Contribution of Protein Kinase D1 on Acute Pulmonary Inflammation and Hypersensitivity Pneumonitis Induced by *Saccharopolyspora rectivirgula*

Tae Won Yoon, Elizabeth A. Fitzpatrick, John D. Snyder, Sangmin Lee, Young-In Kim, Chidi Zacheaus and Ae-Kyung Yi

*ImmunoHorizons* 2022, 6 (3) 224-242
doi: https://doi.org/10.4049/immunohorizons.2200017
http://www.immunohorizons.org/content/6/3/224

This information is current as of March 15, 2022.

Supplementary Material
http://www.immunohorizons.org/content/suppl/2022/03/10/immunohorizons.s2200017.DCSupplemental

References
This article cites 83 articles, 31 of which you can access for free at:
http://www.immunohorizons.org/content/6/3/224.full#ref-list-1

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://www.immunohorizons.org/alerts
Contribution of Protein Kinase D1 on Acute Pulmonary Inflammation and Hypersensitivity Pneumonitis Induced by Saccharopolyspora rectivirgula

Tae Won Yoon,*† Elizabeth A. Fitzpatrick,*† John D. Snyder,*† Sangmin Lee,‡ Young-In Kim,‡ Chidi Zacheaus,§ and Ae-Kyung Yi,*†,§

*Integrated Biomedical Science Graduate Program, The University of Tennessee Health Science Center, Memphis, TN; †Department of Microbiology, Immunology and Biochemistry, The University of Tennessee Health Science Center, Memphis, TN; ‡Department of Pediatrics, The University of Tennessee Health Science Center, Memphis, TN; and §Department of Pharmaceutical Sciences, The University of Tennessee Health Science Center, Memphis, TN

ABSTRACT

Protein kinase D1 (PKD1), a ubiquitously expressed serine/threonine kinase, regulates diverse cellular processes such as oxidative stress, gene expression, cell survival, vesicle trafficking, Ag receptor signaling, and pattern recognition receptor signaling. We found previously that exposure to hypersensitivity pneumonitis (HP) inciting Ag Saccharopolyspora rectivirgula leads to the activation of PKD1 in a MyD88-dependent manner in various types of murine cells in vitro and in the mouse lung in vivo. However, it is currently unknown whether PKD1 plays a role in the S. rectivirgula–induced HP. In this study, we investigated contributions of PKD1 on the S. rectivirgula–induced HP using conditional PKD1-insufficient mice. Compared to control PKD1-sufficient mice, PKD1-insufficient mice showed substantially suppressed activation of MAPKs and NF-kB, expression of cytokines and chemokines, and neutrophilic alveolitis after single intranasal exposure to S. rectivirgula. The significantly reduced levels of alveolitis, MHC class II surface expression on neutrophils and macrophages, and IL-17A and CXCL9 expression in lung tissue were observed in the PKD1-insufficient mice repeatedly exposed to S. rectivirgula for 5 wk. PKD1-insufficient mice exposed to S. rectivirgula for 5 wk also showed reduced granuloma formation. Our results demonstrate that PKD1 plays an essential role in the initial proinflammatory responses and neutrophil influx in the lung after exposure to S. rectivirgula and substantially contribute to the development of HP caused by repeated exposure to S. rectivirgula. Our findings suggest that PKD1 can be an attractive new molecular target for therapy of S. rectivirgula–induced HP. ImmunoHorizons, 2022, 6: 224–242.

INTRODUCTION

Saccharopolyspora rectivirgula is a thermophilic actinomycetes commonly found in moldy hay and is one of the inciting Ags that causes farmer's lung disease, one of the most common types of hypersensitivity pneumonitis (HP) (1). HP, also known as extrinsic allergic alveolitis, is characterized by inflammation of the interstitium, bronchioles, and alveoli of the lung that can

Received for publication February 22, 2022. Accepted for publication February 22, 2022.

Address correspondence and reprint requests to: Dr. Ae-Kyung Yi, Department of Microbiology, Immunology and Biochemistry, The University of Tennessee Health Science Center, 858 Madison Avenue, Suite 501C, Memphis, TN 38163. E-mail address: ayi@uthsc.edu

A.-K.Y. was supported by National Institutes of Health Grants AR064723 and AR069010. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health or The University of Tennessee Health Science Center.

T.W.Y., E.A.F., and A.-K.Y. designed the study; T.W.Y., E.A.F., J.D.S., S.L., Y.-I.K., and C.Z. performed experiments and acquired the data; T.W.Y., E.A.F., J.D.S., S.L., Y.-I.K., and A.-K.Y. analyzed and interpreted the data; T.W.Y., E.A.F., S.L., and A.-K.Y. wrote the manuscript. All authors proofread and gave their approval for the final submitted version.

Abbreviations used in this article: AM, alveolar macrophage; BAL, bronchoalveolar lavage; BALF, BAL fluid; gMFI, geometric mean fluorescence intensity; HP, hypersensitivity pneumonitis; 4-HT, 4-hydroxytamoxifen; ILC, interstitial lung cell; IRAK, IL-1R–associated kinase; KC, keratinocyte chemoattractant; LIX, LPS–induced CXC chemokine; MHC-II, MHC class II; PKC, protein kinase C; PKD, protein kinase D; PKD1KO, PKD1 knockout; PMN, polymorphonuclear neutrophil; R26<sup>Cre/ERT2</sup>, Gt(Rosa)26Sortm1(cre/ERT2)Tyj/J; TRAF6, TNFR-associated factor 6; UTHSC, The University of Tennessee Health Science Center; WT, wild-type.

The online version of this article contains supplemental material.

This article is distributed under the terms of the CC BY 4.0 Unported license.

Copyright © 2022 The Authors

ImmunoHorizons is published by The American Association of Immunologists, Inc.
ultimately lead to irreversible fibrosis. It is caused by repetitive inhalation of HP Ags in a variety of environmental settings in susceptible individuals (2). The pathogenic mechanisms underlying initiation of HP and the interplay between the host immune system, the environment, and the causative microorganisms are complex and not fully understood. Following inhalation of HP-causing Ags (e.g., *S. rectivirgula*), pattern recognition receptors on/in lung-resident innate immune cells are triggered, resulting in the production of TNF-α, IL-6, CXCL2, IL-17, and IFN-γ that contribute to the recruitment of neutrophils and other inflammatory cells into the lung (2–9). Chronic exposure to HP Ags results in fibrosis, which is associated with high morbidity and mortality (1, 10, 11). Thus, identification of host innate immune receptors that are involved in initial detection of HP Ags and careful investigation of signal transduction mediated by those innate immune receptors will provide new and important insight into the pathogenesis of this disease.

The protein kinase D (PKD) family is composed of three structurally related serine/threonine kinases; PKD1 (protein kinase C [PKC]α), PKD2, and PKD3 (PKCβ) (12). PKD family proteins are expressed ubiquitously and implicated in the regulation of a variety of cellular and subcellular processes such as vesicle transport, reactive oxygen species generation, activation of MAPKs and NF-κB, and regulation of cell shape, motility, adhesion, and gene expression (13–20). PKDs can be activated by upstream signaling modulators such as PI3K, phospholipase Cγ, diacylglycerol, and classical/novel PKCs that are activated through diacylglycerol, and classical/novel PKCs that are activated through diacylglycerol, and classical/novel PKCs that are activated through diacylglycerol, and classical/novel PKCs that are activated through diacylglycerol, and classical/novel PKCs that are activated through diacylglycerol, and classical/novel PKCs that are activated through diacylglycerol, and classical/novel PKCs that are activated through diacylglycerol, and classical/novel PKCs that are activated through diacylglycerol, and classical/novel PKCs that are activated through diacylglycerol, and classical/novel PKCs that are activated through diacylglycerol, and classical/novel PKCs that are activated through diacylglycerol, and classical/novel PKCs. PKD family proteins can be activated by TNF receptor–mediated production of proinflammatory cytokines and chemokines (35, 44–48). Previously, we found that *S. rectivirgula* induces activation of PKD1 in an MyD88-dependent manner in the mouse lung in vivo and in various murine cell lines, including MLE12 (alveolar type II epithelial cells), MPRO (promyelocytes differentiated to neutrophils), RAW264.7 (macrophages), and AMJ2-C11 (alveolar macrophages [AMs]) (49). We also found that *S. rectivirgula*–mediated acute lung injury accompanied by neutrophilic alveolitis and increased proinflammatory cytokines and chemokines in these cell lines are significantly suppressed when PKD1 expression is knocked down by PKD1-specific small interfering RNA (49). Furthermore, we demonstrated that *S. rectivirgula*–induced acute lung injury accompanied by neutrophilic alveolitis and increased proinflammatory cytokines and chemokines are significantly inhibited in mice pretreated with Gö6976, a pharmacological inhibitor that inhibits the activity of PKCα, PKCβ, checkpoint kinases 1 and 2 (CHK1/2), PKD1, PKD2, and PKD3 (49–51). These findings suggest that PKD1 is one of the critical factors required for development of proinflammatory reactions in the lung after inhalation of *S. rectivirgula*. However, it is currently unknown whether PKD1 plays any biologic role in acute pulmonary proinflammatory responses to inhaled *S. rectivirgula* and in development of HP. In the current study, we investigated whether PKD1 is essential for mediating acute lung injury after exposure to *S. rectivirgula* and whether PKD1 contributes to the neutrophil and T cell influx, Th1/Th7 related cytokine and chemokine expression, and granuloma formation during the development of HP using inducible PKD1-insufficient mice.

**MATERIALS AND METHODS**

**Mice**

C57BL/6 mice and Gt(Rosa)26Sor<sup>tm1(cre/ERT2)Tg/J</sup> (R26<sup>Cre/ERT2</sup>) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). PKD1<sup>fl/fl</sup> mice (mice with insertedloxP sites in the PKD1 gene to flanking exons 12–14 that encode the kinase function) were provided by Dr. E. Olson (University of Texas Southwestern Medical Center, Dallas, TX) (52). PKD1<sup>Cre/ERT2</sup> mice...
and R26$^{Cre/ERT2}$ mice were backcrossed onto the C57BL/6 for more than nine generations at The University of Tennessee Health Science Center (UTHSC). Subsequent cross-breeding of these PKD1$^{fl/fl}$ (C57BL/6) and R26$^{Cre/ERT2}$ (C57BL/6) mice resulted in PKD1$^{fl/fl}$, R26$^{Cre/ERT2}$ (C57BL/6), which were bred and maintained in a pathogen-free facility at UTHSC. All animal care and housing requirements set forth by the National Institutes of Health Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources were followed. Animal protocols were reviewed and approved by the UTHSC Institutional Animal Care and Use Committee.

**Preparation of 4-hydroxytamoxifen and induction of PKD1 gene deletion**

4-Hydroxytamoxifen (4-HT; H6278; Sigma-Aldrich, St. Louis, MO) was dissolved in ethanol (20 mg/ml) and mixed with an equal volume of sunflower oil (Sigma-Aldrich) by vortexing. The residual ethanol was removed by evaporation in a Speed-Vac vacuum concentrator. To induce deletion of the PKD1 gene, PKD1$^{fl/fl}$, R26$^{Cre/ERT2}$ mice were treated with 100 µl of sunflower oil (wild-type [WT] control, PKD1-sufficient mice) or 100 µl of 4-HT dissolved in sunflower oil (PKD1 knockout [PKDIKO], PKD1-insufficient mice) by i.p. injection on days 0, 1, 3, 4, 6, and 7. To provide a control for 4-HT treatment, PKD1$^{fl/fl}$ mice were also treated with 100 µl of sunflower oil or 100 µl of 4-HT dissolved in sunflower oil by i.p. injection on days 0, 1, 3, 4, 6, and 7. Mice were challenged with *S. rectivirgula* within 2 wk after the last 4-HT treatment.

**S. rectivirgula strain and reagents**

*S. rectivirgula* strain (ATCC 15347) was grown in trypticase soy broth (Sigma-Aldrich) at 55°C in a shaking incubator for 2 d, centrifuged, and washed three times with endotoxin-free water (Sigma-Aldrich). Bacterial cells were lyophilized. The lyophilized bacterial pellet was reconstituted in endotoxin-free saline at a concentration of 10 mg/ml and kept at −80°C until used. Reconstituted *S. rectivirgula* had no detectable endotoxin by Limulus assay (Sigma-Aldrich).

**S. rectivirgula challenge, bronchoalveolar lavage, lung cell isolation, and determination of alveolitis**

Mice were exposed intranasally to *S. rectivirgula* (100 µg/50 µl of endotoxin-free saline) or endotoxin-free saline (50 µl) (AdipoGen Life Sciences, San Diego, CA) for one or three times per week for 5 wk. Mice were euthanized at designated time points (1, 6, 24, 48, or 72 h after the last *S. rectivirgula* challenge). Unless otherwise indicated, control and *S. rectivirgula*–exposed mice were analyzed individually. To perform bronchoalveolar lavage (BAL), mice tracheas were cannulated, and the lungs were washed with 1 ml of 2 mM EDTA/PBS. The typical amount of fluid recovered was ~70% of the input. The recovered BAL fluid (BALF) was centrifuged, and the resulting supernatants were kept at −80°C until use for detection of cytokines and chemokines. The cells recovered from the BALF were counted using trypan blue dye exclusion and used for flow cytometric analysis. Following BAL, the lungs were perfused through the right ventricle with PBS and digested with collagenase (20 U/ml) and DNase I (40 µg/ml) for 45 min at 37°C. Cells were freed by mechanical disruption and filtered through 40-µm nylon mesh. Discontinuous Ficoll-Paque Plus (GE Healthcare Bio-Sciences) gradient centrifugation was used to separate extraneous fibroblasts or epithelial cells. The mononuclear cells were isolated at the 40/80% interface and used for flow cytometric analysis and total cell counts. The degree of alveolitis was determined by counting the number of interstitial lung tissue cells or live cells recovered in BALF using trypan blue exclusion. The cellular composition of the alveolitis was determined by flow cytometric analysis.

**PCR genotyping, RT–PCR, and RT–real-time PCR**

To distinguish PKDI alleles, genomic DNA was isolated using a DNeasy tissue kit (Qiagen, Valencia, CA) and PKDI alleles were analyzed (52). The sequences of PCR primers used are as follows: WT/loxP (366 forward, 5'-GCCCAACAGCTATTGCTCAA-3', 517 reverse, 5'-GGATAAAATGCTCAAGGCACA3') and KO (LAI forward, 5'-GACCTTCACCTGGAGACAGC-3', SA4 reverse, 5'-CTTTGAATCTGGAGCAG-3'). For RT–PCR, DNA-free total RNA was isolated and the relative amounts of selected gene transcripts were analyzed by RT–PCR as described previously (44). Actin or GAPDH was used as a loading control for all RT–PCR reactions. Sequences of RT–PCR primers for PKDI are as follows: forward, 5'-AAGGACTCTGGAGATTGGG-3', reverse, 5'-ACTTGCCAGGGTCC-3' (as published previously in Ref. 34). Sequences of RT–PCR primers for mouse genes were described previously (34, 35, 44, 49, 53). All primers were purchased from Integrated DNA Technologies (Coralville, IA). For RT–real-time PCR (TaqMan assay and SYBR Green assay), total RNA (250 ng) was reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems). Next, quantitative RT–PCR was performed on a QuantStudio 5 (Applied Biosystems) using the TaqMan universal PCR master mix (Applied Biosystems) or SYBR Green master mix (Applied Biosystems). Primers were designed and supplied by Applied Biosystems (for TaqMan assay) and Integrated DNA Technologies (for SYBR Green assay). The product size was initially monitored by agarose gel electrophoresis. Melting curves were analyzed to control for specificity of PCR reactions. The data on genes that were differentially expressed were normalized to the expression of housekeeping gene, actin. The relative units were calculated from a standard curve, plotting three different concentrations against the PCR cycle number at which the measured intensity reaches a fixed value (with a 10-fold increment equivalent to ~3.1 cycles). Fold change comparing *S. rectivirgula*–treated exposed PKDI-sufficient mice and *S. rectivirgula*–treated PKDI-insufficient mice to control PKDI-sufficient mice exposed to saline were calculated by the comparative quantification algorithm–ΔΔCt method (fold difference = 2$^{-∆∆Ct}$). Primer information is listed in Table I.
**Western blot assay, ELISA, and multiplex sandwich immunoassay**

Levels or phosphorylation status of specific proteins in whole-lung tissue extracts were analyzed by Western blot assay as described previously (34, 53). Blots developed in ECL reagents (GE Healthcare, U.K.) were either exposed onto x-ray film followed by processing through an X-OMAT 2000A processor (Kodak) or scanned using a ChemiDoc touch imaging system (Bio-Rad, Hercules, CA). Blots developed with fluorochrome-conjugated secondary Abs were scanned using an Odyssey CLx (LI-COR Biosciences, Lincoln, NE). Actin was used as a loading control for all Western blot assays. All phospho-specific Abs were purchased from Cell Signaling Technology (Beverly, MA). Abs specific for actin, MyD88, TRAF6, and IκBα were purchased from Santa Cruz Biotechnology (Dallas, TX). Abs specific for PKD1 were purchased from Santa Cruz Biotechnology and OriGene (Rockville, MD). Concentrations of IFN-γ, TNF-α, IL-6, IL-12, IL-17A, IL-21, IL-22, and IL-23 in BALF were analyzed by ELISA as described (34, 53). All ELISA kits were purchased from Invitrogen. Concentrations of IL-1α, IL-1β, MCP-1, MIP-2, keratinocyte chemoattractant (KC), IL-2, IL-4, IL-6, and IL-10 in BALF were analyzed using a multiplex sandwich assay kit (MCYTOMAG-70K, EMD Millipore, Billerica, MA) following the manufacturer’s protocols. The fluorescence was measured with Bio-Plex MAGPIX multiplex reader (Bio-Rad, Hercules, CA).

**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 stained with H&E. To determine inflammatory cell influx into the interstitial lung tissue, digital images of whole H&E-stained slides were captured using the Aperio ScanScope XT slide scanner (Aperio Technologies, Vista, CA).

**Flow cytometric analysis**

For splenic immune cell profiling, spleen cells were incubated with anti-CD16/CD32 (2.4G2) Abs and subsequently stained with fluorochrome-conjugated Abs to CD45 (I3/2.3), CD19 (6D5), CD11b (M1/70), and then analyzed using a BD Biosciences LSR II flow cytometer (BD Biosciences, San Diego, CA) and FlowJo flow cytometry data analysis software (FlowJo, Ashland, OR). Debris and doublets were gated out (Supplemental Fig. 1A). In the single-cell gate, the CD19+ population was identified as B cells (CD19+), and the CD45+CD11b+ population was identified as CD11b+ cells. In the single-cell gate, the βTCR+CD3+ population was gated, and then the CD4+ population in the βTCR+CD3+ gate was identified as CD4+ T cells (βTCR+CD4+). The CD8+ population in the βTCR+CD3+ gate was identified as CD8+ T cells (βTCR+CD8+). The frequency of each cell population was expressed as the percentage of spleen cells. To detect neutrophils in the airway after one-time *S. rectivirgula* exposure, cells recovered from BALF were incubated with anti-CD16/CD32 Abs, subsequently stained with fluorochrome-conjugated Abs to CD11b, F4/80 (BM8), Gr-1(RB68-C5), and MHC class II (MHC-II) (M5/114.152), and then analyzed using a BD Biosciences LSR II flow cytometer and FlowJo flow cytometry data analysis software. Debris and doublets were gated out (Supplemental Fig. 1B). In the single-cell gate, the CD11b+Gr1+ population was gated, and then the Gr1+F4/80+ population in the CD11b+Gr1+ gate was identified as neutrophils (CD11b+Gr1+F4/80+). The frequency of CD11b+Gr1+F4/80+ cells was expressed as the percentage of BAL cells. To detect neutrophils among interstitial lung cells (ILCs) recovered from the lungs isolated from mice after one-time *S. rectivirgula* exposure, ILCs were incubated with anti-CD16/CD32 Abs, subsequently stained with fluorochrome-conjugated Abs to CD45, CD11b, F4/80, Gr-1, and MHC-II, and then analyzed using a BD Biosciences LSR II flow cytometer and FlowJo flow cytometry data analysis software. Debris and doublets were gated out (Supplemental Fig. 1C). In the single-cell gate, the CD45+ population was gated and identified as CD45+ cells. In the CD45+ gate, the CD11b+Gr1+ population was gated, and then the Gr1+F4/80+ population in the CD11b+Gr1+ gate was identified as neutrophils (CD45+CD11b+Gr1+F4/80+). Frequency of the CD45+CD11b+Gr1+F4/80+ cell population was expressed as the percentage of ILCs. To determine cellular composition of alveolitis following a 5-wk exposure of mice to *S. rectivirgula,* cells recovered from BALF were incubated with anti-CD16/CD32 Abs, subsequently

---

**TABLE 1. List of real-time PCR primers used**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chemistry</th>
<th>Assay ID</th>
<th>Reference Sequence No. or Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-17A</td>
<td>TaqMan</td>
<td>Mm00439618_m1</td>
<td>NM_010552.3</td>
</tr>
<tr>
<td>IL-17RA</td>
<td>TaqMan</td>
<td>Mm00434214_m1</td>
<td>NM_008359.2</td>
</tr>
<tr>
<td>CXCL9</td>
<td>TaqMan</td>
<td>Mm00439496_m1</td>
<td>NM_008599.4</td>
</tr>
<tr>
<td>CXCL11</td>
<td>TaqMan</td>
<td>Mm00444662_m1</td>
<td>NM_019494.1</td>
</tr>
<tr>
<td>CXCL12</td>
<td>TaqMan</td>
<td>Mm00445553_m1</td>
<td>NM_00102477.2, NM_013655.4, NM_021704.3</td>
</tr>
<tr>
<td>CXCL13</td>
<td>TaqMan</td>
<td>Mm00208154_g1</td>
<td>NM_001159738.1, NM_016960.2, GenBank: AK075973.1</td>
</tr>
<tr>
<td>CCL20</td>
<td>TaqMan</td>
<td>Mm00208154_g1</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>TaqMan</td>
<td>Mm00208154_g1</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>SYBR Green</td>
<td>Mm.PT.58.41769240</td>
<td>5'-CGGAGAAGAGAAGTCTACAC-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>SYBR Green</td>
<td>Mm.PT.39a.22214843.g</td>
<td>5'-CCTCACTGATTCGACTTC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5'-AGGAGGCTGACTTCGACT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5'-GAGGAGGCTGACTTCGACT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5'-GACGACAGACACTTCGAC-3'</td>
</tr>
</tbody>
</table>

https://doi.org/10.4049/immunohorizons.2200017
stained with fluorochrome-conjugated Abs to CD11b, CD11c (N418), F4/80, Gr-1, MHC-II, CD86 (GL-1), βTCR, CD4, CD8, and/or CD69 (HL2F3), and then analyzed using a BD Biosciences LSR II flow cytometer and FlowJo flow cytometry data analysis software. Debris was excluded (Supplemental Fig. 2A).

The single-cell gate, the CD11b+ Gr1+ population was identified as polymorphonuclear neutrophils (PMNs; CD11b+ Gr1+), the CD11c+ F4/80+ population was identified as AMs (CD11c+ F4/80+), the CD4+ βTCR+ population was identified as CD4+ T cells (CD4+ βTCR+), and the CD8+ βTCR+ population was identified as CD8+ T cells (CD8+ βTCR+). The frequency of each cell population was expressed as the percentage of BAL cells. The geometric mean fluorescence intensities (gMFIs) of CD86 and MHC-II on AMs and of CD69 on CD4+ T cells and CD8+ T cells were calculated using FlowJo flow cytometry data analysis software. To determine cellular composition of ILCs following a 5-wk exposure of mice to S. rectivirgula, ILCs were incubated with anti-CD16/CD32 Abs, subsequently stained with fluorochrome-conjugated Abs to CD11b, CD11c (N418), F4/80, Gr-1, MHC-II, βTCR, CD4, CD8, and/or CD69 (HL2F3), and then analyzed using a BD Biosciences LSR II flow cytometer and FlowJo flow cytometry data analysis software. Debris was excluded (Supplemental Fig. 2B). In the single-cell gate, the CD11b+CD11c+ population, CD11b+Gr1+ population, CD11b+F4/80+ population, CD11b+CD11c+ population, CD4+ βTCR+ population, and CD8+ βTCR+ population were identified.

The frequency of each cell population was expressed as the percentage of ILCs. The gMFIs of MHC-II on the CD11b+CD11c+ population, CD11b+Gr1+ population, CD11b+F4/80+ population, and CD11b+CD11c+ population, as well as CD69 on the CD4+ βTCR+ population and CD8+ βTCR+ population, were calculated using FlowJo flow cytometry data analysis software. All Abs were purchased from BioLegend (San Diego, CA) and eBioscience.

Statistical analysis
Data were expressed as mean values ± SD. The differences between two groups were evaluated using a two-tailed Student t test. The differences between multiple groups were evaluated using one-way ANOVA with a Tukey post hoc test. GraphPad Prism statistical software (GraphPad Software, San Diego, CA) was used. Statistically significant differences are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

RESULTS
Tamoxifen-inducible systemic deletion of PKD1
To induce depletion of the PKD1 gene from the mouse genome, PKD1β/β-R26Cre/ERT2 mice were injected i.p. with 4-HT, as indicated in Fig. 1A. The PKD1 gene deletion was confirmed by the presence of a 359-bp knockout band in the 4-HT-treated PKD1β/β-R26Cre/ERT2 mice on PCR analysis of genomic DNA (Fig. 1B, left panel). Expression levels of PKD1 mRNA (Fig. 1B, second panel from left) and protein (Fig. 1B, third panel from left) were substantially inhibited in the lungs of the 4-HT-treated PKD1β/β-R26Cre/ERT2 mice. In contrast, levels of mRNA and protein from other genes tested in the lungs of the 4-HT-treated PKD1β/β-R26Cre/ERT2 mice were comparable to those in the lungs in the vehicle-treated control mice. To evaluate whether 4-HT treatment or PKD1 deletion affected the general immune cell profile, spleen cells were analyzed by flow cytometry. Proportions of B cells, CD4+ T cells, CD8+ T cells, and CD11b+ cells in the spleens of 4-HT-treated PKD1β/β-R26Cre/ERT2 mice were comparable to those of vehicle-treated PKD1β/β-R26Cre/ERT2 mice (Fig. 1B, right panel). Of note, 4-HT treatments neither deleted PKD1 nor altered immune cell profile in PKD1β/β mice (the strain lacks the Cre recombinase) (Fig. 1C). These data demonstrate that our 4-HT treatment regimen effectively deleted PKD1 in PKD1β/β-R26Cre/ERT2 mice and that the 4-HT treatment regimen did not alter the general immune cell profile in mouse spleen.

PKD1 is essential for activation of MAPks and NF-κB and expression of cytokines and chemokines in the lung after S. rectivirgula inhalation
We have previously found that S. rectivirgula induces activation of PKD1 in various murine cell lines in vitro and in the lung in vivo and that expression of various proinflammatory cytokines and chemokines in response to S. rectivirgula is significantly suppressed in PKD1-knockdown cells (49). These findings suggested that PKD1 plays an essential role in the S. rectivirgula–induced pulmonary inflammation. We tested this using tamoxifen-inducible systemic PKD1-insufficient mice. PKD1β/β-R26Cre/ERT2 mice were treated with either vehicle or 4-HT as indicated in Fig. 1A. With these vehicle-treated PKD1β/β-R26Cre/ERT2 mice (WT; PKD1-sufficient mice) and 4-HT–treated PKD1β/β-R26Cre/ERT2 mice (PKDIK0; PKD1-insufficient mice), we investigated whether PKD1 is essential for activation of MAPks and NF-κB and expression of cytokines and chemokines that influence the cytokine milieu and leukocyte influx in the lung following single intranasal exposure to S. rectivirgula. As shown in Fig. 2A, S. rectivirgula induced activation of PKD1, MAPks (JNK, ERK, and p38), and degradation of IκBα (as an indication of NF-κB activation) in the lungs of PKD1-sufficient mice (WT) within 1 h of exposure. However, the S. rectivirgula–mediated activation of PKD1, JNK, ERK, and p38 and degradation of IκBα were substantially inhibited in the lungs of PKD1-insufficient mice (PKDIKO). These results indicate that PKD1 plays an indispensable role in S. rectivirgula–induced activation of JNK, ERK, p38, and NF-κB in the lung. In addition, these results further verify the previous finding (49) that S. rectivirgula induces activation of PKD1 among three PKD family members.

Next, proinflammatory responses in the lungs of PKD1-sufficient mice and PKD1-insufficient mice exposed to S. rectivirgula were assessed by analyzing mRNA levels and protein levels of selected cytokines and chemokines in lungs and BALF, respectively. As demonstrated in Fig. 2B, there was increased expression of TNF-α, IL-6, IFN-β, IL-12p40, keratinocyte chemotactic attractant (KC; CXCL1), LPS-induced CXC chemokine (LIX; CXCL5), MCP-1 (CCL2), MIP-1α (CCL3), MIP-2 (CCL2), and
RANTES (CCL5) in the lungs of PKD1-sufficient mice within an hour after exposure to *S. rectivirgula*. We were not able to detect expression of eotaxin, CCR5, and CXCR4 in the lungs of these mice within 1 h after *S. rectivirgula* exposure (data not shown). Although there were slight variations among individuals, when compared with PKD1-sufficient mice, PKD1-insufficient mice showed substantially reduced mRNA expression levels of cytokines TNF-α, IL-6, and IL-12p40 and chemokines KC, LIX, MCP-1, MIP-1α, MIP-2, and RANTES in the lung in response to *S. rectivirgula* exposure. However, *S. rectivirgula*–induced expression of IFN-β in the lungs was not inhibited in PKD1-insufficient mice. Protein levels of IL-1α, IL-1β, and KC in BALF from *S. rectivirgula*–exposed PKD1-insufficient mice were not significantly different from those in PKD1-sufficient mice. These results indicate that PKD1 is dispensable for the *S. rectivirgula*–induced expression of IFN-β and production of IL-1α, IL-1β, and KC in the lung. Of note, mRNA expression levels of cytokines IL-6 and IL-12 in the lungs and BALF protein levels of cytokines TNF-α, IL-6, and IL-12 in response to *S. rectivirgula* in PKD1/β mice treated with 4-HT were comparable to those in the vehicle-treated PKD1/β mice (Fig. 3A, 3B). These findings demonstrate that the 4-HT treatment regime used in our
FIGURE 2. PKD1 contributes to the proinflammatory responses in the lung that develop after S. recti virgula inhalation.

(A–C) PKD1 

fl/fl–R26 

Cre/ERT2 

mice were treated with vehicle (WT) or 4-HT (PKD1KO) six times by i.p. injection, as described in Materials and Methods. Within 1 wk after the last 4-HT treatment, mice were exposed intranasally to saline or S. recti virgula for 1 h (A and B), 6 h (for KC and MIP-2 in C), or 24 h (for IL-1α, IL-1β, IL-6, IL-12, TNF-α, and MCP-1 in C). (A) Lung lysates were prepared and the activation status of PKD1, JNK, p38, and ERK and protein levels of IκBα and actin in lung lysates were detected by Western blot. Actin was used as a loading control. Each lane represents the samples collected from an individual mouse. (B) Total RNA was purified from lung lobes and mRNA levels of the indicated genes were analyzed by RT-PCR. Each lane represents the samples collected from an individual mouse. GAPDH and actin were used as loading controls. (C) Bronchoalveolar lavage (BAL) was performed. Levels of the indicated cytokines and chemokines in BAL fluid were detected by either ELISA (TNF-α, IL-6, and IL-12) or a multiplex sandwich assay (IL-1α, IL-1β, MCP-1, MIP-2, and KC). Data represent the mean concentration (pg/ml) ± SD. The number (Continued)
experiment does not have immune-suppressive effects. Collectively, these results demonstrated that PKD1 plays an essential role in *S. rectivirgula*–mediated activation of JNK, ERK, p38, and NF-κB and contributes significantly to the subsequent proinflammatory responses in the lung. Our results also imply that pulmonary proinflammatory responses to *S. rectivirgula* might involve a complicated orchestration of multiple signaling pathways.

**Significant contribution of PKD1 to the development of a neutrophilic alveolitis following single time exposure to *S. rectivirgula***

Our results showed significant reduction in *S. rectivirgula*–induced pulmonary expression and production of MIP-2 and MCP-1, chemokines that attract leukocytes into the lungs after *S. rectivirgula* exposure (38, 54), in PKD1-insufficient mice. These results suggest a possibility that PKD1 may contribute to...
FIGURE 4. PKD1 is necessary for neutrophil recruitment into the airways and interstitial lung spaces following S. rectivirgula exposure.

(A) PKD1<sup>fl/fl</sup>-R26<sup>CremERT2</sup> mice were treated with vehicle (WT) or 4-HT (PKD1KO) six times by i.p. injection, as described in Materials and Methods. Within 1 wk after the last 4-HT treatment, mice were exposed intranasally to saline or S. rectivirgula for 6 h or 24 h. BAL was performed, and the (Continued)
the neutrophil influx into the lung observed after exposure to *S. rectivirgula*. Therefore, we further investigated whether deletion of PKD1 also affects leukocyte infiltration into the lung following *S. rectivirgula* exposure. As expected, at 6 or 24 h after exposure to *S. rectivirgula*, PKD1-sufficient mice exhibited dramatic increases in total BAL cell numbers (alveolitis) and total ILC numbers compared with control mice exposed to saline (Fig. 4A). Histological sections of lungs from *S. rectivirgula*-exposed PKD1-sufficient mice also showed the presence of extensive mononuclear cell infiltration in the lungs compared with those from saline-exposed mice (Fig. 4B). In agreement with previous studies (38, 49, 54, 55), neutrophils were the predominant cell type recovered from the airways and lung tissues isolated from PKD1-sufficient mice exposed to *S. rectivirgula* (Fig. 4A). In contrast, significantly fewer BAL cells and ILCs were recovered from the PKD1-insufficient mice at 6 or 24 h after the *S. rectivirgula* exposure compared with those from PKD1-sufficient mice (Fig. 4A). Histological sections of lungs from *S. rectivirgula*-exposed PKD1-insufficient mice also showed significantly less mononuclear cell infiltration in the lungs compared with those from *S. rectivirgula*-exposed PKD1-sufficient mice (Fig. 4B). Similar to the *S. rectivirgula*-exposed PKD1-sufficient mice, the major cell type recovered from the airways and lung tissues isolated from PKD1-insufficient mice was neutrophils. Although they did not reach statistical significance, frequencies of neutrophils in BAL cells (at 6 h, \( p = 0.0903 \); at 24 h, \( p = 0.0705 \)) and ILCs (\( p = 0.0886 \)) from *S. rectivirgula*-exposed PKD1-insufficient mice were slightly lower compared with those from *S. rectivirgula*-exposed PKD1-sufficient mice. The numbers of neutrophils recovered from the BALF or lung tissue from PKD1-insufficient mice were \( \sim \)20 and 28%, respectively, of those in *S. rectivirgula*-exposed PKD1-sufficient mice. Of note, *S. rectivirgula*-mediated neutrophil influx into the lungs of PKD1\(^{fl/fl}\) mice treated with 4-HT were comparable to those in vehicle-treated PKD1\(^{fl/fl}\) mice, indicating that the observed reduction in alveolitis in PKD1-insufficient mice is not due to 4-HT treatment but due to the PKDI insufficiency (Fig. 3C). Taken together, these results demonstrate that PKDI plays a significant role in *S. rectivirgula*-induced neutrophilic alveolitis.

### Indispensable contribution of PKD1 to leukocyte infiltration into the bronchial space following repeated exposures to *S. rectivirgula*

Leukocyte infiltration into the airways and interstitial lung space after exposure to *S. rectivirgula* is a recurring phenotype for individuals with HP (2, 56, 57). To determine whether PKD1 also contribute to the leukocyte influx following repeated intranasal exposures to *S. rectivirgula*, PKD1-sufficient mice and PKD1-insufficient mice were exposed to *S. rectivirgula* three times per week for 5 wk, as indicated in Fig. 5A, and BAL was performed 48 h after the last *S. rectivirgula* exposure. As shown in Fig. 5B, PKD1 insufficiency resulted in significant suppression of alveolitis after repeated *S. rectivirgula* exposures. The numbers of PMNs (CD11b\(^+\)Gr1\(^+\)), AMs (CD11c\(^+\)F4/80\(^+\)), and CD4\(^+\) T cells were significantly reduced in the airway of PKD1-insufficient mice repeatedly exposed to *S. rectivirgula* for 5 wk compared with those in the PKD1-sufficient mice. However, the numbers of CD8\(^+\) T cells were not statistically different in the airway of PKD1-insufficient mice repeatedly exposed to *S. rectivirgula* for 5 wk compared with those in the PKD1-sufficient mice and in the saline-treated control mice. Cellular composition of alveolitis following a 5-wk exposure to *S. rectivirgula* was not different between PKD1-sufficient mice and PKD1-insufficient mice (Table II). The levels of surface expression of a costimulatory molecule CD86 and MHC-II on PMNs and AMs, as well as an early activation marker CD69 on CD4\(^+\) T cells and CD8\(^+\) T cells isolated from bronchial spaces of these mice, were also detected by flow cytometry. As shown in Fig. 5C, levels of surface expression of CD86 on PMNs and AMs, as well as CD69 on CD4\(^+\) T cells and CD8\(^+\) T cells isolated from PKD1-insufficient mice, were comparable to those from PKD1-sufficient mice. There was slight, but significant, reduction in the levels of surface expression of MHC-II in PMNs (\( \sim \)16% reduction in gMFI) and AMs (\( \sim \)20% reduction in gMFI) isolated from airways of PKD1-insufficient mice compared with those from PKD1-sufficient mice. Our results suggest that PKDI is one of major signaling contributors that lead the process of leukocyte infiltration into the bronchial space following repeated exposures to *S. rectivirgula*.
**FIGURE 5.** PKD1 insufficiency results in significant reduction in alveolitis and MHC-II surface expression on alveolar macrophages and polymorphonuclear neutrophils following repeated exposures to *S. rectivirgula.*

(A) Intranasal administration scheme for repeated *S. rectivirgula* exposures. (B and C) PKD1fl/fl, R26Cre/ERT2 mice were treated with vehicle (WT) or 4-HT (PKD1KO) six times by i.p. injection, as described in Materials and Methods. Within 1 wk after the last 4-HT treatment, mice were exposed intranasally to saline or *S. rectivirgula* three times per week for 5 wk and analyzed 48 h after the last *S. rectivirgula* exposure. (B) BAL was performed, and the BAL cells were recovered. (B) BAL cells were counted to determine the degree of alveolitis using trypan blue exclusion. Total cell counts and MHC-II surface expression were performed 48 h after the last 4-HT treatment. BAL was performed, and the BAL cells were recovered. (B) BAL cells were counted to determine the degree of alveolitis using trypan blue exclusion.

(B) **Alveolitis (x10^9)** and **PMNs (x10^6)**

(C) **CD69 (gMFI)/PMNs** and **MHCII (gMFI)/PMNs**

**Effects of PKD1 insufficiency on the cellular composition of ILCs and granuloma formation following repeated exposures to *S. rectivirgula***

Repeated exposures to HP-inciting Ags lead to the formation of granulomas, a focal aggregate of immune cells. To determine whether PKD1 contributes to the immune cell influx into the lungs following repeated exposures to *S. rectivirgula*, ILCs were isolated from PKD1-sufficient mice and PKD1-insufficient mice exposed to *S. rectivirgula* three times per week for 5 wk, and flow cytometric analysis was performed 48 h after the last...
**TABLE II. Cellular composition of alveolitis following 5-wk exposure of WT and PKD1KO mice to S. rectivirgula**

<table>
<thead>
<tr>
<th></th>
<th>PMNs (%)</th>
<th>AMs (%)</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt;βTCR&lt;sup&gt;+&lt;/sup&gt;</th>
<th>CD8&lt;sup&gt;+&lt;/sup&gt;βTCR&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>57.16 ± 13.32</td>
<td>5.74 ± 1.27</td>
<td>10.88 ± 3.49</td>
<td>4.36 ± 2.47</td>
</tr>
<tr>
<td>PKD1KO</td>
<td>61.12 ± 13.60</td>
<td>6.13 ± 4.01</td>
<td>9.10 ± 2.97</td>
<td>4.29 ± 2.16</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD of each group. PKD1-sufficient mice (WT; n = 3) and PKD1-insufficient mice (PKD1KO; n = 6) were exposed to S. rectivirgula (100 μg) three times per week for 5 wk and analyzed 48 h after the last exposure. The frequencies of polymorphonuclear cells (PMNs; CD11b<sup>+</sup>Gr<sup>1+</sup>), alveolar macrophages (AMs; CD11c<sup>+</sup>F4/80<sup>+</sup>), CD4<sup>+</sup> T cells (CD4<sup>+</sup>βTCR<sup>+</sup>), and CD8<sup>+</sup> T cells (CD8<sup>+</sup>βTCR<sup>+</sup>) in BAL cells were measured by flow cytometry followed by analysis using FlowJo software as described in Materials and Methods and expressed as the percentage of BAL cells.

*S. rectivirgula* exposure. As shown in Fig. 6A, compared with the saline-treated control mice, PKD1-sufficient mice repeatedly exposed to *S. rectivirgula* for 5 wk showed significant increases in the percentage of CD11b<sup>+</sup>CD11c<sup>+</sup> cells, CD11b<sup>+</sup>CD11b<sup>+</sup> cells, and CD4<sup>+</sup>βTCR<sup>+</sup> cells, whereas they showed significant decreases in the percentage of CD8<sup>+</sup>βTCR<sup>+</sup> cells, and no significant change in the percentages of CD11b<sup>+</sup>Gr<sup>1+</sup> cells (p = 0.2515) and CD11b<sup>+</sup>F4/80<sup>+</sup> cells. The frequencies of CD11b<sup>+</sup>F4/80<sup>+</sup> cells, CD11b<sup>+</sup>Gr<sup>1+</sup> cells, CD11c<sup>+</sup>CD11b<sup>+</sup> cells, CD4<sup>+</sup>βTCR<sup>+</sup> cells, and CD8<sup>+</sup>βTCR<sup>+</sup> cells within the interstitial lung tissue were not different between PKD1-sufficient mice and PKD1-insufficient mice following repeated exposures to *S. rectivirgula*. However, the frequency of CD11b<sup>+</sup>CD11c<sup>+</sup> cells was significantly lower in PKD1-insufficient mice than in PKD1-sufficient mice. We observed no difference on levels of MHC-II expression in CD11b<sup>+</sup>CD11c<sup>+</sup> cells and CD11c<sup>+</sup>CD11b<sup>+</sup> cells between mice exposed to saline and mice repeatedly exposed to *S. rectivirgula* (Fig. 6B). In contrast, repeated exposures to *S. rectivirgula* led to significant increases in the levels of MHC-II expression on CD11b<sup>+</sup>F4/80<sup>+</sup> cells and CD11b<sup>+</sup>Gr<sup>1+</sup> cells in PKD1-sufficient mice. These increases in levels of MHC-II expression on CD11b<sup>+</sup>F4/80<sup>+</sup> cells and CD11b<sup>+</sup>Gr<sup>1+</sup> cells following repeated exposures to *S. rectivirgula* were almost completely (if not completely) ablated in PKD1-insufficient mice. Our results showed that PKD1 contributes to the increased frequency of CD11b<sup>+</sup>CD11c<sup>+</sup> dendritic cells and increased surface expression of MHC-II on macrophages and PMNs following repeated exposures to *S. rectivirgula*. The surface expression levels of an early activation marker CD69 on CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells in the interstitial lung tissues were significantly increased following repeated exposures to *S. rectivirgula* in PKD1-sufficient mice compared with those in PKD1-sufficient mice exposed to saline. The levels of the surface expression of CD69 on CD4<sup>+</sup> T cells (p = 0.2641) and CD8<sup>+</sup> T cells (p = 0.3438) were not statistically different between PKD1-sufficient mice and PKD1-insufficient mice following repeated exposures to *S. rectivirgula*. To determine whether PKD1 contributes to the development of granulomas, we examined lung tissue sections from PKD1-sufficient mice and PKD1-insufficient mice that had been exposed to *S. rectivirgula* for 5 wk. As shown in Fig. 5C, whereas mice exposed to saline showed normal lung architecture, PKD1-sufficient mice exposed to *S. rectivirgula* for 5 wk exhibited granuloma formation. The PKD1-insufficient mice exposed to *S. rectivirgula* for 5 wk showed substantially reduced granuloma formation compared with PKD1-sufficient mice. Collectively, these results demonstrate that PKD1 contributes to leukocyte infiltration in the lungs, increased expression of MHC-II on myeloid lineage cells, and subsequent granuloma formation following repeated exposures to *S. rectivirgula*.

**Contribution of PKD1 to Th1- and Th17-related cytokine and chemokine expression**

Th1- and Th17-associated cytokines and chemokines are critical to the development and severity of HP (58–60). The regulatory cytokine IL-10 has been known to modulate inflammation and granuloma formation in HP (3). The Th2 cytokine IL-4 inhibits Th1 and Th17 differentiation and is known to play a suppressive role in HP (61–64). To investigate whether PKD1 contributes to the expression of any Th1/Th2/Th17-associated cytokines and chemokines following repeated exposures to *S. rectivirgula* for 5 wk, expression levels of the several selected cytokines and chemokines were assessed either by ELISA (IFN-γ, TNFα, IL-17A, IL-21, and IL-23) or a multiplex sandwich assay (IL-1B, IL-2, IL-4, IL-6, and IL-10). As shown in Fig. 7A, compared with the saline-exposed PKD1-sufficient mice or *S. rectivirgula*–exposed PKD1-insufficient mice, slightly increased levels of IFN-γ in BALF obtained from PKD1-sufficient mice 48 h after the last *S. rectivirgula* exposure were detected (saline versus *S. rectivirgula*–treated PKD1-sufficient mice, p = 0.1102; *S. rectivirgula*–treated PKD1-sufficient mice versus *S. rectivirgula*–treated PKD1-insufficient mice, p = 0.1352). The levels of IFN-γ in BALF obtained from PKD1-insufficient mice exposed to *S. rectivirgula* repeatedly for 5 wk were not different from those from saline-exposed control mice (p = 0.9385). The levels of IL-17A in BALF obtained from PKD1-sufficient mice following repeated exposures to *S. rectivirgula* were significantly increased compared with those from saline-exposed mice. The levels of IL-17A in BALF obtained from PKD1-insufficient mice repeatedly exposed to *S. rectivirgula* were significantly reduced compared with those from *S. rectivirgula*–exposed PKD1-sufficient mice but were not different from those of saline-exposed control mice. Similar to the protein levels in BALF, mRNA levels of IFN-γ and IL-17A in the lungs of PKD1-sufficient mice exposed to *S. rectivirgula* for 5 wk were significantly increased compared with those of PKD1-sufficient mice exposed to saline for 5 wk (Fig. 7B). The levels of IFN-γ expression in the lungs of PKD1-insufficient mice repeatedly exposed to *S. rectivirgula* were not significantly different from those of PKD1-sufficient mice exposed to *S. rectivirgula* (PKD1-sufficient mice versus *S. rectivirgula*–treated PKD1-sufficient mice, p = 0.2591). The levels of IL-17A expression in the lungs from PKD1-insufficient mice repeatedly exposed to *S. rectivirgula* were significantly reduced compared with those of *S. rectivirgula*–exposed PKD1-sufficient mice but were not different from those of saline-exposed control mice.

https://doi.org/10.4049/immunohorizons.2200017
PKD1 KO-R26Cre/ERT2 mice were treated with vehicle (WT) or 4-HT (PKD1KO) six times by i.p. injection, as described in Materials and Methods. Within 1 wk after the last 4-HT treatment, mice were exposed intranasally to saline or S. rectivirgula three times per week for 5 wk and analyzed 48 h after the last S. rectivirgula exposure. (A and B) ILCs were isolated from the whole lung from each mouse. ILCs were stained with fluorochrome-conjugated Abs and then analyzed by flow cytometry and FlowJo flow software as described in Materials and Methods. (A) The frequency of the indicated cell population is expressed as percentage of ILCs. (B) Levels of surface expression of MHC-II and CD69 were determined by geometric mean fluorescence intensity (gMFI) of each marker in the indicated cell population. Data represent the mean ± SD (n = 3–7 mice/group). Significance was determined by one-way ANOVA with a Tukey post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001. (C) Representative H&E staining of the left lung lobe sections from mice exposed to the indicated stimuli are shown (n = 5 mice/group). Each column represents the lung collected from an individual mouse. The Aperio ScanScope XT slide scanner system was used to capture whole-slide digital images. The images presented are x4 original magnification (scale bars, 600 μm) and x20 original magnification (scale bars, 200 μm), SR, S. rectivirgula.

control mice. We were not able to detect cytokines TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-21, and IL-23 above the assay detection sensitivity level in BALF obtained from PKD1-sufficient mice and PKD1-insufficient mice following repeated exposures to saline or S. rectivirgula (data not shown). IL-17RA mRNA levels in the lung were not different between saline-exposed control mice, S. rectivirgula–exposed PKD1-sufficient mice, and S. rectivirgula–exposed PKD1-insufficient
PKD1 IN HYPERSENSITIVITY PNEUMONITIS

FIGURE 7. PKD1 contributes to the increased expression of Th1/Th17-related cytokines and chemokines in lungs following repeated exposures to S. rectivirgula. WT-Sa mouse model of HP demonstrated that PKD1, but not PKD2 or PKD3, through a MyD88-dependent mechanism and that PKD1 is one of the key signaling intermediaries downstream of MyD88 that lead to the expression of proinflammatory cytokines and chemokines in several murine cell lines following exposure to S. rectivirgula (49).

DISCUSSION

The acute phase of HP is characterized by production of proinflammatory cytokines and a neutrophilic influx into the lung within hours of Ag exposure (2, 56, 57). Our previous studies using the S. rectivirgula mouse model of HP demonstrated that these responses are largely dependent on the TLR2-, TLR9-, and TLR/IL-1R–signaling adaptor MyD88 (36, 38). Previously, we have demonstrated that S. rectivirgula induces activation of PKD1, but not PKD2 or PKD3, through a MyD88-dependent mechanism. Levels of CXCL9 (MIG), CXCL11 (I-TAC), and CCL20 (MIP-3α) are known to contribute to the Th1/Th17 polarization (65, 66). We analyzed whether expression of these chemokines in the lung following repeated exposure to S. rectivirgula changed and whether PKD1 contributes to the S. rectivirgula–mediated changes in their expression. We found that the level of CXCL9 mRNA expression was significantly increased in the lungs of PKD1-sufficient mice following repeated exposure to S. rectivirgula (Fig. 7C). Compared to the expression level in PKD1-sufficient mice, expression levels of CXCL9 in the lung of PKD1-insufficient mice following repeated exposure to S. rectivirgula were significantly suppressed. The mRNA expression levels of CXCL11, CXCL12, CXCL13, and CCL20 in the lungs were not different between saline-exposed control mice, S. rectivirgula–exposed PKD1-sufficient mice, and S. rectivirgula–exposed PKD1-insufficient mice under our experimental condition (data not shown). Taken together, these results suggest that PKD1 contributes substantially to the expression of IL-17A and the Th1/Th17–associated chemokine CXCL9 following repeated exposures to S. rectivirgula.

RNA was purified from lung lobes isolated from each individual mouse and reverse transcribed. mRNA levels of the indicated genes were analyzed in triplicates by quantitative RT–PCR using a TaqMan Assay (for IL-17A, CXCL9, and CCL20) or SYBR Green Assay (for IFN-γ). The data on genes that were differentially expressed were normalized to the expression of the housekeeping gene, actin. Fold change comparing S. rectivirgula–treated exposed PKD1-sufficient mice and S. rectivirgula–treated PKD1-deficient mice to control PKD1-sufficient mice exposed to saline were calculated by comparative quantification algorithm–ΔΔCt method (fold difference = 2^−ΔΔCt). Data represent the mean (fold) ± SD. Significance was determined by one-way ANOVA with a Tukey post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 SR, S. rectivirgula.

PKD1suff-R26CreERT2 mice were treated with vehicle (WT) or 4-HT (PKD1KO) six times by i.p. injection, as described in Materials and Methods. Within 1 wk after the last 4-HT treatment, mice (n = 3–6 mice/group) were exposed intranasally to saline or S. rectivirgula three times per week for 5 wk and analyzed 48 h after the last S. rectivirgula exposure. (A) Bronchoalveolar lavage (BAL) was performed. Levels of IFN-γ and IL-17A were detected by either ELISA. Data represent the mean concentration (pg/ml) ± SD. (B and C) Total mice (data not shown). Our results indicate that PKD1 plays a substantial role in the expression of IL-17A in mouse lungs following repeated exposures to S. rectivirgula.

By binding to specific receptors expressed on the surface of leukocytes, chemokines direct leukocyte movement. Chemokines CXCL9 (MIG), CXCL11 (I-TAC), and CCL20 (MIP-3α) are known to contribute to the Th1/Th17 polarization (65, 66). We analyzed whether expression of these chemokines in the lung following repeated exposure to S. rectivirgula is changed and whether PKD1 contributes to the S. rectivirgula–mediated changes in their expression. We found that the level of CXCL9 mRNA expression was significantly increased in the lungs of PKD1-sufficient mice following repeated exposure to S. rectivirgula (Fig. 7C). Compared to the expression level in PKD1-sufficient mice, expression levels of CXCL9 in the lung of PKD1-insufficient mice following repeated exposure to S. rectivirgula were significantly suppressed. The mRNA expression levels of CXCL11, CXCL12, CXCL13, and CCL20 in the lungs were not different between saline-exposed control mice, S. rectivirgula–exposed PKD1-sufficient mice, and S. rectivirgula–exposed PKD1-insufficient mice under our experimental condition (data not shown). Taken together, these results suggest that PKD1 contributes substantially to the expression of IL-17A and the Th1/Th17–associated chemokine CXCL9 following repeated exposures to S. rectivirgula.
Treatment of mice with the pharmacological agent Gö6976, which inhibits all three PKD family members and PKCδ, and checkpoint kinases 1 and 2 (50, 51), ablated *S. rectivirgula*-mediated induction of alveolitis, pointing to a possibility of the essential role of PKD1 in the pulmonary inflammatory response to *S. rectivirgula* (49). However, due to the lack of specificity of the inhibitor for PKD1, contributions of PKD1 to development of the acute pulmonary inflammatory response and HP following exposures to *S. rectivirgula* have not been revealed. To investigate the role of PKD1 in *S. rectivirgula*-induced pulmonary inflammation and the development of HP, we employed tamoxifen-inducible PKD1-insufficient mice. The results from the current study provide evidence that PKD1 contributes substantially to production of proinflammatory cytokines and chemokines, leukocyte infiltration into the lungs, and granuloma formation following exposures to *S. rectivirgula*.

Activation of PKD1 by *S. rectivirgula* and inhibition of *S. rectivirgula*-mediated MAPK activation, NF-κB activation, and subsequent proinflammatory cytokine and chemokine production in vitro and in vivo by inhibitors for PKD, such as Gö6976, strongly suggested the role of PKD1 in *S. rectivirgula*-induced cytokine and chemokine production (49). In agreement with these previous findings, lungs obtained from PKD1-insufficient mice 1 h after exposure to *S. rectivirgula* were deficient in phosphorylation of MAPKs (JNK, ERK, and p38) and reduced IκBα degradation (an indication of NF-κB activation), demonstrating that PKD1 plays an indispensable role in *S. rectivirgula*-induced activation of these signaling modulators that are involved in expression of numerous proinflammatory mediators. Our previous studies demonstrated that induction of TNF-α, IL-6, MCP-1, and MIP-2, but not IL-1 or KC, following exposure to *S. rectivirgula* was completely dependent on MyD88 and partially dependent on TLR2 and TLR9 (36, 38). Using synthetic TLR ligands, we also found previously that PKD1 is indispensable for MyD88-dependent expression of TNFα, IL-6, IL-10, IL-12, IP-10, MCP-1, CCL5, and CD86 but dispensable for MyD88-dependent expression of type I IFNs and TRIF-dependent expression of cytokines and chemokines (34, 35). Similar to these previous findings, PKD1 was required for the optimal mRNA expression and protein production of IL-6, MIP-2, MCP-1, TNF-α, and IL-12 in the lungs in response to *S. rectivirgula* exposure. Especially, PKD1 appeared to be critical for *S. rectivirgula*-induced IL-12 production. However, PKD1 was not necessary for IFN-β mRNA expression or IL-1α, IL-1β, or KC production. These results suggest that PKD1 is a critical component of the TLR/MyD88 signaling pathways leading to proinflammatory cytokine and chemokine production following *S. rectivirgula* stimulation. Our results also indicate the presence of PKD1-independent pathways mediating signals downstream of *S. rectivirgula* and pattern recognition receptors, including TLR2, TLR9, and others yet to be identified. Patients with HP express increased levels of TNF-α, IL-6, and IL-8 in the BALF (4, 7, 9, 67, 68). Although PKD1 activation is not the absolute requirement for proinflammatory response to *S. rectivirgula*, our results suggest that PKD1 significantly contributes to the *S. rectivirgula*-induced proinflammatory response in the lungs, and therapeutics targeting PKD1 can be a valuable option for ameliorating these early inflammatory events in patients with HP.

HP patients develop a neutrophilic alveolitis shortly after Ag exposure. The neutrophils isolated from the lungs of patients with the chronic form of HP express high levels of gelatinase B and collagenase-2 that correlated with fibrosis (69, 70). Inhibition of neutrophil recruitment in a mouse model of HP resulted in a decrease in inflammation, supporting the idea that neutrophils play a role in pathogenesis (38). The in vitro studies demonstrating the importance of PKD1 in induction of cytokines and chemokines that contribute to neutrophil recruitment such as TNF-α, MIP-2, and MCP-1 suggest that PKD1 may play a critical role in neutrophil recruitment during HP. Our previous studies using the PKD- and PKC-specific inhibitor Gö6976 also pointed to a role for the PKD family in neutrophil recruitment into the lungs following exposure to *S. rectivirgula* (49). One-time exposure of PKD1-insufficient mice to *S. rectivirgula* resulted in significantly decreased alveolitis and a decrease in immune cells recruited into the interstitial lung tissue compared with PKD1-sufficient mice. The decrease in immune cells in the lungs and airspaces was attributed to a decrease in neutrophils. This was confirmed in H&E-stained lung sections, which demonstrated that the lungs of PKD1-insufficient mice are similar to those of saline-exposed mice. The reduction in neutrophils in the lungs correlated with a reduction in IL-6, IL-12, TNF-α, MCP-1, and MIP-2 in BALFs and lung tissues. The reduced production of these cytokines and chemokines is likely responsible for the low level of neutrophil recruitment that was observed in the PKD1-insufficient mice. Taken together, our findings demonstrate the importance of PKD1 in acute inflammation in this model.

The initial proinflammatory cytokine/chemokine environment and neutrophil infiltration might affect the outcome of disease development and progress. Our findings in PKD inhibitor-treated mice (49) and PKD1-insufficient mice after single exposure to *S. rectivirgula* raise the possibility that PKD1 may play a pivotal regulatory role in the pathogenesis of HP caused by repeated pulmonary exposures to *S. rectivirgula*. In agreement with this prediction, repeated exposures of PKD1-insufficient mice to *S. rectivirgula* resulted in significantly decreased alveolitis and a substantial reduction in granuloma formation compared with PKD1-sufficient mice. In our experimental setting, repeated exposures of PKD1-sufficient mice to *S. rectivirgula* for a 5-wk period resulted in significantly increased frequencies of dendritic cells (CD11b+CD11c+ cells and CD11b+CD11c– cells) and CD4+ T cells in interstitial lung spaces. However, the increased frequency of monocyte-derived dendritic cells (CD11b+CD11c+ cells) was not observed in PKD1-insufficient mice exposed to *S. rectivirgula* repeatedly for 5 wk. In addition, although it is not statistically significant, frequencies of CD11b+CD11c– cells and CD4+ T cells in interstitial lung spaces of PKD1-insufficient mice following repeated exposures to *S. rectivirgula* were slightly reduced compared with those in PKD1-sufficient mice. Although the mechanism by which PKD1 is involved in *S. rectivirgula*
induced influx of monocyte-derived dendritic cells into the lung is yet to be revealed, it may be related to reduced production of MCP-1, one of chemokines that can attract dendritic cells to the site of inflammation, in the PKD1-insufficient mice following *S. rectivirgula* exposures. These findings suggest that in addition to the initial neutrophil infiltrations, PKD1 might play a critical role in subsequent recruitment of monocyte-derived dendritic cells into the lungs following repeated exposures to *S. rectivirgula*, which will affect the process of Ag presentation and granuloma formation during the pathogenic course of *S. rectivirgula*-mediated HP.

MHC-II, the molecules that present extracellular Ag-derived peptides to CD4⁺ T cells, is constitutively expressed in APCs (dendritic cells and macrophages) (71, 72). Under certain disease conditions, certain non-APCs acquire MHC-II expression and Ag presentation capacity (73). Tissue-infiltrated blood monocytes acquire MHC-II expression progressively as they mature to macrophages (74, 75). Expression of MHC-II can be induced in mouse neutrophils (76). MHC-II–expressing neutrophils function as a professional APC specializing in driving generation of proinflammatory Th1 and Th17 effector cells (76). Our results show that repeated exposures to *S. rectivirgula* led to the increases in MHC-II surface expression on macrophages and PMNs in the lungs of PKD1-sufficient mice. These increases in MHC-II surface expression following repeated exposures to *S. rectivirgula* appear to be influenced by PKD1. MHC-II surface expression levels on macrophages and PMNs at 6 and 24 h following one-time exposure to *S. rectivirgula* were not different between PKD1-sufficient mice exposed to saline and PKD1-sufficient mice and PKD1-insufficient mice exposed to *S. rectivirgula* (data not shown), indicating that these newly infiltrated cells may acquire MHC-II expression and Ag presentation ability on the site during the periods while the host is repeatedly exposed to *S. rectivirgula*. It is yet to be delimited how PKD1 is involved in regulation of MHC-II expression in macrophages and neutrophils following repeated exposures to *S. rectivirgula*. There are several possible mechanisms by which PKD1 regulates surface expression of MHC-II. PKD1 regulates the fission from the trans-Golgi network of cargos that are destined to the cell surface (77). Several TLR ligands, especially the TLR2 ligand, induces surface expression of MHC-II in porcine monocytes and monocyte-derived dendritic cells (78). Thus, it is possible that PKD1 activated by *S. rectivirgula* (presumably through TLR2) promotes transportation of MHC-II–containing cargo from trans-Golgi network to the cell surface. This possibility is warranted to be tested in the future. Inflammatory cytokine IFN-γ plays a critical role on maturation of newly infiltrated blood monocytes, which are MHC-II–negative cells, to functional APCs that express MHC-II on their surfaces (73). IFN-γ is also a potent inducer of MHC-II in both APCs and non-APCs, allowing them to acquire Ag presentation capacity (79). Thus, it is possible that PKD1 contributes to the expression of MHC-II by directly and/or indirectly involving IFN-γ expression in the lungs following exposure to *S. rectivirgula*. IFN-γ expression in the lungs of mice following repeated *S. rectivirgula* exposures is dependent on TLR9, one of the TLRs that use PKD1 as a key signaling intermediary (36). *S. rectivirgula*–induced expression of IFN-γ in MLE12 cells (a murine bronchial epithelial cell line) is dependent on PKD1 (49). *S. rectivirgula*–induced expression of IL-12, a cytokine that can induce expression of IFN-γ (80), in MPRO (promyelocytes differentiated to neutrophils) and AMJ2-C11 (AMs) is dependent on PKD1 (49). Our study also shows that PKD1 plays an indispensable role in IL-12 expression, a cytokine that is involved in MHC-II expression by upregulating IFN-γ expression (81), in mouse lungs following exposure to *S. rectivirgula*. Taken together, these findings support the possibility that PKD1 contributes to the increased expression of MHC-II on macrophages and neutrophils following repeated exposures to *S. rectivirgula* by directly and/or indirectly involving IFN-γ production. It will be interesting to further pursue whether and how PKD1 plays a role in *S. rectivirgula*–induced IFN-γ expression and whether IFN-γ is responsible for increased surface expression of MHC-II on macrophages and neutrophils following repeated exposures to *S. rectivirgula*. Inducible MHC-II expression is often associated with inflammation. The TLR/IL-1R signaling adaptor MyD88 is necessary for neutrophil recruitment and pulmonary inflammation following exposures to *S. rectivirgula* (38). Although it is not complete abolition similar to what was observed in MyD88-deficient mice, PKD1 contributes significantly to neutrophil infiltration into the lungs and pulmonary inflammation following exposure to *S. rectivirgula*. Thus, we cannot rule out the possibility that the increased MHC-II surface expression on PMNs and macrophages in the lungs of mice following repeated exposures to *S. rectivirgula* could be the result of increased inflammation that was significantly inhibited in the absence of PKD1.

Th1- and Th17–associated cytokines and chemokines are critical to development of HP and the disease severity (58–60). TLR2, TLR6, and TLR9 contribute significantly to expression of IL-17A and Th17 cell generation following repeated exposure to *S. rectivirgula* (36, 82). Similar to these previous findings, our study demonstrates that PKD1, which can act as a MyD88-dependent TLR/IL-1R signaling modulator, also contributes significantly to expression of IFN-γ, IL-17A, and Th1/Th17-polarizing chemokine CXCL9 following repeated exposures to *S. rectivirgula*. No significant change in CXCL11 and CCL20 expression in lungs following repeated exposures to *S. rectivirgula* was detected under our experimental condition. Unlike CXCL9/CXCR3 binding, CXCL11/CXCR3 binding induces Foxp3-negative IL-10-bearing regulatory T cells (Tr1) and IL-4-bearing (Th2) cells (83). IL-10 and IL-4 were also not detected in BALF obtained from PKD1-sufficient mice and PKD1-insufficient mice following repeated exposures to *S. rectivirgula*. These results imply that the reduced Th1/Th17–associated responses observed in PKD1-insufficient mice is not skewing *S. rectivirgula*–induced Th1/Th17 immune response toward Th2 activity.

In summary, using PKD1-insufficient mice, we found that PKD1 contributes substantially for *S. rectivirgula*–induced production of proinflammatory cytokines TNF-α, IL-6, IL-12, IFN-γ, and IL-17A and chemokines MIP-2, MCP-1, and CXCL9 in the
lungs in response to *S. rectivirgula*. PKD1 contributes to the development of an alveolitis, increased expression of MHC-II in myeloid lineage cells, and granuloma formation following repeated exposures to *S. rectivirgula*. Taken together, our findings imply that PKD1 activation may play an essential role in pulmonary proinflammatory responses and HP caused by *S. rectivirgula* and that inhibiting PKD1 activity or expression could be a valuable adjunctive therapeutic option for controlling HP at an early stage.

**ACKNOWLEDGMENTS**

We thank Dr. E. Olson (University of Texas Southwestern Medical Center, Dallas, TX) for providing PKD1KO mice. The Flow Cytometry Facility in Research Service at the Memphis V.A. Medical Center is also gratefully acknowledged.

**DISCLOSURES**

The authors have no financial conflicts of interest.

**REFERENCES**


https://doi.org/10.4049/immunohorizons.2200017