Pneumocystis carinii Activates the NF-κB Signaling Pathway in Alveolar Epithelial Cells

Jing Wang, Francis Gigliotti, Sanjay Maggirwar, Carl Johnston, Jacob N. Finkelstein, and Terry W. Wright

Department of Pediatrics and Department of Microbiology and Immunology, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, New York 14642

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Pneumocystis carinii pneumonia (PCP) is a clinically important infection of immunocompromised patients. Although the interaction of Pneumocystis with the alveolar epithelium has been well documented, very little information regarding the epithelial response to Pneumocystis is currently available. In order to study Pneumocystis-alveolus interactions, a murine cell line derived specifically from an alveolar epithelial cell (AEC) was utilized. The coculture of murine AECs with mouse Pneumocystis induced a dose- and time-dependent release of the CXC chemokine MIP-2. Importantly, the specific removal of Pneumocystis from the preparation, or the pretreatment of AECs with sulphasalazine, a potent and specific inhibitor of NF-κB, nearly completely abrogated the chemokine response to Pneumocystis. Since the murine MIP-2 promoter contains consensus κB binding sequences, the ability of Pneumocystis to stimulate NF-κB signaling in AECs was examined. Pneumocystis stimulation of an AEC line stably transfected with a κB-dependent reporter construct triggered the NF-κB signaling pathway and reporter production. These data were confirmed in gel shift assays, providing direct evidence that Pneumocystis induces the nuclear translocation of the p50/p65 heterodimeric form of NF-κB. Maximal NF-κB activation was dependent upon direct contact with viable Pneumocystis organisms. These data demonstrate that Pneumocystis activates NF-κB signaling in AECs and establish a reporter cell line for studying NF-κB activation in AECs. Given the global regulatory functions of the NF-κB family, these findings suggest that Pneumocystis directly alters AEC gene expression in a manner that promotes pulmonary immune and inflammatory responses.

Pneumocystis carinii is an opportunistic fungal pathogen that causes life-threatening pneumonia in patients suffering from various congenital or acquired immunodeficiencies. These include patients with certain genetic diseases, malignancies requiring chemo- or radiation therapy, and AIDS (10, 54, 56, 58). Despite improved treatments for human immunodeficiency virus infection and improved prophylaxis for P. carinii infection, PCP remains one of the most common AIDS-defining illnesses and is directly responsible for significant morbidity and mortality (12, 16, 17, 58, 62). Although significant progress has been made in our understanding of PCP, the exact mechanisms of lung injury are still largely undetermined. Because recent evidence has demonstrated that lung injury during PCP is not only a consequence of the direct effects of the organism but also a function of the host’s response to infection (22, 23, 47, 48, 63, 67), the identification of signals that promote inflammation and contribute to injury is critical to understanding and treating this disease. Early studies demonstrated that the interaction of P. carinii with the alveolar epithelium is one of the initial events in the infectious process (28, 35, 38, 68). However, very little knowledge exists concerning how the pulmonary epithelium responds to P. carinii infection or what signaling pathways might be involved. An increasing body of evidence supports an immunomodulatory function for alveolar epithelial cells (AECs) (7, 15, 49, 52, 55, 61) and suggests that

\[ \text{the interaction of } P. \text{ carinii} \text{ with alveolar epithelial cells could induce signals that help initiate and target the inflammatory response. Importantly, when } P. \text{ carinii}-\text{infected severe combined immunodeficient (SCID) mice are immunologically reconstituted, inflammatory cells migrate specifically to infected regions of the alveolar epithelium while uninfected regions are not involved (64, 65). This finding suggests that the } P. \text{ carinii}-\text{alveolus interactions occurring at sites of infection produce signals that direct the inflammatory response only to infected alveoli. In addition, it has been reported that a human pulmonary epithelial cell line (A549) secretes the proinflammatory cytokines interleukin-6 (IL-6), IL-8, and MCP-1 following stimulation with rat } P. \text{ carinii} (2, 44) \text{ and that } P. \text{ carinii} \beta\text{-glucan stimulates MIP-2 secretion from primary cultures of rat AECs through a lactosylceramide-mediated mechanism (21). Together, these findings suggest that } P. \text{ carinii} \text{ stimulates changes in AEC gene expression that may function to initiate immunity in immunocompetent hosts and/or contribute to injury in susceptible hosts.} \]

The NF-κB family of transcriptional regulators is involved in the rapid regulation of many immune- and inflammation-associated genes, including cytokines, chemokines, and adhesion molecules (5, 18, 31). NF-κB consists of at least five distinct subunits (p105/p50, RelA, p100/p52, RelB, and c-Rel), and various homo- and heterodimeric combinations of these subunits have DNA binding and transcriptional regulatory functions. NF-κB exists as a preformed complex with its inhibitor, IκB, in the cytoplasm of many cell types, and a variety of stimuli can induce the release of NF-κB from its inhibitor and the translocation of functional NF-κB to the nucleus for gene
regulation. Although kB binding activity was originally identified in lymphocytes, NF-kB-like activity has been reported in many cell types. Stimulation of alveolar macrophages with whole P. carinii or purified cell wall β-glucan induces the nuclear translocation of NF-κB and may be an important signal transduction pathway regulating the macrophage response to P. carinii (29, 69). NF-κB is also an important mediator of signal transduction in epithelial cells, and cytokines, bacterial products, and toxicants have been reported to induce NF-κB activation in pulmonary epithelial cells (6, 20, 46, 51). Importantly, the promoters of all the AEC genes reportedly regulated by P. carinii stimulation contain consensus kB binding sites, suggesting that P. carinii could regulate AEC gene expression through this global transcription factor.

In this study, we hypothesized that the interaction of P. carinii with AECs induces the expression of proinflammatory genes that promote the generation and targeting of the immune response through a pathway that involves the activation of NF-κB signaling. Therefore, to test this hypothesis we developed an in vitro coculture system of mouse P. carinii and a murine AEC line. In addition, an AEC line that is stably transfected with a kB-dependent reporter construct was generated and used in conjunction with gel shift assays to directly test the ability of P. carinii to stimulate NF-κB activation in AECs.

MATERIALS AND METHODS

Cell culture. The murine AEC line MLE-15 was derived from a pulmonary adenocarcinoma generated in a transgenic mouse expressing the simian virus 40 large T antigen under transcriptional control of the human surfactant protein C (SPC) promoter (24, 60). MLE-15 cells maintain morphological and gene expression characteristics of type II AECs, including a typical polygonal morphological appearance, secretion of mRNA, and production of SPC proprotein (24, 60). However, it has also been demonstrated that these cells express aquaporin-5, suggesting that they may also have some type I cell characteristics (3). MLE-15 cells were maintained at 37°C, 10% CO2 in Dulbecco’s modified Eagle’s medium nutrient mixture F-12 Ham (GIBCO, NY) supplemented with 10% FBS, 0.5% glutamine, 20 mM HEPES buffer, 1% penicillin, and streptomycin. Cells were grown to confluency, washed with Hank’s balanced salt solution, 0.5% glutamine, 20 mM HEPES buffer, 1% penicillin, and streptomycin. Cells were grown to confluency, washed with Hank’s balanced salt solution, 0.5% glutamine, 20 mM HEPES buffer, 1% penicillin, and streptomycin. Cells were grown to confluency, washed with Hank’s balanced salt solution, 0.5% glutamine, 20 mM HEPES buffer, 1% penicillin, and streptomycin.

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incubated at 37°C, 6% CO₂ for 6 h, and then the medium was replaced with regular growth medium. Eighteen hours later, the cells were stimulated as described. In separate experiments, transfection of MLE-15 cells with a green fluorescent protein (GFP)-expressing construct demonstrated a transfection efficiency of >75% for these cells. MIP-2 ELISA. Culture supernatants from stimulated MLE-15 cells were collected, centrifuged at 12,000 × g for 5 min to remove debris, and then stored at −80°C. MIP-2 concentrations were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). Luciferase assays. Cells were assayed for luciferase activity using the commercially available luciferase assay system from Promega (Madison, WI). Briefly, MLE-PLUC/cb cells were grown to confluency in 24-well tissue culture dishes and then stimulated for 6 h with the indicated amounts of freshly isolated P. carinii or with P. carinii-depleted preparations. The cells were then washed twice with 1 ml of PBS and dissolved in 60 μl of passive lysis buffer. The lysed cells were centrifuged at 12,000 × g for 15 seconds at room temperature, and 20 μl of the cell lysate supernatant was mixed with 100 μl of the luciferase assay substrate in a luminometer tube. The light intensity was determined with a luminometer. Data were expressed as fold increase over nonstimulated cells transfected with the same construct. EMSAs. Nuclear extracts were prepared from stimulated and unstimulated MLE-15 cells as described previously (50). Briefly, cells were washed in ice-cold PBS, resuspended in 50 μl of ice-cold lysis buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.4% NP-40, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 1× protease/phosphatase inhibitor cocktail [Sigma]), and then placed on ice for 2 min. Lysates were centrifuged at 20,000 × g for 5 min, and the supernatant with cytoplasmic proteins was removed. The pellets were resuspended in 40 μl of cold hypotonic buffer (20 mM HEPES [pH 7.6], 0.4 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1× protease/phosphatase inhibitor cocktail [Sigma]), incubated for 10 min at 4°C shaking, and then centrifuged for 1 min. Nuclear protein-containing supernatants were removed, quantified with the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA), and then stored at −80°C. For electrophoretic mobility shift assays (EMSA), 5 μg of nuclear extract was mixed with a radiolabeled, double-stranded oligonucleotide probe encoding a consensus κB binding site (5′-CAACGCGA GGGGAATCCCTCCTCCTCCT-3′). The reaction was carried out in EMSA reaction buffer (12 mM HEPES [pH 7.9], 100 mM NaCl, 0.25 mM EDTA, 1 mM DTT, and 1 mM PMSF) at room temperature for 10 min, followed by resolution of the protein-DNA complexes on non-denaturing 6% polyacrylamide gels. The gels were dried and placed against XAR film (Kodak, Rochester, NY) to detect the shifted bands. The nuclei were exposed and the vasculature was perfused with sterile PBS, and then stored at 4°C and resuspended in DMEM supplemented with 25 mM HEPES and 10% FBS. The recovered cells were then incubated with biotinylated anti-CD32/CD16 and anti-CD45 antibodies for 30 min at 37°C. The cells were collected by centrifugation and resuspended in DMEM with 25 mM HEPES and 10% FBS. The cells were placed in 60-mm tissue culture dishes for 4 to 6 h to remove any mesenchymal cells. Nonadherent cells were recovered and enumerated, and viability was assessed. The purity of the type II cell preparation was assessed by modified Papanicolaou staining and intracellular staining for pro-SPC. Using this procedure, we typically recover >95% viability and >75% purity based on SPC expression. For P. carinii stimulated primary AECs, 10% freshly isolated AECs were incubated with or without P. carinii (P. carinii cyst/AEC ratio of 3:1) for 2 h in a 12-well tissue culture dish. The cells were recovered by centrifugation at 130 × g for 8 min at 4°C, and nuclear extracts were isolated as described above. Statistical analysis. One-way and two-way analyses of variance (ANOVA) were performed with the SigmaStat 2.0 software (Jandel, San Rafael, Calif.) to determine the significance of variations in chemokine protein and mRNA levels in AEC cultures. The Student-Newman-Keuls method was used for all pairwise multiple comparisons of experimental groups. RESULTS P. carinii induces MIP-2 secretion from murine AECs. Confluent monolayers of the murine lung epithelial cell line MLE-15 were inoculated with freshly isolated murine P. carinii at cyst to AEC ratios of 0.0, 0.1, 0.2, 0.5, 1.0, 2.0, and 4.0. At 2, 6, 24, and 48 h postinoculation, the culture supernatants were removed and assayed for MIP-2 levels by ELISA. Stimulation of AECs with P. carinii induced a dose- and time-dependent increase in MIP-2 secretion (Fig. 1A). MIP-2 concentrations were significantly elevated in AECs treated with 2.0 and 4.0 ratios of P. carinii by 24 h postinoculation compared to unstimulated cells (P ≤ 0.002). At 24 h, MIP-2 secretion from AECs cocultured with 0.5, 1.0, 2.0, and 4.0 ratios of P. carinii were significantly elevated compared to unstimulated controls (P ≤ 0.003), and by 48 h the MIP-2 concentrations in all P. carinii-stimulated cultures were significantly elevated (P ≤ 0.018). To further support the significance of these data, the mean MIP-2 values for the 0.0, 0.5, 1.0, 2.0, and 4.0 ratios from eight independent 24-h experiments were pooled (n = 18 for each ratio), and two-way ANOVA was performed using the Student-Newman-Keuls method for pairwise multiple comparisons. The mean MIP-2 value ± 1 standard error measurement for AECs cocultured with a 4:1 ratio of P. carinii to AEC was 223 ± 14 pg/ml. This value was statistically different from the mean MIP-2 values from AECs treated with 2:1 (109 ± 15 pg/ml; P < 0.001), 1:1 (40 ± 16 pg/ml; P < 0.001), and 0.5:1 (12 ± 16 pg/ml; P < 0.001) ratios of P. carinii, as well as untreated AECs (2 ± 14 pg/ml; P < 0.001). The mean MIP-2 value for the
PCR, demonstrated that this method removed cysts before and after *P. carinii* studies of the of rat AECs, and suggest that these cells are useful for in vitro stimulation with MIP-2 production, similar to primary cultures demonstrating that the *MLE-15* cell line responds to (Fig. 3A and B) and not a copurified contaminant. These data showing that the AECs are responding to the *P. carinii* to AEC, and RNA was isolated 2, 6, 12, and 24 h later. The steady-state MIP-2 and L32 mRNA levels were then determined by RPA (B). The experiment shown is a representative of at least three separate experiments.

Since there is no reliable in vitro culture system for mouse *P. carinii*, the organisms used in these experiments were purified from the lungs of infected SCID mice. Therefore, to ensure that MIP-2 secretion by AECs was a response to *P. carinii* and not to potentially copurified contaminants, such as lipopolysaccharide (LPS) or mouse lung proteins, an antibody and magnetic bead-based technique for the specific removal of *P. carinii* from the preparation was employed. The purified preparation was depleted of *P. carinii* by using magnetic beads coated with a pool of anti- *P. carinii* antibodies. Enumeration of cysts before and after *P. carinii* depletion, as well as real-time PCR, demonstrated that this method removed >96% of the *P. carinii* organisms (Fig. 2A, B, and C). Equal amounts of *P. carinii* and of depleted preparations were used to stimulate AECs. As expected, stimulation of AECs with a 3:1 ratio of *P. carinii* to epithelial cells caused a significant increase in MIP-2 mRNA expression and a significant release of MIP-2 protein (Fig. 3A and B). In contrast, antibody-mediated removal of the *P. carinii* from the preparation abolished the MIP-2 response, demonstrating that the AECs are responding to the *P. carinii* (Fig. 3A and B) and not a copurified contaminant. These data demonstrate that the *MLE-15* cell line responds to *P. carinii* stimulation with MIP-2 production, similar to primary cultures of rat AECs, and suggest that these cells are useful for in vitro studies of the *P. carinii*-epithelium interaction.

**SAA blocks *P. carinii*-stimulated MIP-2 secretion.** The murine MIP-2 promoter contains consensus κB binding sites that are important for the inducible expression of this gene (26). Therefore, to determine whether *P. carinii*-stimulated activation of NF-κB signaling was involved in AEC chemokine production, the NF-κB inhibitor sulfasalazine (SAA) was utilized. Recent studies have demonstrated that SAA is a potent and specific inhibitor of NF-κB that directly inhibits IKK-α and -β (57, 59). Preliminary studies found that a concentration of 2 mM SAA had no effect on the viability of MLE-15 cells compared to untreated cells but nearly completely abrogated tumor necrosis factor (TNF)-stimulated NF-κB activation (data not shown). When MLE-15 cells were pretreated with SAA for 2 h prior to *P. carinii* stimulation, inducible MIP-2 synthesis was almost completely blocked (Fig. 4) (*P < 0.05*). In contrast, 2 mM SAA treatment in the absence of *P. carinii* stimulation had no demonstrable effect on the AEC line. Together, these data indicate that *P. carinii*-stimulated MIP-2 production by AECs could be inhibited by SAA and suggested that the interaction of *P. carinii* with AECs induces activation of the important transcription factor NF-κB.

**P. carinii induces κB-dependent reporter gene expression in AECs.** To study NF-κB-dependent gene expression in *P. carinii*-stimulated murine AECs, MLE-15 cells were stably transfected with a κB-dependent luciferase reporter construct derived from the human IL-8 promoter (39, 40). In order to characterize the NF-κB response in these cells and to determine if they were suitable for use as reporter cells, they were stimulated with recombinant murine TNF, a known inducer of NF-κB activation and signaling. As expected, TNF induced the NF-κB-dependent production of luciferase in the MLE-pLUC/κB cell line (Fig. 5A). Importantly, luciferase production by TNF-stimulated cells was blocked by transient transfection of cells with a constitutively expressed dominant
negative form of IκB but unaffected by transient transfection
with parent vector (Fig. 5A). Additionally, the NF-κB inhibitor
SSA also blocked luciferase production by TNF-stimulated
MLE-pLUC/κB cells (Fig. 5B). These data demonstrated that
MLE-pLUC/κB cells could serve as a reporter cell line to study
NF-κB activation and signaling in response to P. carinii.

To determine whether P. carinii is capable of inducing NF-
κB-dependent gene expression in murine AECs, the MLE-
pLUC/κB cell line was utilized. P. carinii stimulation resulted
in a dose-dependent increase in NF-κB-dependent luciferase
activity. MLE-pLUC/κB cells stimulated with P. carinii to AEC
ratios of 0.5, 1.0, 2.0, and 3.0 demonstrated respective 2-fold,
3-fold, 4.25-fold, and 4.5-fold increases in luciferase activity
compared to unstimulated cells (Fig. 5C) (P < 0.05 for all
groups). Similar to the data obtained for inducible MIP-2 pro-
duction, luciferase induction was dependent upon the presence
of P. carinii, since luciferase activity in MLE-pLUC/κB cells
were performed on nuclear extracts isolated from MLE-15 cell cultures
treated with preparations depleted of P. carinii was not statis-
tically different from that of untreated cells (Fig. 5C). Finally,
the NF-κB inhibitor SSA also blocked luciferase reporter ex-
pression by P. carinii-stimulated cells (Fig. 5C). Together,
these data suggested that P. carinii induces NF-κB activation
and κB-dependent gene expression in AECs.

P. carinii stimulates κB binding activity in the nucleus of
AECs. To directly demonstrate that P. carinii induces κB bind-
ing activity in the nucleus of AECs, gel shift assays were per-
fomed on nuclear extracts isolated from MLE-15 cell cultures
at 0, 1, 1.5, 2, 2.5, 3, 4, and 6 h postinoculation. There was
low-level baseline κB binding activity in the nucleus of un-
treated AEC cultures. In contrast, P. carinii treatment induced
a significant increase in a slower-migrating κB binding activity
in the nucleus. Elevated κB binding activity was detected by 1 h
postinoculation, peaked at 1 to 2 h, and was declining by 6 h
(Fig. 6). To identify the subunit composition of the induced κB
binding activity, supershift assays were performed with control
antibodies and antibodies specific for the p50, p65, and RelB
subunits of NF-κB. These assays demonstrated that the main
P. carinii-stimulated κB binding activity was composed of p50/
p65 heterodimers, while the faster-migrating activity present at
low levels in the untreated AECs consisted of p50/p50 ho-
modimers (Fig. 6B).

Direct contact of viable P. carinii with AECs is critical for
maximal activation of NF-κB. In order to further characterize
the nature of NF-κB activation in treated AECs, the require-
ment of direct P. carinii-AEC contact, and of P. carinii viability,
was examined. EMSAs were performed in triplicate on stimu-
lated and unstimulated cells (Fig. 7A), and the intensity of
each band corresponding to the p50/p65 heterodimer was
quantified using a PhosphorImager (Fig. 7B). As expected,
unstimulated cells exhibited little κB binding activity (Fig. 7,
lanes 1), while treatment of AECs with P. carinii induced
significant NF-κB nuclear translocation (Fig. 7, lanes 2) (P <
0.05). Specific antibody-magnetic bead removal of P. carinii
from the preparation demonstrated that inducible p50/p65 nu-
clear translocation is dependent upon the presence of P. carinii
organisms (Fig. 7, lanes 3). In addition, to determine whether
direct P. carinii-AEC contact was required for NF-κB activa-
tion, experiments were performed in which the P. carinii orga-
isms were separated from the AECs by Transwell inserts.
Although soluble mediators were freely diffusible between the
AEC and P. carinii compartment, the lack of direct contact
prevented NF-κB activation (Fig. 7, lanes 4). Finally, the re-
quirement of P. carinii viability for the NF-κB response in
AECs was assessed. Five cycles of freezing and thawing P.
carinii in serum-free medium reduced the viability of the iso-
lated P. carinii by approximately 90% based on incorporation
of the vital dye 2′,7′-bis-(2-carboxyethyl)-5′-(and-6)-carbox-
fluorescein (data not shown). When the killed P. carinii was
used to stimulate AECs, NF-κB activation was significantly
reduced compared to freshly isolated live P. carinii (Fig. 7,
lanes 5). These data demonstrated that NF-κB activation in P.
carinii-stimulated AECs requires the direct contact of viable P.
carinii with the AEC monolayer.
NF-κB activation is associated with increased IκBα expression in *P. carinii*-stimulated AECs. The activation and nuclear translocation of NF-κB involves the degradation of its cytoplasmic inhibitor, IκBα. In order to control stimulus-mediated alterations in cellular gene expression, the activation of NF-κB induces the transcription and resynthesis of IκBα. As additional evidence confirming the induction of IκBα-dependent gene expression in *P. carinii*-stimulated AECs, steady-state levels of IκBα mRNA were examined. When AECs were treated with *P. carinii*, a greater-than-10-fold increase in the steady-state level of IκBα compared to unstimulated cells was observed at 2 and 6 h postinfection (Fig. 8) \( P < 0.05 \). These data supported our conclusion that many NF-κB-responsive genes are altered in *P. carinii*-stimulated AECs and suggested that resynthesis of IκBα is likely an attempt to control the NF-κB response of AECs to *P. carinii*.

*P. carinii* stimulates κB binding activity in the nucleus of primary murine AECs. The data presented thus far demonstrated that *P. carinii* activates NF-κB signaling in a murine AEC line. Therefore, to validate that primary AECs respond to *P. carinii* in a similar fashion to the AEC line, EMSA was performed on nuclear extracts from primary type II cells following *P. carinii* stimulation. Nuclear extracts from unstimulated primary type II cells contained no detectable κB binding activity as assessed by EMSA. In contrast, κB binding activity was detected in type II cells following 2 h of coculture with *P. carinii* at a *P. carinii/AEC* ratio of 3:1 (Fig. 9). Furthermore, the major form of NF-κB induced in primary cells is consistent with the migration pattern of p50/p65 heterodimers. These data support the validity of the results obtained using the MLE-15 cell line and strengthen our data suggesting that *P. carinii* induces NF-κB signaling in AECs.

**DISCUSSION**

Given that *P. carinii* is acquired by aerosol spread, the interaction of *P. carinii* with the alveolar epithelium is likely the initial step in the infectious process leading to PCP. Our data and reports from other groups suggest that this interaction induces changes in AEC gene expression that may 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...
To assess the effect of pretreated with 2 mM SSA for 2 h were stimulated with 10 ng/ml TNF (A). In a separate experiment, untreated cells and cells transiently transfected with control vector were stimulated with 5 ng/ml TNF (B). To assess the effect of P. carinii stimulation on reporter expression, MLE-pLUC/κB cells were stimulated with P. carinii or equivalent amounts of P. carinii-depleted preps. In addition, cells pretreated with 2 mM SSA were stimulated with P. carinii (C). For all experiments the cells were recovered and assayed for luciferase activity after 6 h of stimulation. Untreated cells were used as controls in all experiments, and the values are expressed as the mean fold change in luciferase activity ± 1 standard error measurement (n = 3, except for the “TNF + vector” condition in panel A [n = 2]). *, P < 0.05 compared to unstimulated cells, cells stimulated with equivalent amounts of P. carinii-depleted preps, and cells pretreated with 2 mM SSA and then stimulated with equivalent amounts of P. carinii.

贡献到免疫介导的肺损伤。这些研究呈现了最近的报告，表明P. carinii刺激了MIE-15细胞系响应P. carinii，支持我们的 contention that the MIE-15 cell line responds to P. carinii. 预先接触可用的乳糖脂在AECs中刺激NF-κB信号的诱导，促进NF-κB活化。此外，它表明NF-κB依赖的信号传导途径在AECs中的作用是关键的在P. carinii诱导NF-κB依赖的基因表达的改变。虽然没有我们还没有确定特定的分子相互作用的需求，为了刺激NF-κB的途径，我们已经证明了直接接触可用的P. carinii. 是必要的。在某些情况下的相互作用之间P. carinii和AECs的相互作用已经发生。直接与P. carinii. 引起AECs为契机的β-glucan，因为它已被报道。Hahn等. 报道了纯化的P. carinii. β-glucan诱导的MIP-2的分泌，AECs通过乳糖脂配体介导的机制的。然而，他们没有评估的NF-κB对这个过程的贡献。虽然我们的研究没有改变乳糖脂在AEC NF-κB的活化，因为我们没有研究可用的P. carinii. 组织被报道需要最大NF-κB的活化。我们的研究发现β-glucan不是唯一能通过AECs对P. carinii的响应的β-glucan，这阻碍了其他机制的P. carinii-AEC相互作用的存在。然而，它不排除β-glucan单独的可能的作用，因为缺乏针对P. carinii. 的反应P. carinii. 组织是重要的。这个主要的技术差异使它不可能直接
compare the results of these two studies. Furthermore, although the studies by Hahn et al. used defined quantities of β-glucan, it is difficult to normalize this for a specific number of *P. carinii* organisms. Therefore, it is highly probable that Hahn et al. used an amount of free β-glucan that greatly exceeds the amount contained in the *P. carinii* organisms we used to stimulate the AECs. The establishment of this in vitro model will allow further studies to examine the critical interactions that lead to NF-κB activation.

The proinflammatory responses of alveolar macrophages (AMs) to *P. carinii* stimulation has also been well documented, and *P. carinii* β-glucan plays an important role in the AM response (29, 53). However, our data suggest that AECs and AMs may recognize *P. carinii* via distinct receptor-ligand interactions. Although *P. carinii* β-glucan is quite efficient in activating NF-κB in macrophages, the data presented herein suggest that the AEC response to *P. carinii* may not be exclusively to β-glucan. There are many documented differences in the response of AECs and macrophages to a variety of stimuli. For example, macrophages respond much more vigorously to LPS than do cells of epithelial origin, probably owing to differential receptor expression. We believe that this may also be the situation with macrophage and AEC responses to *P. carinii* β-glucan. A recent report has demonstrated that a newly identified β-glucan receptor, Dectin-1, is critical to the macrophage response to *P. carinii* (53). However, Dectin-1 expression is specific to phagocytic cells, and there is no evidence that Dectin-1 is expressed on AECs. Furthermore, it has been reported that the AEC response to β-glucan is mediated by lactosylceramide (21). Therefore, our data are consistent with the existence of distinct pathogen recognition mechanisms utilized by AECs and AMs.

While the main focus of the current study was the direct

FIG. 6. *P. carinii* stimulation induces nuclear translocation of the p50/p65 heterodimeric form of NF-κB. MLE-15 cells were stimulated with a 3:1 ratio of *P. carinii* to AEC, and EMSAs were performed on nuclear extracts taken from the cells at 1, 1.5, 2, 2.5, 3, 4, and 6 h after inoculation (lanes 1 to 7, respectively) (A). Unbound probe (lane P) and unstimulated cells (lane C) were used as controls. The gel is representative of at least three independent experiments. (B) Representative supershift assay on the nuclear extract from MLE-15 cells stimulated with a 3:1 ratio of *P. carinii* for 2 h. Antibodies specific for p50, the C-terminal portion of p65 (anti-p65c), the N-terminal portion of p65 (anti-p65n), and RelB were used to determine the composition of the *P. carinii*-inducible form of NF-κB.

FIG. 7. Specificity of NF-κB activation in *P. carinii*-stimulated AECs. Nuclear extracts were isolated from MLE-15 cells stimulated for 2 h with equal amounts (a 3:1 ratio) of either *P. carinii* (lanes 2), *P. carinii*-depleted preps (lanes 3), *P. carinii* separated from the AECs by Transwell inserts (lanes 4), or killed *P. carinii* (lanes 5). Unstimulated cells were used as controls. EMSAs were performed on nuclear extracts with a labeled κB probe (A). The dried gel was then used to expose PhosphorImager screens, and the relative intensity of each p50/p65 band was quantified using ImageQuant software (B). Values are means ± 1 standard error measurements. *, P < 0.05 compared to untreated cells.
able TNF is present in the lungs of cells (6, 20). Prior studies have demonstrated that little detect-
that TNF induces NF-
B activation in pulmonary epithelial P. carinii, it is also well documented
B activation in AECs proximal to
also contribute to NF-
B activation in the AECs of
P. carinii-infected SCID mice, immune and
lung injury. We have also demonstrated that TNF-induced NF-
B activation in AECs is blocked by the potent and specific
NF-
B inhibitors have been developed, and this signaling pathway is becoming an important target for antiinflammatory therapeutics to alleviate many different dis-
B in AECs, it has also
been demonstrated that P. carinii and P. carinii β-glucan stim-
ulate NF-κB in AMs (29, 69). Furthermore, it has been well
documented that NF-κB is important for lymphocyte activation and proliferation, and a direct role for lymphocytes in PcP-related lung injury has been demonstrated (63, 66). There-
fore, the inhibition of 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. Several classes of NF-κB inhibitors have been developed, and this signaling pathway is becoming an important target for antiinflammatory therapeutics to alleviate many different dis-
eases (11). For example, SSA is used therapeutically in humans to alleviate the symptoms of inflammatory bowel disease and rheumatoid arthritis (4, 13, 14, 25, 27). Therefore, further studies should be performed to determine whether appropriate anti-inflammatory treatments, including NF-κB inhibitors, could provide a valuable therapeutic adjunct for PcP treat-
ment.

In vitro studies of P. carinii-AEC interactions are made significantly more difficult because of the inability to grow and culture significant quantities of P. carinii in vitro. Instead, or-
ganisms purified from the lungs of SCID mice must be used for these studies. While significant improvement has been made to the purification protocol, there always exists the possibility that a non-P. carinii contaminant will be copurified and contribute to the observed AEC response. Therefore, in addition to rou-
tinely testing P. carinii preparations for endotoxin contamina-
tion and the presence of other microorganisms, we have de-
volved a MAb-linked magnetic bead method by which greater than 96% of the P. carinii is removed from the preparations in a specific manner (Fig. 2). The remaining non-P. carinii con-
taminants (including LPS, bioactive inflammatory particles, or

activation of NF-κB by P. carinii, it is also well documented that TNF induces NF-κB activation in pulmonary epithelial cells (6, 20). Prior studies have demonstrated that little detect-
able TNF is present in the lungs of P. carinii-infected SCID mice (8, 64). However, following immune reconstitution a large spike in TNF mRNA levels in the lung and protein levels in bronchoalveolar lavage fluid is observed (64). Importantly, increased TNF production coincides with immune-mediated pulmonary dysfunction (63). Therefore, in addition to direct stimulation of NF-κB by P. carinii, local TNF production may also contribute to NF-κB activation in AECs proximal to P. carinii infection and contribute to inflammatory cell targeting and lung injury. We have also demonstrated that TNF-induced NF-κB activation in AECs is blocked by the potent and specific inhibitor SSA (57, 59) (Fig. 5). While further in vivo studies are required to determine the contribution of NF-κB signaling in AECs to PcP-related immune-mediated lung injury, these data do suggest that NF-κB inhibitors could have some beneficial effects for individuals suffering from PcP.

The consequences of NF-κB activation in the AECs of P. carinii-infected animals remain unclear. However, it is plausible that altered AEC gene expression could promote the immune-mediated lung injury associated with PcP (63, 66, 67). We have demonstrated that following the immunological re-
constitution of P. carinii-infected SCID mice, immune and inflammatory cells are recruited specifically to alveolar regions of infection and not to uninfected alveoli (64, 65). This finding suggests that the interaction of P. carinii with AECs in vivo produces signals that direct the inflammatory response to in-
fected alveoli. Therefore, if the recruitment of inflammatory cells to the lung were a direct consequence of NF-κB-mediated signal transduction in AECs, then blockade of NF-κB might alleviate the lung injury resulting from immune-mediated mechanisms. While the present study has focused on the P. carinii-stimulated activation of NF-κB in AECs, it has also

FIG. 8. Expression of IκBα mRNA in P. carinii-stimulated AECs. Total RNA was extracted from MLE-15 cells stimulated with 1.0, 2.0, and 4.0 ratios of P. carinii to AEC for the indicated times, and IκBα mRNA levels were assessed by RPA. Values are means ± 1 standard error measurements (n = 3). * P < 0.05 compared to untreated cells.

FIG. 9. P. carinii stimulation induces nuclear translocation of NF-κB in primary murine AECs. Primary type II cells were stimulated with a 3:1 ratio of P. carinii to AECs, and EMSAs were performed on nuclear extracts taken from the cells at 2 h after inoculation (C). Unbound probe (A) and unstimulated primary cells (B) were used as controls. The gel is representative of at least three independent experiments, and the samples shown were performed in duplicate.
ECM breakdown products) are then used to stimulate AEC cultures. Using this depletion technique it was demonstrated that the removal of P. carinii from the preparation nearly completely abolished both P. carinii-induced NF-κB activation and MIP-2 secretion. We have also validated this technique in vivo by demonstrating that cytokine responses to intratracheally inoculated P. carinii are blocked when P. carinii is first depleted in this manner (data not shown). Thus, we are confident in concluding that P. carinii is directly responsible for the observed alterations in AEC gene expression.

Although the dose-dependent MIP-2 response of P. carinii-stimulated AECs exhibited a very small degree of variability in each individual experiment, it must be noted that we often observed greater variability among replicate samples from independent experiments (Fig. 1, 3, and 4). The most likely explanation for this observation is variation in the enumeration and/or viability of individual P. carinii preparations. To quickly enumerate the P. carinii organisms isolated from SCID mouse lungs, we performed cyst counts on silver-stained slides. While this provides an easy, rapid method for determining a relative count, it does not give an absolute number of all P. carinii organisms, as our isolation procedure recovers both cyst and trophozoite forms. Importantly, we have found that the cyst/trophozoite ratio can vary from 1:10 to 1:15 in the lungs of SCID mice, depending upon the level of infection. Therefore, it is possible that one experiment may actually use more total P. carinii (or more trophozoites) than another experiment because we have normalized the inocula to cyst counts only. These differences would likely produce the observed variation, especially if there were a difference in the capacity of a cyst or trophozoite to interact with an AEC. However, in our experience this variation has not been so great that it creates an obstacle to our data interpretation, since we typically use a range of P. carinii inocula. Furthermore, to ensure that the MIP-2 results were statistically relevant, the 24-h data from eight independent experiments, utilizing eight different P. carinii preparations and four different P. carinii doses, were pooled and analyzed by two-way ANOVA using the Student-Newman-Keuls method for pairwise multiple comparisons. This analysis demonstrated that the difference in mean MIP-2 values of AECs treated with different P. carinii ratios was statistically significant when allowing for variation in the individual P. carinii preparations (P < 0.001) and also confirmed the dose dependence of the MIP-2 response at P. carinii-to-AEC ratios of 4.0 and 2.0.

The temporal pattern of NF-κB activation, IκB resynthesis, and gene regulation has been extensively studied in several model systems. However, in the current study we did not specifically examine transcriptional regulation in P. carinii-stimulated AECs. While the elevated levels of MIP-2 mRNA and protein at 24 h (Fig. 1) may seem counterintuitive to our data showing that κB binding activity is decreasing and IκB expression is increasing at 6 h, there are several possible explanations for this. First, the amount of κB binding activity in the nucleus at 6 h is decreasing but still elevated above controls. Thus, NF-κB-dependent mRNA and protein production and accumulation could continue to later time points. A second possibility is that following stimulation, MIP-2 mRNA exhibits increased stability and remains present even at 24 h. Since we are measuring steady-state levels of MIP-2 mRNA, and not specific transcriptional activity of the MIP-2 promoter (which is directly controlled by NF-κB), the persistence of MIP-2 mRNA could continue in the absence of further NF-κB-dependent transcription. Finally, this phenomenon could be explained by the continued presence of P. carinii in the culture. Because there is no method to easily remove adherent P. carinii from the cultured AECs, the stimulus remains throughout the experimental time course. Thus, we may be observing a second wave of NF-κB activation after the first wave has been blunted by NF-κB-stimulated IκB resynthesis.

Although we report data obtained using a murine AEC line, it is important to note that similar results were obtained using another pulmonary epithelial cell line (2), and also primary rat AECs (21). In addition, PpP-related MIP-2 production in rodents and IL-8 production in humans have been demonstrated in vivo. Thus, since our data are in agreement with both pulmonary epithelial cell line and in vivo experiments, we are optimistic that this will provide a valuable in vitro model for studying the interaction of P. carinii with AECs. In addition, this cell line can be genetically modified to perform signal transduction studies that may not be possible in vivo, or with primary epithelial cells. For example, we were able to construct an NF-κB reporter cell line by stable transfection of MLE-15 cells with a κB-dependent reporter construct. This cell line should prove valuable for studies evaluating the mechanism of P. carinii-induced NF-κB signaling.

The data presented herein demonstrate that direct interaction with P. carinii induces NF-κB activation in type II-like AECs. In contrast, the majority of in vivo studies have described the physical association between P. carinii and type I cells (28, 35, 38, 68), with less common reports of binding to type II cells (35). However, we believe that the in vivo interaction of P. carinii with the type II cell may be unrepresented. Since the vast majority of the lung surface area is covered by type I cells, the structure of the lung makes it much more likely that in vivo studies would identify P. carinii in direct contact with these cells. However, since the structure of the alveoli is such that type I cells are in close proximity to type II cells, it is likely that ample opportunity exists for P. carinii to contact the apical surface of the type II cell. Furthermore, activation of signal transduction pathways may not necessarily require firm attachment to the type II cell, and direct contact with P. carinii may induce NF-κB activation without the firm adherence characteristic of the P. carinii-type I cell interaction. In support of our contention that P. carinii-type II interactions may be important during in vivo infection, several other groups have reported that cells with type II-like characteristics bind and respond to P. carinii (2, 21, 33, 34, 42, 44). Alternatively, it is possible that, because type II cells are precursors to type I cells, the MLE-15 and other cell lines can take on some characteristics of type I cells in culture, making them more responsive to direct interaction with P. carinii. More definitive studies will be required to determine the differential responses of type I and type II cells to P. carinii.

In summary, we have demonstrated that stimulation of AECs with viable P. carinii activates the NF-κB signaling pathway and alters chemokine gene expression. The P. carinii-stimulated activation and nuclear translocation of the classic p50/p65 form of NF-κB suggests that in addition to MIP-2, the expression of other immune- and inflammatory-related genes.
may also be altered in AECs following \textit{P. carinii} stimulation. More in-depth studies of the role of NF-kB in AECs may provide insight into how AECs affect immunity to \textit{P. carinii} and also the role of AECs in promoting and maintaining the immune-mediated lung injury observed during PCP.

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