that PKD1 is indispensable for S. rectivirgula-mediated activation of MAPKs and NF-kB and the expression of various proinflammatory cytokines and chemokines. In addition, compared with controls, mice pretreated with G66976 showed significantly decreased myeloperoxidase activity in the lung after pulmonary exposure to S. rectivirgula. These results demonstrate that PKD1 is essential for S. rectivirgula-mediated proinflammatory immune responses and neutrophil influx in the lung. Our findings also imply the possibility that PKD1 is one of the critical factors that play a regulatory role in the development of hypersensitivity pneumonitis caused by microbial Ags and that inhibition of PKD1 activation could be an effective way to control microbial Ag-induced hypersensitivity pneumonitis. The Journal of Immunology, 2010, 184: 3145–3156.

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Abbreviations used in this paper: BAL, bronchoalveolar lavage; BMDM, bone marrow-derived macrophage; IP, IFN-inducible protein; IRAK, IL-1R–associated kinase; ITAC, IFN-inducible T cell α chemotractant; KC, keratinocyte-derived chemokine; LIX, LPS-induced CXC chemokine; Mgp, monokine induced by IFN-γ; MPO, myeloperoxidase; NT, nontarget; PAMP, pathogen-associated molecular pattern; PGN, peptidoglycan; PRC, polynosinic-polycyti-dylic acid; PRR, recognition receptor; siRNA, small interfering RNA; TAK1, TGFβ–activated kinase 1; TLR, Toll-like receptor; TRAF6, TNF-αR–associated factor 6; TIR, Toll–

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hypothesis that the early inflammatory response to hypersensitivity pneumonitis-causing Ags results in the production of chemokines and proinflammatory cytokines that likely play a role in the induction and progression of the disease. Although the role of inflammatory mediators and the contributions of immune cells in the development of hypersensitivity pneumonitis have been intensively studied and relatively well documented, the mechanisms involved in the initiation of the host proinflammatory responses to the hypersensitivity pneumonitis-inciting agents, such as *S. rectivirgula*, are not completely understood. Identification of host innate immune receptors that are involved in the initial detection of the inciting Ags of hypersensitivity pneumonitis and careful investigation of signal transduction mediated by these innate immune receptors will provide new and important insights into the disease pathogenesis.

Cells in the innate immune system express a family of evolutionarily conserved PRRs, such as TLRs, nucleotide-binding oligomerization domain–like receptor–family proteins, helicase domain–containing antiviral proteins, and cytosolic DNA sensors (21–26). Recognition of the evolutionarily conserved structures in microorganisms (pathogen–associated molecular pattern [PAMP]), including LPS, peptidoglycan (PGN), and microbial nucleic acids by PRRs on/in innate immune cells initiates the complex signaling cascades leading to secretion of proinflammatory cytokines and mediators. The most extensively studied PRRs are TLRs, which are type I transmembrane proteins containing N-terminal leucine-rich repeats that are responsible for binding to PAMPs, a transmembrane domain, and a C-terminal Toll/IL-1 receptor (TIR) domain that is responsible for signaling. At least 13 TLRs have been identified in mice, and 10 have been identified in humans (27). Each TLR forms homodimers or heterodimers and has different binding specificity to PAMPs (28). After binding to a specific PAMP, TLRs recruit one or more TIR domain–containing adaptor molecules that orchestrate a complexity of downstream signaling cascades and biologic outcomes. Among the five identified TIR domain–containing adaptor molecules, MyD88 is used by all TLRs except TLR3 (29, 30). In MyD88–dependent TLR signaling, recruitment of MyD88 to a TLR through interaction between TIR domains leads to the recruitment of IL–1R–associated kinase (IRAK) family members IRAK4 and IRAK1 to the TLR/MyD88 signaling complex through interaction between the death domains of MyD88 and IRAKs (22, 31, 32). IRAK1 becomes rapidly phosphorylated by IRAK4, resulting in recruitment of TNF–αR–associated factor 6 (TRAF6) to the receptor complex (31, 33–35). Phosphorylated IRAK1 and TRAF6 are thought to dissociate from the receptor, which is followed by TRAF6 autoubiquitination with K63–linked polyubiquitin chains and subsequent polyubiquitination of IRAK1 by TRAF6 (36–39). Ubiquitinated TRAF6 binds to and activates a signaling complex composed of TGF–β–activated kinase 1 (TAK1) and TAK1–binding protein 2, which initiates signaling cascades that lead to activation of NF–κB and MAPKs, and subsequent expression of proinflammatory cytokines and chemokines (32, 39–44). In addition to this well-known MyD88–dependent pathway, we recently found that a serine/threonine kinase protein kinase D (PKD)1 is activated by all TLRs that transduce their signal through MyD88 (45, 46). PKD1 is recruited to and activated in the TLR/MyD88 receptor complex via interaction with IRAK4, IRAK1, and TRAF6. PKD1 is required for ubiquitination of TRAF6 and the subsequent activation of TAK1. Activation of PKD1 by TLRs is essential for MyD88–dependent expression of proinflammatory cytokines and mediators in innate immune cells. Using an animal model of hypersensitivity pneumonitis, a recent study demonstrated that the initial expression of proinflammatory cytokines and chemokines in the lung and the subsequent neutrophil infiltration into the lung after pulmonary exposure to *S. rectivirgula* are largely dependent on MyD88 (4). These findings suggest the possibility that *S. rectivirgula* activates PKD1, and PKD1 may play a regulatory role in *S. rectivirgula*–induced proinflammatory responses. In the current study, we investigated whether *S. rectivirgula*, a microbial Ag that causes farmer’s lung disease, induces the activation of PKD1 in various innate immune cells in the lung in vitro and in vivo in an MyD88–dependent manner and whether PKD1 plays a biologic role in *S. rectivirgula*–mediated proinflammatory responses in vitro and in vivo.

**Materials and Methods**

**Mice**

*C57BL/6* mice were obtained at 4–5 wk of age from The Frederick Cancer Research and Development Center, National Cancer Institute (Frederick, MD) and were used within 3 wk. MyD88 gene–deficient (MyD88<sup>−/−</sup>) mice were provided by Dr. S. Akira (Osaka University, Osaka, Japan). All animal care and housing requirements set forth by the National Institutes of Health Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources were followed, and animal protocols were reviewed and approved by the University of Tennessee Animal Care and Use Committee.

**Cells and culture conditions**

Bone marrow–derived macrophages (BMDMs) were isolated as described (45, 46). Murine cell lines RAW264.7 (macrophages), AM2–C11 (alveolar macrophages), MPRO (premyelocytes), and MLE12 (bronchial epithelial cells) were purchased from the American Type Culture Collection (Manassas, VA). FLAG-tagged PKD–expressing RAW264.7 cells were generated as described (46). BMDM, RAW264.7, and FLAG-tagged PKD–expressing RAW264.7 cells were cultured in DMEM supplemented with 10% (v/v) heat–inactivated FCS. AM2–C11 cells were cultured in DMEM supplemented with 5% (v/v) heat–inactivated FCS. MLE cells were cultured in DMEM/F-12 supplemented with 2% (v/v) heat–inactivated FCS. MPRO cells were cultured in Iscove’s modified Dulbecco’s medium supplemented with 5% (v/v) heat–inactivated FCS and 10 ng/ml GM–CSF.

The morphologic differentiation of MPRO promyelocytes to neutrophils (MPRO–Neut) was induced by supplementing 10 μM retinoid acid (R2625, Sigma–Aldrich, St. Louis, MO) for 3 d. To confirm morphologic changes, cells were stained with Diff–Quik (IMEB, San Marcos, CA), according to the manufacturer’s protocol. All cells were incubated in the indicated FCS–containing medium supplemented with 1.5 mm L–glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified incubator with 5% CO<sub>2</sub>. All culture reagents were purchased from Invitrogen (Carlsbad, CA) and Sigma–Aldrich.

*S. rectivirgula–Ag*, TLR ligands, and reagents

*S. rectivirgula* (strain designation A1313; ATCC number 29034) was purchased from the American Type Culture Collection. *S. rectivirgula* was grown in tryptase soy broth at 55°C in a shaking incubator for 2 d, centrifuged, and washed three times with endotoxin–free water. The bacterial cells were lysed by sonication and kept at –80°C until used. The bacterial cell pellet (*S. rectivirgula–Ag*) was reconstituted in endotoxin–free saline at a concentration of 10 mg/ml and kept at –80°C until used. Reconstituted *S. rectivirgula–Ag* had no detectable endotoxin by Limulus assay (Sigma–Aldrich). Nuclease–resistant phosphorothioate oligodeoxynucleotides 1826 (Cpg DNA) were purchased from Coley Pharmaceutical Group (Kanata, Ontario, Canada). PGN and polyinosinic:polycytidylic acid (PIC) were purchased from Invivogen (San Diego, CA). PMA, G6976, and G6983 were purchased from Calbiochem (San Diego, CA).

**S. rectivirgula–Ag exposure protocol, bronchoalveolar lavage, and lung cell isolation**

Female mice (*C57BL/6; MyD88<sup>−/−</sup>; three to five mice/group) were exposed intranasally to *S. rectivirgula–Ag* (200 μg) or endotoxin–free saline. In some experiments, *C57BL/6* mice were administered DMSO (vehicle), G6976 (2.3 mg/kg body weight), or G6983 (2.3 mg/kg body weight) by i.p. injection and intranasal inhalation at 4 and 1 h prior to *S. rectivirgula–Ag* exposure and then were exposed intranasally to endotoxin–free saline or *S. rectivirgula–Ag* (200 μg) plus DMSO, G6976 (1.15 mg/kg body weight), or G6983 (1.15 mg/kg body weight). Mice were sacrificed at designated...
time points (3, 6, or 24 h). Unless indicated, control and S. rectivirgula-Ag–exposed mice were analyzed individually. Bronchoalveolar lavage (BAL) was performed by intratracheal injection of 1 ml PBS into the lungs with immediate vacuum aspiration. The typical amount of fluid recovered was ~70% of the input. The recovered BAL fluid (BALF) was centrifuged. The supernatants were kept at ~80°C until used for the detection of cytokines and chemokines. The cells recovered from BALF were used to determine the degree of alveolitis. To obtain interstitial lung tissue cells, lungs were perfused with PBS to remove blood, and lung lobes were removed from the mouse. Lung tissues were digested with collagenase (20 U/ml) and DNase I (40 μg/ml) for 45 min at 37°C. Cells were freed by mechanical disruption using a Stomacher tissue processor (Seward, Worthing, U.K.). Mononuclear cells were isolated at the 40/80% interface following discontinuous Fico/Lite-M (Atlanta Biologicals, Norcross, GA) gradient centrifugation and used for flow cytometric analysis and total cell counts.

**Small interfering RNA transfection**

AMJ2-C11 cells or MLE12 cells were plated at 2 × 10^5 cells/10 ml in a 100-mm petri dish, incubated overnight, and then transfected with 100 nM nontarget (NT) small interfering RNA (siRNA; Dharmacon, Lafayette, CO) or PKD1-specific siRNA (5′-CTCCTGATGTCTAAGGTGA-3′ and 5′-CCATTGATCTTATCAATAA-3′) using lipofectamine (Invitrogen), according to the manufacturer’s protocol.

**In vitro kinase assays**

Each FLAG-tagged PKD protein in whole-cell lysates was immunoprecipitated with anti-FLAG Abs. The resulting immune complexes were subjected to in vitro kinase assay using Syntide-2 (Sigma-Aldrich) as a PKD substrate, as previously described (47).

**RT-PCR, ELISA, and Western blot assay**

Levels of the selected cytokine and chemokine genes in cells or lung tissue, concentrations of the selected cytokines and chemokines in culture supernatants or BALF, and levels or phosphorylation status of specific proteins in whole-cell extracts were analyzed by RT-PCR, ELISA, and Western blot assay, respectively, as described previously (46, 48). Actin or GAPDH was used as a loading control for all RT-PCR. Actin was used as a loading control for all Western blot assays. All primers for RT-PCR were purchased from Integrated DNA Technologies (Corvalle, IA); sequences of primers are listed in Table I or were described previously (45, 46, 48). All recombinant murine cytokines, MIP-2, and Abs specific for murine cytokines or MIP-2 were purchased from BD Biosciences (San Jose, CA), R&D Systems (Minneapolis, MN), or ebioscience (San Diego, CA). Ab specific for TLR9 was purchased from Imgenex (San Diego, CA). Abs specific for TLR4, MyD88, TRAF6, PKD, IκBα, IκBβ, or actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All phospho-specific Abs were purchased from Cell Signaling Technology (Beverly, MA).

**Determination of alveolitis**

The degree of alveolitis was determined by counting the number of interstitial lung cells or live cells recovered in BALF using trypan blue exclusion. The cellular composition of the alveolitis was determined by differential staining of the BAL cells with Diff-Quik, according to the manufacturer’s protocol. Differential cell counts were made on a total of 300 cells/sample using standard morphological criteria to determine the percentages of lymphocytes, eosinophils, neutrophils, and macrophages per total cell count.

**Multiplex sandwich immunoassay**

Unconcentrated BALF samples were incubated in triplicate with beads coupled with the specific Abs to the selected chemokines (KC and MCP-1). Then the beads were incubated with biotinylated Abs followed by streptavidin-PE. The fluorescence was measured with a BioPlex array reader (Bio-Rad, Hercules, CA). Chemokine standards (Bio-plex Pro mouse cytokine standard group I 23-plex, Bio-Rad), ranging from 5–20,000 pg/ml, were prepared to determine the concentration of chemokines in the samples. For data analysis, a curve fit was applied to the standards, and the sample concentrations were extrapolated from the standard curve using the Logistic SPL model in the BioPlex manager 3.0 software (Bio-Rad). Multiplex kits for chemokines were purchased from Bio-Rad.

**Measurement of myeloperoxidase activity**

One lung lobe from each mouse was homogenized at a concentration of 50 mg/ml in hexadecyl trimethylammonium bromide buffer. The resulting lung homogenates were centrifuged at 16,000 × g for 20 min. After centrifugation, the supernatants were mixed with 50 nmol phosphate buffer containing O-dianisidine hydrochloride and H2O2. The samples were read spectrophotometrically at 460 nm for 1–20 min.

**Lung histology**

The left lung lobes removed from the mice were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin. The paraffin-embedded lungs were sectioned longitudinally at 5 μm and then stained with H&E. To determine inflammatory cell influx into the interstitial lung tissue, digital images (×40 objective) of whole H&E-stained slides were captured using the Aperio ScanScopeXT Slide Scanner system (Aperio Technologies, Vista, CA).

**Flow cytometric assay**

Isolated lung mononuclear cells were stained with fluorochrome-conjugated Abs to identify neutrophils (CD45+/Ly6G+/7/4+) and macrophages (CD45+/Ly6G−/F4/80−). To exclude dead cells from the analysis, cells were also stained with the DNA-binding dye DAPI. A minimum of 30,000 live events/sample were collected on a BD Biosciences LSR II flow cytometer and analyzed using FACSDiva software (BD Biosciences). A gate was set to exclude dead cells (DAPI+), and an additional gate was set to include only CD45+ hematopoietic cells. The gated cells were analyzed to determine the percentage of each cell population per total CD45+ cells from lungs of individual mice in each group.

**Statistical analysis**

All in vitro experiments were repeated three times before analysis. Data are expressed as mean ± SD. The differences between the control and experimental groups were evaluated using the Student t test. Statistical differences with p < 0.05 were considered significant.

**Results**

S. rectivirgula-Ag induces activation of PKD1 in innate immune cells in the lung in vitro and in vivo

Recently, we found that all TLR ligands (with the exception of the TLR3 ligand PIC) and proinflammatory cytokines IL-1β and -18 induce activation of PKD1 and that PKD1 is essential for MyD88-dependent proinflammatory gene expression in innate immune cells (45, 46). However, it is not known whether PKD1 is activated in innate immune cells in response to any whole microorganism or complex components of microorganisms or what role it may play in the proinflammatory response to these stimuli. Therefore, we investigated whether S. rectivirgula-Ag, an inciting Ag for farmer’s lung disease, induces the activation of PKD1 in innate immune cells. RAW264.7 cells stably expressing empty vector, FLAG-tagged PKD1, FLAG-tagged PKD2, or FLAG-tagged PKD3 were stimulated with S. rectivirgula-Ag. As demonstrated in Fig. 1A, S. rectivirgula-Ag induced kinase activity and phosphorylation of PKD1; however, it did not induce kinase activity and phosphorylation of PKD2 or PKD3. Of note, control PMA induced kinase activity and phosphorylation of all three PKD family members. We further investigated whether S. rectivirgula-Ag induces the activation of PKD1 and/or other signaling modulators, such as MAPKs and NF-κB, in innate immune cells involved in lung inflammation in vitro and in vivo. First, we stimulated AMJ2-C11 (a murine alveolar macrophage cell line), MPRO-Neut, and MLE12 (a murine bronchial epithelial cell line) cells with various concentrations of S. rectivirgula-Ag. As shown in Fig. 1B, S. rectivirgula-Ag induced the activation of PKD1, as judged by phosphorylation at serine 744/748 and 916 residues, in all three types of cells. In addition, S. rectivirgula-Ag induced the activation of MAPKs (JNK, ERK, and p38), as judged by phosphorylation, and transcription factor NF-κB, as evidenced by degradation of IκBα and IκBβ, in those cells. Second, to further investigate whether S. rectivirgula-Ag induces activation of PKD1 and/or those signaling modulators in the lung in vivo, C57BL/6 mice were exposed intranasally to S. rectivirgula-Ag and then whole-lung lysates were prepared. As demonstrated in Fig. 1C, S. rectivirgula-Ag inhalation resulted in the activation of
PKD1, JNK, ERK, and p38 and the phosphorylation of IκBα (indication of NF-κB activation) in the lung of C57BL/6 mice. Taken together, these results demonstrated that *S. rectivirgula*-Ag induces the activation of PKD1 and other signaling modulators (MAPKs and NF-κB) critical for the expression of proinflammatory genes in cells involved in inflammation in the lung in vitro and in vivo.

*S. rectivirgula*-Ag induces activation of PKD1, MAPKs, and NF-κB through a pathway involving MyD88

As with other microorganisms, various components of *S. rectivirgula* might interact with various PRRs on/in the host innate immune cells. A study demonstrated that MyD88 plays a substantial role in *S. rectivirgula*-Ag–induced production of proinflammatory cytokine TNF-α and chemokine MIP-2 and recruitment of neutrophils into the lungs (4). Therefore, we investigated whether *S. rectivirgula*-Ag–mediated PKD1 activation is dependent on MyD88. BMDMs isolated from wild-type control or MyD88<sup>2/2</sup> mice were stimulated with *S. rectivirgula*-Ag, and activation of PKD1 and MAPKs and degradation of IκBα (as an indication of NF-κB activation) were assayed. As shown in Fig. 2A, *S. rectivirgula*-Ag, as well as CpG DNA, induced the phosphorylation of PKD1, JNK, ERK, and p38 and the degradation of IκBα in BMDMs isolated from wild-type mice. *S. rectivirgula*-Ag failed to induce the activation of PKD1, JNK, ERK, and p38 and the degradation of IκBα in BMDMs isolated from wild-type mice. *S. rectivirgula*-Ag failed to induce the activation of PKD1, JNK, ERK, and p38 and the degradation of IκBα in BMDMs isolated from wild-type mice. Therefore, we investigated whether *S. rectivirgula*-Ag induces the activation of PKD1 and other signaling modulators (MAPKs and NF-κB) critical for the expression of proinflammatory genes in cells involved in inflammation in the lung in vitro and in vivo.

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**FIGURE 1.** *S. rectivirgula*-Ag induces activation of PKD1 in innate immune cells in the lung in vitro and in vivo. A, RAW264.7 cells (murine macrophages) stably expressing empty vector and FLAG-tagged PKD1, PKD2, or PKD3 were stimulated with medium, *S. rectivirgula*-Ag (2 μg/ml), or PMA (10 ng/ml) for 45 min. Whole-cell lysates were prepared, and each PKD family protein was immunoprecipitated with anti-FLAG Ab. Kinase activity of PKDs was analyzed in vitro using syntide-2 as a PKD substrate (top). The expression and phosphorylation status of PKDs were analyzed by immunoblotting with anti-FLAG or anti–phospho-PKD Abs (pPKDs744/748 and pPKDs916) (bottom). B, AMJ2-C11 (alveolar macrophages), MPRO-Neut (neutrophils developed from MPRO), and MLE12 (bronchial epithelial cells) cells were stimulated with medium or the indicated concentration of *S. rectivirgula*-Ag. The phosphorylation status of PKD1, JNK, p38, and ERK and the degradation of IκBα and IκBβ were analyzed by Western blot (top). MPRO and MPRO-Neut cells were stained with Diff-quick (bottom) (original magnification ×40). MPRO-Neut cells showed standard morphologic features of neutrophils. C, C57BL/6 mice were intranasally exposed to saline or *S. rectivirgula*-Ag (200 μg) for 3 h. Lung lysates were prepared, and the activation status of PKD1, JNK, p38, and ERK and the phosphorylation of IκBα in lung lysates was detected by Western blot. Actin was used as a loading control. Each lane represents an individual mouse. All experiments were done two to four times with similar results.
or those signaling modulators in a manner dependent on MyD88 in the lung in vivo. Wild-type control or MyD88−/− mice were intranasally exposed to saline or S. rectivirgula-Ag. Lung lysates were prepared, and the activation of PKD1 and MAPKs was detected. S. rectivirgula-Ag consistently failed to induce the activation of PKD1, MAPKs (JNK, ERK, and p38) and NF-κB (judged by the phosphorylation of IκBα) in the lungs of MyD88−/− mice (Fig. 2B). Taken together, these results indicate that S. rectivirgula-Ag leads to the activation of PKD1, JNK, ERK, p38, and NF-κB by using MyD88, the common signaling adaptor molecule for the TLR/IL-1R superfamily.

Effect of pharmacological PKD inhibitor Go6976 on S. rectivirgula-Ag–mediated activation of MAPKs and NF-κB and expression of cytokines and chemokines in vitro

Although the pharmacological inhibitor that specifically inhibits only PKD1 has yet to be developed, careful use of several pharmacological inhibitors can provide insight into the biologic role of PKD1. Pharmacological PKD/protein kinase C (PKC) inhibitor Go6976 selectively inhibits PKCα (IC50 = 2.3 nM), β1 (IC50 = 6.2 nM), and PKD (IC50 = 20 nM), but it does not inhibit PKCβ, ε, and ζ. Pharmacological PKC inhibitor Go6938 selectively inhibits PKCα (IC50 = 7 nM), β (IC50 = 7 nM), γ (IC50 = 6 nM), δ (IC50 = 10 nM), and ζ (IC50 = 60 nM), but it does not inhibit PKD and is used with Go6976 to differentiate PKD from other PKC isoforms (49, 50). Recently, we found that Go6976 selectively inhibits TLR ligand-mediated PKD1 activation in vitro and in vivo, whereas Go6938 has no effect (45, 46). To investigate the biologic role of S. rectivirgula-Ag–activated PKD1, cells (AMJ2-C11, MPRO-Neut, and MLE12) were pretreated with vehicle (DMSO), PKD inhibitor Go6976, or PKC inhibitor Go6938 and then stimulated with S. rectivirgula-Ag. Activation of PKD1 by S. rectivirgula-Ag in these cells was inhibited by Go6976 but not by Go6983 (Fig. 3A). In contrast, activation of PKDs by PMA, which occurs in a PKC-dependent manner, was suppressed similarly by Go6976 or Go6983 in all three types of cells. In addition, the phosphorylation of pan PKCs (an indication of activation of conventional PKC isoforms) by PMA in AMJ2-C11 cells and MPRO-Neut cells was ablated by Go6976 or Go6983. These results demonstrate that Go6976 and Go6983 effectively inhibit conventional PKC isoforms, but only Go6976 effectively and selectively inhibits S. rectivirgula-Ag–mediated PKD1 activation, which presumably occurs through the conventional PKC-independent pathway. We further investigated whether this inhibition of PKD1 activation affects S. rectivirgula-Ag–mediated activation of MAPKs and NF-κB. As shown in Fig. 3A, S. rectivirgula-Ag failed to induce the activation of JNK, ERK, and p38 or the degradation of IκBα and IκBβ in all three types of cells pretreated with Go6976. In contrast, S. rectivirgula-Ag–mediated activation of JNK, ERK, and p38 and degradation of IκBα and IκBβ in these cells was not affected by the presence of Go6983. As expected, Go6976 and Go6983 inhibited PMA-induced activation of JNK and ERK in all three types of cells to a similar degree. Neither Go6976 nor Go6983 inhibited the activation of JNK, ERK, p38, or STAT1 or the degradation of IκBα and IκBβ induced in response to PIC, which does not activate PKD1 in AMJ2-C11 and MPRO-Neut cells, indicating that neither Go6976 nor Go6983 was toxic to cells at the concentration used in this experiment. Of note, MLE12 cells did not respond to PIC. Although we cannot completely rule out the possible involvement of other PKC isoforms on S. rectivirgula-Ag–mediated activation of MAPKs and NF-κB in these types of cells, our results suggest that Ca2+-dependent conventional PKCs and PKCβ and ζ (which can be inhibited by Go6983) may not be critical for S. rectivirgula-Ag–mediated activation of MAPKs and NF-κB. Because MAPKs and NF-κB play a pivotal regulatory role in the expression of various cytokines and chemokines that are critical for S. rectivirgula-Ag–mediated leukocyte recruitment and proinflammatory responses, we investigated whether PKD1 plays a role in the S. rectivirgula-Ag–induced expression of selected proinflammatory cytokines and chemokines in AMJ2-C11, MPRO-Neut, and MLE12 cells. Fig. 3B shows that S. rectivirgula-Ag, as well as TLR2 ligand PGN or TLR9 ligand CpG DNA, induced the expression of cytokines TNF-α and IL-1β, -6, -10, -12p40, and IL-23p19 and chemokines MIP-1α, -1β, and -2, MCP-1, KC, IFN-inducible protein (IP)-10, RANTES, IFN-inducible T cell α chemoattractant (ITAC), and LPS-induced CX3 chemokine (LIX) in AMJ2-C11 and MPRO-Neut cells. In MLE12 cells, S. rectivirgula-Ag or PGN induced the expression of cytokines IFN-γ and IL-6 and -23p19 and chemokines MIP-1α and -2, MCP-1, KC, IP-10, RANTES, ITAC, Eotaxin, LIX, and monokine induced by IFN-γ (Mig). MLE12 cells did not respond to PGN DNA. Expression of these cytokines and chemokines in response to S. rectivirgula-Ag, as well as PGN or CpG DNA, was ablated in the presence of PKD inhibitor Go6976 in all three types of cells. In contrast, PKC inhibitor Go6983 did not affect the expression of the cytokines and chemokines in response to S. rectivirgula-Ag, PGN, or CpG DNA in any of the cells. Similar to mRNA expression, the production of selected cytokine (TNF-α, IFN-γ, and IL-6, -10, and -12) and chemokine (MIP-2) proteins in response to S. rectivirgula-Ag, as well as to PGN or CpG DNA, was almost, if not, completely inhibited by Go6976, but not by Go6983, in all three types of cells (Fig. 3C). These results suggest that PKD family members (presumably PKD1) play a critical role in the
FIGURE 3. Effect of pharmacological PKC/PKD inhibitor Go6976 on *S. rectivirgula*-Ag–mediated activation of MAPKs and NF-κB and the expression of cytokines and chemokines in vitro. AMJ2-C11, MPRO-Neut, and MLE12 cells were pretreated with DMSO, Go6976 (250 ng/ml), or Go6983 (250 ng/ml) for 1 h and then stimulated with medium, *S. rectivirgula*-Ag (1 μg/ml for AMJ2-C11, 5 μg/ml for MPRO-Neut, and 10 μg/ml for MLE12), PMA (10 ng/ml), PIC (100 μg/ml), CpG DNA (12 μg/ml for AMJ2-C11 and 24 μg/ml for MPRO-Neut and MLE12), PGN (1 μg/ml for AMJ2-C11, 2 μg/ml for
S. rectivirgula-Ag–induced expression of cytokines and chemokines. Of note, control stimulus IFN-γ, which does not activate PKD1 but is critical for the pathogenesis of hypersensitivity pneumonitis, induced the expression of cytokines TNF-α in AMJ12-C11 and MPRO-Neut cells and IFN-γ in MLE12 cells (Fig. 3B). IFN-γ also induced the expression of chemokines IP-10, RANTES, ITAC, and Mig in AMJ12-C11 cells and IP-10, RANTES, ITAC, Eotaxin, and Mig in MPRO-Neut and MLE12 cells. Neither Go6976 nor Go6983 affected the IFN-γ–mediated expression of cytokines and chemokines in all three types of cells. Go6976 or Go6983 also failed to inhibit the IFN-γ–mediated production of TNF-α protein in AMJ12-C11 and MPRO-Neut cells (Fig. 3C). These results indicate that neither Go6976 nor Go6983, at the concentration used for these experiments, is toxic to or affects the basic biology of cells. Collectively, these results suggest that PKD1 plays an indispensable role in S. rectivirgula-Ag–mediated activation of MAPKs and NF-κB and the subsequent expression and production of cytokines/chemokines.

PKD1 is required for S. rectivirgula-Ag–induced activation of MAPKs and NF-κB and expression of inflammatory cytokines and chemokines in vitro

To further confirm the findings with pharmacological PKD inhibitor Go6976 that PKD1 is required for S. rectivirgula-Ag–mediated innate immune cell activation, we silenced PKD1 expression in AMJ12-C11 and MLE12 cells by transiently transfecting these cells with NT siRNA (control cells) or PKD1–specific siRNA (PKD1-knockdown cells). The expression of PKD1 mRNA and protein was almost completely inhibited in PKD1-knockdown cells (Fig. 4A, 4B). In contrast, mRNA and protein levels of the other genes tested in PKD1-knockdown cells were comparable to those in control cells. In addition, S. rectivirgula-Ag failed to induce phosphorylation of PKDs in PKD1-knockdown cells (Fig. 4C), further supporting our finding that S. rectivirgula-Ag activates PKD1 but not PKD2 or PKD3. These results demonstrate that PKD1–specific siRNA specifically and effectively silenced PKD1 expression in these cells. Using these PKD1-knockdown cells, we further investigated whether PKD1 plays a role in S. rectivirgula-Ag–mediated activation of signaling modulators and subsequent cytokine/chemokine expression and production. Compared with control cells, the activation of MAPKs (JNK, ERK, and p38) and transcription factor NF-κB (judged by degradation of IκBα and IκBβ) in response to S. rectivirgula-Ag were ablated in PKD1-knockdown alveolar macrophages and bronchial epithelial cells (Fig. 4C). In addition, mRNA expression of selected cytokines (TNF-α, IFN-γ, and IL-1β, -6, -10, -12p40, and -23p19) and chemokines (MIP-1α, -1β, and -2; MCP-1 and -2; KC; IP-10; RANTES; ITAC; LIX; and Mig), in response to S. rectivirgula-Ag, were ablated in these PKD1-knockdown cells (Fig. 4D). Furthermore, cytokine (TNF-α, IFN-γ, and IL-6, -10, and -12) and chemokine (MIP-2, MCP-1, and KC) protein production in response to S. rectivirgula-Ag was almost completely, if not completely, inhibited in these PKD1-knockdown cells (Fig. 4E). Of note, the activation of STAT1, gene expression, and protein production in response to IFN-γ were not altered in these PKD1-knockdown cells, indicating that the absence of PKD1 specifically affects the S. rectivirgula-Ag–mediated signaling pathway, but not the general biology of the cell. These results demonstrate that PKD1 plays an indispensable role in the S. rectivirgula-Ag–mediated activation of MAPKs and NF-κB and the subsequent expression and production of inflammatory cytokines/chemokines.

Inhibition of PKD1 activation by Go6976 in mice prior to intranasal exposure to S. rectivirgula-Ag results in decreased expression of proinflammatory cytokines/chemokines in lungs

Initial proinflammatory responses in the lung after pulmonary exposure to S. rectivirgula play a critical role in the initiation of farmer’s lung disease, a prototype hypersensitivity pneumonitis (1). Because the inhibition of PKD1 activation by Go6976 or the inhibition of PKD1 expression by siRNA resulted in the inhibition of S. rectivirgula-Ag–mediated activation of MAPKs and NF-κB, as well as the subsequent expression and production of various cytokines and chemokines in the types of cells critical for lung inflammatory responses, we further investigated whether PKD1 plays a role in S. rectivirgula-Ag–induced proinflammatory responses in the lung in vivo. To inhibit activation of PKD1, we pretreated C57BL/6 mice with Go6976, exposed them intranasally to S. rectivirgula-Ag, and then examined them for proinflammatory responses and development of alveolitis in the lungs. As shown in Fig. 5A, the systemic administration of Go6976 effectively inhibited S. rectivirgula-Ag–induced activation of PKD1 in lung cells. In contrast, control Go6983 did not inhibit S. rectivirgula-Ag–induced activation of PKD1 in lung cells. Similar to what we observed in vitro, S. rectivirgula-Ag failed to induce the activation of MAPKs (JNK, ERK, and p38) and the phosphorylation of IκBα in lung cells isolated from C57BL/6 mice pretreated with Go6976. The activation of MAPKs and the phosphorylation of IκBα in lung cells after exposure to S. rectivirgula-Ag were not suppressed by Go6983. These results indicate that the systemic administration of Go6976 effectively inhibits the S. rectivirgula-Ag–mediated PKD1 activation in the lung and that PKD1 plays a key role in the S. rectivirgula-Ag–induced activation of MAPKs and NF-κB in the lung. Proinflammatory responses in the lungs of C57BL/6 mice exposed to S. rectivirgula-Ag were assessed by analyzing mRNA levels of the selected cytokines and chemokines in lungs and protein levels in bronchial lavage fluids. As demonstrated in Fig. 5B, S. rectivirgula-Ag induced the expression of cytokines TNF-α, IFN-γ, and IL-1β, -6, -10, -12p40, and -23p19 and chemokines (MIP-1α, -1β, and -2; MCP-1 and -2; KC; IP-10; RANTES; ITAC; LIX; and Mig), in the lung. Although there were slight variations among individuals compared with vehicle (DMSO)-pretreated control mice, mice pretreated with Go6976 showed substantially suppressed expression of all cytokines and chemokines analyzed in response to S. rectivirgula-Ag in the lung. In contrast, the mRNA expression levels of these cytokines and chemokines in response to S. rectivirgula-Ag in mice pretreated with Go6983 were comparable to those in vehicle-pretreated control mice. Cytokine (TNF-α and IL-6) and chemokine (KC, MIP-2, and MCP-1) levels in BALF were substantially increased in mice exposed to S. rectivirgula-Ag (Fig. 5C). However, they were significantly decreased in mice pretreated with PKD inhibitor Go6976 but not in mice pretreated with PKC inhibitor Go6983. These results indicate that PKD1 is essential for the S. rectivirgula-Ag–induced proinflammatory responses in the lung.

MPRO-Neut, and 4 μg/ml for MLE12), or IFN-γ (10 ng/ml) for 45 min (A), 2 h (for IFN-γ in B), 4 h (B), or 24 h (C). Phosphorylation of pan-PKC, PKD1, JNK, p38, ERK, and STAT1 and the presence of IκBα and IκBβ in cell lysates were detected by Western blot analysis (A). mRNA levels of the indicated cytokines and chemokines in cells were analyzed by RT-PCR (B). Levels of the cytokines and chemokines in culture supernatants were analyzed by ELISA (C). Data represent the mean concentration ± SD (pg/ml) of triplicates. Actin was used as a loading control. Experiments were done three times with similar results.
Inhibition of PKD1 activation by Go6976 in mice prior to intranasal exposure to \textit{S. rectivirgula}-Ag results in decreased alveolitis and neutrophil recruitment in the lung

Because our results showed that \textit{S. rectivirgula}-Ag failed to induce the expression and production of chemokines that attract leukocytes into the lungs in mice pretreated with PKD inhibitor Go6976, we further investigated whether the inhibition of PKD1 also prevents leukocyte infiltration into the lung in response to \textit{S. rectivirgula}-Ag exposure. As expected, at 24 h postexposure to \textit{S. rectivirgula}-Ag, mice exhibited dramatic increases in total BAL cell numbers and total interstitial lung cell numbers (alveolitis) compared with control mice exposed to saline (Fig. 6A, Table II). Histological sections of lungs from \textit{S. rectivirgula}-Ag–exposed mice also showed the presence of extensive mononuclear cell infiltration in the lungs compared with saline-exposed mice (Fig. 6B). Neutrophils were the predominant cell type recovered

**FIGURE 4.** PKD1 is essential for \textit{S. rectivirgula}-induced activation of MAPKs and NF-κB and the subsequent expression and production of cytokines and chemokines in vitro. AMJ2-C11 and MLE12 cells were transiently transfected with 100 nM of NT siRNA (control) or PKD1-specific siRNA (PKD1-knockdown) using lipofectamine. A, mRNA levels of the indicated genes were analyzed by RT-PCR. B, PKD1 protein levels of the indicated genes were examined using Western blot analysis. C–E, Control or PKD1-knockdown cells were stimulated with medium, \textit{S. rectivirgula}-Ag, or IFN-γ for 45 min (C), 2 h (for IFN-γ in D), 4 h (for IFN-γ in E). The activation status of PKD1, MAPKs, STAT1, and NF-κB (assessed by degradation of IxBα and IxBβ) was detected by Western blot (C). Messenger mRNA levels of the indicated cytokines and chemokines were analyzed by RT-PCR (D). Levels of cytokines and chemokines in culture supernatants were analyzed by ELISA (TNF-α, IFN-γ, IL-6 and -12, and MIP-2) or multiplex sandwich assay (KC and MCP-1) (E). Data represent the mean concentration ± SD (pg/ml) of triplicates. Actin and GAPDH were used as loading controls. All experiments were done three times with similar results.
from airways and lung tissues isolated from mice exposed to S. rectivirgula-Ag (Fig. 6C, Table II). In addition, S. rectivirgula-Ag–exposed mice showed significant increases in myeloperoxidase (MPO) activity in the lung, reflecting increased neutrophil influx (Fig. 6D). In contrast, PKD inhibitor Go6976 pretreatment ablated S. rectivirgula-Ag–mediated induction of alveolitis and neutrophil influx in airways and interstitial lung tissues (Fig. 6A–C, Table II). Also, the Go6976-pretreated mice showed significantly decreased MPO activity in the lung after S. rectivirgula-Ag exposure compared with the vehicle-pretreated mice, reflecting significantly decreased neutrophil influx into the lung (Fig. 6D). Of note, PKC inhibitor Go6983 pretreatment did not have any significant effect on S. rectivirgula-Ag–induced alveolitis and neutrophil influx in airways and interstitial lung tissues. These results indicate that PKD1 is essential for S. rectivirgula-Ag–induced alveolitis and that pharmacological inhibitors that suppress PKD1 activity can be useful therapeutic agents for S. rectivirgula-Ag–induced hypersensitivity pneumonitis.

Discussion
Using pharmacological PKD inhibitor Go6976, several recent studies suggested that PKD family proteins might be involved in certain cytokine production mediated through TLR4 and TLR5 signaling (33, 50, 51). Using more defined approaches, we demonstrated that various synthetic or purified ligands for TLR/IL-1R superfamiliy members that use the TIR domain-containing adaptor molecule MyD88 induce the activation of PKD1 in human and murine macrophages (45, 46). PKD1 activation by TLR ligands is dependent on MyD88, IRAK4, and IRAK1 but is independent of TRAF6. Although the precise role of PKD1 in the TLR/IL-1R signaling pathway is unknown, PKD1 seems to be involved in MyD88-dependent ubiquitination of TRAF6 and the subsequent activation of TAK1, which eventually leads to the activation of MAPKs and NF-κB. The activation of PKD1 by TLRs is essential for the MyD88-dependent expression of proinflammatory cytokines and mediators in innate immune cells. It is not known whether any microorganism induces the activation of PKD1 or whether PKD1 plays a regulatory role in the inflammatory responses induced by pathogenic microorganisms. We demonstrated in this paper that the farmer’s lung disease-inciting agent S. rectivirgula induces the activation of PKD1 and that PKD1 accounts for the S. rectivirgula-Ag–mediated activation of MAPKs and NF-κB and the subsequent expression and production of inflammatory cytokines and chemokines that are critical for the recruitment of leukocytes. Indeed, the S. rectivirgula-Ag–mediated induction of alveolitis was ablated in the lungs of mice pretreated with a pharmacological inhibitor of

![FIGURE 5.](http://www.jimmunol.org/...)

The Journal of Immunology 3153

by guest on April 18, 2017 http://www.jimmunol.org/ Downloaded from
PKD, pointing to the essential role of PKD1 in pulmonary inflammation in response to *S. rectivirgula*-Ag.

The precise mechanism through which innate and adaptive immune cells are activated and recruited into the lungs during the course of hypersensitivity pneumonitis, as well as the contribution of different types of innate immune cells to the initiation and progression of the disease, have yet to be elucidated. Although the initial responding innate immune cells in the lungs after exposure to *S. rectivirgula* have not been identified, it is expected that lung resident dendritic cells/macrophages and bronchial epithelial cells might be the type of cells that provide the first line of defense in the lung. Airway epithelial cells play critical roles in homeostasis and host defense by acting as a physical barrier, secreting and modifying the airway surface liquid, and removing particulates via mucociliary transport (52). In addition, they detect and respond to PAMPs and secrete various cytokines and chemokines that are critical for innate host defense. A study demonstrated that epithelial cell line A549 expresses and releases IL-8 in response to *S. rectivirgula* (7). Our results also showed that *S. rectivirgula*-Ag directly acted on and induced the activation of PKD1, MAPKs, and transcription factor NF-κB and the expression and production of proinflammatory cytokines and chemokines.

### Table II. Comparison of cells in BAL fluid from mice at 24 h postexposure

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Stimulation</th>
<th>Total Cells (10⁶)ᵃ</th>
<th>Macrophages (%)</th>
<th>Neutrophils (%)</th>
<th>Lymphocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>Saline</td>
<td>1.2 ± 1.17</td>
<td>89.0 ± 3.20</td>
<td>5.1 ± 1.50</td>
<td>6.0 ± 4.70</td>
</tr>
<tr>
<td>G60976</td>
<td><em>S. rectivirgula</em>-Ag</td>
<td>73.7 ± 25.85</td>
<td>15.4 ± 7.60</td>
<td>79.5 ± 6.00</td>
<td>5.1 ± 1.70</td>
</tr>
<tr>
<td>Saline</td>
<td>2.5 ± 2.03</td>
<td>74.1 ± 3.15</td>
<td>9.1 ± 7.93</td>
<td>18.6 ± 8.60</td>
<td>5.1 ± 0.80</td>
</tr>
<tr>
<td>G60983</td>
<td><em>S. rectivirgula</em>-Ag</td>
<td>17.0 ± 15.2*</td>
<td>61.7 ± 3.30**</td>
<td>28.9 ± 1.00**</td>
<td>9.4 ± 4.40</td>
</tr>
<tr>
<td>Saline</td>
<td>2.6 ± 1.97</td>
<td>64.8 ± 8.27</td>
<td>20.6 ± 11.95</td>
<td>14.5 ± 4.67</td>
<td>7.0 ± 0.80</td>
</tr>
<tr>
<td><em>S. rectivirgula</em>-Ag</td>
<td>73.2 ± 3.88</td>
<td>22.5 ± 11.70</td>
<td>70.6 ± 10.90</td>
<td>7.0 ± 0.80</td>
<td></td>
</tr>
</tbody>
</table>

C57BL/6 mice were administered DMSO (vehicle), G60976 (2.3 mg/kg body weight), or G60983 (2.3 mg/kg body weight) by i.p. injection and intranasal inhalation at 4 and 1 h prior to *S. rectivirgula*-Ag exposure and then were exposed intranasally to endotoxin-free saline or *S. rectivirgula*-Ag (200 µg) plus DMSO, G60976, or G60983 (1.15 mg/kg body weight). Mice were euthanized at 24 h after *S. rectivirgula*-Ag exposure. BAL was performed, and the cells were recovered. The severity of alveolitis was determined by counting the number of live cells recovered in BAL fluid using trypan blue exclusion. Cellular composition of the alveolitis was determined by differential staining of the BAL cells with Diff-Quik.

Differential cell counts were performed on a total of 300 cells/sample using standard morphological criteria to determine the percentages of lymphocytes, eosinophils, neutrophils, and macrophages per total cell count.

ᵃDerived from five individual mice per group (mean ± SD).

ᵇ*p < 0.05; ᵃᵇ*p < 0.005. G60976/S. rectivirgula-Ag or G60983/S. rectivirgula-Ag groups were compared with control DMSO/S. rectivirgula-Ag group.
chemokines in bronchial epithelial cells, as well as alveolar macrophages and neutrophils. The profiles of cytokines and chemokines expressed in bronchial epithelial cells in response to *S. rectivirgula*-Ag are different from those in alveolar macrophages or neutrophils. We were not able to detect the expression of TNF-α and IL-1β, -10, and -12 in MLE12 cells in response to *S. rectivirgula*-Ag under our experimental conditions. However, MLE12 cells expressed IFN-γ, IL-6 and -23p19; MIP-1α and -2; MCP-1; KC; IP-10; RANTES; ITAC; Eotaxin; LIX; and Mig in response to *S. rectivirgula*-Ag. These cytokines and chemokines are known to be critical for Th1 cell development and the recruitment of macrophages, neutrophils, and T cells. These results suggest that airway epithelial cells might play more than a negligible role in the development and progress of hypersensitivity pneumonitis caused by pulmonary exposure to *S. rectivirgula*.

Similar to various synthetic or purified TLR ligands, the activation of PKD1 by *S. rectivirgula*-Ag in vivo and in vitro is also dependent on MyD88 signaling, indicating that the recognition of *S. rectivirgula*-Ag by one or more of the MyD88-dependent TLR/IL-1R superfamily members accounts for PKD1 activation. Using TLR-overexpressing HeLa cells, a study showed that *S. rectivirgula*-Ag is recognized by TLR2 but not by TLR4, TLR5, or TLR7 (4). However, experiments using TLR2<sup>−/−</sup> mice revealed that TLR2 only partially accounts for MIP-2 production and is not required for the production of TNF-α and KC and the infiltration of neutrophils into the lungs following *S. rectivirgula*-Ag exposure (4). In agreement with these previous findings, we also found that the contribution of TLR2 to *S. rectivirgula*-Ag–mediated activation of PKD1, as well as the activation of MAPKs and NF-κB, in vitro and in vivo was only partial to minimal under our experimental conditions (data not shown), indicating the involvement of additional PRRs in the *S. rectivirgula*-Ag–mediated activation of those signaling modulators. It is possible that genomic DNA released from *S. rectivirgula* can be detected by TLR9 and leads to the activation of PKD1 and other downstream signaling modulators. However, we found that the *S. rectivirgula*-Ag–mediated activation of PKD1, MAPKs, and NF-κB in TLR9<sup>−/−</sup> mice is comparable to that in wild-type mice (data not shown). These results indicate that the contribution of TLR9 to the *S. rectivirgula*-Ag–mediated activation of those signaling modulators is minimal to none, although we cannot completely rule out the possibility that TLR2 compensates for the lack of TLR9 and vice versa. The assessment of PKD1 activation in response to *S. rectivirgula*-Ag in TLR2/TLR9 double knockout mice might provide insight. To the best of our knowledge, the natural ligands for TLR10–13 are not known. It is possible that one or more of those TLRs may contribute to the detection of *S. rectivirgula*-Ag. Another possibility is that inflammasome-activating PRRs detect parts of *S. rectivirgula*-Ag and lead to the maturation and secretion of pre-existing precursors of IL-1β, -18, and/or -33. The resulting mature IL-1 family cytokines may be responsible for the MyD88-dependent activation of PKD1. Further investigations on these aspects are warranted.

Farmer’s lung disease, a prototypical hypersensitivity pneumonitis, is caused by repeated pulmonary exposure to *S. rectivirgula* present in moldy hay (1). The initial stage (or acute form) of the disease is characterized by the increased production of proinflammatory cytokines and chemokines and subsequent alveolitis, accompanied by an influx of cells predominantly composed of neutrophils. A recent study demonstrated that the initial expression of proinflammatory cytokines and chemokines in the lung and subsequent neutrophil infiltration into the lung after pulmonary exposure to *S. rectivirgula*-Ag are dependent on MyD88 (4). Recently, we found that PKD1 is indispensable for the MyD88-dependent activation of MAPKs and NF-κB and the expression and production of proinflammatory cytokines and chemokines, but it is dispensable for MyD88-dependent type I IFN expression (45, 46). These findings suggest the possibility that PKD1 plays a role in the *S. rectivirgula*-Ag–induced proinflammatory response in the lung. Indeed, *S. rectivirgula*-Ag failed to induce the activation of MAPKs and NF-κB and the expression and production of proinflammatory cytokines and chemokines in PKD1-knockdown cells in vitro. These findings demonstrate that PKD1 activation in innate immune cells in response to *S. rectivirgula*-Ag is essential for the induction of proinflammatory cytokines and chemokines that are necessary for the recruitment of neutrophils and lymphocytes to the site of *S. rectivirgula*-Ag exposure. Pharmacological inhibitors that inhibit the function or activation of a specific signaling molecule can be useful tools to uncover the biological role of the signaling molecule, as well as effective therapeutic agents if the signaling molecule is involved in the pathogenesis of certain diseases. Although the pharmacological inhibitor that specifically inhibits only PKD1 has yet to be developed, Gö6976 was shown to effectively inhibit PKD1 activation by ligands for TLR/IL-1R superfamily members (33, 45, 46, 50, 51). Similar to these previous findings, *S. rectivirgula*-Ag–mediated PKD1 activation was selectively inhibited by Gö6976, but not by Gö6983, in vitro and in vivo. This indicates that *S. rectivirgula*-Ag–mediated PKD1 activation is independent of conventional PKC and that Gö6976 can be a useful agent to suppress *S. rectivirgula*-Ag–mediated PKD1 activation and downstream biologic events. Indeed, in vivo activation of MAPKs and NF-κB, the expression and production of cytokines/chemokines in the lung, and subsequent neutrophil infiltration into the lung after *S. rectivirgula*-Ag exposure were significantly suppressed by systemic inhibition of PKD1 activity using Gö6976. Although long-term treatment with Gö6976 would require caution and careful monitoring of adverse effects that might develop, a single-course treatment at the concentration we used in this experimental setting does not seem to be toxic to mice. Gö6976–pretreated mice showed normal TIR-domain-containing adaptor-inducing IFN-β-dependent responses to PIC or LPS (45). Collectively, our results indicate that the activation of PKD1 by *S. rectivirgula*-Ag is a necessary step leading to activation of MAPKs and NF-κB, expression and production of various cytokines/chemokines, and inflammatory cell infiltration into the lungs. Our results also suggest that PKD1 might be an attractive candidate as a therapeutic target for hypersensitivity pneumonitis caused by microbial Ags. Considering the increasing roles of MyD88 in acute and chronic inflammatory diseases and the critical role of PKD1 in MyD88-dependent proinflammatory gene expression, the development of an effective and specific pharmacological inhibitor for PKD1, or a pathway-specific inhibitor for PKD1, is desired.

In summary, we found that in addition to alveolar macrophages and neutrophils, bronchial epithelial cells respond directly to *S. rectivirgula*-Ag and produce various cytokines and chemokines (IL-6 and -23, IFN-γ, KC, MIP-1α and -2, MCP-1, KC, IP-10, RANTES, ITAC, Eotaxin, LIX and Mig) that are critical for the recruitment of leukocytes and the development of the Th17 response and hypersensitivity pneumonitis. In addition, we demonstrated that *S. rectivirgula*-Ag induces the activation of PKD1, but not PKD2 or PKD3, in various types of innate cells in the lung through a MyD88-dependent mechanism. Activation of PKD1 is required for the expression of proinflammatory cytokines and chemokines in bronchial epithelial cells, alveolar macrophages, and neutrophils, as well as for neutrophil infiltration into the lung in response to *S. rectivirgula*-Ag exposure. Taken together, our findings indicate that PKD1 might be an attractive molecular target for therapy of microbial Ag-induced hypersensitivity pneumonitis.