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The Alveolar Epithelial Cell Chemokine Response to *Pneumocystis* Requires Adaptor Molecule MyD88 and Interleukin-1 Receptor but Not Toll-Like Receptor 2 or 4

Sheila N. Bello-Irizarry,[a] Jing Wang,[b] Keith Olsen,[a] Francis Gigliotti,[a,b] and Terry W. Wright[a,b]

Department of Microbiology and Immunology, School of Medicine and Dentistry, University of Rochester,[a] and Department of Pediatrics, Infectious Diseases, University of Rochester Medical Center,[b] Rochester, New York, USA

***Pneumocystis* is an opportunistic fungal pathogen that causes pneumonia in a variety of clinical settings. An early step in *Pneumocystis* infection involves the attachment of organisms to alveolar epithelial cells (AECs). AECs produce chemokines in response to *Pneumocystis* stimulation, but the upstream host-pathogen interactions that activate AEC signaling cascades are not well-defined. MyD88 is an adaptor molecule required for activation of proinflammatory signaling cascades following Toll-like receptor (TLR)-dependent recognition of conserved molecular patterns on pathogens. To determine whether the TLR/MyD88 pathway is required for the AEC chemokine response to *Pneumocystis*, wild-type (WT) and MyD88-deficient AECs were incubated with *Pneumocystis*. As expected, WT AECs produced CCL2 and CXCL2 following *Pneumocystis* stimulation. In contrast, MyD88-deficient AECs were severely impaired in their ability to respond to *Pneumocystis*. MyD88-deficient AECs did not display *Pneumocystis*-induced Jun N-terminal protein kinase activation and produced much less chemokine than *Pneumocystis*-stimulated WT AECs. Using a panel of TLR agonists, primary murine AECs were found to respond vigorously to TLR2 and TLR4 agonists. However, the AEC chemokine response to *Pneumocystis* did not require TLR2 or TLR4. Surprisingly, the interleukin-1 receptor (IL-1R) was required for an AEC chemokine response to *Pneumocystis*. The role of MyD88 in early responses during *Pneumocystis* infection was supported by *in vivo* studies demonstrating that MyD88-deficient mice showed impaired *Pneumocystis*-stimulated chemokine production and impaired inflammatory cell recruitment. These data indicate an important role for MyD88 in the AEC inflammatory response to *Pneumocystis*.**

Pneumocystis is an opportunistic pathogen that takes advantage of immunocompromised hosts, causing severe, frequently fatal pneumonia. *Pneumocystis* pneumonia (PCP) remains the most common AIDS-defining illness and remains a common opportunistic infection and a leading cause of death in HIV-infected patients (13, 21). PCP is also becoming more prevalent in a growing population of non-HIV-infected patients, such as patients undergoing long-term anti-tumor necrosis factor (anti-TNF) therapy for rheumatoid arthritis and Crohn's disease (14). Thus, determining the mechanisms by which *Pneumocystis* causes disease will provide opportunities to improve current treatment.

Although antibiotics effective against *Pneumocystis* are available, they do not always produce rapid clinical improvement, probably because host inflammation is a key contributor to PCP-related lung injury (19, 33). Early clinical studies of patients with PCP found that the degree of pulmonary inflammation rather than organism burden correlated with the severity of disease (19). In humans and animal models, PCP is associated with elevated numbers of T cells, alveolar macrophages (AMs), and neutrophils in the alveoli (16, 25, 33, 36). Cytokines and chemokines are also produced in the lungs in response to *Pneumocystis* infection. Lung levels of tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), CCL2, CXCL2, and CCL5 (RANTES) are all elevated during *Pneumocystis* infection (34, 35). CCL2 or monocyte chemoattractant protein-1 (MCP-1) is a member of the CC chemokine family that mainly recruits AMs and T cells to sites of infection and inflammation. CCL2 contributes to the pathophysiology of chronic lung disease and also modulates the pulmonary immune response to infectious agents (2, 4, 22). Our laboratory has found that CCL2 levels increase in bronchoalveolar lavage (BAL) fluid of mice after *Pneumocystis* infection and that alveolar epithelial cells (AECs) express CCL2 *in vivo* during *Pneumocystis* infection (30). These findings suggest that production of chemokines by AECs initiates and/or sustains inflammatory injury during *Pneumocystis* infection by recruiting immune cells to the alveoli.

In response to infectious agents and other stimulants, AECs can modulate the pulmonary immune/inflammatory response by secreting cytokines and chemokines. One of the earliest events following entry of *Pneumocystis* into the lung is the attachment to the alveolar epithelium (18, 26, 38, 39). Importantly, our laboratory has found that *Pneumocystis* stimulates a proinflammatory AEC response that includes secretion of the chemokines CCL2 and CXCL2 (10, 30, 31, 36). Other studies have reported that purified *Pneumocystis* β-glucan also induces AEC chemokine production (11). Thus, the interaction of *Pneumocystis* and AECs is an important early event which likely leads to subsequent immune-mediated inflammatory lung injury. CCL2 release by *Pneumocystis*-stimulated AECs is mediated through an NF-κB- and mitogen-activated protein kinase (MAPK)-dependent pathway (30, 31). However, the upstream receptors or adaptor proteins involved in

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Editor: G. S. Deepe, Jr.
Address correspondence to Terry W. Wright, terry_wright@urmc.rochester.edu.

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this response have not been identified (31). Recent studies have shown that human AECs express certain pathogen recognition receptors (PRRs), including Toll-like receptors (TLRs) (3, 9). Most TLR signaling requires the adaptor molecule myeloid differentiation factor 88 (MyD88), which signals downstream molecules belonging to the NF-κB and MAPK pathways that ultimately control expression of TLR- and MyD88-dependent proinflammatory genes (15).

The current study utilizes *in vitro* and *in vivo* approaches to demonstrate that the AEC chemokine response to *Pneumocystis* is mediated through a MyD88-dependent pathway. These results enhance our understanding of the innate immune pathway utilized by AECs in the early inflammatory responses to *Pneumocystis*. Understanding the mechanisms by which early events occur during *Pneumocystis* infection in the lung will identify more specific therapeutic targets that might be used to improve current therapy and modulate inflammatory responses in the lung.

MATERIALS AND METHODS

Mice. CB.17 severe combined immunodeficient (SCID) and C57BL/6 wild-type mice were bred at the University of Rochester. C57BL6 MyD88$^{-/-}$, C57BL/6 TLR2$^{-/-}$, C57BL/6 TLR4$^{-/-}$, and C57BL/6 mice with a TIR-domain-containing adaptor protein inducing IFN-β (TRIF) knockout (TRIF$^{-/-}$) were previously generated and generously provided by S. Akira (Osaka University, Japan) (1, 32). TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were crossed to generate TLR2/4$^{-/-}$ double-knockout mice on the C57BL/6 background. All animal protocols were approved by the University Committee for Animal Research (UCAR) at the University of Rochester Medical Center.

Isolation and culture of primary murine AECs. Primary type II AECs were isolated from mouse lungs as previously described with few modifications (30). Briefly, the lungs were perfused with 1× Hank's balanced salt solution (HBSS). A catheter was inserted into the trachea and 2 ml of dispase solution (BD Biosciences) was instilled, followed by slow instillation of 0.45 ml of low-melting-point agarose (Gibco-BRL) at 45°C. The lungs were cooled on ice for 2 min, removed, and incubated in dispase for 45 min at room temperature. The lung tissue was microdissected and incubated for 10 min in Dulbecco's modified Eagle medium (DMEM) with 0.01% DNase at room temperature. The cell suspensions were filtered through nylon monofilament screens (pore sizes, 100 and 40 μm [BD Falcon] and 25 μm [Spectrum Labs]) and centrifuged at 150 × *g* for 8 min at 4°C. Immune and inflammatory cells were removed from the AEC preparation by magnetic selection. Cell suspensions were incubated with biotin-conjugated antibodies against CD16/32 and CD45 to remove hematopoietic cell-derived cells and leukocytes, followed by removal of positive cells with streptavidin-conjugated magnetic beads in a magnetic separator. AECs were centrifuged at 150 × *g* for 8 min at 4°C and incubated in tissue culture treated plates (Corning) at 37°C for 4 to 12 h, after which nonattached AECs were centrifuged again and enumerated. With this procedure, the AEC yield was approximately 1 × 10^6 cells/mouse. Typically, the AECs were >85% viable and >90% pure, as assessed by Papanicolaou staining. In addition, approximately 95% of the isolated cells were typically positive for surfactant protein C (SP-C) expression. AECs were cultured on Matrigel/rat tail collagen-coated plates (ratio, 70/30, vol/vol; BD Biosciences) under conditions previously described by Rice et al. (27) or on precoated mouse alveolar primary cell culture extracellular matrix plates (Celprogen). Cells were maintained at 37°C with 5% CO_2 in bronchial epithelial cell growth medium (BEGM) without hydrocortisone (Cambrex) supplemented with 5% charcoal-stripped fetal bovine serum (HyClone, Logan, UT) and 10 ng/ml keratinocyte growth factor (KGF; Calbiochem).

***In vitro* AEC treatments.** Primary AECs were cultured to approximately 90% confluence. Culture medium was replaced with BEGM without KGF 6 h before treatment. Cells were treated with freshly isolated *Pneumocystis* diluted in serum-free DMEM at the indicated *Pneumocystis* cyst-to-AEC ratios. Other controls used were TNF-α (10 ng/ml), lipopolysaccharide (LPS; 10 ng/ml), and Pam3CSK4 (0.5 μg/ml). In some experiments, the following Toll-like receptor agonists were used to treat the cells: TLR1/2 agonist (Pam3CSK4), TLR2 agonist (heat-killed *Listeria monocytogenes*), TLR2/6 agonist (FSL1), TLR3 agonist [poly(I·C)], TLR4 agonist (*Escherichia coli* K-12 LPS), TLR5 agonist (*Salmonella enterica* serovar Typhimurium flagellin), TLR7 agonist (single-stranded RNA 40 [ssRNA40]), and TLR9 agonist (ODN1826) at the concentrations shown in Table 1. After treatment, supernatants were collected and stored at −80°C until used.

TABLE 1 TLR agonists used to stimulate primary murine AECs[a]

Control treatment[b]	Treatment designation	Value
TLR1/2 agonist Pam3CSK4	A	0.1 μg/ml
	B	0.5 μg/ml
	C	1 μg/ml
TLR2 agonist HKLM	A	10^6 cells/ml
	B	10^7 cells/ml
	C	10^8 cells/ml
TLR2/6 agonist FSL1	A	10 ng/ml
	B	100 ng/ml
	C	1 μg/ml
TLR3 agonist poly(I·C)	A	1 μg/ml
	B	10 μg/ml
	C	100 μg/ml
TLR4 agonist LPS	A	1 ng/ml
	B	10 ng/ml
	C	100 ng/ml
TLR5 agonist ST-FLA	A	10 ng/ml
	B	100 ng/ml
	C	1 μg/ml
TLR7 agonist ssRNA40	A	0.25 μg/ml
	B	2.5 μg/ml
	C	10 μg/ml
TLR9 agonist ODN1826	A	0.5 μM
	B	1 μM
	C	5 μM
TNF-α		10 ng/ml
Pneumocystis-to-AEC ratios of:		
0.5:1		0.75 × 5 *Pneumocystis* cysts/well
Pc 1:1		1.5× 10^5 *Pneumocystis* cysts/well
Pc 3:1		4.5 × 10^5 *Pneumocystis* cysts/well
Pc depleted		

[a] Primary murine AECs were isolated from C57BL/6 (WT) mice. Confluent monolayers were stimulated for 6 h with a panel of TLR ligands used at the indicated concentrations. CCL2 and CXCL2 levels were measured in supernatants (Fig. 5).
[b] Pam3CSK4, Pam3CysSerLys4; HKLM, heat-killed preparation of *L. monocytogenes*; FSL1, Pam2CGDPKHPKSF; ST-FLA, flagellin from *S.* Typhimurium; ODN1826, CpG synthetic oligonucleotide; Pc depleted, supernatant of *Pneumocystis*-depleted preparation.

Isolation and enumeration of mouse *Pneumocystis*. *Pneumocystis* was isolated from the lungs of heavily infected SCID mice and enumerated by Gomori's methenamine silver (GMS) staining as previously described (31). In order to ensure that the AEC response was specific to *Pneumocys-*

tis and not to any non-*Pneumocystis* contaminant that might have been coisolated from the lung tissue, a cocktail of *Pneumocystis*-specific antibodies linked to magnetic beads was used to remove *Pneumocystis* organisms from the purified *Pneumocystis* preparation as previously described (31). The remaining preparation with any non-*Pneumocystis* contaminants that may have been present, but depleted of *Pneumocystis*, was then used to treat AECs as controls. This is referred to as "*Pneumocystis* depleted."

Phospho-JNK ELISA. Primary AECs from WT and MyD88$^{-/-}$ mice were grown to confluence and were exposed to medium only or 2×10^5 *Pneumocystis* cysts for 0.5, 1, and 2 h. As a control, AECs were treated with TNF-α (10 ng/ml) for 10 min. After treatment, cells were fixed with 4% formaldehyde and phosphorylation of Jun N-terminal protein kinase (JNK) was measured by a cellular activation of signaling enzyme-linked immunosorbent assay (ELISA) (CASE) kit for JNK T183/Y185, according to the manufacturer's instructions (SuperAray Bioscience Corporation, Frederick, MD).

RNA isolation and RPAs. Primary murine alveolar epithelial cells were grown to confluence and then stimulated for 6 h. Total RNA isolation was performed using TRIzol reagent according to the manufacturer's instructions (Life Technologies, Grand Island, NY). For the RNase protection assay (RPA), a custom RPA template was used to transcribe radiolabeled, antisense riboprobes for murine CCL2, murine ribosomal protein L32, and glyceraldehyde-3-phosphate dehydrogenase as previously described (31, 35).

***In vivo Pneumocystis* infection and BAL.** Mice were intratracheally inoculated with 1×10^6 freshly isolated *Pneumocystis* cysts. After 3, 8, and 24 h, BAL fluid was recovered from experimental mice by lavaging both lungs with three, 1-ml aliquots of 1× HBSS. The recovered BAL fluid was centrifuged for 10 min at 1,110 rpm at 4°C to pellet cells. Cell-free BAL fluid was used for chemokine ELISA. Recovered cells were counted using a hemacytometer (Hausser Scientific).

Cytokine and chemokine ELISA. Culture supernatants and BAL fluid were collected and then centrifuged at 12,000 × *g* for 10 min to remove cell debris. Supernatants and BAL fluid were kept at −80°C until used. CCL2 and CXCL2 chemokine concentrations were measured using a commercially available ELISA kit (R&D Systems) and utilized according to the manufacturer's instructions.

Statistical analysis. Data are presented as the mean ± 1 standard error measurement (SEM). Differences between treatments were analyzed using one-way analysis of variance (ANOVA) and Tukey's multiple-comparison test as a posttest. Differences between treatments at different doses or at different time points were analyzed using two-way ANOVA and Bonferroni's multiple-comparison test as a posttest. Differences were considered significant at P values of <0.05. All data were analyzed using GraphPad Prism (version 5.00) software (GraphPad Software, San Diego, CA).

RESULTS

AEC chemokine responses to *Pneumocystis* require MyD88. AECs produce chemokines in response to *Pneumocystis* in a time- and dose-dependent manner (30). To determine whether MyD88 plays a role in the signaling cascade leading to chemokine production by *Pneumocystis*-stimulated AECs, primary murine AECs isolated from WT and MyD88-deficient mice were exposed to *Pneumocystis*. Confluent monolayers were treated with freshly isolated murine *Pneumocystis* at cyst-to-AEC ratios of 1:1 and 3:1. CCL2 levels in the supernatants at 6 h and 12 h were measured by ELISA. CCL2 responses in WT cells after 6 h of treatment with *Pneumocystis* at cyst-to-AEC ratios of 1:1 or 3:1 were 13- and 25-fold higher than those in untreated controls, respectively (Fig. 1A). Similarly, CCL2 levels of *Pneumocystis*-treated AECs after 12 h of stimulation were 22- and 24-fold higher than those in untreated controls, respectively (Fig. 1B). In contrast, MyD88$^{-/-}$ AECs were severely impaired in their ability to produce CCL2 in response to *Pneumocystis* stimulation at both doses and both time points (Fig. 1A and B). WT and MyD88$^{-/-}$ AECs displayed similar increases in CCL2 production in response to treatment with TNF-α, demonstrating that MyD88$^{-/-}$ AECs do not have an intrinsic deficiency in CCL2 responses. Furthermore, MyD88$^{-/-}$ AECs were also impaired in their ability to respond to MyD88-dependent TLR agonists LPS and Pam3SCK4, confirming that these cells are MyD88 deficient.

Murine AECs have also been shown to produce the chemokine CXCL2 in response to *Pneumocystis* stimulation (31). To determine whether MyD88 is also necessary for the upregulation of this chemokine, we measured CXCL2 in the culture supernatants of stimulated AECs. As shown in Fig. 1C, *Pneumocystis*-stimulated WT AECs had greater than 3- and 6-fold increases in CXCL2 production after 6 h and 12 h stimulation, respectively. In contrast, *Pneumocystis*-stimulated MyD88$^{-/-}$ AECs had no upregulation of CXCL2 production and had responses comparable to those of the untreated cells. Overall, these data show that CCL2 and CXCL2 chemokine production by AECs is dependent on signaling through the adaptor molecule MyD88.

Our data demonstrated impaired production and release of CCL2 from *Pneumocystis*-treated MyD88$^{-/-}$ AECs compared to WT AECs. In order to determine if MyD88 plays a role in the transcription of *Pneumocystis*-mediated CCL2, we sought to determine if the steady-state levels of CCL2 RNA were different between WT and MyD88$^{-/-}$ *Pneumocystis*-stimulated AECs. RNA was isolated from WT and MyD88$^{-/-}$ AECs after 6 h of *Pneumocystis* stimulation, and RPAs were performed. CCL2 mRNA was not detectable in untreated cells from either strain of mice (Fig. 2A and B). Similar to ELISA data, CCL2 mRNA levels were increased >10-fold in *Pneumocystis*-stimulated WT AECs. However, the CCL2 mRNA level in *Pneumocystis*-stimulated MyD88$^{-/-}$ AECs was not elevated above untreated control levels (Fig. 2A and B). These data show that MyD88 controls CCL2 protein production by *Pneumocystis*-stimulated AECs through a transcriptional control mechanism.

CCL2 production by *Pneumocystis*-stimulated AECs is independent of the adaptor molecule TRIF. CCL2 production by AECs in response to *Pneumocystis* is dependent on NF-κB and JNK. NF-κB is also known to be activated by a MyD88-independent pathway, the TRIF (37). TRIF is the main adaptor molecule utilized by TLR3 and the MyD88-independent pathway from TLR4 signaling (37). Thus, to determine if the response to *Pneumocystis* was specific to MyD88 or was codependent on TRIF and MyD88, confluent monolayers of primary AECs were isolated from WT or TRIF$^{-/-}$ mice and treated with freshly isolated *Pneumocystis* for 6 h. Supernatants were collected and analyzed by ELISA. Interestingly, CCL2 responses from *Pneumocystis*-treated TRIF$^{-/-}$ AECs were comparable to responses from *Pneumocystis*-treated WT AECs at both *Pneumocystis* cyst-to-AEC ratios tested (Fig. 3). CCL2 responses to TNF-α (10 ng/ml) were also comparable to those of WT AECs. These data show that the CCL2 responses to *Pneumocystis* are independent of the adaptor molecule TRIF and specifically dependent on MyD88.

***Pneumocystis*-stimulated JNK activation requires MyD88-dependent signaling in alveolar epithelial cells.** Our group has previously reported that CCL2 production by *Pneumocystis*-stimulated AECs is dependent on JNK activation (30). To determine whether JNK activation is dependent on MyD88, primary murine

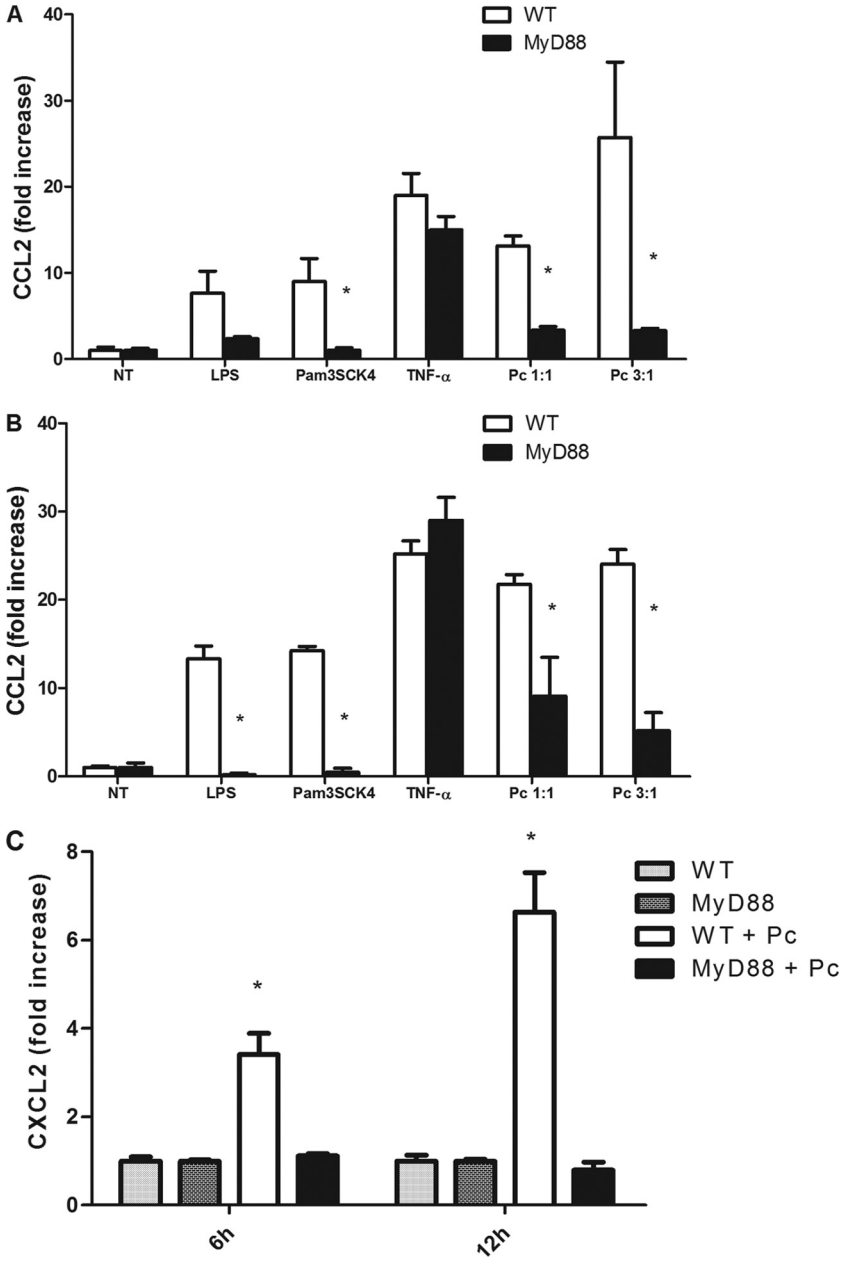

FIG 1 CCL2 and CXCL2 responses to *Pneumocystis* (Pc) in primary alveolar epithelial cells are dependent on MyD88. Primary mouse AECs were isolated and cultured from C57BL/6 mice (WT) and C57BL/6 Myd88$^{-/-}$ mice. Confluent monolayers were treated with freshly isolated murine *Pneumocystis* at cyst-to-AEC ratios of 1:1 and 3:1. AECs were also treated with either medium alone as a negative control (NT) or TNF-α (10 ng/ml) as a non-MyD88-dependent positive control. Cells were also treated with LPS (10 ng/ml) and Pam3CSK4 (0.5 μg/ml). CCL2 levels in the supernatants at 6 h (A) and 12 h (B) were measured by ELISA (*, $P < 0.05$ compared to the same treatment in WT). (C) CXCL2 levels in supernatants were measured at 6 h and 12 h (*, $P < 0.05$ compared to all other groups at each time point). Results are shown as the fold increase relative to medium-only treatment. Bars represent means ± SEMs ($n = 3$) from a representative experiment that was repeated four times.

pneumocytes were isolated from WT and MyD88$^{-/-}$ mice. Confluent monolayers were treated with *Pneumocystis* or with medium only. After 30 min, 1 h, and 2 h, AECs were tested for JNK phosphorylation by ELISA. As expected, WT AECs exhibited increased JNK activation at 30 min after *Pneumocystis* stimulation (Fig. 4A) (30). In contrast, *Pneumocystis* stimulation did not induce JNK signaling in AECs lacking MyD88 (Fig. 4A). This lack of JNK phosphorylation in MyD88$^{-/-}$ AECs was specific to the treatment with *Pneumocystis*, as JNK activation was observed in both WT and MyD88$^{-/-}$ AECs following stimulation with TNF-α, which signals through a MyD88-independent pathway (Fig. 4B). These data suggest that the MyD88-dependent mechanism through which *Pneumocystis*-stimulated AECs produce CCL2 involves activation of the JNK pathway, leading to increased gene transcription.

Stimulation through TLR2, TLR3, TLR4, and TLR5, but not TLR7 or TLR9, induces chemokine production by AECs. Our studies demonstrate that the AEC chemokine response to *Pneu-*

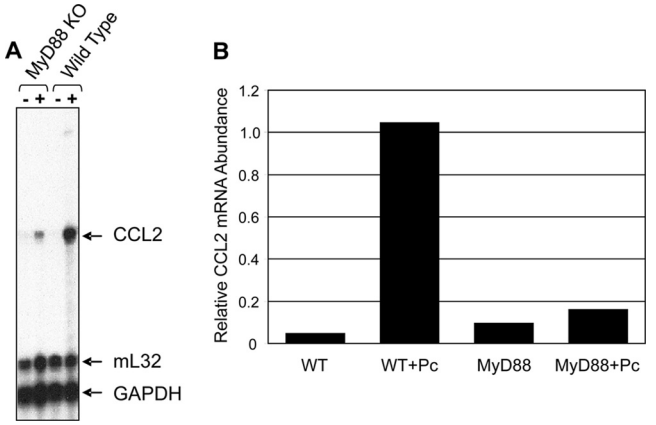

FIG 2 MyD88 is required for *Pneumocystis*-stimulated CCL2 mRNA expression in AECs. Primary murine pneumocytes were isolated from C57BL/6 WT and Myd88$^{-/-}$ mice. Confluent monolayers were exposed to freshly isolated murine *Pneumocystis* at a *Pneumocystis*-to-AEC ratio of 3:1. AECs were also treated with medium alone as a negative control (WT or MyD88$^{-/-}$). (A) RNA was collected after 6 h of treatment and analyzed by RPA. KO, knockout. (B) MCP-1 mRNA levels were quantified and normalized to the levels of the housekeeping gene *mL32*.

mocystis requires the adaptor molecule MyD88. MyD88 is required for signaling initiated by most TLRs, with the exception of TLR3 and the MyD88-independent pathway of TLR4 (15). However, little is known about the responsiveness of primary murine AECs to various TLR ligands. Thus, we sought to elucidate which TLR ligands induce chemokine production in primary murine AECs. Confluent cultures of WT murine AECs were stimulated with a panel of TLR ligands specific for TLR1/2 (Pam3CSK4), TLR2 (heat-killed *Listeria monocytogenes*), TLR2/6 (FSL1), TLR3 [poly(I·C) and poly(I·C) low molecular weight (LMW)], TLR4 (*E. coli* K-12 LPS), TLR5 (*S. enterica* serovar Typhimurium flagellin), TLR7(ssRNA40), and TLR9 (ODN1826) (Table 1; Fig. 5). There was a significant increase in CCL2 production in response to the ligands for TLR1/2, TLR2/6, TLR3, TLR4, and TLR5 but not to the

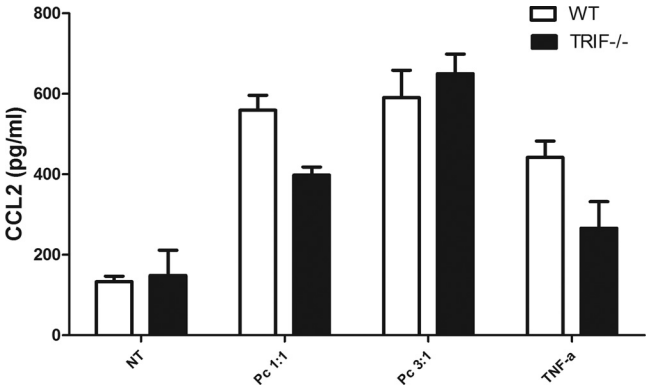

FIG 3 CCL2 response of *Pneumocystis*-stimulated AECs is independent of TRIF. Primary murine pneumocytes were isolated from C57BL/6 WT and TRIF$^{-/-}$ mice. Confluent monolayers were exposed to freshly isolated murine *Pneumocystis* at cyst-to-AEC ratios of 1:1 and 3:1. AECs were also treated with either medium alone (negative control [NT]) or TNF-α (10 ng/ml). CCL2 levels in the supernatants at 6 h were measured by ELISA ($P > 0.05$). Bars represent means ± SEs ($n = 3$) from a representative experiment that was repeated twice.

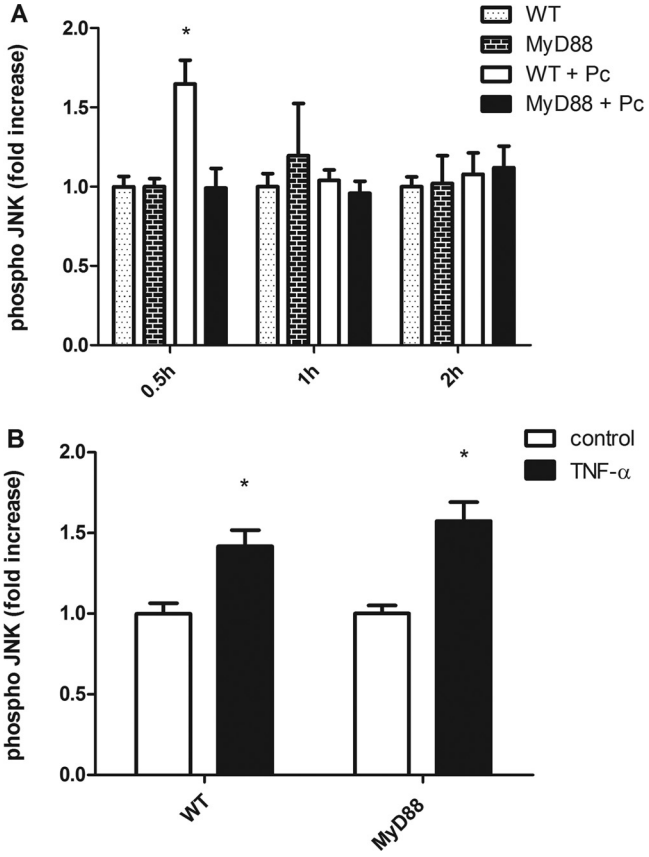

FIG 4 MyD88 signaling is required for *Pneumocystis*-stimulated JNK activation in primary murine AECs. Primary murine pneumocytes were isolated from C57BL/6 WT and Myd88$^{-/-}$ mice. Confluent monolayers were exposed to freshly isolated murine *Pneumocystis* (WT + Pc or MyD88 + Pc) (A). AECs were also treated with either medium alone as a negative control (WT or MyD88 in panel A and control in panel B) or TNF-α (10 ng/ml) (B) for 10 min. After 0.5, 1, or 2 h of stimulation, phospho-JNK was measured by ELISA in cells. *, $P < 0.05$ compared to each group at a specific time point (A) or compared to the control from the same strain (B). Bars represent means ± SEs from a representative experiment that was repeated two times.

ligands for TLR2 only, TLR7, or TLR9 (Fig. 5A). Moreover, AEC CXCL2 production was increased by TLR2/6, TLR4, and TLR5 ligands but not TLR1/2, TLR2, TLR3, TLR5, TLR7, or TLR9 ligands (Fig. 5B). As expected, the WT AECs responded to *Pneumocystis* at *Pneumocystis*-to-AEC ratios of 1:1 and 3:1. To ensure that the AEC response was specific to *Pneumocystis* and not to any non-*Pneumocystis* contaminant that might have been coisolated from the lung tissue, a cocktail of *Pneumocystis*-specific antibodies was used to deplete *Pneumocystis* from lung homogenates. AECs were treated with the *Pneumocystis*-depleted preparation, and no CCL2 or CXCL2 responses were observed, confirming that the observed responses were due to *Pneumocystis*. These data suggest that murine primary AECs express TLRs, respond to various TLR ligands, and are able to respond to pathogen-associated molecular patterns with chemokine secretion. These results imply that AECs in the lung are capable of initiating innate immune responses to pathogens in the lung.

Chemokine production by *Pneumocystis*-stimulated AECs does not require TLR2 or TLR4. Recent studies have suggested

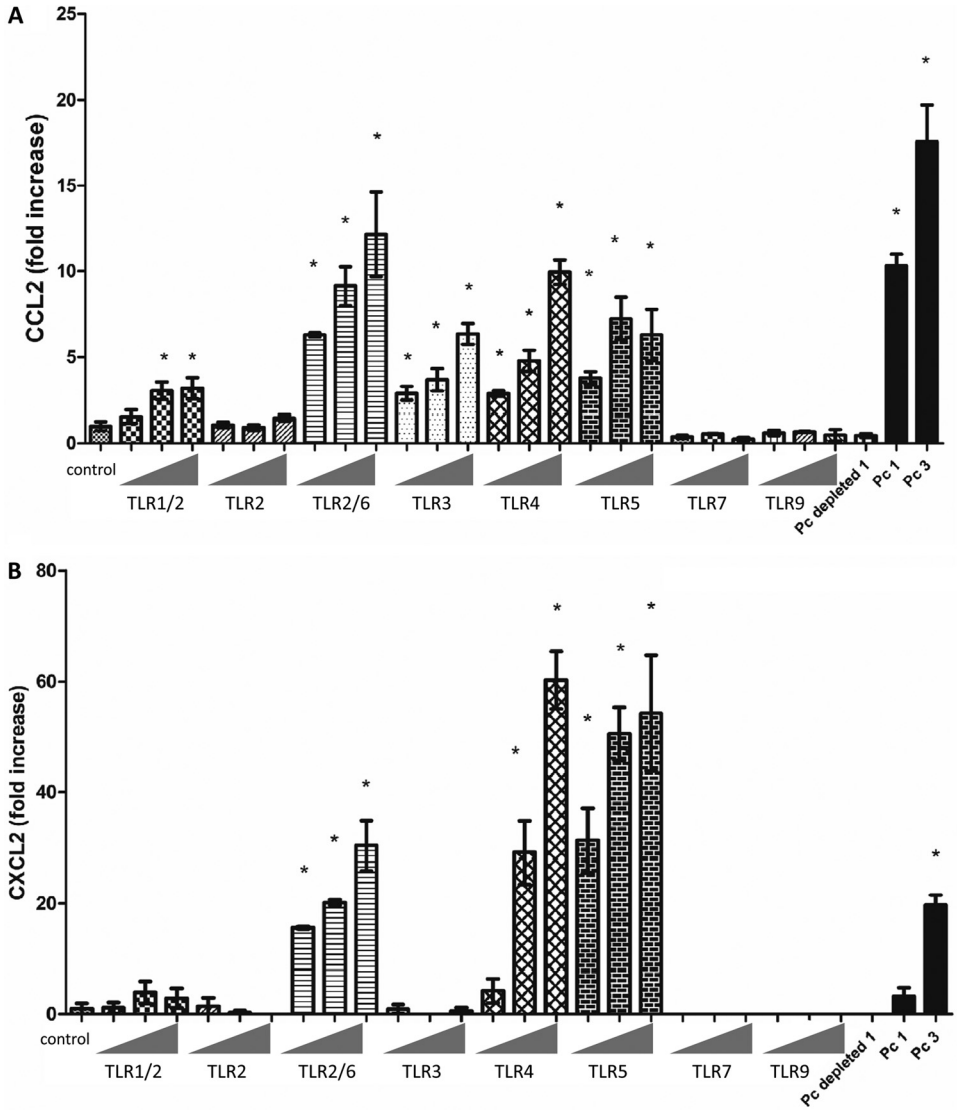

FIG 5 Primary murine AECs respond to TLR agonists. Primary murine AECs were isolated from C57BL/6 WT mice, and confluent monolayers were stimulated with a panel of TLR ligands for 6 h at increasing concentrations (Table 1): TLR1/2 (Pam3CSK4), TLR2 (heat-killed *Listeria monocytogenes*), TLR2/6 (FSL1), TLR3 [poly(I·C) and poly(I·C) LMW], TLR4 (*E. coli* K-12 LPS), TLR5 (*S. enterica* serovar Typhimurium flagellin), TLR7(ssRNA40), and TLR9 (ODN1826). AECs were also exposed to freshly isolated murine *Pneumocystis* at cyst-to-AEC ratios of 1:1 (Pc 1) and 3:1 (Pc 3), antibody-depleted *Pneumocystis* preparations (Pc depleted), and medium alone as negative control (control). CCL2 (A) and CXCL2 (B) levels in the supernatants at 6 h were measured by ELISA. Results are shown as the fold increase relative to the medium-only treatment. Bars represent means ± SEs ($n = 3$) from a representative experiment that was repeated two times in triplicate. (*, $P < 0.05$ compared to control).

that alveolar macrophages respond to *Pneumocystis* in a proinflammatory manner, releasing cytokines and chemokines in a TLR2- and TLR4-dependent manner (7, 40). We have shown that primary murine AECs respond to several TLR ligands, including TLR2 and TLR4 agonists. Thus, we hypothesized that the MyD88-dependent AEC chemokine response to *Pneumocystis* was also dependent on the interaction of TLR2 or TLR4 with *Pneumocystis* organisms. Primary AECs were isolated from WT, TLR2$^{-/-}$, TLR4$^{-/-}$, and TLR2/4$^{-/-}$ mice on the C57BL/6 background. Cells were then exposed to *Pneumocystis* at cyst-to-AEC ratios of 1:1 and 3:1. TNF-α (10 ng/ml), LPS (10 ng/ml), and Pam3CSK4 (0.5 μg/ml) were used as controls. At 6 h posttreatment, cell supernatants were collected and CCL2 chemokine responses were tested by ELISA. As expected, WT AECs produced CCL2 in response to TNF-α and *Pneumocystis*, as well as in response to the TLR2 and TLR4 agonists Pam3CSK4 and LPS, respectively (Fig. 6). MyD88-deficient AECs produced CCL2 in response to TNF-α stimulation but were significantly impaired in their ability to respond to *Pneumocystis*, LPS, or Pam3CSK4. Surprisingly, TLR2- and TLR4-deficient AECs also responded to *Pneumocystis* stimulation with significantly elevated CCL2 production compared to that by untreated controls. In order to address whether the presence of either TLR2 or TLR4 might compensate for the absence of the other, we also generated TLR2/4 double-knockout mice and isolated primary AECs. TLR2/4 double-deficient mice did not respond to the TLR2 and TLR4 agonists Pam3CSK4 and LPS, respectively, but did produce CCL2 in response TNF-α and *Pneumocystis* stimulation (Fig. 6). TLR2, TLR4, and TLR2/4 dou-

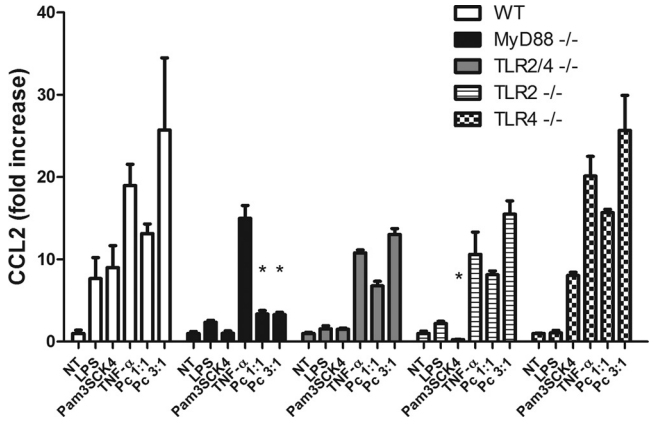

FIG 6 CCL2 production by *Pneumocystis*-stimulated AECs is independent of TLR2 and/or TLR4. Primary murine AECs were isolated from WT and MyD88$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$, and TLR2/4 double-knockout mice all on the C57BL/6 background. Confluent monolayers were exposed to *Pneumocystis* (at cyst-to-AECs ratios of 1:1 and 3:1), TNF-α (10 ng/ml), LPS (10 ng/ml), or Pam3CSK4 (0.5 μg/ml). At 6 h poststimulation, cell supernatants were collected and CCL2 chemokine responses were tested by ELISA. Bars represent means ± SEs ($n = 3$) from a representative experiment that was repeated three times (*, $P < 0.05$ compared to the same treatment in WT).

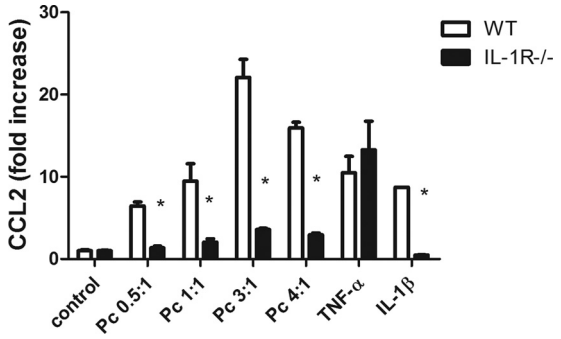

FIG 7 The CCL2 response to *Pneumocystis* in primary alveolar epithelial cells is dependent on IL-1R. Primary murine AECs were isolated from C57BL/6 WT and IL-1R$^{-/-}$ mice. Confluent monolayers were exposed to freshly isolated murine *Pneumocystis* at cyst-to-AEC ratios of 0.5:1, 1:1, 3:1, and 4:1. AECs were also treated with either medium alone (control), TNF-α (10 ng/ml), or IL-1β (10 ng/ml). CCL2 levels in the supernatants at 6 h were measured by ELISA. Bars represent means ± SEs ($n = 3$) from a representative experiment that was repeated twice (*, $P < 0.05$ compared to same treatment in WT).

ble-deficient AECs also produced CXCL2 in response to *Pneumocystis* stimulation (data not shown). These data show that the MyD88-dependent chemokine response of *Pneumocystis*-stimulated AECs does not require TLR2 or TLR4.

Chemokine production by *Pneumocystis*-stimulated AECs requires the IL-1 receptor. Since the likeliest candidate TLRs (TLR2 and TLR4) were found to not be required for chemokine production by *Pneumocystis*-stimulated AECs, other MyD88-dependent pathways were explored. MyD88 is also required for signaling through the interleukin-1 (IL-1) receptor complex (IL-1R). Therefore, the chemokine response following *Pneumocystis* stimulation was evaluated in primary AECs isolated from WT and IL-1R-knockout mice. As expected, WT AECs produced CCL2 in response to stimulation with *Pneumocystis*, TNF, or IL-1β (Fig. 7). In contrast, IL-1R-deficient AECs produced CCL2 in response to TNF but did not respond to *Pneumocystis* or IL-1β stimulation (Fig. 7). These data indicate that the MyD88-dependent mechanism through which *Pneumocystis* stimulates chemokine production by AECs requires a functional IL-1R.

MyD88 signaling is required for early chemokine release and cell recruitment following *Pneumocystis* infection *in vivo*. AECs are the first cells in the lungs that interact with *Pneumocystis* and likely the cells that first detect and respond to *Pneumocystis* infection. MyD88-dependent signaling was found to play a major role in the AEC chemokine response to *Pneumocystis in vitro* (Fig. 1). To determine whether MyD88 signaling controls the chemokine response to *Pneumocystis* infection *in vivo*, WT and MyD88-deficient mice were intratracheally inoculated with 1×10^6 *Pneumocystis* cysts. At 3 h, 6 h, and 24 h postinfection, BAL was performed on individual mice and chemokine levels and total cells in the BAL fluid were determined. At 3 h postinfection, CCL2 and CXCL2 levels were significantly elevated in the lungs of WT mice compared to MyD88-knockout mice (Fig. 8A and B). However, by 8 h and 24 h, CCL2 levels declined in WT mice and were not significantly different from those in MyD88-knockout mice. Since AECs express CCR2, the receptor for CCL2, we speculate that the decline in CCL2 levels is at least partly due to a negative-feedback loop caused by high CCL2 levels in the lung at 3 h postinoculation. Additionally, the recruitment of CCR2-positive macrophages to the lung in response to CCL2 has been shown to downregulate local CCL2 production (20). *In vivo*, these mechanisms would result in a decrease in CCL2 levels in BAL fluid because of diffusion of CCL2 into the lung and circulation. CXCL2 levels remained elevated in WT mice at 8 and 24 h and were greater than those in MyD88-knockout mice. The number of cells recovered in the BAL fluid of *Pneumocystis*-infected WT mice was higher than that recovered from infected MyD88-knockout mice at all time points (Fig. 8C). Importantly, the number of cells in the BAL fluid of infected MyD88-knockout mice was not significantly different from that in the BAL fluid of uninfected control mice. These data demonstrate that MyD88 is important for chemokine production and early cell recruitment to the airspaces following *Pneumocystis* infection *in vivo*.

DISCUSSION

Pneumocystis is an extracellular pathogen that interacts closely with the alveolar epithelium. Ultrastructural studies have shown interdigitation between *Pneumocystis* and the membrane of host AECs, and it has been suggested that attachment to AECs is required to establish infection, leading to the development of PCP (18, 26). Previously, AECs were mainly recognized as barrier cells and producers of surfactant. However, there is an increasing body of evidence suggesting an important role for AECs in host defense. Because of their barrier position in the lung, AECs are likely to mediate the initial encounter and response to inhaled pathogens. Furthermore, since AECs are uniquely situated in close proximity to the blood supply, they can affect the recruitment of inflammatory cells to the alveoli. Our laboratory has demonstrated that the direct interaction of viable *Pneumocystis* with lung epithelial cells induces the rapid production of proinflammatory chemokines, such as CCL2 and CXCL2, through a JNK-dependent mechanism (30, 31). In this study, we expanded upon those results by demonstrating that *Pneumocystis*-stimulated JNK activation and subsequent chemokine production require the adaptor molecule MyD88. Surprisingly, the AEC chemokine response was indepen-

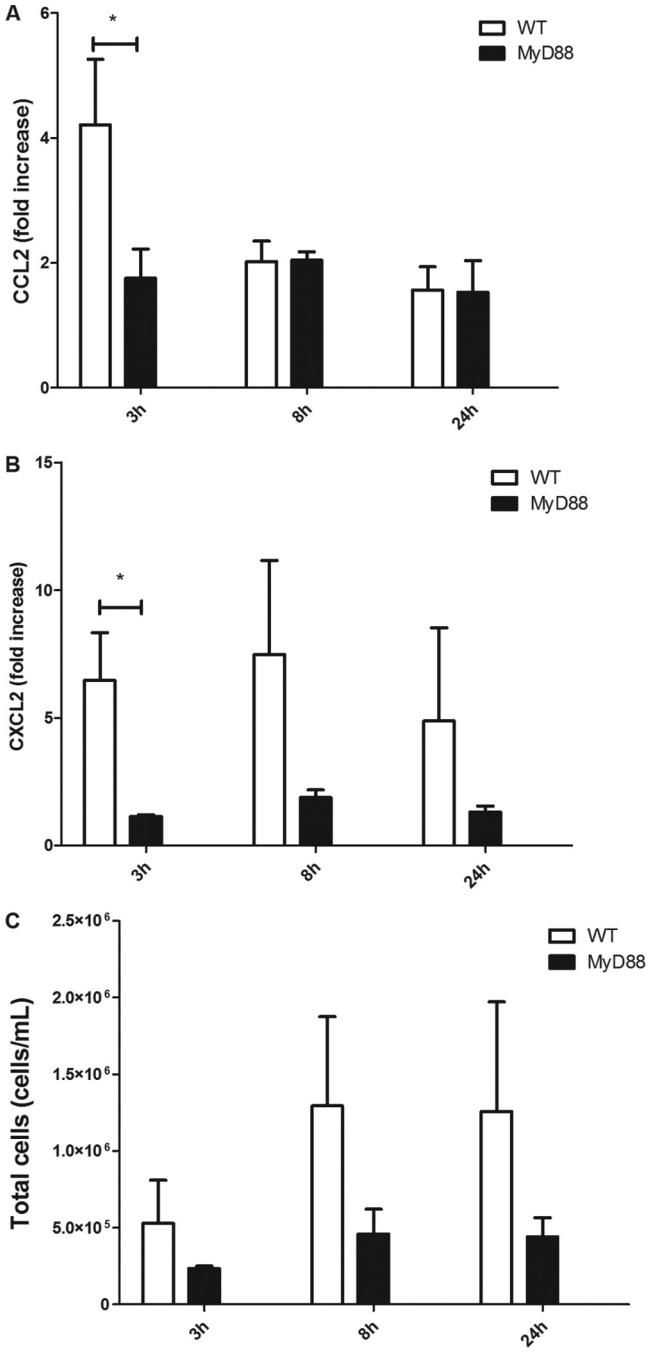

FIG 8 Early *Pneumocystis*-stimulated chemokine release and cell recruitment to the lung are dependent upon MyD88 signaling. C57BL/6 WT and MyD88$^{-/-}$ mice were intratracheally inoculated with 1×10^6 freshly isolated *Pneumocystis* cells. After 3 h, 8 h, and 24 h of *Pneumocystis* infection, BAL fluid from lungs were obtained to measure protein levels of CCL2 (A) and CXCL2 (B) by ELISA and for assessment of total cell recovery (C) (*, $P < 0.05$). Bars represent means ± SEs from pooled data from two independent experiments ($n \geq 6$ mice per group).

dent of TLR2 or TLR4 but did require AEC expression of IL-1R, which also signals through a MyD88-dependent mechanism. *In vivo* studies revealed that MyD88 is involved in the early pulmonary chemokine response to *Pneumocystis* infection and is also important for the early recruitment of inflammatory cells into the lung. Together, these data demonstrate a role for MyD88 in the generation of *Pneumocystis*-induced pulmonary inflammation and suggest that the AECs may modulate organism clearance in immunocompetent hosts and/or lung injury caused by an exacerbated inflammatory response in susceptible hosts.

It is well documented that professional phagocytes such as macrophages have important proinflammatory and host defense roles in the lung. These cells utilize TLRs and other PRRs such as dectin-1 to detect infectious agents and initiate inflammatory responses. TLRs signal through MyD88, and the alveolar macrophage response to *Pneumocystis* β-glucan is at least partly dependent upon MyD88 signaling (17). In addition, recent studies have suggested a role for TLR2 in the alveolar macrophage CXCL2 and TNF-α response to *Pneumocystis* organisms *in vitro* and *in vivo* (40). Moreover, a study by Ding et al. showed that TLR4-deficient AMs have impaired CXCL2, IL-10, and IL-12p40 responses to *Pneumocystis in vitro* and *in vivo* (7). However, they unexpectedly found that TLR4-deficient mice showed increased levels of TNF-α and IL-6 in BAL fluid, as well as higher lung injury scores and weight loss. These data suggested that other cell populations, such as AECs, may be involved in the host response to *Pneumocystis* infection *in vivo*. In the current study, we have shown that murine AECs respond to TLR2 and TLR4 agonists with CCL2 and CXCL2 release. However, we have also shown that the AEC response to *Pneumocystis* is independent of TLR2 or TLR4, suggesting that an alternate MyD88-dependent pathway is utilized by AECs in response to *Pneumocystis*.

Prior studies have identified several receptors that may be involved in the recognition of *Pneumocystis* and subsequent activation of proinflammatory pathways. Dectin-1 recognizes β-glucans found in cell walls of fungi and has been implicated in the AM response to *Pneumocystis* β-glucan (29). However, AECs express neither dectin-1 nor the integrin CD11b/CD18 (Mac-1, CR3) β-glucan receptor. Lactosylceramide (CDw17) has been identified as the alternate glucan receptor on AECs (8, 11). Interestingly, our group found that CCL2 production by *Pneumocystis*-stimulated murine primary AECs is not mediated by β-glucan (12), suggesting that other mechanisms that mediate the AEC response to viable *Pneumocystis* organisms exist. Benfield et al. reported that a nonglucan component of *Pneumocystis*, surface glycoprotein A, induces the production of IL-8 and CCL2 by the human alveolar epithelial cell line A549 (5). In our experiments, we stimulated AECs with freshly isolated *Pneumocystis*, with the aim of mimicking the interactions that occur *in vivo*. Our studies further enhance our understanding of the pathways that AECs utilize to recognize *Pneumocystis* and signal proinflammatory chemokine release.

It has been suggested that IL-1/IL-1 receptor signaling plays an important role in *Pneumocystis* clearance (6, 34), but the role of IL-1R in the AEC response to *Pneumocystis* has not been determined. We have now shown that IL-1R is required for the AEC chemokine response to *Pneumocystis* (Fig. 7), and there are several possible mechanisms through which IL-1R may be involved in the *Pneumocystis* inflammatory response. It is possible that *Pneumocystis* or a *Pneumocystis* product could directly interact with IL-1R to stimulate MyD88-dependent signaling. Alternatively, *Pneumocystis* may activate the inflammasome to induce production and maturation of IL-1, which then stimulates AECs in an autocrine manner. Some cell types require two signals for IL-1β to be secreted: (i) an initial step that is activated through TLRs, C-type lectins, or other signaling pathways to produce intracellular pro-

IL-1β and (ii) a second step where caspase-1 is activated to cleave pro-IL-β into mature IL-1β that can be secreted (23, 24, 28). In respiratory syncytial virus and *Francisella novicida* infection models, TLR2 activation precedes the second step of inflammasome activation for IL-1β maturation and release (12, 28). Based on previous results that suggest a role for TLR2 in the host response to *Pneumocystis* and our results that show a slightly reduced chemokine response in TLR2-deficient AECs compared to WT AECs, we speculate that TLR2 plays a role in the initial step required for pro-IL-1 production. Thus, perhaps such a dramatic reduction in *Pneumocystis*-stimulated AEC chemokine release is observed in MyD88-deficient AECs because MyD88 is required for both the initial TLR2 signaling and the subsequent IL-1R-dependent signaling cascades.

In order to prevent and treat inflammation-mediated lung injury caused by *Pneumocystis* infection, it is necessary to understand how different lung cell populations interact with and respond to the organism. Understanding the contribution of AECs to the host's inflammatory response will aid in the development of improved treatments by targeting anti-inflammatory therapy to specific cell types and specific pathways utilized by the host. Further studies exploring the specific mechanisms by which the MyD88 and IL-1R pathway is exploited by *Pneumocystis* in AECs will help target appropriate treatment.

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