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Protein kinase D3 promotes neutrophil migration during viral infection

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Abstract

Protein kinase D (PKD) is a serine/threonine kinase family with three isoforms (PKD1–3) that are expressed in most cells and implicated in a wide array of signaling pathways, including cell growth, differentiation, transcription, secretion, polarization and actin turnover. Despite growing interest in PKD, relatively little is known about the role of PKD in immune responses. We recently published that inhibiting PKD limits proinflammatory cytokine secretion and leukocyte accumulation in mouse models of viral infection, and that PKD3 is highly expressed in the murine lung and immune cell populations. Here we focus on the immune-related phenotypes of PKD3 knockout mice. We report that PKD3 is necessary for maximal neutrophil accumulation in the lung following challenge with inhaled polyinosinic: polycytidylic acid, a double-stranded RNA, as well as following influenza A virus infection. Using reciprocal bone marrow chimeras, we found that PKD3 is required in the hematopoietic compartment for optimal neutrophil migration to the

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AUTHOR CONTRIBUTIONS

Janelle M Veazey: Conceptualization; data curation; formal analysis; investigation; methodology; project administration; validation; visualization; writing – original draft; writing – review and editing. **Gordon S Wong**: Data curation; formal analysis; methodology; visualization. **Sophia I Eliseeva**: Conceptualization; methodology. **Timothy R Smyth**: Conceptualization. **Timothy J Chapman**: Conceptualization; methodology; supervision; writing – review and editing. **Kihong Lim**: Data curation; formal analysis; methodology; validation; writing – review and editing. **Minsoo Kim**: Conceptualization; funding acquisition; resources; writing – review and editing. **Steve N Georas:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; project administration; resources; supervision; visualization; writing – original draft; writing – review and editing.

CONFLICT OF INTEREST

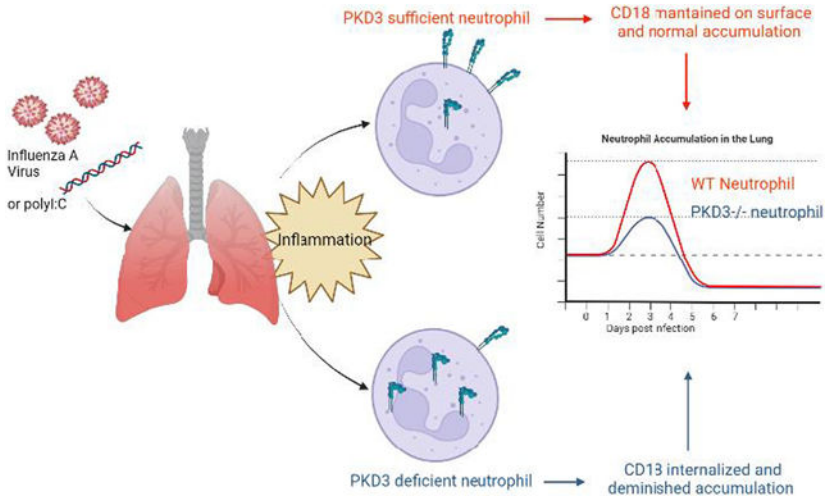
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

lung. *Ex vivo* transwell and chemokinesis assays confirmed that PKD3$^{-/-}$ neutrophils possess an intrinsic motility defect, partly because of reduced surface expression of CD18, which is critical for leukocyte migration. Finally, the peak of neutrophilia was significantly reduced in PKD3$^{-/-}$ mice after lethal influenza A virus infection. Together, these results demonstrate that PKD3 has an essential, and nonredundant, role in promoting neutrophil recruitment to the lung. A better understanding of the isoform-specific and cell type–specific activities of PKD has the potential to lead to novel therapeutics for respiratory illnesses.

Graphical Abstract



In this study, we identified a novel role for protein kinase 3 (PKD3) in neutrophil migration. Using mouse models of respiratory viral infection, we found that fewer PKD3-deficient neutrophils accumulated in the lung compared with wild-type controls. PKD3-deficient neutrophils were also defective in *in vitro* assays of both directional and nondirectional migration and exhibited lower levels of CD18 on the surface. Taken together, these data demonstrate that PKD3 inhibitors might limit neutrophil accumulation at sites of inflammation. This figure was created using BioRender.

Keywords

Influenza A; neutrophil migration; protein kinase D; viral infection

INTRODUCTION

Protein kinase D (PKD) is a serine/threonine kinase family consisting of three isoforms, namely, PKD1, PKD2 and PKD3, each localized to different chromosomes.[1] PKD has been implicated in a wide range of cellular signaling pathways, including growth, differentiation, motility, vesicle secretion, reactive oxygen species generation and cytokine induction. PKD signaling outcomes are highly cell-type, stimuli and isoform specific (reviewed in Rozengurt[2] and Ellwanger and Hausser[3]).

Despite growing interest in the role of PKD in different cell types, relatively little is known about the expression or function of PKD in the immune system. Early studies revealed

that PKD2 was involved in lymphocyte activation and cytokine secretion.[4–8] More recently, PKD has been shown to play a role in MyD88-dependent signaling in innate immune cells including macrophages and monocytes during bacterial and fungal infection,[9–14] as well participate in inflammasome signaling.[15] PKD has also been implicated in promoting production of neutrophil chemoattractants in the lung[16] and facilitating neutrophil activation and migration.[17] We recently reported that PKD3 is highly expressed in airway epithelial cells, where it contributes to chemokine production, neutrophil accumulation and barrier dysfunction in response to polyinosinic:polycytidylic acid [poly(I:C)], a synthetic double-stranded RNA (dsRNA), and viral infection.[18]

Furthermore, while there is growing literature implicating PKD activity during inflammation, little is known about the signaling mechanism of different PKD isoforms in different cells following dsRNA challenge (a mimic of viral infection). What is known about PKD isoforms often tells a complex story. For example, PKD isoforms have been shown to promote cell motility by regulating actin dynamics and cell polarization with different (even opposing) mechanisms being described.[19] Even with the same isoform in the same cell type, such as PKD1 in neutrophils, the results vary depending on the experimental model studied. For example, PKD1 has been reported to promote neutrophil accumulation and cytokine production during bacterial challenge in the lung,[9,16] and PKD1 has been implicated in actin polymerization within neutrophils to promote migration.[20] However, Ittner *et al.*[21] reported that myeloid-specific PKD1 deletion surprisingly enhanced lung neutrophilia. This apparent difference in reported PKD activity on neutrophil accumulation highlights an ongoing need to investigate PKD activity in neutrophil recruitment.

We and others have found that PKD3 is highly expressed in the lung tissue and immune cells.[4,18,22] We therefore hypothesized that PKD3 may play an underappreciated role in immune cell accumulation in the lung. Using a global PKD3 knockout (KO) mouse, we report that PKD3 is necessary for maximal neutrophil accumulation following dsRNA challenge and influenza A virus (IAV) infection in mice *in vivo*. Furthermore, *ex vivo* analysis of PKD3$^{-/-}$ neutrophils uncovered a previously unreported role of PKD3 in neutrophil chemotactic ability.

RESULTS

PKD3 is required for neutrophil accumulation, but not cytokine levels or barrier disruption, in the lung following poly(I:C) challenge

We recently reported that inhibiting PKD promotes airway barrier integrity and limits cytokine production and neutrophil accumulation into the airway following dsRNA inhalation with poly(I:C).[18] However, the PKD inhibitor used in that study (CRT0010166, referred to as CRT from here on) blocks all three isoforms of PKD, leaving the question as to which isoform regulates these processes. As PKD3 is highly expressed in the lung and in immune cells,[4,18,22] we hypothesized PKD3 was the predominant isoform regulating barrier integrity and neutrophil accumulation. We obtained a global PKD3 KO mouse strain (PKD3$^{-/-}$) and confirmed that homozygous KO mice were indeed deficient in PKD3 (Supplementary figure 1). These PKD3$^{-/-}$ mice are on a mixed C57Bl/6 and 129SvEv^{Brd} background, have normal fecundity and exhibit no gross phenotypic abnormalities beyond

mild defects in trabecular bone formation (Lexicon characterization[23]) and a susceptibility to liver fibrosis.[24]

We next challenged wild-type (WT) and PKD3$^{-/-}$ mice with inhaled poly(I:C), a synthetic dsRNA and viral mimetic that causes airway inflammation and neutrophilia in a dose-dependent manner. Similar to our previously published data with PKD inhibitor, neutrophil recruitment to the lungs was significantly reduced in PKD3$^{-/-}$ mice compared with WT controls (22 ± 12% versus 38 ± 16%, $P < 0.01$) (Figure 1a). By contrast, there was no reduction in the primary neutrophil chemoattractant chemokine (C–X–C motif) ligand 1 in bronchoalveolar lavage fluids (BALFs) from PKD3$^{-/-}$ mice, nor were other proinflammatory cytokines affected by PKD3 deletion (Figure 1b).

In addition to promoting airway inflammation, inhaled poly(I:C) causes epithelial barrier dysfunction in a Toll-like receptor 3- and PKD-dependent manner.[18,25] Epithelial barrier dysfunction can be estimated by measuring the accumulation of serum proteins in BALFs ("inside/out" barrier), or by monitoring the rapid translocation of inhaled fluorescein isothiocyanate (FITC)–dextran out of the airspaces and into serum ("outside/in" barrier).[25] We previously reported that PKD inhibition with CRT blocked both inside/out and outside/in barrier dysfunction in poly(I:C)-challenged mice. To determine the role of PKD3 in this process, we next measured epithelial barrier function in WT and PKD3$^{-/-}$ mice. In contrast to the barrier protection and reduced inflammation observed with CRT-treated mice,[18] PKD3$^{-/-}$ mice were not protected from poly(I:C)-mediated insideout barrier disruption as seen by total protein and serum albumin leak into the airspace (Figure 2a). PKD3$^{-/-}$ mice were also not protected from outside-in barrier disruption as seen by loss of inhaled 4-kDa FITC–dextran from the airspace and accumulation in the serum (Figure 2b). Taken together, this suggests that PKD3 is responsible for neutrophil recruitment to the lung after poly(I:C) challenge, while other PKD isoforms are responsible for regulating airway epithelial barrier integrity.

PKD3 in the hematopoietic compartment is necessary for neutrophil accumulation in the lung

We next investigated why PKD3$^{-/-}$ mice have less neutrophil accumulation in the lung despite similar levels of neutrophil-attracting chemokines as their WT counterparts. We first asked whether the defect in neutrophil accumulation could be attributed to either hematopoietic or nonhematopoietic compartments. We generated WT and PKD3$^{-/-}$ reciprocal bone marrow chimeras (see Methods), challenged them with poly(I:C) as in Figure 1 and assessed levels of neutrophils in BALF. In the control transfers (WT ➔ WT and KO ➔ KO; Figure 3, left panel), poly(I:C)-induced neutrophil recruitment was essentially abrogated in KO ➔ KO mice, confirming a key role for PKD3 in this process. In the mixed transfers, neutrophil recruitment to the lung was significantly attenuated in WT recipients of KO bone marrow (KO ➔ WT mice, Figure 3, right panel), but not KO recipients of WT bone marrow (WT ➔ KO mice). Taken together, these results indicate that PKD3 in the hematopoietic compartment promotes neutrophil migration *in vivo* (Figure 3).

Neutrophil-specific PKD3 deletion has little effect on neutrophil recruitment to the lung

To determine whether PKD3 has a neutrophil-intrinsic role in promoting neutrophil recruitment to the lung following dsRNA challenge, we generated a new line of mice by breeding PKD3-floxed mice[18] with mice expressing Cre recombinase driven by the Ly6G promoter. Resulting dual transgenic PKD3-floxed × Ly6G-Cre mice were challenged with inhaled poly(I:C) and compared with single transgenic controls. Interestingly, similar numbers and percentages of neutrophils were recovered from the lungs of both strains (Figure 4).

PKD3$^{-/-}$ neutrophils have an intrinsic motility defect *ex vivo* and have reduced surface CD18 levels

We next asked whether PKD3 was directly involved in neutrophil motility. To do this we subjected neutrophils isolated from the bone marrow of WT and PKD3$^{-/-}$ mice to multiple *ex vivo* motility assays. First, we studied directional chemotaxis using transwell assays. We found that PKD3$^{-/-}$ neutrophils failed to migrate toward the neutrophil chemoattractant *N*-formylmethionyl-leucyl-phenylalanine (Sigma, St Louis, MO, USA; hereafter referred to as fMLP; Figure 5a). Next, we quantified nondirected neutrophil migration on intercellular adhesion molecule–coated plates using time-lapse microscopy. PKD3-deficient neutrophils exhibited reduced fMLP-stimulated chemokinesis *in vitro* compared with WT neutrophils (Figure 5b and Supplementary videos 1 and 2). Together, these results indicate that PKD3 regulates neutrophil motility in a cell-intrinsic manner.

Others previously demonstrated a role for PKD1 in neutrophil motility *via* regulating actin dynamics.[20,21] We therefore hypothesized that PKD3 may direct neutrophil motility through a similar mechanism of actin regulation. To investigate whether PKD3$^{-/-}$ neutrophils had a defect in actin polarization, we quantified f-actin mean fluorescence intensity using fluorescently labeled phalloidin in WT and PKD3$^{-/-}$ splenic neutrophils. We found that the mean fluorescence intensity of f-actin was similar between WT and PKD3$^{-/-}$ neutrophils both at baseline and after stimulation with fMLP, suggesting that PKD3 is not regulating migration *via* actin turnover (Figure 6a). Next, we asked whether the motility defect might be a consequence of differences in cell surface integrin expression. To test integrin levels, we measured both total and surface levels of the integrin CD18 (part of LFA1) and CD11b using flow cytometry (gating scheme shown in Supplementary figure 2). PKD3$^{-/-}$ neutrophils displayed a significant decrease in surface CD18 level compared with WT neutrophils (4652 ± 639 versus 6721 ± 1077 mean fluorescence intensity; $P < 0.05$). The total levels of CD18 were equivalent between PKD3$^{-/-}$ and WT neutrophils, suggesting that PKD3 helps maintain CD18 on the surface rather than altering overall protein production (Figure 6b). Interestingly, there was no global defect in integrin surface expression as total and surface levels of CD11b were similar in PKD3$^{-/-}$ and WT neutrophils (Figure 6c).

PKD3$^{-/-}$ mice exhibit reduced neutrophilia following influenza A infection

To determine whether the neutrophil-intrinsic motility defect we observed using poly(I:C) inhalation challenge was biologically relevant during a respiratory viral infection, we infected WT and PKD3$^{-/-}$ mice with IAV. IAV causes substantial morbidity and mortality worldwide, and induces neutrophilic airway inflammation in humans and mice. Kinetics

of neutrophil accumulation were similar between WT and PKD3$^{-/-}$ mice as neutrophil numbers peaked on day 3 after the infection in both groups (Figure 7a). However, peak neutrophil levels were attenuated in PKD3$^{-/-}$ mice compared with WT mice (75.5 ± 12.8% *versus* 52.0 ± 3.5% in WT *versus* PKD3$^{-/-}$ mice 3 days post infection), suggesting that the intrinsic motility defect in PKD3$^{-/-}$ neutrophils is maintained during replicating viral infection (Figure 7a). Importantly, PKD3$^{-/-}$ mice are still able to clear the virus as seen by similar levels of viral RNA 6 days after the infection (Figure 7b).

DISCUSSION

Although there is growing interest in the role of PKD during immune reactions, little is known about the role of different PKD isoforms in specific immune cells. Studies have identified a role for PKD1 in neutrophil recruitment and activation,[9,16,20] whereas PKD2 has been implicated in lymphocyte activation and cytokine secretion.[4–8] We found that PKD3 was highly expressed in the both the lung and immune cells[18] and hypothesized that PKD3 may have an underappreciated role in regulating immune responses. Using a PKD3 KO mice, here we report that PKD3 is necessary for neutrophil recruitment to the lung in response to inhalation challenge with the dsRNA poly(I:C). Reciprocal bone marrow chimeras between PKD3$^{-/-}$ and WT mice indicate that the defect in neutrophil accumulation was primarily within the hematopoietic compartment, suggesting that PKD3 may have a neutrophil-intrinsic role. However, neutrophil recovery from the lungs was not attenuated by myeloid-specific PKD3 conditional deletion. Subsequent *ex vivo* migration and chemokinesis assays confirmed that PKD3 has a cell-intrinsic and necessary role in neutrophil motility. Finally, we show that during infection with replicating IAV, fewer neutrophils accumulate in the lung following lethal IAV infection in PKD3$^{-/-}$ mice.

Using a global PKD3-deficient mouse, we determined that PKD3 is the isoform responsible for the observed reduction in neutrophil accumulation, as PKD3$^{-/-}$ mice phenocopied mice treated with the pan-PKD inhibitor CRT.[18] To our knowledge, this is the first report demonstrating a role for PKD3 in neutrophil migration. This was surprising as others have published PKD1 to be the isoform most involved in neutrophil migration.[20,21,26] It is possible that PKD1 and PKD3 play nonredundant roles in neutrophil migration, whereby the absence of either greatly diminishes neutrophil chemotaxis ability. For example, PKD1 has been reported to control neutrophil polarization and motility by regulating actin turnover proteins slingshot 2 and cofilin.[20] By contrast, PKD3$^{-/-}$ neutrophils did not show differences in f-actin levels at baseline or after fMLP stimulation (Figure 5), suggesting that PKD3 controls neutrophil motility *via* a different mechanism than PKD1. PKD3$^{-/-}$ neutrophils exhibited lower surface CD18 levels, suggesting that PKD3 may play a role in integrin recycling, for example, by facilitating integrin trafficking from the Golgi to the plasma membrane. It is important to remember that PKD activity is highly isoform and cell-type specific. Therefore, our finding that PKD3 promotes surface integrin expression in neutrophils does not necessarily contrast with the report from Matthews *et al.*[27] who demonstrated that PKD is dispensable for integrin-dependent migration in lymphocytes.

Using reciprocal bone marrow chimeras, it was clearly apparent that PKD3 expressed in hematopoietic cells was required for neutrophil recruitment to the lung following

poly(I:C) challenge (Figure 3). In a recent publication, we reported that lung neutrophil recruitment was not dependent on PKD3 expression in epithelial cells, as determined using epithelial-specific PKD3 conditional deletion.[18] Therefore we expected that neutrophil recruitment to the lung would be attenuated using myeloid-specific PKD3 conditional deletion, but this was not the case, because neutrophil influx was similar in PKD3-floxed × Ly6G-Cre mice compared with controls (Figure 4). This result is strikingly reminiscent of experiments performed using PKD1 conditional deletion. PKD1 had been reported to promote neutrophil accumulation and cytokine production during bacterial challenge in the lung,[9,16] and PKD1 has also been implicated in actin polymerization within neutrophilia.[20] However, Ittner *et al.*[21] reported that myeloid-specific PKD1 deletion actually enhanced lung neutrophilia. Therefore, there is precedence for the idea that global *versus* conditional KO approaches reveal different results when it comes to understanding how PKD isoforms regulate neutrophil recruitment *in vivo*. The fact that PKD3-floxed × Ly6G-Cre mice did not recapitulate results using PKD3-deficient hematopoietic precursors in reciprocal bone marrow chimeras suggests that other hematopoietic cells are involved, perhaps by priming neutrophils for migration *in vivo via* secreted mediators. This and other possibilities will need to be explored in future experiments.

Although PKD3 was required for neutrophil recruitment to the lung, as well as neutrophil migration and motility *in vitro*, we found that it was not involved in regulating epithelial barrier function in response to poly(I:C) inhalation challenge (Figure 2). Using this model, we reported that poly(I:C)-induced epithelial barrier dysfunction was dependent on the dsRNA sensor Toll-like receptor 3, whereas neutrophil recruitment was more dependent on melanoma differentiation–associated protein 5 (MDA5).[25] Poly(I:C)-induced epithelial barrier dysfunction is thought to be caused by defective expression and function of junctional proteins that normally help cells form a tight monolayer. The fact that PKD3 KO mice developed similar degrees of barrier dysfunction as their WT counterparts was surprising, because (1) the PKD inhibitor CRT was strikingly effective at blocking poly(I:C)-induced barrier dysfunction, and (2) PKD3 appears to be the major isoform expressed by airway epithelial cells. One possibility is that other epithelial PKD isoforms (e.g. PKD1/2) are more important for promoting junctional protein dysfunction in response to poly(I:C) challenge. If so, then future studies investigating how PKD isoforms couple with dsRNA sensors will be highly insightful.

During IAV infection, we found that the magnitude of neutrophil accumulation in the lung was reduced in PKD3$^{-/-}$ mice comparted with WT mice (Figure 7). We also reported that the PKD inhibitor CRT reduced neutrophil influx into the lungs in IAV-infected mice.[18] Neutrophils play a complex role in respiratory viral infections, having both immunoregulatory and proinflammatory effects in different models.[28] During infection with IAV, unchecked neutrophil activation is thought to contribute to tissue damage by disrupting lung tissue architecture and impairing gas exchange. Because IAV titers were not appreciably affected by PKD3 deficiency, targeting PKD3 may allow for reduced immunopathology during severe influenza infections without compromising a patient's ability to clear the virus.

In summary, we conclude that PKD3 has an important and previously underappreciated role in promoting neutrophilic lung inflammation in response to poly(I:C) inhalation challenge and IAV infection, and does so in a hematopoietic cell–intrinsic manner. Our results also suggest that inhibitors targeting PKD3 may have anti-inflammatory effects *in vivo*.

METHODS

Mouse strains

C57Bl/6NCr mice were obtained from the National Cancer Institute, USA. PKD3-deficient mice [PKD3$^{-/-}$; B6;129S5-Prkd3Gt(OST191038) Lex/Mmucd, identification number RRID:MMRRC_ 011761-UCD] were obtained from the Mutant Mouse Regional Resource Center, an NCRR–NIH (National Center for Research Resources–National Institutes of Health)–funded strain repository, and donated to the MMRRC by Lexicon Genetics Incorporated. These mice are phenotypically normal with only a mild trabecular bone defect[23] and liver fibrosis susceptibility reported.[24] WT mice were maintained on the same mixed background as the PKD3$^{-/-}$ mice. Experimental and control animals were maintained independently and were age and sex matched. PKD3-floxed mice were generated by homologous recombination using targeting vectors with lox P sites flanking exon 3 at the Mouse Genomics Core services at the University of Rochester (NY, USA). Genotyping and characterization were previously described in Veazey *et al.*[18] Ly6G-Cre mice were a generous gift from Dr Minsoo Kim and previously described in Hasenberg *et al.*[29] All animals were treated according to the Institutional Animal Care and Use Committee approva (Table 1).

Influenza A virus infection and quantification

The IAV strain A/PR/8/1934(H1N1) was a generous gift from Dr Dave Topham at the University of Rochester (NY, USA). Viral genome was quantified *via* RT-PCR using the strategy published in Kawakami *et al.*[30] In brief, lung samples from infected mice were lysed in TRIzol and RNA was extracted with the Qiagen RNeasy Mini kit (Hilden, Germany). The initial reverse transcription step was carried out with a unique primer (GGCCGTCATGGTGGCGAATACCATAATGACCGAT GGCCCAAGT) and 1-h extension at 42°C to allow for discrimination of viral genome *versus* viral messenger RNA. The cDNA product was then subjected to real-time PCR of 40 cycles of 95°C, 15 s, 60°C 60 s, with SYBER Green (Bio-Rad, Hercules, CA, USA) and forward primer: CTCAATATGAGTGCAGACCGTGCT, and reverse primer: GGCCGTCATGGTGGCGAAT.

Assay of “inside-out” leak

C57BL/6 mice were given varying doses of the water-soluble competitive PKD inhibitor CRT 2 days prior to receiving 10 μg poly(I:C), (a dsRNA that mimics viral replication) days 0–2 oropharyngeally (o.p.). Mice were killed 24 h after final the poly(I:C) challenge and BALF was analyzed for total protein (*via* Bradford) and albumin (*via* ELISA). Chemokine (C–X–C motif) ligand 1 levels in BALF were analyzed *via* ELISA, and all other cytokines were analyzed *via* multiplex. Leukocytes in BALF were spun onto slides and stained with hematoxylin and eosin (Table 1).

Assay of "outside-in" leak

This assay of outside/in leak is described in depth by Veazey *et al.*[18] Briefly here, C57/B6 mice were administered 10 μg poly(I:C) (InVivoGen, San Diego, CA, USA) on days 0–2 to induce inflammation. On day 3, 0.2 mg of 4 kDa FITC–dextran (Sigma, St. Louis, MO, USA) was administered by oropharyngeal aspiration and mice were killed 1 h after 4 kDa FITC–dextran administration. BALF was collected as described earlier. Blood was collected *via* cardiac puncture and serum analyzed for FITC–dextran levels with a plate reader (Beckman Coulter DTX 880 Multimode Plate Reader).

Western blot analysis

All primary antibodies (Table 2) were probed overnight at 4°C. Secondary antibodies were probed for 2 h at room temperature in 5% milk. Bands were visualized with Clarity Enhanced Chemiluminescence (Bio-Rad, Hercules, CA, USA).

Bone marrow chimera

The 8–12-week-old female WT and PKD3$^{-/-}$ mice were irradiated with 13-Gy spilt over 4 h (6.5 Gy, 4-h rest, 6.5 Gy). Bone marrow from 5–7-week-old female WT and PKD3$^{-/-}$ mice was isolated and 6×10^6 cells were transferred retro-orbitally to irradiated recipients. Recipients were rested 6 weeks to allow reestablishment of the immune system before challenge with poly(I:C) as described earlier.

Neutrophil isolation and *ex vivo* migration

Neutrophils were isolated from bone marrow of WT and PKD3$^{-/-}$ mice with a negative selection magnetic separation kit. Uncoated 5-μm transwells were allowed to equilibrate to serum-free media containing 0–0.5 μM fMLP (Sigma, St. Louis, MO, USA) in the basolateral chamber at 37°C. Then, 5×10^5 enriched cells were added to the apical side of a 5-μm transwell (uncoated) and allowed to migrate for 2 h before counting the number of cells in the basolateral chamber. For chemokinesis assay using videomicroscopy, enriched cells were transferred to intercellular adhesion molecule–coated coverslips and incubated with 1 μM fMLP for 10 min (photos every 20 s). Migratory velocity and distance (the straight distance between start and end of the neutrophil track) were analyzed using Volocity software (PerkinElmer).

Flow cytometric analysis

Spleens were harvested from naïve WT and PKD3-deficient mice, homogenized, red blood cell lysed and stained for flow cytometric analysis (antibodies are listed in Table 3).

Statistical analysis

All values are expressed as means ± standard deviation. Statistical analyses were performed using an unpaired *t*-test for two groups and ANOVA followed by the unpaired Tukey's multiple comparison test for multiple groups. A *P*-value of 0.05 or less was considered statistically significant. All data were analyzed using GraphPad Prism (Version 9).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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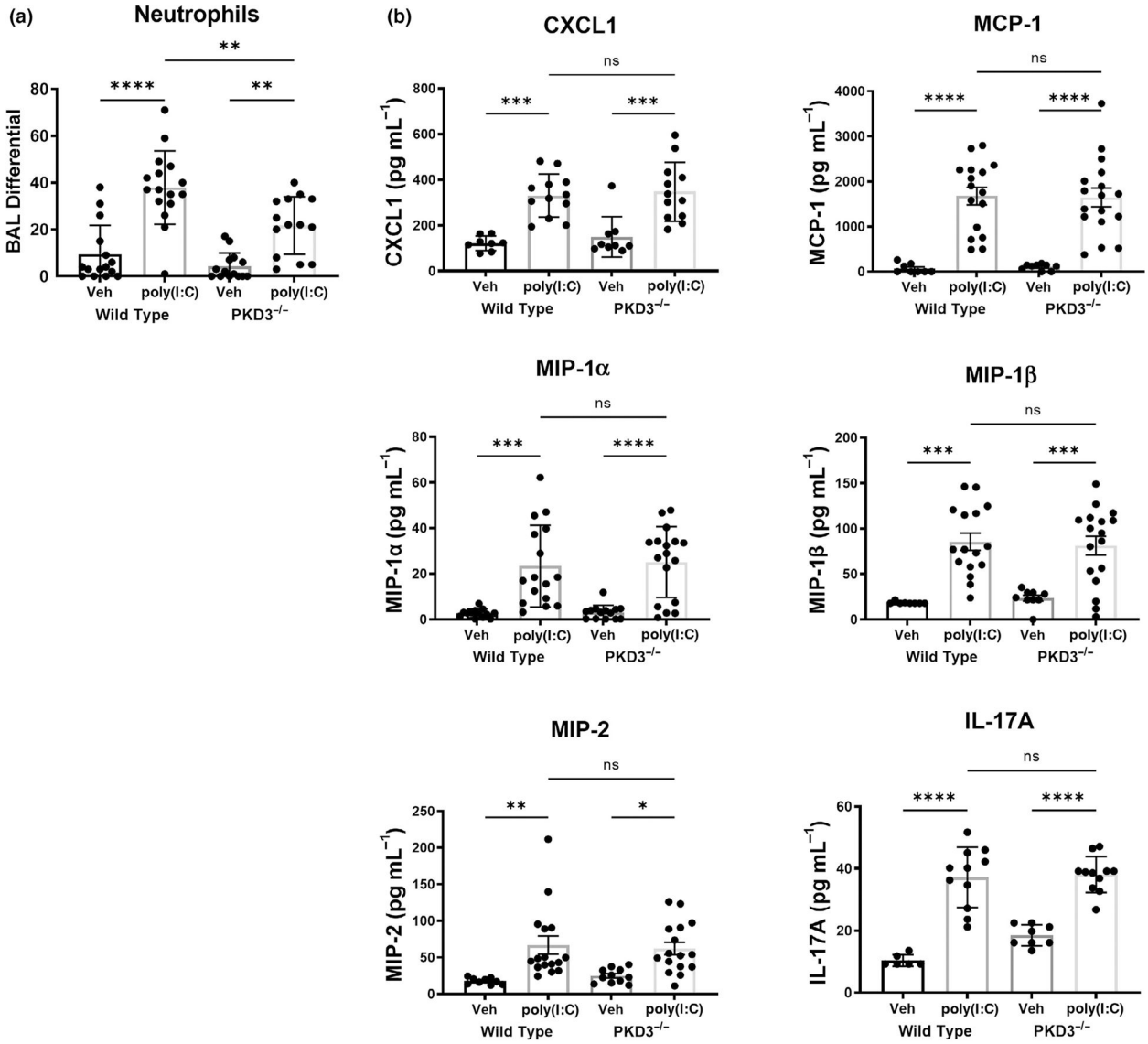

Figure 1.

Protein kinase D3 (PKD3) promotes neutrophil accumulation, but is dispensable for cytokine levels. PKD3$^{-/-}$ or wild-type control mice were administered 10 μg polyinosinic:polycytidylic acid [poly(I:C)] on days 0–2. On day 3, bronchoalveolar lavage (BAL) fluid was harvested and **(a)** neutrophils were assessed *via* cytospins, and **(b)** cytokine levels were assessed with ELISA/multiplex. Data are pooled from three independent experiments ($n = 6$ mice per group per experiment) and results are mean ± standard deviation. One-way ANOVA followed by the unpaired Tukey's multiple comparisons test. *$P < 0.05$, **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. CXCL1, chemokine (C-X-C motif) ligand 1; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MIP, macrophage inflammatory protein; ns, not significant; Veh, vehicle.

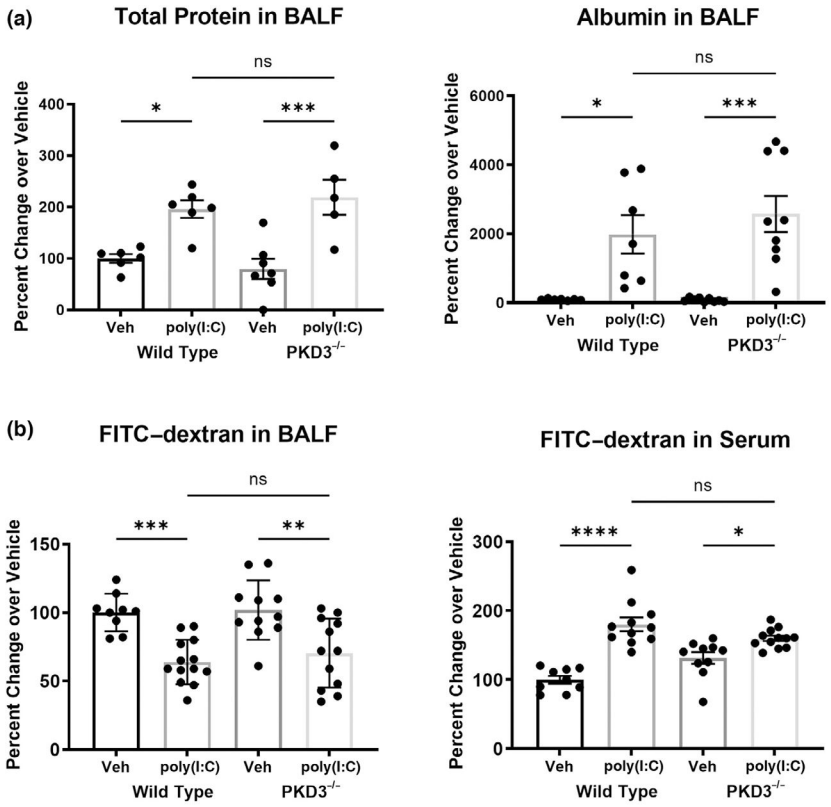

Figure 2.

Protein kinase D3 (PKD3) is dispensable for barrier integrity. PKD3$^{-/-}$ or wild-type control mice were administered 10 μg polyinosinic: polycytidylic acid [poly(I:C)] on days 0–2. On day 3, inside/out barrier integrity was assayed *via* quantification of total protein (Bradford) and albumin (ELISA) accumulation into bronchoalveolar lavage fluid (BALF). **(a)** Outside/in barrier was assessed *via* quantification of 4-kDa fluorescein isothiocyanate (FITC)–dextran loss from the BALF and accumulation into the serum. **(b)** Data are pooled from two independent experiments (n = 4 or 6 mice per group per experiment) and are mean ± standard deviation. One-way ANOVA followed by the unpaired Tukey's multiple comparisons test. *P < 0.05, **P < 0.01; ***P < 0.001; ****P < 0.0001. ns, not significant; Veh, vehicle.

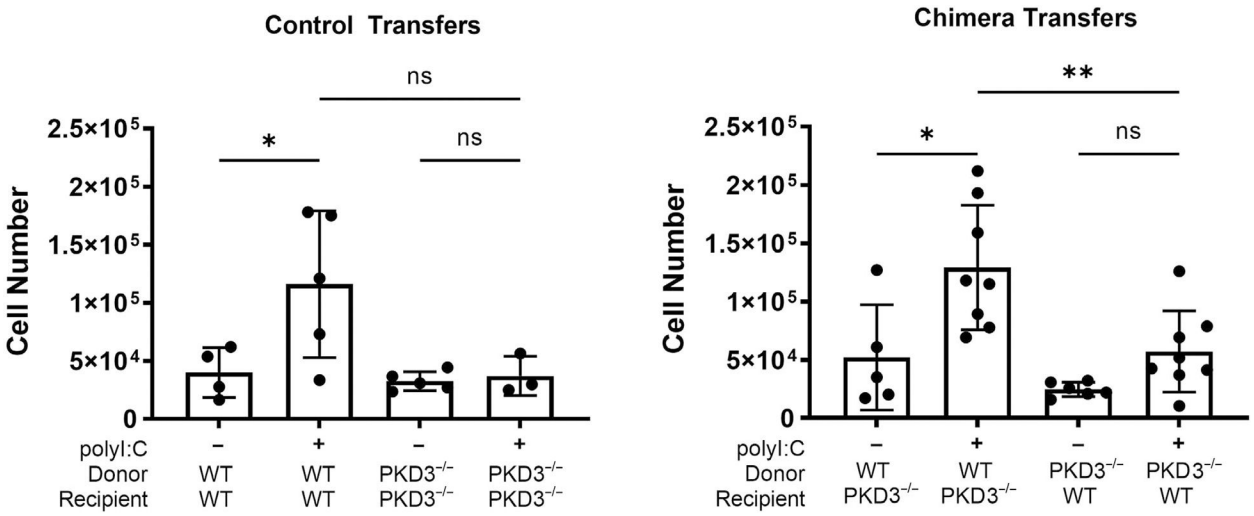

Figure 3.

PKD3$^{-/-}$ in the hematopoietic compartment is necessary for neutrophil accumulation in the lung. Bone marrow chimeras were established between PKD3$^{-/-}$ and wild-type (WT) mice prior to challenge with 10 μg polyinosinic:polycytidylic acid [poly(I:C)] on days 0–2. On day 3, bronchoalveolar lavage fluid was harvested and assayed for neutrophils *via* cytospin. Data are from one representative experiment of two independent experiments (n = 3 or 5 mice per group in each experiment) and values are reported as mean ± standard deviation. One-way ANOVA followed by the unpaired Tukey's multiple comparisons test. *P < 0.05, **P < 0.01. PKD3, protein kinase D3; ns, not significant.

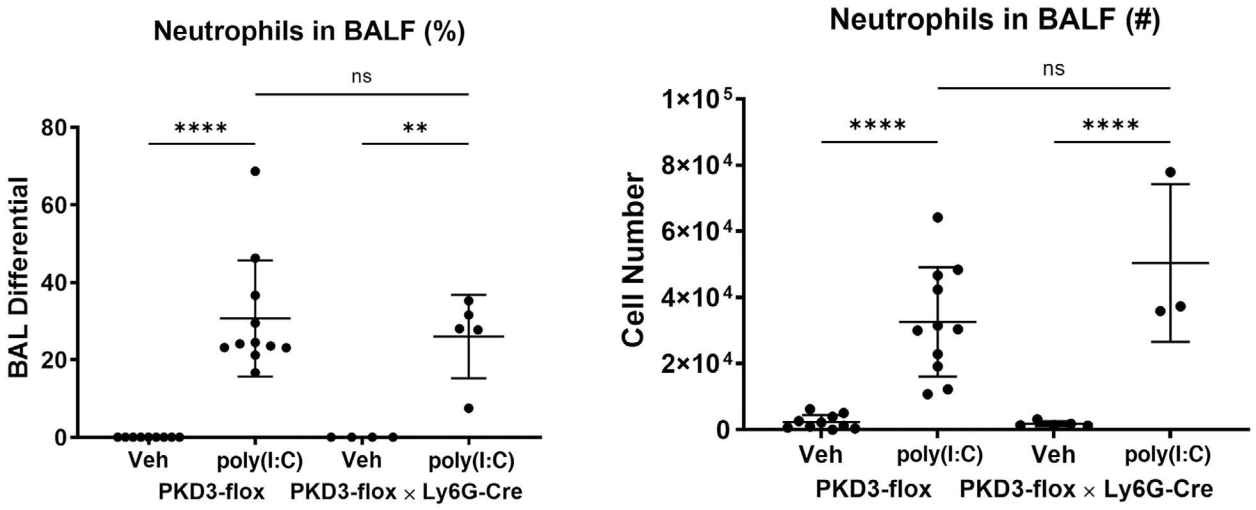

Figure 4.

Protein kinase D3 (PKD3) conditional knockout mice do not have a defect in neutrophil accumulation. PKD3-floxed mice were generated by homologous recombination using targeting vectors with lox P sites flanking exon 3 at the Mouse Genomics Core services at the University of Rochester (NY, USA). Ly6G-Cre mice 29 were a generous gift from Dr Minsoo Kim at the University of Rochester (NY, USA). All animals were treated according to the Institutional Animal Care and Use Committee at the University of Rochester. $PKD3^{fl/fl}$ or $PKD3^{+/+}$ littermate mice were administered 10 μg polyinosinic:polycytidylic acid [poly(I:C)] on days 0–2. On day 3, bronchoalveolar lavage (BAL) fluid (BALF) was harvested and neutrophils were assessed *via* cytospins. Data are pooled from three independent experiments ($n = 4$ or 6 mice per group per experiment) and results are mean ± standard deviation. One-way ANOVA followed by the unpaired Tukey's multiple comparisons test. $^{**}P < 0.001$; $^{****}P < 0.0001$. ns, not significant.

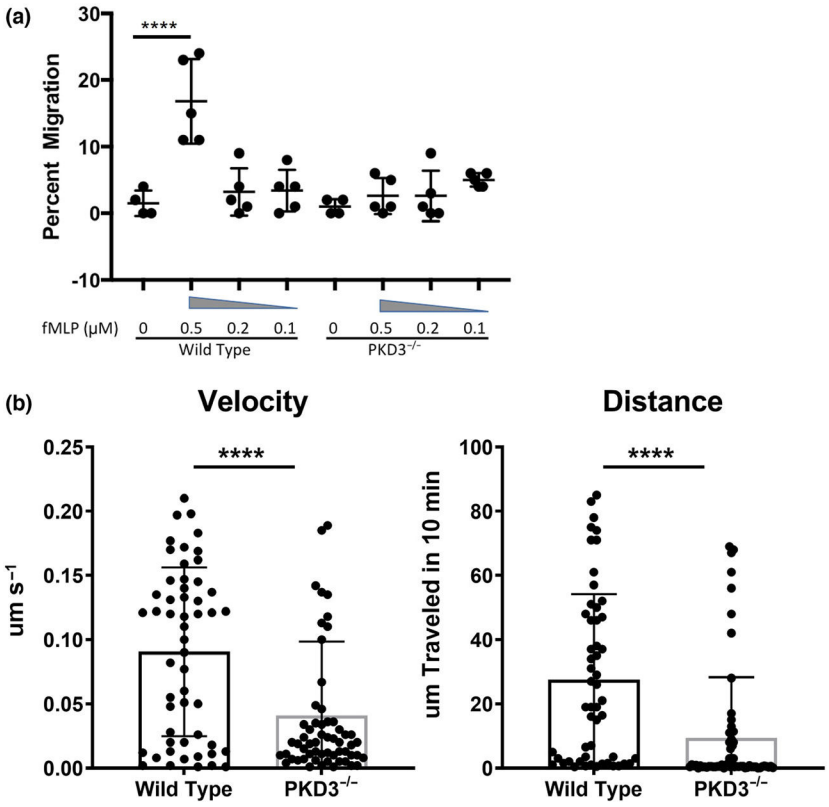

Figure 5.

PKD3$^{-/-}$ neutrophils have an intrinsic motility defect *ex vivo*. **(a)** Neutrophils were isolated from bone marrow of wild-type or PKD3$^{-/-}$ mice and plated on the apical surface of a transwell. Migration toward fMLP was assayed *via* hemacytometer counts of the basal chamber after 2 h. Data are from one trial (n = 5 replicates) and reported as mean ± standard deviation. One-way ANOVA followed by the unpaired Tukey's multiple comparisons test. ****P < 0.0001. **(b)** Neutrophils were isolated from bone marrow of wild-type or PKD3$^{-/-}$ mice (n = 3 mice per group), incubated on intercellular adhesion molecule–coated coverslips at 37°C and imaged for 10 min in the presence of 1 μM fMLP. Migratory velocity and distance (the straight distance between start and end of the neutrophil track) were analyzed using Volocity software (PerkinElmer). Frequency of migrating neutrophils (cells that moved more than one cell body distance (defined as 10 μm)) was 59% and 19.4% for wild type and PKD3$^{-/-}$, respectively. Data are pooled from three independent experiments and reported as mean ± standard deviation. Unpaired t-test. ****P < 0.0001. fMLP, *N*-formylmethionyl-leucyl-phenylalanine; PKD3, protein kinase D3.

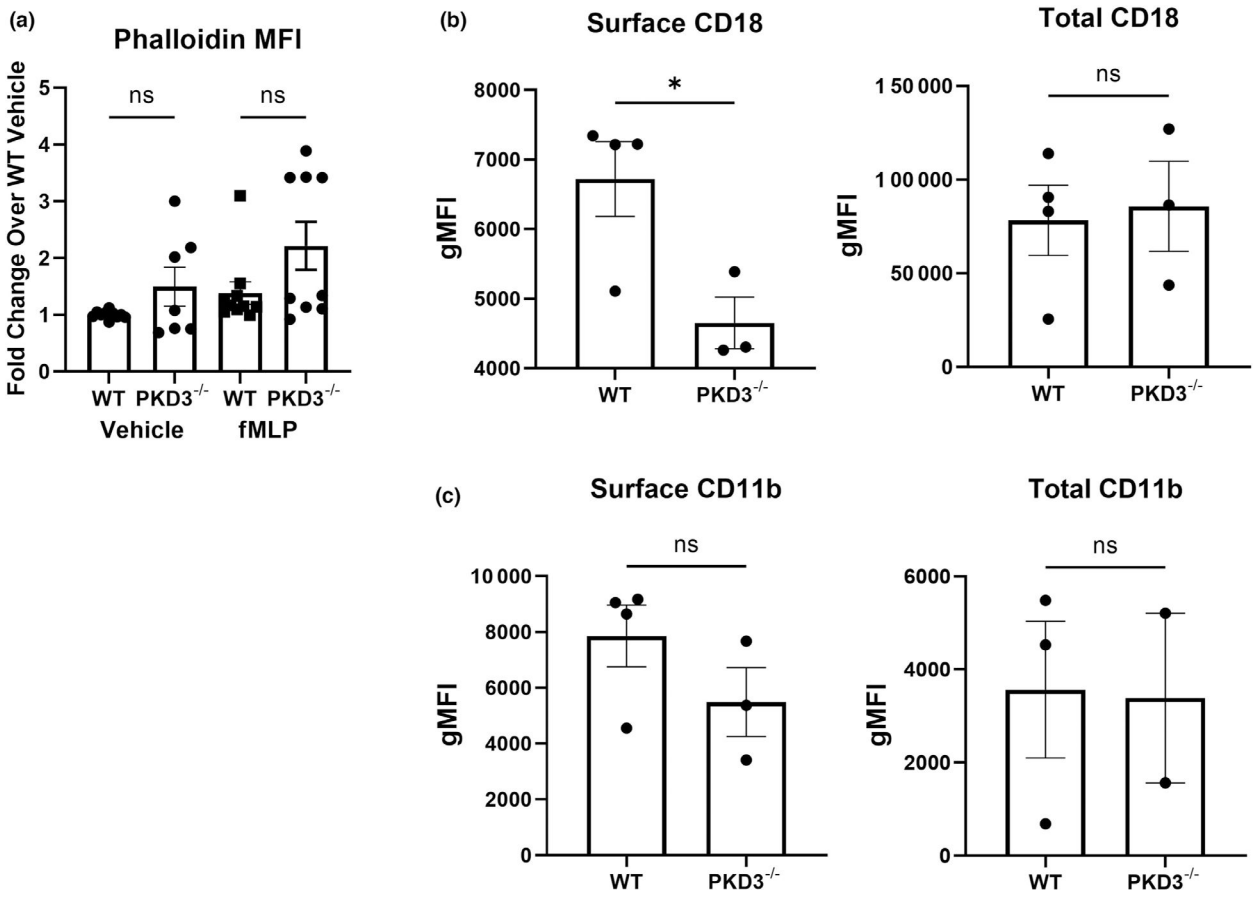

Figure 6.

PKD3$^{-/-}$ neutrophils have reduced surface CD18 levels. Splenocytes were harvested from PKD3$^{-/-}$ and wild-type mice and stimulated with fMLP for 1 min. **(a)** Reactions were stopped by fixation in 4% paraformaldehyde (PFA) followed by intracellular staining for total phalloidin. Data are pooled from four independent experiments (n = 2 or 3 mice per experiment) and reported as mean ± standard deviation. **(b, c)** Splenocytes were harvested from PKD3$^{-/-}$ and wild-type mice and levels of surface and total CD18 assessed with flow cytometry. Data are n = 3 or 4 biological replicates and values are reported as mean ± standard deviation. Unpaired t-test, $^{*}P < 0.05$. fMLP, *N*-formylmethionyl-leucyl-phenylalanine; gMFI, geometric mean fluorescence intensity; ns, not significant; PKD3, protein kinase D3; WT, wild type.

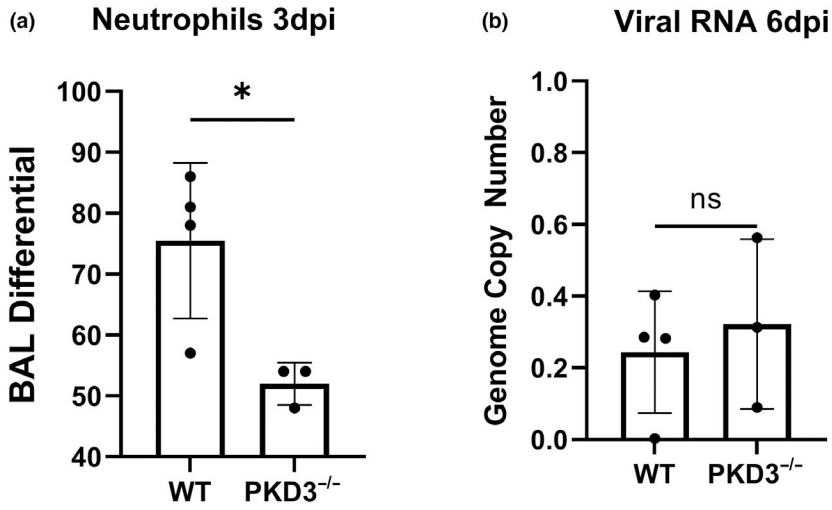

Figure 7.

PKD3$^{-/-}$ mice exhibit reduced neutrophilia following replicating influenza A virus (IAV) infection. Wild-type (WT) and PKD3$^{-/-}$ mice were infected with IAV (500 plaque-forming units; PR/8 strain). **(a)** Bronchoalveolar lavage (BAL) fluid was harvested 3 days post infection (3dpi) (peak of neutrophil accumulation) and analyzed for neutrophils *via* cytospin. **(b)** Lungs were harvested on 6 days post infection (6pdi) and homogenized in TRIzol for RNA analysis of viral genome. Data are $n = 3$ or 4 mice per group from one experiment (representative of two independent experiments of 3 or 4 mice per group per experiment) and values reported as mean ± standard deviation. Unpaired *t*-test, $*P < 0.05$. ns, not significant; PKD3, protein kinase D3.

Table 1.

Reagents and kits. All plate-based assays were used according to the manufacturer's instructions

Reagent	Company	Catalog no.
High molecular weight poly(I:C)	InvivoGen, San Diego, CA, USA	tlrl-pic; Version#11C21-MM
PKD inhibitor CRT0066101 (CRT)	Tocris, Mnneapolis, MN, USA	4975
Murine CXCL1 ELISA	R&D Systems, Minneapolis, MN, USA	DY453
Murine Albumin ELISA	Abcam, Cambridge, UK	Ab108792
Th1/Th2 cytokine 8-plex mouse ProcartaPlex 2	Invitrogen, San Diego, CA, USA	EPX080-20832-901
4-kDa FITC–dextran	Sigma, St. Louis, MO, USA	D588 4, CAS 9025-70-1
Hema3 Stain	Fisherbrand, Waltham, MA,	122–911
MojoSort neutrophil (negative) isolation kit	BioLegend, San Diego, CA, USA	480058
N-formylmethionyl-leucyl-phenylalanine	Sigma, St. Louis, MO, USA	59880–97-6
mRNeasy mini kit	Qiagen, Hilden, Germany	7
iScript cDNA synthesis kit	Bio-Rad, Hercules, CA, USA	1708891
iQ SYBR Green Supermix	Bio-Rad, Hercules, CA, USA	1708882

FITC, fluorescein isothiocyanate.

Table 2.

Antibodies used for western blot analysis

Antibody	Company	Catalog no.	Dilution
Anti-PKD3	Cell Signal Technology, Danvers, MA, USA	5655	1:1000
Anti-GAPDH	Abcam, Cambridge, UK	8245	1:50 000
Anti-Rabbit-HRP	Abcam, Cambridge, UK	ab205718	1:10 000
Anti-Mouse-HRP	Abcam, Cambridge, UK	ab205724	1:10 000

Table 3.

Antibodies used for flow cytometry analysis

Marker	Color/Format	Clone	Company	Catalog no.	Dilution
F4/80	FITC	BM8	BioLegend, San Diego, CA, USA	123108	1:400
CD45	PE-Dazzle 594	30-F11	BioLegend, San Diego, CA, USA	103146	1:400
Ly6G	PE-Cy7	1A8	BD Biosciences, Franklin Lakes, NJ, USA	560601	1:400
CD11b (surface)	APC-Fire 750	M1/70	BioLegend, San Diego, CA, USA	101262	1:400
CD11b (intracellular)	Alexa Fluor 700	M1/70	eBioscience, Waltham, MA, USA	56-0112-82	1:200
Live/Dead Fix Aqua	Live/Dead Fix Aqua	N/A	Thermo Fisher Scientific, Waltham, MA, USA	L34957	1:200
CD18 (LFA1)	PE	M18/2	Invitrogen, San Diego, CA, USA	12-0181-82	1:200
Phalloidin	Alexa Fluor 680		Invitrogen, San Diego, CA, USA	A22286	1:300

APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.