Pneumocystis stimulates MCP-1 production by alveolar epithelial cells through a JNK-dependent mechanism

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Wang J, Gigliotti F, Bhagwat SP, Maggirwar SB, Wright TW. Pneumocystis stimulates MCP-1 production by alveolar epithelial cells through a JNK-dependent mechanism. Am J Physiol Lung Cell Mol Physiol 292: L1495–L1505, 2007. First published February 16, 2007; doi:10.1152/ajplung.00452.2006.—Pneumocystis carinii is an opportunistic fungal pathogen that causes pneumonia (PCP) in immunocompromised individuals. Recent studies have demonstrated that the host’s immune response is clearly responsible for the majority of the pathophysiological changes associated with PCP. P. carinii interacts closely with alveolar epithelial cells (AECs); however, the nature and pathological consequences of the epithelial response remain poorly defined. Monocyte chemotactic protein-1 (MCP-1) is involved in lung inflammation, immunity, and epithelial repair and is upregulated during PCP. To determine whether AECs are an important source of MCP-1 in the P. carinii-infected lung, in vivo and in vitro studies were performed. In situ hybridization showed that MCP-1 mRNA was localized to cells with morphological characteristics of AECs in the lungs of infected mice. In vitro studies demonstrated that P. carinii stimulated a time- and dose-dependent MCP-1 response in primary murine type II cells that was preceded by JNK activation. Pharmacological inhibition of JNK nearly abolished MCP-1 production, while ERK, p38 MAPK, and TNF receptor signaling were not required. Furthermore, delivery of a JNK inhibitor peptide specifically to pulmonary epithelial cells using a recombinant adenovirus vector blocked the early lung MCP-1 response following intratracheal instillation of infectious P. carinii. JNK inhibition did not affect P. carinii-stimulated production of macrophage inflammatory protein-2 in vitro or in vivo, indicating that multiple signaling pathways are activated in P. carinii-stimulated AECs. These data demonstrate that AECs respond to P. carinii in a proinflammatory manner that may contribute to the generation of immune-mediated lung injury.

inflammation; epithelial; AIDS

Pneumocystis carinii (PCP) continues to be the most common AIDS-defining illness as well as an important cause of morbidity and mortality in patients with a wide array of immunosuppressive conditions. Most recent studies indicate that, although the presence of P. carinii is obviously a factor in the development of lung injury, pulmonary inflammation is a major determinant of the severity of PCP (5, 27, 53, 54). In AIDS patients with profound reductions in CD4+ T cell numbers, bronchoalveolar lavage (BAL) fluid IL-8 and neutrophil concentrations, but not organism numbers, correlate with severity of PCP (5, 23, 28). In addition, clinical studies of immune-reconstituted PCP patients and controlled animal studies have both demonstrated that inflammatory mediators are released, and immune and inflammatory cells are recruited to the lung in response to P. carinii (2, 25, 38, 39). More defined studies in mice have identified specific T cell subsets as having prominent roles in the lung injury associated with PCP (54, 57). For example, CD8+ T cells are responsible for much of the PCP-associated lung injury that occurs in CD4-deficient hosts (17). Therefore, recent studies have focused on the mechanisms by which T cells accumulate in the lung during PCP.

While the close interaction of P. carinii with the alveolar epithelium was one of the first observations offering insight into the pathogenesis of PCP (26, 32, 58, 59), very little is known about the epithelial response to P. carinii. In vivo studies have most often noted the attachment of P. carinii to the type I pneumocyte. However, this observation does not preclude an important role for type II cells in the response to P. carinii. Type II cells are closely positioned near the type I cells and have been reported to interact with P. carinii in vivo (29). These findings are particularly relevant given that the type II cell is becoming increasingly recognized as an immune effector cell in the alveolus (16, 46). Our prior studies have found that immune and inflammatory cells are recruited specifically to alveolar sites of P. carinii infection in mice, suggesting that the interaction of P. carinii with the alveolar epithelium targets the immune response (55, 56). Other groups have found that disruption of alveolar epithelial cell (AEC) signaling in vivo modifies pulmonary immune and inflammatory responses (21, 46). Evidence has also accumulated that transformed human lung epithelial-like cell lines can produce inflammatory mediators in response to P. carinii stimulation (4, 41, 60). In addition, a murine AEC line and primary rat and mouse type II cells undergo NF-κB-dependent macrophage inflammatory protein-2 (MIP-2) production when stimulated with P. carinii or purified P. carinii glucan (15, 52). However, recent evidence indicates that neutrophils are not critical to the development of lung injury during PCP (47). Thus, focus has shifted to CC chemokines, including monocyte chemotactic protein-1 (MCP-1), which have a role in the tissue recruitment of T cells. Pulmonary epithelial cells are capable of secreting MCP-1 in response to infectious stimulation, and epithelial MCP-1 mediates the pulmonary recruitment of CD8+ T cells (42, 62). In addition, a role for MCP-1/CC chemokine receptor 2 (MCP-1/CCR2) signaling in the repair of damaged pulmonary epithelium has been suggested (10). These findings, combined with the fact that lung MCP-1 levels are dramatically elevated in mice with PCP, make this chemo-
kine of interest for its contribution to the pathological T cell response that is critical to the progression of PCP.

Identification of epithelial-specific responses to *P. carinii,* and the signaling cascades leading to these responses, will aid in understanding the role of AECs as immune effector cells in the generation of pulmonary immune and inflammatory responses. The hypothesis of the current study is that the interaction of *P. carinii* with type II AECs induces MCP-1 production through NF-κB and mitogen-activated protein kinase (MAPK)-dependent mechanisms. To test this hypothesis, primary murine type II cell cultures, in vivo mouse models, and specific pharmacological and protein inhibitors of NF-κB, p38, JNK, and ERK signaling were utilized. This study will help determine the mechanism by which *P. carinii*-epithelial interaction promotes and targets the pathological immune/inflammatory response and also determine whether this interaction might be exploited as a therapeutic intervention.

**MATERIALS AND METHODS**

**Animals.** CB.17 wild-type and severe combined immunodeficient (SCID) mice were purchased from Taconic. C57BL/6 mice were also purchased from the Jackson Laboratory. These mice were then crossed to produce mice deficient in both TNFR1 and TNFR2 ("TNFR-deficient") on a 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 type II cells.** Primary type II pneumocytes were isolated from mouse lungs using a modification of the method of Corti et al. (11). Briefly, the lung was perfused with saline. Two milliliters of dispase solution (BD Biosciences) was instilled by tracheal catheter, followed immediately by slow insertion of 0.45 ml of low-melting-point agarose (GIBCO-BRL) at 45°C. The lungs were cooled briefly on ice and then incubated at room temperature in dispase for 45 min. The lung tissue was microdissected, incubated briefly in DMEM with 0.01% DNase at room temperature, filtered through nylon monofilament screens (100, 40, and 25 μm; BD Falcon), and centrifuged at 150 g at 4°C. Type II cells were purified from inflammatory cells by incubation with biotin-conjugated antibodies against CD32 and CD45 followed by recovery with streptavidin-conjugated magnetic beads in a magnetic separator. With this procedure, type II cell yield was ~1 × 10⁶ cells/mouse. Typically the type II cells were >95% viable and >92% pure as assessed by papanicolaou staining. In addition, >95% of the isolated cells were typically positive for surfactant protein C (SP-C) expression as assessed by intracellular staining and fluorescence-activated cell sorting (FACS) analysis.

The isolated type II cells were cultured under conditions previously demonstrated to maintain a type II phenotype as described by Rice et al. (43). The cells were cultured on Matrigel/rat tail collagen-coated plates (ratio 70/30, vol/vol) (BD Biosciences). Cells were maintained at 37°C with 6% CO₂ in bronchial epithelial cell growth medium (BEGM) without hydrocortisone (Cambrex), supplemented with 5% charcoal-stripped FBS (Hyclone, Logan, UT) and 10 ng/ml keratinocyte growth factor (KGF) (Calbiochem) to promote maintenance of a type II cell phenotype.

**Intracellular SP-C staining.** Intracellular SP-C expression was measured to determine the purity of type II cells. Briefly, primary AECs were gently dislodged from wells and washed with PBS + 1% FBS staining buffer, spun at 250 g for 10 min, and resuspended in staining buffer. The cells were incubated with Cytofix/Cytoperm (BD Biosciences) solution on ice for 20 min. The cells were stained with rabbit-anti-human SP-C (Chemicon International), which cross-reacts with mouse SP-C, followed by goat anti-rabbit conjugated with allopurinol or primary antibody alone. Unstained cells were used as an additional negative control. SP-C expression was measured by a FACS caliber cytometer and analyzed by CellQuest software (Becton-Dickinson, San Jose, CA).

**In situ hybridization.** In situ hybridization was performed as previously described (54, 56). Murine clones for MCP-1 were subcloned into the plasmid vector, pBluescript II SK+ (Stratagene, La Jolla, CA), for the in vitro transcription of RNA. Concentrations were measured using a commercially available ELISA kit according to the manufacturer’s instructions (R&D, Minneapolis, MN).

**Cytokine enzyme-linked immunosorbent assay (ELISA).** Culture supernatants were collected, centrifuged at 12,000 g for 5 min to remove debris, and then stored at −80°C. MCP-1 and MIP-2 concentrations were measured using a commercially available ELISA kit according to the manufacturer’s instructions (R&D, Minneapolis, MN).

In **in situ hybridization.** In situ hybridization was performed as previously described (54, 56). Murine clones for MCP-1 were subcloned into the plasmid vector, pBluescript II SK+ (Stratagene, La Jolla, CA), for the in vitro transcription of RNA. Sense and antisense orientations were confirmed by DNA sequencing. MCP-1 antisense riboprobes were created by limited alkaline hydrolysis was performed to create riboprobes ranging in
length from 0.1 to 0.3 kb. Hydrolyzed transcripts were sized by denaturing agarose gel electrophoresis.

The lungs from *P. carinii*-infected SCID mice were infection-fixed with 15 cmH2O gravity flow pressure of 10% buffered formalin (Sigma-Aldrich, St. Louis, MO). Tissue sections of 4-μm thickness were cut, then treated by the method of Angerer et al. (1a) with modification as previously described (54, 56).

**RNA isolation and RPA**. Primary AECs were grown to confluence in 24-well plates, then stimulated for the indicated times. Total RNA was isolated from the cells using Trizol reagent according to the manufacturer’s instructions (Life Technologies, Grand Island, NY). A custom RPA template was purchased (BD Biosciences) and used to transcribe radiolabeled, antisense riboprobes for murine MCP-1 and the murine ribosomal protein L32, as previously described (56).

**Western blot analysis**. Primary type II cells were cultured and treated in 12-well cell tissue culture plates. At the indicated time points, cells were washed with 1× cold PBS and lysed in EBL buffer (50 mM HEPES, pH 7, 250 mM NaCl, 0.1% NP-40, 5 mM EDTA, 1 mM NaF, 0.1 mM Na2VO4, 50 μM ZnCl2, supplemented with 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and a mixture of protease and phosphatase inhibitors), and cellular debris was removed by high-speed centrifugation. Cell lysates containing equal amounts of total protein were fractionated by 10% SDS-PAGE and electrophoretically transferred to methanol-pretreated polyvinylidene difluoride membrane. Immunoblot assays were performed as described previously (30). Phospho-JNK primary antibodies were purchased from Cell Signaling Technology and diluted 1:1,000 in PBS with 5% Tween and nonfat dry milk. Secondary anti-mouse and anti-rabbit antibodies were purchased from Amersham and used at a dilution of 1:2,000.

**Admixture of recombinant adenovirus**. Replication-deficient recombinant adenovirus (rAd) type 5 was used to construct a rAd vector overexpressing the Jun binding domain (JBD) of JNK interacting protein 1 (Ad-JBD). This peptide interrupts the activation of the downstream JNK signal. A rAd-vector expressing firefly luciferase (Ad-LUC) was used as a control. Mice were intratracheally instilled with rAd vectors or an equal volume of sterile PBS under anesthesia. Each mouse was given a single dose of 10⁹ plaque-forming units of Ad-JBD or Ad-LUC diluted in sterile PBS. Recent data has demonstrated that intratracheal instillation of this dose of rAd delivers genes selectively to the lung epithelium (46). Forty-eight hours after virus infection, mice were intratracheally inoculated with 10⁶ purified *P. carinii* or an equal volume of sterile PBS as a control. Three hours after *P. carinii* inoculation, mice were euthanized and lungs were lavaged with four 1-ml aliquots of sterile PBS. MCP-1 levels in the lavage fluid were determined by ELISA.

**Statistical analysis**. A one-way analysis of variance was performed with the SigmaStat 2.0 software (Jandel, San Rafael, CA) to determine the confidence intervals of observed variations in chemokine protein and mRNA levels in the experimental animals. The Student-Newman-Keuls method was used for all pair-wise multiple comparisons of experimental groups.

**RESULTS**

*P. carinii* stimulates epithelial MCP-1 production in vivo. It has been demonstrated that MCP-1 levels are elevated in the lungs of mice with active PCP (56, 57). However, the intensity of the inflammatory response accompanying PCP has made it difficult to identify the cell types responsible for MCP-1 production. To examine the role of resident lung cells in the MCP-1 response to *P. carinii*, normal and CD4-depleted mice were intratracheally inoculated with *P. carinii* and examined for MCP-1 production at 1, 3, 8, 24, 48, and 72 h postinoculation. While MCP-1 was not detected in the BAL fluid of mice inoculated with saline alone, MCP-1 levels were significantly elevated in the BAL fluid of *P. carinii* inoculated mice at 1, 3, and 8 h postinoculation and had declined toward baseline levels by 24 h (Fig. 1A). MCP-1 protein levels were not different in normal and CD4-depleted mice, demonstrating that CD4⁺ T cells were not required for MCP-1 production. Importantly, MCP-1 was produced at time points that precede inflammatory cell infiltration into the lungs following *P. carinii* inoculation. Because neutrophils are the first cells recruited to the lung following the instillation of *P. carinii*, the percentage of BAL fluid neutrophils was used as a marker of *P. carinii*-induced inflammatory response. MCP-1 levels were clearly elevated prior to the appearance of neutrophils in the lung (Fig. 1B). Importantly, inoculation of mice with preparations that were depleted of *P. carinii* did not elicit an MCP-1 response, showing that the organism was driving the response (Fig. 1C). Together, these data showed that resident lung cells were capable of mounting an MCP-1 response to *P. carinii*.

To more specifically identify the cells producing MCP-1 in vivo, in situ hybridization was performed on inflation-fixed *P. carinii*-infected and uninfected SCID mouse lungs. Sections did exhibit less cellular infiltration in response to *P. carinii* infection, thereby facilitating localization of mRNA hybridization to cells with histological characteristics of epithelial cells. A radiolabeled MCP-1-specific antisense riboprobe was constructed and used as previously described (54, 56). While only low background hybridization was observed in the uninfected lung (Fig. 2C), focal hybridization was shown in the alveolar epithelium in *P. carinii*-infected mice (Fig. 2, A, B, D–F). The appearance and positioning of cells expressing MCP-1 RNA was consistent with that of type II AECs. Gomori methenamine silver staining of a serial section demonstrated that *P. carinii* cysts colocalized to the same region of the lung that exhibited an increased steady-state level of MCP-1 mRNA (Fig. 2, G and H). Black asterisks in Fig. 2, A, B, and G mark the same structures in each micrograph. Red asterisks in Fig. 2, G and H, mark the same alveoli under lower and higher power magnification. These data demonstrated that AECs in *P. carinii*-infected regions of the lung produce MCP-1 in vivo.

*P. carinii* stimulates MCP-1 production by primary type II pneumocytes. To directly assess the ability of AECs to respond to *P. carinii*, primary type II cells were purified and cultured under conditions that promote a type II phenotype (43). Confluent monolayers of primary murine type II cells were inoculated with freshly isolated murine *P. carinii* at cyst-to-AEC ratios of 0, 0.5, 1.0, and 2.0. At 6, 12, and 24 h postinoculation, the culture supernatants were removed and assayed for MCP-1 by ELISA, and total RNA was isolated from the cells for RPA analysis. Stimulation of AECs with *P. carinii* induced a dose- and time-dependent increase in MCP-1 secretion (Fig. 3A). MCP-1 concentrations were significantly elevated in AECs treated with *P. carinii* at 6, 12, and 24 h postinoculation compared with unstimulated cells (P < 0.05). Because the mouse *P. carinii* must be isolated from lung tissue, a *P. carinii*-depleted preparation was used to control for the presence of non-*P. carinii* contaminants that could contribute to the AEC response. Antibody-mediated removal of the *P. carinii* from the preparation completely abolished the inducible MCP-1 response, demonstrating that the AECs are responding to *P. carinii* (Fig. 3A) and not a copurified contaminant.
A

Control IgG + Pc
Anti-CD4 + Pc

B

% BALF PMNs

C

MCP-1 (pg/ml)

Fig. 1. *Pneumocystis carinii* induces monocyte chemotactic protein-1 (MCP-1) production by resident lung cells in vivo. Nondepleted and CD4⁺ T-cell-depleted CB.17 mice were intratracheally inoculated with 1 × 10⁶ purified *P. carinii* (Pc), and then euthanized 1, 3, 8, 24, 48, and 72 h postinoculation. The bronchoalveolar lavage (BAL) fluid ("BALF") was collected, and MCP-1 levels were determined by ELISA (A). The percentage of neutrophils (PMNs) recovered in the BAL fluid was also determined at the same time points (B). The label "C" on the abscissa indicates the very small bar that shows the percentage of PMNs in the BAL fluid of uninfected mice. C: the BAL fluid MCP-1 levels of CB.17 mice that were inoculated with 1 × 10⁶ *P. carinii* or an equal amount of the same *P. carinii* preparation that had been depleted of viable organisms (52). BAL fluid was collected at 3, 8, and 24 h postinoculation, and MCP-1 levels were determined by ELISA. Values are means ± SE. *P < 0.05 compared with mice treated with *P. carinii*-depleted preparations; n ≥ 3 for each group at each time point.

RPA analysis demonstrated that the *P. carinii*-stimulated increase in MCP-1 protein secretion was accompanied by a concomitant increase in the steady-state level of MCP-1 mRNA (Fig. 3, B and C). MCP-1 mRNA was elevated at 6 h postinoculation in AECs stimulated with cyst-to-AEC ratios of 0, 0.5, 1.0, and 2.0. In contrast, unstimulated cells exhibited no detectable MCP-1 mRNA expression. steady-state MCP-1 mRNA was not significantly elevated at 12 or 24 h (data not shown). These data demonstrate that the primary AECs respond to *P. carinii* stimulation with MCP-1 production and suggest that primary AECs have the capacity to serve as important inflammatory modulators in response to *P. carinii*.

**JNK signaling is required for *P. carinii*-stimulated MCP-1 production by type II AECs.** Primary type II cells were isolated and cultured as described above, then stimulated with freshly isolated mouse *P. carinii* in the absence or presence of the MAPK inhibitors SB203580, PD98059, and SP600125, or the IKK inhibitor SSA. Unstimulated cells and cells stimulated with *P. carinii*-depleted preparations were used as controls. Experiments were terminated at the 6 h time point to assess MCP-1 levels in the culture supernatants by ELISA. Inhibition of either JNK/SAPK1 with SP600125 or IKK with SSA nearly completely abolished MCP-1 protein secretion by *P. carinii*-stimulated AECs (Fig. 4). In contrast, neither inhibition of p38 MAPK with PD98059 nor p44/42 MAPK with SB203580 had any effect on *P. carinii*-stimulated MCP-1 production (Fig. 4).

Since it has been reported that SP600125 may not have absolute specificity for JNK, additional experiments were performed using L-JNKI1 as a specific, cell-permeable inhibitor of JNK. Similar to SP600125, L-JNKI1 also nearly completely blocked the MCP-1 response of *P. carinii*-stimulated AECs (Fig. 5). Western blot analysis of cell lysates from type II AECs confirmed that *P. carinii* induced increased cellular levels of activated phospho-JNK (Fig. 6). Together, these results demonstrated that *P. carinii* stimulates the JNK signaling pathway in AECs, leading to MCP-1 production.

**TNFR signaling is not required for *P. carinii*-stimulated MCP-1 production by type II AECs.** It has been reported that *P. carinii* β-glucan can induce a low level of TNF by AECs (15). Therefore, to determine whether the MCP-1 response of *P. carinii*-stimulated AECs was dependent upon autocrine stimulation by TNF, we assessed the MCP-1 response of primary AECs isolated from wild-type and TNFR-deficient mice. Importantly, the MCP-1 response to *P. carinii* did not require TNFR signaling. Both TNFR-deficient and wild-type AECs exhibited elevated MCP-1 protein secretion under recombinant TNF-α stimulation (data not shown). Overall, this data demonstrates that both *P. carinii* and TNF are potent inducers of MCP-1 production by AECs. Furthermore, the majority of MCP-1 production by *P. carinii*-stimulated AECs is TNFR-independent, it suggests that in vivo direct *P. carinii*-AEC interactions contribute to MCP-1 production.

**β-Glucan does not induce MCP-1 production in murine primary type II cells.** Prior studies have demonstrated that purified β-glucan is a more potent inducer of epithelial MIP-2 secretion than whole *Pneumocystis* (15). Therefore, the relative potency of β-glucan and whole *Pneumocystis* for the induction of MCP-1 production in primary murine type II cells was...
assessed. As expected, whole *Pneumocystis* induced significant MCP-1 secretion at 24 h. In contrast, AECs treated with 200 μg/ml of β-glucan did not exhibit increased MCP-1 production (Fig. 8). These data suggested that *P. carinii* β-glucan was likely not responsible for the type II AEC MCP-1 response to whole *P. carinii*. Thus, it is likely that multiple mechanisms of *P. carinii*-AEC interaction exist.

**P. carinii-stimulated MIP-2 production by AECs is JNK independent.** Prior studies have demonstrated that *P. carinii* stimulates MIP-2 production by an immortalized murine AEC line through an NF-κB-dependent signaling mechanism (52). Consistent with these previous findings, SSA also blocked MIP-2 production by primary murine type II cells (Fig. 9). In addition, SSA also significantly inhibited MCP-1 production by primary AECs, suggesting that NF-κB is involved (Fig. 4). However, JNK inhibition had no effect on MIP-2 production by type II AECs, suggesting that different signaling requirements exist for *P. carinii*-induced MIP-2 and MCP-1 production by AECs (Fig. 9). NF-κB activation regulates MIP-2 gene expression, while both the JNK and NF-κB pathway are involved in MCP-1 production by AECs in response to *P. carinii*.

**Blockade of epithelial JNK signaling blocks the early MCP-1 response in vivo.** Prior studies have demonstrated that recombinant adenovirus vectors can deliver genes specifically to airway and AECs in vivo (44). Therefore, this technique was utilized to deliver DNA encoding the JBD of JIP-1 to the pulmonary epithelium of mice. This peptide acts as a dominant negative inhibitor of JNK signaling. Either PBS, Ad-JBD, or Ad-LUC was intratracheally instilled into the lungs of mice. Mice instilled with PBS were used as uninfected controls. Forty-eight hours later, the mice were inoculated with 1 × 10⁶ *P. carinii*. At 3 h postinfection, at the time of peak MCP-1 levels in the lung (Fig. 1), the mice were lavaged, and cell-free BAL fluid was collected. Mice pretreated with either PBS or Ad-LUC had similar levels of MCP-1 in the lavage fluid.
In contrast, mice pretreated with Ad-JBD exhibited a dramatic reduction in lavage MCP-1 levels (Fig. 10A; \( P < 0.05 \)). In vitro studies found that JNK inhibition did not affect MIP-2 secretion by type II AECs (Fig. 9). This finding was confirmed in vivo. Mice pretreated with either PBS, Ad-JBD, or Ad-LUC all had similar levels of MIP-2 in the lavage fluid (Fig. 10B), indicating that JNK signaling was not required for epithelial production of MIP-2 in vitro or in vivo.

**DISCUSSION**

Located at the boundary between the environment and internal tissues, lung epithelial cells are an important component of the host defense. AECs function not only as a physical barrier but also as biological sensors for invading microorganisms and their products, by producing cytokines, chemokines and other potent inflammatory mediators. The close interaction of \textit{P. carinii} with the type I pneumocyte is characteristic of PCP (32, 51, 58). In addition, direct and indirect interactions...
between \( P. \textit{carinii} \) and type II pneumocytes have been demonstrated. Importantly, an increasing body of evidence supports a role for type II AECs in the initiation and targeting of pulmonary inflammation through secretion of proinflammatory cytokines and chemokines. While AEC responses are critical for protective host responses against many infectious agents, the immunomodulatory potential of these cells is also likely to directly impact the immune-mediated lung injury characteristic of PCP. Many studies have focused on the ability of \( P. \textit{carinii} \) to directly damage the alveolar epithelium, but few have examined the potential of AECs to function as cell mediators of \( P. \textit{carinii} \)-driven inflammatory responses. In addition, the difficulties associated with isolating and culturing primary type II AECs have dictated that most of the studies involving \( P. \textit{carinii} \)-AEC interactions utilize transformed cell lines. In the present study we have successfully isolated highly purified primary murine type II cells, and cultured them under conditions that promote the maintenance of a type II phenotype. Using our in vitro system, we have demonstrated that \( P. \textit{carinii} \) stimulates MCP-1 production from type II AECs through a JNK and NF-\( \kappa \)B-dependent signaling pathway. This finding was corroborated by in situ hybridization studies demonstrating that \( P. \textit{carinii} \)-stimulated AECs express MCP-1 mRNA in vivo, and also by in vivo studies demonstrating that delivery of a gene encoding the JBD of JIP-1 specifically to the lung epithelium blocked the early MCP-1 response to \( P. \textit{carinii} \).

This study demonstrated that \( P. \textit{carinii} \) activates the JNK signaling pathway, and alters gene expression in murine primary type II AECs. JNK, p38, and ERK1/2 belong to a superfamily of stress-induced MAPK kinases used by cells to transduce extra-cellular signals into a cellular response (14).

![Fig. 5. JNK inhibitor 1, \( \mathbf{L} \)-stereoisomer (\( \mathbf{L} \)-JNKI1), blocks MCP-1 production by \( P. \textit{carinii} \)-stimulated type II AECs. Primary type II cells were isolated and cultured until >90\% confluent as described above. The cells were then treated with a 2:1 cyst-to-AEC ratio of \( P. \textit{carinii} \) in the absence or presence of SP600125, the highly specific JNK inhibitor, \( \mathbf{L} \)-JNKI1, or a control peptide, \( \mathbf{L} \)-TAT. Culture supernatants were collected 6 h or 24 h later for MCP-1 ELISA. Untreated AECs and AECs treated with inhibitors alone were used as controls. AECs were pretreated for 2 h with 10 \( \mu \)M SP600125, or for 1 h with 20 \( \mu \)M \( \mathbf{L} \)-JNKI1 or 20 \( \mu \)M control peptide \( \mathbf{L} \)-TAT. Values are means ± SE. *\( \mathbf{P} < 0.05 \) compared with untreated controls, cells treated with inhibitors alone, and \( P. \textit{carinii} \)-stimulated cells treated with SP600125 or \( \mathbf{L} \)-JNKI1; \( n = 3 \) for each condition. The data are from one of three independent experiments with separate AEC and \( P. \textit{carinii} \) isolations.](http://ajplung.physiology.org/)

![Fig. 6. \( P. \textit{carinii} \) stimulates JNK activation in primary type II AECs. Cells were isolated and cultured as described above. When cells were 80\% confluent, they were treated with \( P. \textit{carinii} \) at a cyst-to-AEC ratio of 2:1. Cells were washed and harvested 30 min, 1 h, and 2 h later. Cell lysates were separated on a 10\% SDS-PAGE gel and electrotransferred to polyvinylidene difluoride membranes. Immunoblotting was performed with anti-phospho-JNK and anti-\( \beta \)-actin primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibody and detection with electrochemical luminescence. One representative experiment out of three is shown.](http://ajplung.physiology.org/)

![Fig. 7. MCP-1 production by \( P. \textit{carinii} \)-stimulated type II AECs is TNFR independent. Primary type II AECs from wild-type (WT) and TNFR-deficient mice were isolated and cultured as described above. AECs were grown to >90\% confluence and then treated with a 2:1 cyst-to-AEC ratio of \( P. \textit{carinii} \). Culture supernatants were collected 6 h later for MCP-1 ELISA. Untreated wild-type and TNFR-deficient AECs were used as controls. Values are means ± SE (\( n = 3 \) for each condition). **\( \mathbf{P} < 0.05 \) compared with both untreated controls and \( P. \textit{carinii} \)-stimulated TNFR\( ^{-/-} \) AECs. *\( \mathbf{P} < 0.05 \) compared with untreated controls. The data are from one of three individual experiments.](http://ajplung.physiology.org/)
induction of proinflammatory responses and, in contrast to ERK1/2 MAPK, have not been strongly associated with proliferation, transformation and differentiation (24). Consistent with previous data that JNK is involved in MCP-1 production from LPS-stimulated primary microglia (49), our data showed that the JNK specific inhibitors SP600125 and L-JNKI1 block MCP-1 production in *P. carinii*-stimulated primary AECs.

While other studies have demonstrated that *P. carinii* can stimulate AEC production of the PMN-specific chemokines IL-8 in humans and MIP-2 in rodents (4, 15, 19, 20), a direct role for neutrophils in damaging the lung has not been shown (27, 47). In contrast, MCP-1 is a CC chemokine with the potential to attract and activate T lymphocytes and macrophages, which are critical to both effective immunity and the generation of immune-mediated lung injury. Our prior studies have found that immune and inflammatory cells are recruited specifically to alveolar sites of *P. carinii* infection in mice, suggesting that the interaction of *P. carinii* with the alveolar epithelium targets the immune response (55, 56). In vivo studies aimed at defining the role of epithelial MCP-1 production in the generation of PCP-related lung injury are currently underway. Together, these data suggest that the *P. carinii*-AEC interaction induces changes in AEC gene expression that contribute to the generation and targeting of the host’s immune response. In a normal host these changes may lead to the generation of effective immunity, while in a compromised host they may contribute to the immunopathogenesis of PCP.

Consistent with our previous work demonstrating that *P. carinii* stimulates NF-κB signaling in AECs, this study found that blockade of NF-κB activation reduced MCP-1 production. Activation of NF-κB regulates the expression of a network of mediators involved in activation of inflammatory cells and their recruitment to extravascular tissues. The finding that *P. carinii* induces MAPK and NF-κB signaling suggests that in addition to the known genes that are altered by the
interaction of *P. carinii* with AECs many other MAPK and NF-κB responsive genes are also regulated. Several groups have shown that there is a crosstalk between the NF-κB and JNK pathways (34). The relevance of the JNK cascade to TNFR-mediated apoptosis is highlighted by the finding that activation of this cascade is controlled by NF-κB (9). The NF-κB-mediated attenuation of JNK signaling is crucial for numerous physiological processes, such as the response of the liver to injury and the survival of cells during an inflammatory reaction, as well as for chronic inflammatory diseases and cancers (34, 37). However, it is still not clear how NF-κB and JNK signaling pathway cooperate in chemokine production during inflammatory responses. Our studies have found that NF-κB signaling is required for both MCP-1 and MIP-2 production by AECs (Figs. 4 and 9) (52). However, JNK inhibition was critical to MCP-1 production by *P. carinii*-stimulated AECs, but had no affect of MIP-2 production. Thus, multiple epithelial signaling pathways that differentially regulate gene expression are activated by the *P. carinii*-AECs interaction.

Although the biology of *P. carinii* has been extensively investigated, the molecular mechanisms by which *P. carinii* induces inflammatory responses in AECs are not clear. TLRs are a group of transmembrane receptors known to be activated by conserved molecular patterns of microbes such as fungi (18, 35). It has been shown TLR2 and TLR4 are known to share a close relationship with AECs (12, 61). Thus, TLRs may mediate *P. carinii* induced activation of JNK and NF-κB signaling pathways in AECs. The alternate glucan receptor, lactosylceramide, has also been shown to mediate NF-κB activation in response to *P. carinii* glucan (15). Therefore, it is possible that the interaction of *P. carinii* with lactosylceramide could also promote AEC inflammatory responses. However, our data indicate that intact *P. carinii*, but not purified glucan, stimulates an MCP-1 response in purified murine type II cells (Fig. 8).

The consequences of NF-κB and JNK activation in the AECs of *P. carinii*-infected animals remain unclear. However, it is plausible that altered AEC gene expression promotes the immune-mediated lung injury associated with PCP (54, 55, 56). We have demonstrated that following the immunological reconstitution of *P. carinii*-infected SCID mice, immune and inflammatory cells are recruited specifically to alveolar regions of infection, and not to uninfected alveoli (54, 56). This finding suggests that the interaction of *P. carinii* with AECs in vivo produces signals that target the inflammatory response to infected alveoli. Therefore, if the recruitment of inflammatory cells to the lung is a direct consequence of NF-κB and JNK-mediated signal transduction in AECs, then blockade of NF-κB and JNK may alleviate some of the damage caused by immune-mediated lung injury. Several NF-κB inhibitors are already used in humans, including SSA, which is used therapeutically to alleviate the inflammatory consequences of inflammatory bowel disease and rheumatoid arthritis (13, 40). Furthermore, it has been well-documented that NF-κB is important for lymphocyte activation and proliferation (3), and lymphocytes are directly involved in PCP-related lung injury (54). MAPK inhibition is also a very active area of research with regards to the treatment of inflammatory disorders (6, 24). Our previous studies have shown that MCP-1 levels correlate with lung injury during PCP. Therefore, the inhibition of MAPK and/or NF-κB signaling may interfere with the generation of an injurious host response to *P. carinii* on several fronts, and could provide a promising therapeutic intervention to lessen immune-mediated respiratory impairment during PCP.

In summary, we have demonstrated that stimulation of primary type II AECs with *P. carinii* induces the production of MCP-1 through JNK- and NF-κB-dependent mechanisms. This finding provides evidence that AECs are potentially important modulators in the lung during PCP. The interaction of *P. carinii* with AECs may promote the development of immune-mediated lung injury through the production of chemokines and subsequent recruitment of immune cells to infected alveoli. More in-depth studies of the role of epithelial MCP-1, JNK, and NF-κB are needed to provide insight into how AECs may affect immunity to *P. carinii* and also the role of AEC in promoting the immune-mediated lung injury observed during PCP.

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