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ORIGINAL ARTICLE

Roles of Host Phospholipase D during *Aspergillus fumigatus* Infection in Mice

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Abstract

Objective: *Aspergillus fumigatus* infection in the lungs is accompanied by the recruitment of innate immune cells, phagocytosis, and the release of inflammatory factors. Phospholipase D (PLD) is a key regulator of cell migration and phagocytosis, but the effect of PLD deficiency on antifungal infection in animals is unknown. This study aims to investigate the impact of PLD on the host immune response to *A. fumigatus* infection under either immunocompetent or immunosuppressed status.

Methods: The invasive pulmonary aspergillosis mouse model was created using a modified protocol with immunosuppression by steroids. For collection of bronchoalveolar lavage fluid (BALF) from mice, the lungs were washed eight times with 0.5 ml of PBS. Total cell counts in BALF were determined using a Coulter Counter. The content of alveolar macrophages, neutrophils, and monocytes in BALF was examined by flow cytometry and analyzed by FlowJo V10 software. Multiplex immunoassays were used to determine the concentrations of inflammatory cytokines in BALF.

Results: In immunocompetent mice, alveolar macrophages were the major cell population in BALF after *A. fumigatus* infection, and a number of neutrophils and monocytes were recruited in the alveoli. Loss of both *pld1* and *pld2* genes did not affect the content of alveolar macrophages, neutrophils, or monocytes in BALF. Under immunosuppression induced by hydrocortisone acetate, $pld1^{-/-}pld2^{-/-}$ mice showed higher mortality after *A. fumigatus* infection and had a higher fungal burden and much lower number of prominent focal areas of dense inflammatory infiltrates in lung tissue than wild type mice. Moreover, interleukin (IL)-12p40 significantly decreased, and IL-10 markedly increased, in BALF from $pld1^{-/-}pld2^{-/-}$ mice after infection.

Conclusion: Our findings revealed that, during *A. fumigatus* infection, deficiency in both *pld1* and *pld2* in mice was not conducive to the infiltration of inflammatory cells into lung tissue but promoted the release of IL-10 and blocked the release of IL-12, thereby increasing fungal burden and mortality.

Key words: *Aspergillus fumigatus*, phospholipase D, macrophages, neutrophils, inflammatory cytokines

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INTRODUCTION

Aspergillus fumigatus is widely found in the environment, particularly in the air, water, plants, and soil, and is the main pathogen causing aspergillosis; *A. fumigatus* can be isolated from more than 80% of samples from patients with aspergillosis [1]. Conidia of *A. fumigatus*, owing to their small size (diameter of 2–3 μm), are

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inhaled by humans and animals and travel deep into the lower respiratory tract; these conidia may be responsible for a variety of clinical cases. However, invasive aspergillosis, which is found primarily in immunocompromised people, remains the most dangerous because of its high mortality rates, which range from 30% to 100% [2-4].

Alveoli are the principal origin of systemic infections of *Aspergillus*, although infection may also arise from other anatomical sites, such as the sinuses. Alveolar macrophages represent 90% of the resident leucocytes in the lungs, and they home to areas just beneath the alveolar surfactant film [5]. The alveolar macrophages engulf *A. fumigatus* conidia and subsequently produce cytokines and chemokines, such as TNF-α, IL-12, IFN-γ, IL-18, IL-6, IL-1β, MIP-1, MIP-1α, MIP-2, G-CSF, and GM-CSF [6]. Consequently, professional phagocytes, such as circulating macrophages, neutrophils, monocytes, and dendritic cells, are massively recruited to infection sites. These professional phagocytes play essential roles in host defense against fungal infection through various mechanisms, such as efficiently binding and ingesting *A. fumigatus* conidia, secreting proinflammatory cytokines, and producing reactive oxygen species [7,8]. Furthermore, damaged tissues release chemokines during inflammation, which attract white blood cells to injury sites [9,10].

Phospholipase D (PLD) is an important phospholipase that catalyzes the hydrolysis of phosphatidylcholine, the most abundant membrane phospholipid, thus producing phosphatidic acid and choline [11]. In mammals, two PLD isoforms, PLD1 and PLD2, are found on the cell membrane surface and are responsible for the phosphatidylcholine-hydrolyzing activity [12]. Host PLD1 and PLD2 are central signaling proteins, and their deficiency in mice leads to complicated developmental, behavioral, and immunological effects, such as defects in T cell signaling and expansion, Fc-mediated phagocytosis in macrophages, and activation of NADPH oxidase in neutrophils [13]. Previous studies have shown that PLD is activated in early stages of β2 integrin-mediated phagocytosis in neutrophils [14], and the two isoforms of PLD—PLD1 and PLD2—coordinately regulate macrophage phagocytosis [15,16] and cell migration [17].

The activity of PLD enzymes in host cells is associated with antimicrobial activities [18]. PLD enzymes in the host can be targeted to halt viral replication during infection. PLDs in macrophages participate in the phagocytosis of *Yersinia pseudo tuberculosis* and the elimination of *Salmonella typhimurium*. PLD inhibitors such as VU0285655 block the proliferation of *Plasmodium falciparum* (a pathogenic factor in malaria) and that of *Toxoplasma gondii* (the pathogenic factor in toxoplasmosis), on the basis of *in vitro* experiments. According to our previous research, PLD in lung epithelial cells is activated not only by β-1,3-glucan on the surfaces of *A. fumigatus* conidia but also by gliotoxin, the most potent toxin produced by *A. fumigatus*. The activation of both PLD1 and PLD2, two subtypes of mammalian PLD, is important for the efficient internalization of *A. fumigatus* into lung epithelial cells *in vitro* [19,20]. However, little is known regarding the consequences of PLD deficiency in the host (through deletion of both the *pld1* and *pld2* genes) on antifungal ability during *A. fumigatus* infection in animal models.

In this study, we used *pld1*$^{-/-}$*pld2*$^{-/-}$ mice to analyze the function of PLD during *A. fumigatus* infection under immunocompetent or immunosuppressed conditions. Deficiency in both *pld1* and *pld2* genes in mice had only moderate effects on the number of innate immune cells in BALF. Under immunosuppression, PLD deficiency inhibited the production of the cytokine IL-12 and increased production of the anti-inflammatory cytokine IL-10. This response was associated with an increase in *A. fumigatus* invasion and growth in lung tissue, and faster death in *pld1*$^{-/-}$*pld2*$^{-/-}$ mice than wild type mice.

MATERIALS AND METHODS

A. fumigatus strains and culture

The *A. fumigatus* wild type strain B5233 was a kind gift from Dr. K. J. Kwon-Chung (National Institutes of Health, Bethesda, Maryland). Conidia were inoculated on Sabouraud dextrose agar and propagated for 5–8 days at 37°C. Then the newly growing conidia were collected with 0.1% Tween-20 and passed through a 40 μm filter to remove hyphal fragments. Finally, the conidia were washed three times in PBS and counted with a hemocytometer before being stored at 4°C.

Mouse infections

All animal studies were approved by the Laboratory Animal Welfare and Ethics Committee (IACUC-13-2016-002) and were conducted according to relevant guidelines and regulations for laboratory animals. C57BL/6 mice were obtained from Fengtai Animal Center of Military Medical Science Academy. *pld1*$^{-/-}$*pld2*$^{-/-}$ mice were obtained from Prof. Dr. Bernhard Nieswandt (University Hospital Wurzburg, Germany). During the normal feeding process, the hair, activity, mental state, balance, and basic vitality *pld1*$^{-/-}$*pld2*$^{-/-}$ mice were comparable to those of wild type mice, and the weights of mice at 10 weeks of age did not significantly differ. With increased age, *pld1*$^{-/-}$*pld2*$^{-/-}$ mice generally became heavier than wild type mice. The invasive pulmonary aspergillosis mouse model was created through modified immunosuppression with steroids, according to a protocol described by Sugui et al. [21]. The dosage and administration of hydrocortisone acetate were as described by Zhang et al. [22]. Image-Pro Express 6.0 was used to process the images (Media Cybernetics Inc., MA, USA). The lungs were homogenized, the homogenates were cultured on Sabouraud dextrose agar medium, and the numbers of colonies were counted after 18 h of culture.

Collection of BALF and alveolar cells

BALF was collected after mice were anesthetized with 25% (v/v) ethyl carbamate intraperitoneally. The lungs were washed eight times with 0.5 ml of PBS, as previously described [6]. The supernatant was frozen at −80°C for subsequent use for the quantification of inflammatory cytokines. All BALF was collected in 50 ml centrifuge tubes and centrifuged for 8 min at 500 g, 4°C. Red blood cells in cell pellets were lysed with erythrocyte lysis buffer. For subsequent flow cytometry, total cell counts in BALF were determined with a Coulter Counter (Cellometer Auto 2000).

Flow cytometry assays

Total cells were collected from BALF in mice and incubated with Fc block anti-mouse CD16/CD32 (Cat:101319), followed by anti-mouse antibodies, including anti-Ly6G-PE (Cat:127608), anti-CD11b-FITC (Cat:101205), and anti-F4/80-PE (Cat:123110), all from BioLegend. Total cells were examined with a BD FACSCanto II flow cytometer and analyzed by FlowJo V10 software.

Multiplex immunoassay for inflammatory cytokines

As previously described, the BALF supernatant was collected from mice at the indicated time points. Multiplex immunoassays were used to determine the concentrations of inflammatory cytokines in the BALF, according to the manufacturer's instructions (eBioscience).

Statistical analysis

Data are presented as mean ± standard error of the mean for three independent replicates. The significance of differences was assessed with log-rank analysis (for comparative survival), one-way ANOVA, or unpaired Student's t-tests with a 95% confidence interval in GraphPad Prism software. *p < 0.05, **p < 0.01, and ***p < 0.001.

RESULTS

Deletion of *pld1* and *pld2* genes increases death and pulmonary fungal burden after *A. fumigatus* infection in mice with hydrocortisone acetate induced immunosuppression. *A. fumigatus* is an airborne pathogen that causes life-threatening conditions in patients receiving immunosuppressive therapy or people with congenital neutrophil dysfunction [23]. Immunocompetent mice and *pld1*$^{-/-}$*pld2*$^{-/-}$ mice have been reported not to die after *A. fumigatus* infection [24]. The immunosuppressed mouse model for the development of invasive pulmonary aspergillosis after *A. fumigatus* infection was established in the current study with hydrocortisone acetate [21]. The survival rate of *pld1*$^{-/-}$*pld2*$^{-/-}$ mice with *A. fumigatus* infection was lower than that of wild type mice after immunosuppression with hydrocortisone acetate (Fig 1A); however, loss of *pld1* and *pld2* genes did not affect the survival rate of immunocompetent mice. In terms of immunosuppression, *pld1*$^{-/-}$*pld2*$^{-/-}$ mice had significantly higher pulmonary fungal burden (Fig 1B), faster growth and invasion of *A. fumigatus* conidia in lung tissue, and more severe lung tissue damage than wild type mice. Microscopic examination of the lungs of both genotypes of mice after *A. fumigatus* infection revealed large areas of bronchopneumonia consisting of hyphae surrounded by extensive inflammatory infiltrates, and the pulmonary architecture was effaced by zones of necrosis and inflammation with hemorrhage and edema (Fig 1C). Greater numbers of prominent focal areas of dense inflammatory infiltrates were observed in the lungs of wild type mice than *pld1*$^{-/-}$*pld2*$^{-/-}$ mice; moreover, the necrosis, hemorrhage, and edema in the lung tissue was more severe in *pld1*$^{-/-}$*pld2*$^{-/-}$ mice than wild type mice, and the hyphae in the lung tissue were longer in *pld1*$^{-/-}$*pld2*$^{-/-}$ mice than wild type mice at the same time point after infection (Fig 1C). These data suggested that loss of both *pld1* and *pld2* genes made immunosuppressed mice more susceptible to *A. fumigatus* infection, but had less of an effect on the survival rate of immunocompetent mice.

Effects of deficiency in both *pld1* and *pld2* genes on innate immune cell populations in BALF from immunocompetent mice infected by *A. fumigatus*. Professional phagocytes, such as macrophages, neutrophils, and monocytes, play important roles in preventing fungal infection [5]. Phagocytosis and bactericidal function in these phagocytes are inhibited *in vitro* when PLD function is deficient [15,25,26]. However, the effect of PLD deficiency on professional phagocytes during *A. fumigatus* infection was unclear. Here, the cell populations in the BALF from mice were analyzed by flow cytometry with F4/80, CD11b, and Ly6G, which are surface markers of macrophages, monocytes, and neutrophils, respectively.

Alveolar macrophages (F4/80^{+}CD11b^{-}) were the predominant cell populations in BALF from immunocompetent mice before intranasal administration of *A. fumigatus* conidia. The proportion of alveolar macrophages in *pld1*$^{-/-}$*pld2*$^{-/-}$ mice (93.5%) was not significantly less than that (95.9%) in wild type mice (Fig 2A). At 6 h after administration of conidia, alveolar macrophages remained the major cell populations in BALF from both wild type and *pld1*$^{-/-}$*pld2*$^{-/-}$ mice (Fig 2B). From 0 h to 6 h, the proportions of monocytes (F4/80^{+}CD11b^{+}, approximately seven fold increase, from 0.28% to 1.97%) and neutrophils (Ly6G^{+}CD11b^{+}, approximately three fold increase, from 0.58% to 1.72%) increased significantly in wild type mice, whereas the proportions of neutrophils and monocytes in *pld1*$^{-/-}$*pld2*$^{-/-}$ mice decreased slightly (Fig 2A, 2B). The proportion of neutrophils and monocytes in alveolar lavage fluid from wild type mice was 2.1 times and 1.9 times higher than that from *pld1*$^{-/-}$*pld2*$^{-/-}$ mice, respectively (Fig 2B). At 24 h after conidial administration, alveolar macrophages remained the major cell population in BALF from both wild type and *pld1*$^{-/-}$*pld2*$^{-/-}$ mice (Fig 2C). From 0 h to

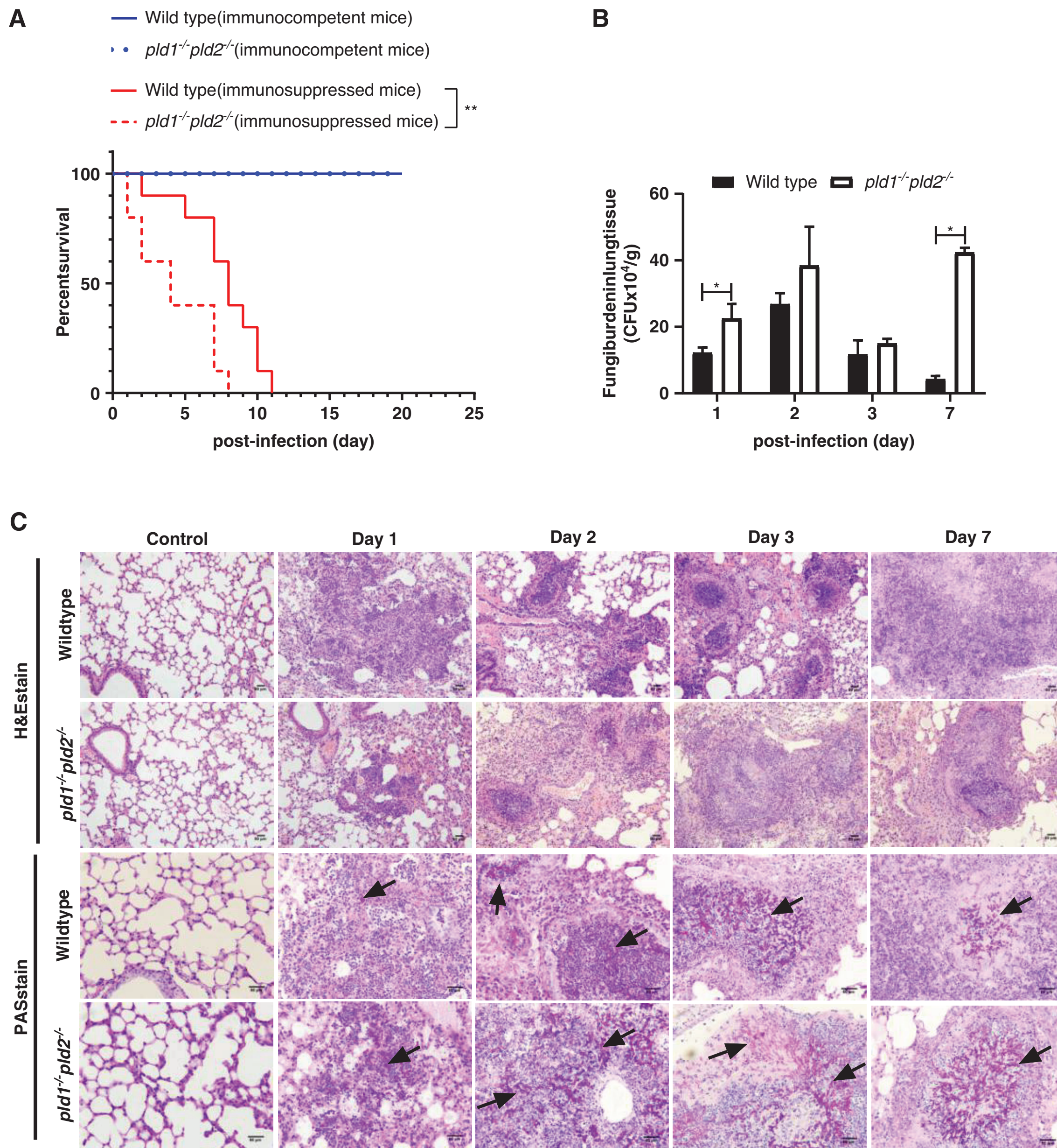

FIGURE 1 | After hydrocortisone acetate induced-immunosuppression, *pld1*$^{-/-}$*pld2*$^{-/-}$ mice die earlier and have higher pulmonary fungal burden than wild type mice after *A. fumigatus* infection.
Intranasally, the mice were inoculated with 5×10^{6} CFU conidia of *A. fumigatus* wild type B5233 after being immunosuppressed with hydrocortisone acetate. The immunocompetent mice were inoculated intranasally with 1×10^{7} CFU conidia. (A) For survival analysis, ten mice were weighed every 24 h from the day of infection and were visually inspected twice daily. (B) Mice were sacrificed at post-infection day 0 (control, three mice), day 1 (five mice), day 2 (four mice), day 3 (three mice), and day 7 (three mice). Fungal burden in the lung tissue was measured by counting CFUs. (C) Lung tissues were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS), and observed by light microscopy (Olympus BX51) at a magnification of 200× or 400×. Scale bar = 50 µm. Black arrows indicate hyphae (red). The images were later processed in Image-Pro Express 6.0 (Media Cybernetics Inc., MA, USA). Three independent experiments with three individual replicates were performed. The significance of differences was assessed with log-rank analysis (for comparative survival) or unpaired Student's t-tests with a 95% confidence interval in GraphPad Prism 8.0.2 software. $^{*}p < 0.05$, $^{**}p < 0.01$.

24 h, the proportion of alveolar macrophages in wild type mice decreased from 95.9% to 88.2%, and the proportion of alveolar macrophages in *pld1*$^{-/-}$*pld2*$^{-/-}$ mice decreased from 93.5% to 87.7%; however, the proportion of monocytes and neutrophils increased from 0.28% to 4.49% and from 0.58% to 5.18% in wild type mice and *pld1*$^{-/-}$*pld2*$^{-/-}$ mice, respectively (Fig 2A, 2C). Notably, the proportions of the three types of cells were higher in wild type mice than *pld1*$^{-/-}$*pld2*$^{-/-}$ mice (Fig 2C). Moreover, the numbers of neutrophils and monocytes in BALF gradually significantly increased after infection, whereas the number of total cells and macrophages did not change significantly in both genotypes of mice. The numbers of total cells and the three types of immune

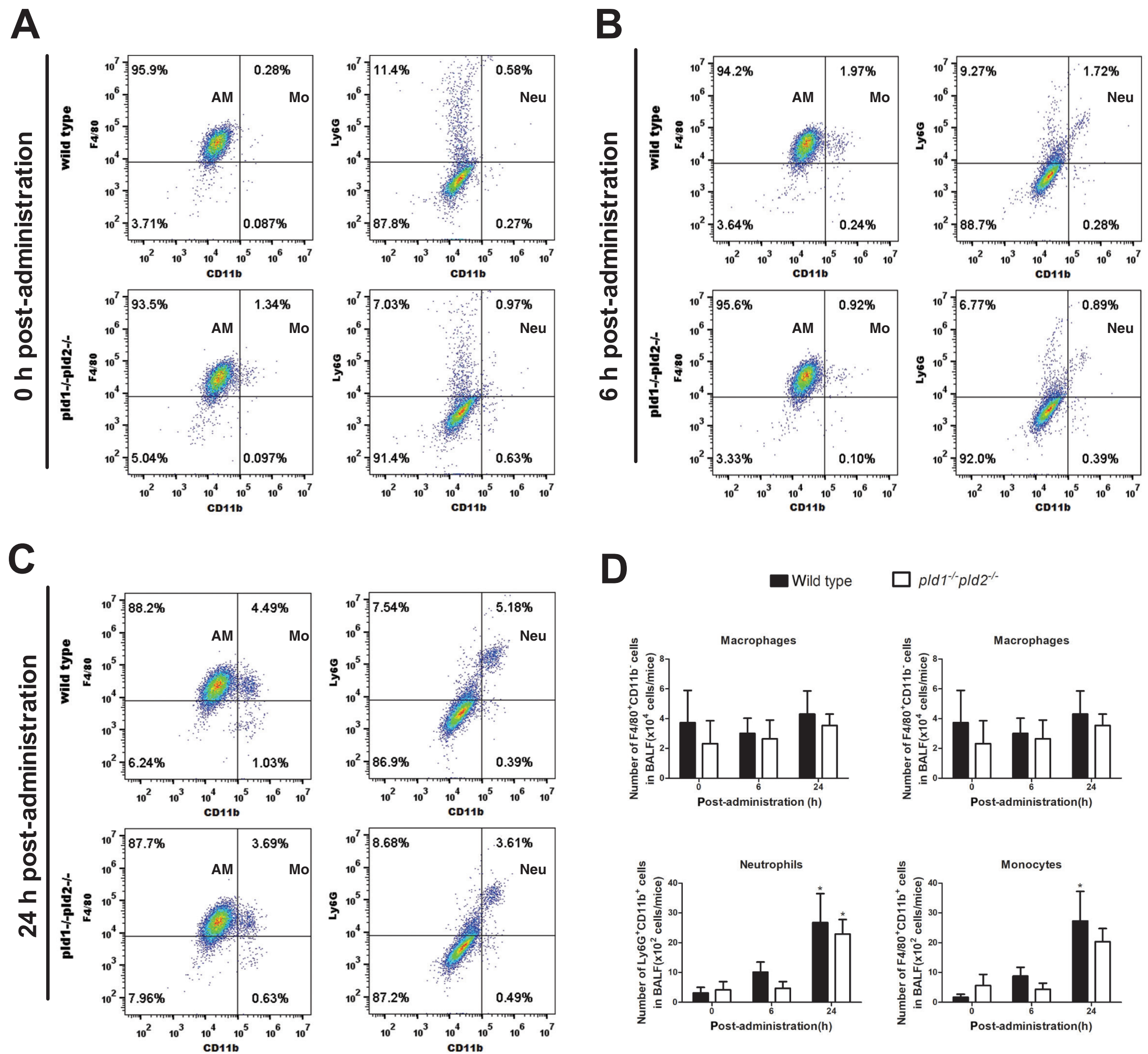

FIGURE 2 | In immunocompetent mice, alveolar macrophages are the major cell populations in BALF after intranasal administration of *A. fumigatus* conidia.

Immunocompetent mice were inoculated intranasally with 1×10^7 CFU conidia and anesthetized with 25% (v/v) ethyl carbamate intraperitoneally. BALF was collected at 0 h, 6 h, and 24 h after administration. Each group in each experiment included seven mice. The alveolar lavage fluid of the seven mice was mixed in one 50 ml sterile centrifuge tube. Total cells were collected after centrifugation and incubated with Fc block anti-mouse CD16/CD32 (Cat:101319), followed by anti-mouse antibodies, including anti-Ly6G-PE (Cat:127608), anti-CD11b-FITC (Cat:101205), and anti-F4/80-PE (Cat:123110). (A–C) The content of alveolar macrophages, neutrophils and monocytes in BALF at post-administration 0 h, 6 h and 24 h was examined by BD FACSCanto II flow cytometer and analyzed by FlowJo V10 software. (D) The number of total cells in BALF were determined using a Coulter Counter (Cellometer Auto 2000), and the number of alveolar macrophages, neutrophils and monocytes in BALF was calculated based on total cell number and their respective proportions. Note: The total cells were mixed cells from the alveolar lavage fluid of seven mice in each group. Two independent experiments were performed. $p < 0.05$, * infected vs. uninfected mice. Abbreviations: AM, alveolar macrophages; Neu, neutrophils; Mo, monocytes.

cells in the *pld1*$^{-/-}$*pld2*$^{-/-}$ mice were slightly lower than those in the wild type mice, but no significant difference was observed between groups (Fig 2D).

These findings suggested that macrophages are the major cells in the pulmonary alveoli initially after *A. fumigatus* infection, and neutrophils and monocytes were recruited into the pulmonary alveoli in response to *A. fumigatus* infection in immunocompetent mice, but knocking out both *pld* genes had little effect on the observed cellular recruitment.

Effects of deficiency in both *pld1* and *pld2* genes on innate immune cell populations in BALF from immunosuppressed mice infected by *A. fumigatus*. Next, we explored the possible effects of deletion of both *pld1* and *pld2* genes in immunosuppressed mice on the recruitment of major innate immune cells in the lungs without *A. fumigatus* infection. The mice were treated with hydrocortisone acetate to establish the immunosuppression model [21]. Alveolar macrophages remained

the major cell populations in BALF from both wild type and *pld1*$^{-/-}$*pld2*$^{-/-}$ mice under immunosuppression, but their proportion was lower than that in immunocompetent mice (Fig 3A, compared with Fig 2A). At day 1 post-infection, in BALF from both wild type and *pld1*$^{-/-}$*pld2*$^{-/-}$ mice, the proportion of alveolar macrophages descended sharply, from 76.2% to 0.26% and from 77.9% to 0.58%, respectively; however, neutrophils increased markedly, from 0.29% to 87.0% and from 0.77% to 81.4%, respectively, and became the major cell populations in BALF from both wild type and *pld1*$^{-/-}$*pld2*$^{-/-}$ mice (Fig 3A, 3B and 3D). A consistent decrease in both the proportion (days 1 and 3) and number of neutrophils (day 1) was observed in the *pld1*$^{-/-}$*pld2*$^{-/-}$ mice, as compared with wild type mice (Fig 3B–3D); however, these differences were not significant. In addition, no statistical difference in the number of total cells and the three types of immune cells in BALF was found between groups (Fig 3D).

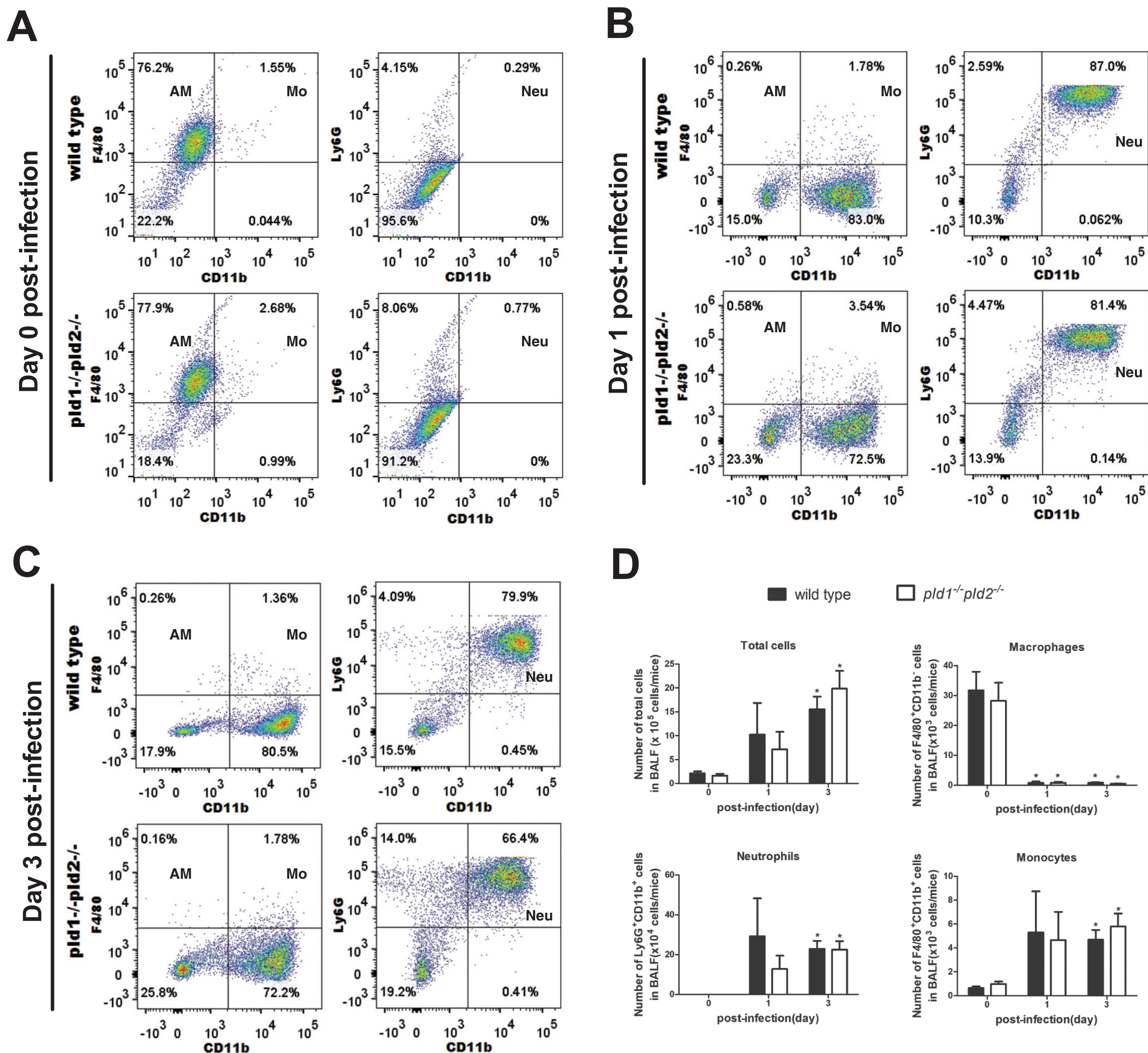

FIGURE 3 | In mice with hydrocortisone acetate-induced immunosuppression, many neutrophils are recruited into BALF and become the major cell populations during *A. fumigatus* infection.

Mice with immunosuppression were inoculated intranasally with 5×10^{6} CFU conidia and anesthetized by intraperitoneal administration of 25% (v/v) ethyl carbamate. BALF was collected at post-infection day 0, day 1, and day 3. The number of mice in each group ranged from five to seven in each experiment. The alveolar lavage fluid of each group was mixed in one 50 ml sterile centrifuge tube. Total cells were collected after centrifugation and incubated with Fc block anti-mouse CD16/CD32 (Cat:101319), followed by anti-mouse antibodies, including anti-Ly6G-PE (Cat:127608), anti-CD11b-FITC (Cat:101205), and anti-F4/80-PE (Cat:123110). (A–C) The content of alveolar macrophages, neutrophils and monocytes in BALF at post-infection Day 0, Day 1, and Day 3 was examined by BD FACSCanto II flow cytometer and analyzed by FlowJo V10 software. (D) The number of total cells in BALF were determined using a Coulter Counter (Cellometer Auto 2000), and the number of alveolar macrophages, neutrophils and monocytes in BALF was calculated based on total cell number and their respective proportions. Note: The total cells were mixed cells from alveolar lavage fluid of seven, six and five mice in the day 0, day 1, and day 3 post-infection groups, respectively. Three independent experiments were performed. $p < 0.05$, * infected vs. uninfected mice. Abbreviations: AM, alveolar macrophages; Neu, neutrophils; Mo, monocytes.

These findings indicated that in both genotypes of mice treated with hydrocortisone acetate and infected with *A. fumigatus*, many neutrophils and some monocytes were recruited into the lungs. However, deficiency in both *pld1* and *pld2* genes had little effect on the recruitment of neutrophils and monocytes into pulmonary alveoli.

Effects of deficiency in both *pld1* and *pld2* genes on the release of inflammatory factors in BALF from immunosuppressed mice infected by *A. fumigatus*. Next, we examined the effects of deficiency in PLD on the release of inflammatory factors in BALF from immunosuppressed mice with *A. fumigatus* infection. During *A. fumigatus* infection, the concentrations of the chemokine MCP-1, and the pro-inflammatory cytokines TNF-α, IL-6, and Gro-αKC (IL-8) in BALF increased significantly in wild type and $pld1^{-/-}pld2^{-/-}$ mice; the increase was clearer in $pld1^{-/-}pld2^{-/-}$ mice at day 1 and day 3 after *A. fumigatus* infection (Fig 4A-D). The concentration of IL-10, an anti-inflammatory cytokine, was higher in $pld1^{-/-}pld2^{-/-}$ mice than wild type mice at day 1 and remained high through day 5 (Fig 4E). Unexpectedly, the concentration of IL-12p40 increased dramatically on day

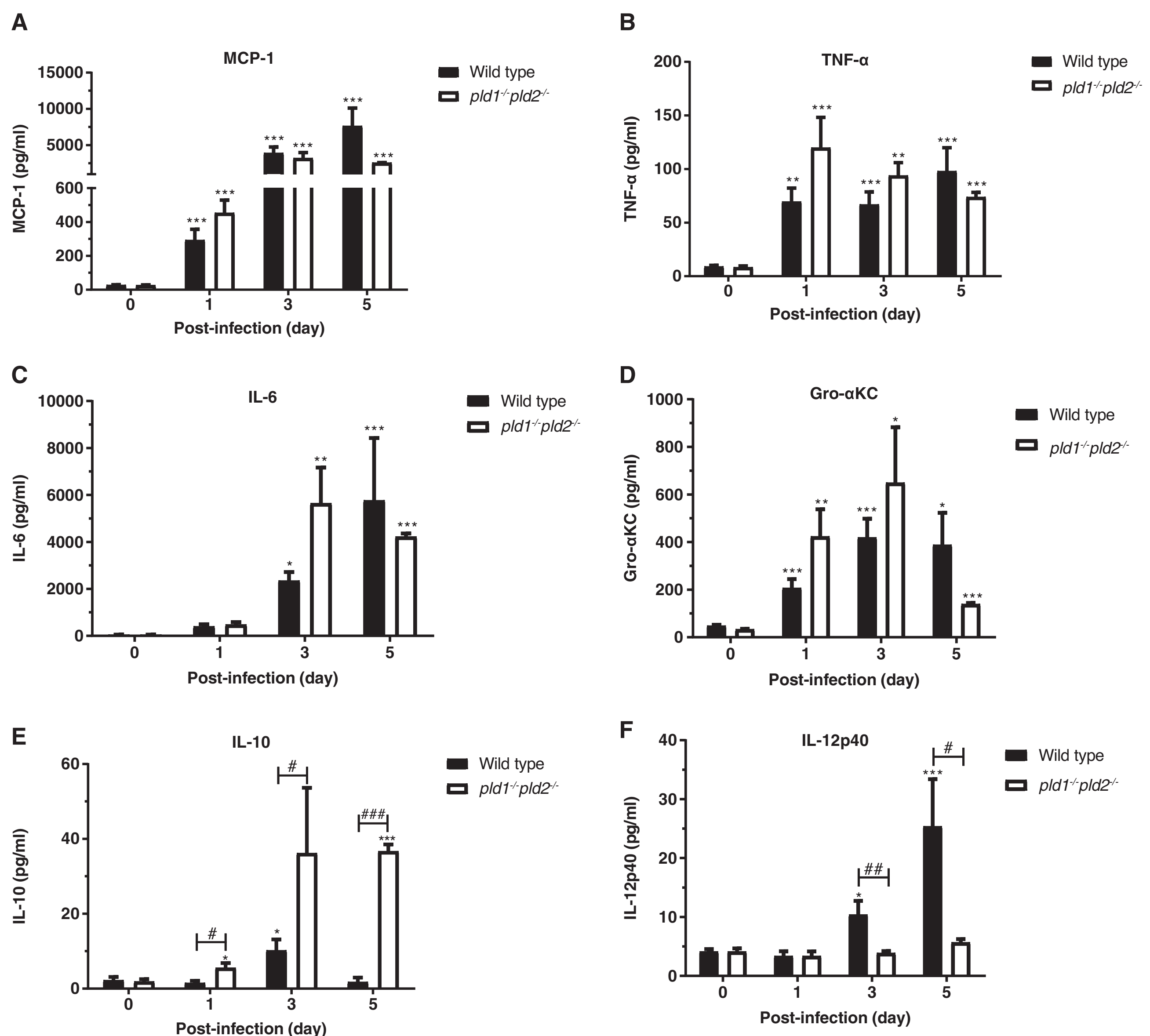

FIGURE 4 | In immunosuppressed mice, deficiency in both *pld1* and *pld2* genes significantly suppresses the production of IL-12p40 and promotes the production of IL-10 in BALF during *A. fumigatus* infection.
Mice with immunosuppression were inoculated intranasally with 5×10^6 CFU conidia and anesthetized by intraperitoneal administration of 25% (v/v) ethyl carbamate. BALF was collected at post-infection day 0, day 1, day 3, and day 5. Each group included six or seven mice. The concentrations of inflammatory cytokines MCP-1 (A), TNF-α (B), IL-6 (C), Gro-αKC (D), IL-10 (E) and IL-12p40 (F) in BALF were detected with multiplex immunoassays according to the manufacturer's instructions (eBioscience). Statistically significant differences were determined with one-way ANOVA followed by Tukey post-hoc test (*vs* day 0, $^{***}p < 0.01$, $^{**}p < 0.01$ and $^{*}p < 0.05$). The significance of differences was determined with unpaired Student's t-tests with a 95% confidence interval (*vs* wild type, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$) in GraphPad Prism 8.0.2 software.

3 and day 5 in wild type mice but not *pld1*$^{-/-}$*pld2*$^{-/-}$ mice (Fig 4F).

These findings suggested that *A. fumigatus* infection significantly increased the content of the inflammatory factors MCP-1, TNF-α, IL-6, Gro-αKC, and IL-10 in the alveolar lavage fluid of mice under hydrocortisone acetate induced immunosuppression. Deficiency in both *pld1* and *pld2* genes in mice promoted the release of IL-10 and suppressed the release of IL-12p40 during *A. fumigatus* infection.

DISCUSSION

Despite being discovered several decades ago, the role of PLD continues to be more precisely elucidated. Most studies have investigated the functions of PLD in cancer, aging-associated diseases, thrombosis, and multiple sclerosis, thus indicating its potential as a target for therapeutic intervention [13]. Most mammalian PLD research has focused on PLD1 and PLD2. Other potential mammalian isoforms have been reported, including PLD3 (also called Sam-9 or HUK4), PLD4, PLD5, and PLD6 (also called mitochondrial PLD, MitoPLD, Zucchini, or Zuc). To date, no studies have been reported whether PLDs in mammalian cells are involved in host antifungal infections. This study presents the first *in vivo* data on PLD function against *Aspergillus* infection in mice.

In immunocompetent and immunosuppressive mice, no significant difference was observed in the number of alveolar macrophages in BALF between wild type and *pld1*$^{-/-}$*pld2*$^{-/-}$ mice before and after *A. fumigatus* infection, thus indicating that the absence of PLD did not alter the number of macrophages in the alveoli. However, in our previous study, the fungal burden in lung tissue and BALF was significantly higher in *pld1*$^{-/-}$*pld2*$^{-/-}$ mice than wild type mice at 6 h post-administration in immunocompetent mice, and the phagocytosis and the fungicidal ability of isolated bone marrow-derived macrophages were significantly lower in *pld1*$^{-/-}$*pld2*$^{-/-}$ mice than wild type mice [24]. In addition, before *A. fumigatus* infection, the content and number of alveolar macrophages in BALF from immunosuppressed mice were significantly lower than those in mice with normal immune function, but no such changes were observed in neutrophils and monocytes. After *A. fumigatus* infection, the content and number of alveolar macrophages in BALF sharply decreased, whereas the content and number of neutrophils and monocytes sharply increased in immunosuppressed mice. Moreover, other studies have demonstrated that corticosteroids impair the killing of *A. fumigatus* conidia by macrophages [18,27,28]. After phagocytosis by macrophages, another important fungal pathogen, *Candida albicans*, transitions from the yeast to the hyphal stage, and causes macrophage death and fungal escape [29]. Hence, macrophage damage by hydrocortisone acetate and phagocytosis-associated death might possibly explain the rapid and irreversible decrease in alveolar macrophages in BALF in hydrocortisone acetate-immunosuppressed mice during *A. fumigatus* infection. Moreover, the damage might have contributed to the increased susceptibility observed in the *pld1*$^{-/-}$*pld2*$^{-/-}$ mice in response to *A. fumigatus* infection.

Neutrophils play critical roles in host defense against invading microorganisms. Previous studies have shown that PLD activation is an early event in β2 integrin-mediated phagocytosis in neutrophils [14], and both PLD1 and PLD2 coordinately regulate cell migration [17]. PLD enzyme activity in neutrophils and leukocytes is associated with antimicrobial functions, such as phagocytosis, chemotaxis, and membrane ruffling [18]. *pld1*$^{-/-}$ and *pld2*$^{-/-}$ neutrophils exhibit impaired migration *in vitro*, but only *pld1*$^{-/-}$ neutrophils have impaired tissue extravasation [15]. In this study, in both immunocompetent and immunosuppressive states, the number of neutrophils in BALF increased significantly in response to *A. fumigatus* infection, particularly in mice with immunosuppression induced by hydrocortisone acetate, and the content and number of neutrophils in BALF sharply increased after *A. fumigatus* infection. However, no significant difference was observed in the number of neutrophils of BALF between wild type mice and *pld1*$^{-/-}$*pld2*$^{-/-}$ mice. Loss of PLD probably did not affect the recruitment of neutrophils to the alveoli in mice during *A. fumigatus* infection, but instead directly modulated the phagocytosis and fungicidal activity of neutrophils toward *A. fumigatus*. In mouse models of sepsis, PLD2 in neutrophils is essential for the pathogenesis of experimental sepsis. Bactericidal activity is significantly elevated in *pld2*$^{-/-}$ mice, and is mediated by increased neutrophil extracellular trap formation and citrullination of histone 3 through peptidylarginine deiminase activation [25]. The exact mechanism of regulation of PLD on the function of neutrophils against *A. fumigatus in vivo* requires further exploration. Isolating primary neutrophils from *pld1*$^{-/-}$*pld2*$^{-/-}$ mice for further investigation of aspects such as phagocytosis, chemotaxis, and extracellular traps should prove interesting.

In immunocompetent states, after inhalation of *A. fumigatus* conidia, the content of inflammatory cytokines IL-6, Gro-αKC, and TNF-α in BALF from *pld1*$^{-/-}$*pld2*$^{-/-}$ mice is significantly higher than that in wild type mice, particularly at 6 h after administration [24]. Similarly, we observed that, in immunosuppressive states, the content of these inflammatory cytokines in BALF from *pld1*$^{-/-}$*pld2*$^{-/-}$ mice was also slightly higher than that in wild type mice at day 1 and/or day 3 post-infection, although no statistical difference was observed. This phenomenon might be explained by deficiency in PLD inducing greater secretion of inflammatory cytokines, which in turn recruit immune cells to combat *A. fumigatus* infection. In contrast, an excessive inflammatory response is also unfavorable to the host and may cause acute inflammatory injury [30,31]. In addition, the cytokine response was likely to be proportional to the higher fungal burden observed in the *pld1*$^{-/-}$*pld2*$^{-/-}$ mice than wild type mice.

IL-12—consisting of two subunits, IL-12p40 and IL-12p35—is an important immunoregulatory cytokine with multiple biological functions. It is produced mainly by phagocytes (monocytes/macrophages and neutrophils) and dendritic cells, in response to pathogens (bacteria, viruses, intracellular parasites, and fungi) [32,33]. IL-12 protects mice against *A. fumigatus* and *C. albicans* infection. Moreover, recombinant human interleukin-12 (IL-12) enhances the ability of human monocytes to elicit an oxidative burst and damage the hyphae of *A. fumigatus* [34]. Another two members of IL-12 family, IL-23 and IL-27, are involved in the response to *A. fumigatus* infection [32,35]. However, whether loss or abnormal function of host PLD affects the production of IL-12 during *A. fumigatus* infection is unknown. In this study, the deletion of two major PLDs, PLD1 and PLD2, significantly inhibited the production of IL-12p40 in mice in response to *A. fumigatus* infection. However, the exact mechanism of this inhibition by PLD on IL-12p40 release during *A. fumigatus* infection must be further studied.

IL-10, an immunosuppressive and anti-inflammatory cytokine, is essential in maintaining peripheral immunological tolerance and limiting inflammation-associated tissue damage. Blocking IL-10 signaling enhances fungal clearance during cryptococcal infection in the lungs, and IL-10 blockade might be a potential therapy for the treatment of fungal lung infections [36,37]. IL-10 is deleterious during systemic aspergillosis infection, thereby increasing host susceptibility to lethal infection [38]. During *A. fumigatus* infection, the concentration of IL-10 in the BALF from $pld1^{-/-}pld2^{-/-}$ mice was significantly higher than that from wild type mice; this aspect was likely to be another essential factor in the higher fungal burden and earlier death of $pld1^{-/-}pld2^{-/-}$ mice than wild type mice.

In summary, this was the first study using $pld1^{-/-}pld2^{-/-}$ mice to investigate the function of PLD against fungal infection in either immunocompetent or immunosuppressed mice. The findings indicated that deficiency in both the *pld1* and *pld2* genes in mice *in vivo* makes immunosuppressed mice more susceptible to *A. fumigatus* infection and significantly affects the release of inflammatory factors during *A. fumigatus* infection.

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CONFLICTS OF INTEREST

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

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