Selective Ablation of Lung Epithelial IKK2 Impairs Pulmonary Th17 Responses and Delays the Clearance of Pneumocystis

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Selective Ablation of Lung Epithelial IKK2 Impairs Pulmonary Th17 Responses and Delays the Clearance of Pneumocystis

Nelissa Perez-Nazario,* Javier Rangel-Moreno, † Michael A. O’Reilly, ‡ Manolis Pasparakis, § Francis Gigliotti,*,# and Terry W. Wright*,#

Pneumocystis is an atypical fungal pathogen that causes severe, often fatal pneumonia in immunocompromised patients. Healthy humans and animals also encounter this pathogen, but they generate a protective CD4+ T cell–dependent immune response that clears the pathogen with little evidence of disease. Pneumocystis organisms attach tightly to respiratory epithelial cells, and in vitro studies have demonstrated that this interaction triggers NF-kB–dependent epithelial cell responses. However, the contribution of respiratory epithelial cells to the normal host response to Pneumocystis remains unknown. IκB kinase 2 (IKK2) is the upstream kinase that is critical for inducible NF-κB activation. To determine whether IKK2-dependent lung epithelial cell (LEC) responses contribute to the anti-Pneumocystis immune response in vivo, transgenic mice with LEC-specific deletion of IKK2 (IKK2<sup>−/−</sup>LECs) were generated. Compared to wild-type mice, IKK2<sup>−/−</sup>LECs mice exhibited a delayed onset of Th17 and B cell responses in the lung and delayed fungal clearance. Importantly, delayed Pneumocystis clearance in IKK2<sup>−/−</sup>LECs mice was associated with an exacerbated immune response, impaired pulmonary function, and altered lung histology. These data demonstrate that IKK2-dependent LEC responses are important regulators of pulmonary adaptive immune responses and are required for optimal host defense against Pneumocystis infection. LECs likely set the threshold for initiation of the pulmonary immune response and serve to prevent exacerbated lung inflammation by promoting the rapid control of respiratory fungal infection. The Journal of Immunology, 2013, 191: 000–000.

Pneumocystis is an opportunistic fungal pathogen with specific tropism for the mammalian lung. Pneumocystis organisms recovered from different mammalian hosts are genetically distinct, and attempts at cross-species transmission have not been successful (1–3). Furthermore, the requirements for Pneumocystis growth in vitro have not been determined, making the study of life cycle and biology a significant challenge.

The environmental reservoir for human Pneumocystis is unknown, but Pneumocystis organisms have been found in lungs of healthy individuals (4). Additionally, most children become seropositive for anti-Pneumocystis Abs at a young age (5, 6), making them a potential reservoir for infection (7). Studies performed in experimental models of infection have found that Pneumocystis is capable of proliferating and establishing short-term infection in immunocompetent mice. Although infected immunocompetent mice can transmit Pneumocystis infection to other mice, a cell-mediated adaptive immune response clears the pathogen rapidly with minimal health consequences (8). These studies suggest that most people at some point in their lives become infected with Pneumocystis without presenting with any obvious or long-term clinical manifestations. The individual’s normal adaptive immune system resolves infection and confers protective immunity.

Although most people are exposed to Pneumocystis (4, 6, 9), it only causes the disease known as Pneumocystis pneumonia (PCP) in immunocompromised hosts. Typically the onset of PCP correlates with CD4+ T cell counts $<200$ cells/μl (10), emphasizing the key role of this lymphocyte subset in lung defense against Pneumocystis infection. Populations at risk for PCP are AIDS patients, cancer patients undergoing chemotherapy, organ recipients, and persons with other primary or acquired immunodeficiency. Animal studies have clearly demonstrated that CD4+ T cells are critical for host defense against Pneumocystis infection (11–13). However, the specific mechanisms through which an appropriate CD4+ T cell response is initiated, as well as the specific process by which the organisms are cleared, remain only partially understood. A recent study determined that the ultimate effector mechanism for CD4+ T cell–dependent removal of Pneumocystis from the lung in vivo is macrophage phagocytosis (14).

One of the earliest events during Pneumocystis lung infection is the tight attachment of Pneumocystis to lung epithelial cells (LECs). This early interaction is necessary for Pneumocystis growth and for the establishment of pulmonary infection. In vitro studies have shown that the interaction of Pneumocystis with LECs activates the NF-κB signaling cascade, resulting in the production of chemokines and cytokines that may accelerate the development of adaptive immunity in immunocompetent hosts and/or contribute to PCP-related immunopathogenesis in compromised hosts (15–18). LECs have also been shown to produce chemokines in vivo during
Pneumocystis infection, and pulmonary chemokine expression is associated with both protective immune responses and the development of PcP-related immunopathogenesis (18, 19). However, the specific contributions of NF-κB–dependent LEC responses to either host defense against Pneumocystis infection, or the development on immunopathogenesis, remain unexplored.

To study the role of NF-κB–dependent LEC responses during Pneumocystis infection in vivo, the cre-lox system was used to generate tissue-specific knockout mice. IkB kinase 2 (IKK2) is an important signaling kinase that is critical for inducible activation of the NF-κB pathway, and blockade of IKK2 activity effectively inhibits NF-κB activation (20). Therefore, conditional ablation of IKK2 has been used to study the role of inducible NF-κB activation in normal immune responses, as well as in inflammatory disease models. Transgenic mice in which the IKK2 gene was flanked by loxP recombination sites were crossed with mice expressing Cre recombinase under the control of the surfactant protein C (SFTPC) promoter to create mice that had specific and exclusive deletion of IKK2 in LECs. These mice were used to determine how IKK2-dependent LEC responses contribute to host defense against Pneumocystis infection.

Materials and Methods

Mice

LEC-specific IKK2-deficient mice (IKK2<sup>ΔLEC</sup>) on the C57BL/6 background were generated by crossing mice with loxP-flanked Ik2 alleles (IKK2<sup>Δ</sup>, provided by Dr. Manolis Pasparakis) (21) with mice expressing Cre recombinase under the control of the surfactant protein C (SFTPC) promoter to create mice that had specific and exclusive deletion of IKK2 in LECs. These mice were used to determine how IKK2-dependent LEC responses contribute to host defense against Pneumocystis infection.

DNA isolation and genotyping

Genomic DNA was isolated from tail snips or isolated cell populations using the DNeasy blood and tissue kit (Qiagen, Germantown, MD) or the KAPA mouse genotyping kit (Kapa Biosystems, Boston, MA) following the manufacturer's instructions. Primers for the Cre gene (forward, 5′-GAT CTT CGG CTG CTA GCA ACC GGG-3′, reverse, 5′-GAT CTC GAT GCA ACG AGT GAT GAG-3′), as well as primers for the IKK2 region of interest (IKK2, forward, 5′-CAC CAT ACT AGC TGA ACT GC-3′, reverse, 5′-AGG TAA GTG CTG AGA TGA GC-3′) were purchased from Integrated DNA Technologies (Corvalis, OR). PCR reactions were performed using PuReTaq Ready-To-Go PCR beads (GE Healthcare, Buckingharnshire, U.K.) and KAPA2G fast genotyping PCR mix (Kapa Biosystems).

Alveolar macrophage isolation

Lungs from WT and IKK2<sup>ΔLEC</sup> mice were lavaged with three 1-ml aliquots of isolation buffer (1× PBS, 1% glucose, 0.35 mg/ml gentamicin, 0.2 mM EGTA). The lavage fluid was centrifuged for 10 min at 250 × g. The cell pellet was used for protein extraction or DNA isolation.

Primary murine LEC isolation

Primary alveolar epithelial cells (AECs) were isolated following published protocols (17). Briefly, mice were euthanized with sodium pentobarbital. Using sterile technique, the peritoneal cavity was exposed to cut portal vein for exsanguination. The chest cavity was opened and the lungs were perfused with 10 ml heparinized 1× HBSS. A 20-gauge catheter was inserted into the trachea and the lungs were filled with 2 ml disase. Immediately, 0.45 ml 1% low-melting-point agarose was also introduced into the lungs, and a small bag of ice was placed in the chest to cool the lungs for 2 min. Lungs were removed into tubes containing 2 ml disase and were incubated for 45 min at room temperature. Lungs were transferred to a 100-mm dish with DMEM supplemented with 25 nM HEPES and 0.01% DNase I and minced into small pieces. The suspension was swirled for 10 min at room temperature followed by filtration through 100-, 40-, and 25-μm filters. Cells were pelleted and resuspended in DMEM supplemented with 10% FBS and incubated with biotinylated anti-CD45 and anti-CD16/32 for 30 min at 37°C to bind hematopoietic cells. Cells were pelleted and resuspended in serum-free media and incubated with streptavidin-coated magnetic beads for 30 min at room temperature. Sample tubes were placed against a magnet for 15 min to remove unbound cells in the supernatant. Cell suspensions were incubated at 37°C for 4-16 h in DMEM supplemented with 10% FBS and 10 ng/ml keratinocyte growth factor (Calbiochem) to allow mesenchymal cells to adhere to the tissue culture dish. Following incubation, the nonadherent epithelial cells are removed and cultured. Type II AEC purity, as assessed by Papanicolaou staining and Sftp expression, was typically >90%.

Protein extraction and Western blots

Protein from primary AECs and from alveolar macrophages was extracted using mammalian protein extraction reagent following the manufacturer’s instructions (Thermo Scientifie/Pierce, Rockford, IL). Total protein was run in NuPAGE 4–12% Bis-Tris polyacrylamide gels from Invitrogen (Life Technologies, Grand Island, NY). Upon transfer to nitrocellulose membrane, the protein of interest was detected using primary goat anti-IKK2 polyclonal Ab and secondary donkey anti-goat IgG HRP. Ab to actin was used as a control. All Abs were from Santa Cruz Biotechnology (Santa Cruz, CA).

Pneumocystis isolation and enumeration

Pneumocystis organisms were propagated in CB.17 SCID mice. Heavily infected mice were euthanized and organisms were isolated from the lungs following our published protocol (15). Briefly, lungs were perfused and aseptically removed into a glass tissue grinder in Pneumocystis isolation buffer (1× HBSS, 0.5% glutathione, 20 mM HEPES buffer, 1% penicillin and streptomycin, pH 7.2). Following homogenization, the preparation was passed sequentially through a series of needles decreasing in size from 18 to 22 to 26 gauge. The homogenate was centrifuged at 52 × g to remove larger tissue pieces in the pellet. The supernatant was then removed and centrifuged at 2000 × g for 20 min to pellet Pneumocystis organisms. The pellet was resuspended in sterile water for [35S] to lyse RBCs, followed by addition of 2× PBS. The preparation was incubated for 30 min at 37°C with 30 μl/ml DNAase-containing media and then centrifuged at 500 × g for 20 min. The supernatant was passed through a 20-μm filter and then centrifuged at 2000 × g for 20 min. The pellet was resuspended in serum-free media and plated for I.5 h at 37°C in tissue culture dishes coated with anti-Ly-6G/6C, anti-CD16/CD32, and anti-CD45 Abs to remove remaining hematopoietic cells. Nonadherent Pneumocystis organisms were removed, centrifuged, and passed through a 26-gauge needle three times to disperse clumps. Cysts were enumerated by bright field examination of Gomori methenamine silver (GMS)–stained slides. Typical preparations consist of a mixed population of 10% cysts and 90% trophic forms.

Pneumocystis infection

Experimental mice were anesthetized with isoflurane, and Pneumocystis organisms in a volume <0.1 ml were inoculated directly into the trachea. Mice received 5 × 10<sup>6</sup> Pneumocystis (based on cyst count) for experiments.

Pulmonary physiology

At each time point, live anesthetized mice were intubated, placed in a whole body plethysmograph (Buxco Electronics, Wilmington, NC), and connected to a rodent ventilator (Harvard Apparatus, Southhampton, MA). Dynamic lung compliance and lung resistance measurements were collected and analyzed using a BioSystem XA software package.

Sample collection

Following pulmonary function measurement, the peritoneal and chest cavities were opened. Blood was obtained from a cardiac puncture using a 1-ml syringe for isolation of sera. Mice were exsanguinated by cutting the portal vein, and lungs were perfused with 10 ml HBSS. The left lobes of the lung (or whole lungs in some cases) were lavaged with three 1-ml aliquots of 1× HBSS. Lavaged lungs were quickly frozen in liquid nitrogen and stored at −80°C for later analysis. In some experiments the right lobe was an infection model. Cysts were harvested and analyzed for tissue culture dishes coated with anti-Ly-6G/6C, anti-CD16/CD32, and anti-CD45 Abs to remove Pneumocystis infection.

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Lung leukocyte isolation

Lungs were excised, placed in a petri dish, and quickly minced using scissors. One-half of each lung was flash-frozen and stored at −80°C for later DNA analyses. The remaining tissue was dispersed through a 70-μm nylon filter using a syringe plunger. Cells were resuspended in RPMI 1640 media supplemented with 10% FBS, 1% penicillin and streptomycin, 1% sodium pyruvate, 20 mM HEPES, and 2-ME. All reagents were purchased from Life Technologies. After centrifugation at 300 × g for 10 min, the cells were incubated in media containing contain collagenase and DNAse for 1 h at 37°C to dissociate extracellular matrix. Cells were passed through a 40-μm filter and subjected to RBC lysis. After washing with media, cells were ready for restimulation and/or staining. Flow cytometry

Cellular fractions obtained from BAL and lung homogenates were incubated with Fc block (BD Biosciences, San Diego, CA) followed by incubation with fluorescently labeled Abs against CD4 (RM4-5), CD8α (53-6.7), CD11c (HL3), CD19 (1D3), and GR-1 (RB6-8C5) (all from BD Biosciences). For intracellular cytokine staining, cells were restimulated with 50–100 ng/ml PMA and 1 μM ionomycin for 4 h in the presence of GolgiPlug (BD Biosciences), a protein transport inhibitor containing brefeldin A. Cells were washed and incubated with Live/Dead fixable aqua stain (Molecular Probes, Eugene, OR) for 30 min followed by surface staining. Cytofix/Cytoperm kit (BD Biosciences) reagents and directions were used for intracellular staining. Data were collected using a BD LSR II flow cytometer with BD FACSDiva software (BD Biosciences) and analyses were performed using FlowJo (Tree Star, Ashland, OR).

Pneumocystis burden

Pneumocystis burden was determined as described (14). Lung homogenates from experimental mice were subjected to three cycles of freezing and thawing followed by boiling for 20 min. Samples were then centrifuged at 13,000 rpm for 20 min to remove cell debris. The supernatant was removed and Pneumocystis burden was determined by quantitative real-time PCR for the single copy Pneumocystis kexin gene. Quantitative real-time PCR was performed using TaqMan primer/fluorogenic probe chemistry detected with an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) as previously described (14).

Cytokine and chemokine measurement

Concentrations of IFN-γ, TNF-α, IL-1β, IL-4, IL-10, IL-17, MCP-1, MIP-2, and RANTES were determined by ELISA using kits purchased from R&D Systems (Minneapolis, MN) and used according to the manufacturer’s instructions.

Detection of anti-Pneumocystis serum Abs

Pneumocystis-specific Abs were measured in sera samples using an ELISA technique as previously described (8, 24). Briefly, flat-bottom microtiter plates (ICN Biomedicals, Aurora, OH) were coated overnight at room temperature with Pneumocystis Ag (in 0.05 M carbonate buffer [pH 9.6]) obtained from infected SCID mouse lungs. After a 2 h blocking step with 5% powdered milk in PBS, sera from experimental mice was diluted 1:50 in PBS plus 0.1% Tween 20 and incubated on plates for 2 h at 37°C. After washing, secondary goat anti-mouse IgG and IgM conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA) was incubated on plates for 1 h at 37°C. After washing, the chromogenic substrate was added and the assay was developed. OD was read in a SpectraMax M5 microplate reader and analyzed with SoftMax Pro. 5.2 software (Molecular Devices, Sunnyvale, CA). Controls included hyperimmune sera of immunocompetent mice immunized with whole Pneumocystis. Samples were also tested against noninfected normal lung Ag preparations for data normalization.

Statistics

All statistical analyses and graphs were made using Sigma Plot version 10 (Systat Software, San Jose, CA) and GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA). Data are presented as means ± SEM. A 2 × 2 contingency test was performed and statistical significance was determined for p values <0.05. For analysis of Pneumocystis burden in the lungs of experimental mice, multiple t tests with Holm–Sidak correction were performed. A p value ≤0.05 was considered statistically significant.

Results

Generation of IKK2<sup>ΔLEC</sup> transgenic mice

Mice with LEC-specific loss of IKK2 were generated by crossing mice with loxP-flanked IKK2 alleles (IKK2<sup>b/b</sup>) with mice expressing Cre recombinase under the control of human Sftpc (Sftpc-cre). The resulting offspring, designated IKK2<sup>ΔLEC</sup>, had floxed IKK2 genes and carried at least one copy of the Cre recombinase gene (IKK2<sup>b/b-cre<sup>−/−</sup></sup>). The new strain was viable, fertile, and appeared phenotypically normal.

The floxed IKK2 gene has loxP sites flanking a 2-kb region containing exons 6 and 7. Deletion of this region results in an IKK2-null phenotype. Disruption of the IKK2 gene in LECs was confirmed by PCR amplification using primers external to the loxP-flanked region (Fig. 1A). Genomic DNA was isolated from primary AECs from IKK2<sup>ΔLEC</sup> and IKK2<sup>b/b</sup> mice. As expected, a large PCR product (~2.9 kb) was amplified from the genomic DNA of IKK2<sup>b/b</sup> AECs, which contained an intact IKK2 gene. In contrast, a much smaller PCR product (~500 bp) was amplified from the genomic DNA of IKK2<sup>ΔLEC</sup> AECs, indicating successful deletion of exons 6 and 7 of the IKK2 gene (Fig. 1B). The IKK2 gene was not deleted from the genome of alveolar macrophages or spleen cells of IKK2<sup>ΔLEC</sup> mice (Fig. 1B and data not shown), demonstrating the specificity of cre-mediated deletion.

To confirm the PCR results, primary LECs and alveolar macrophages were isolated from IKK2<sup>b/b</sup> and IKK2<sup>ΔLEC</sup> mice and used for total protein isolation. Western immunoblotting demonstrated that IKK2 protein was present in IKK2<sup>b/b</sup> AECs, whereas IKK2 protein was absent from the IKK2<sup>ΔLEC</sup> AECs (Fig. 1C). To demonstrate the specificity of this deletion, protein was also isolated from alveolar macrophages of both strains. Importantly, alveolar macrophages from both IKK2<sup>b/b</sup> and IKK2<sup>ΔLEC</sup> mice expressed IKK2 protein. These results demonstrate that IKK2<sup>ΔLEC</sup> mice have LEC-specific deficiency of IKK2 and are consistent with published work demonstrating that the Sftpc promoter drives cre recombinase expression in lung epithelial precursor cells during embryogenesis (22, 23, 25).

**FIGURE 1.** Generation of mice with selective deletion of IKK2 in LECs (IKK2<sup>ΔLEC</sup> mice). (A) Schematic IKK2 gene region and primer genotyping strategy for conditional deletion of IKK2 in LECs. (B) DNA and (C) protein were extracted from primary AECs and alveolar macrophages (AM) isolated from IKK2<sup>b/b</sup> and IKK2<sup>ΔLEC</sup> mice. Genomic deletion of IKK2 was confirmed by PCR and Western immunoblotting with anti-IKK2 Ab.
Delayed Pneumocystis clearance in IKK2<sup>ΔLEC</sup> mice

Immunocompetent WT mice exposed to Pneumocystis develop a mild infection and are able to clear Pneumocystis organisms from the lung without obvious health effects (8). To determine whether loss of IKK2 signaling in LECs affects the course of infection or the kinetics of pathogen clearance, WT and IKK2<sup>ΔLEC</sup> mice were inoculated with freshly isolated Pneumocystis organisms. At different time points spanning the establishment, immune response, and clearance phases of infection, the Pneumocystis lung burden was measured. The Pneumocystis burden remained similar in both WT and IKK2<sup>ΔLEC</sup> mice for the first 18 d postinfection, with little reduction in organism number (Fig. 2). However, WT mice showed a 1 log reduction in total burden by day 21 and a 2 log reduction by day 25 (Fig. 2A). In contrast, IKK2<sup>ΔLEC</sup> mice showed little reduction in Pneumocystis burden at days 21 and 25, and the harbored 1–2 logs more Pneumocystis than did WT mice at these time points. IKK2<sup>ΔLEC</sup> mice were able to eventually clear the pathogen by 32 d postinfection. The same trend was confirmed by Pneumocystis cyst counts of GMS-stained lung homogenate slides (Fig. 2B). These data demonstrate that IKK2-dependent LEC responses are required to promote the efficient clearance of Pneumocystis from the lungs of immunocompetent mice.

B cell recruitment to the lung is delayed in Pneumocystis-infected IKK2<sup>ΔLEC</sup> mice

The clearance of Pneumocystis is dependent on CD4<sup>+</sup> T cells (26). B cells (27–29), and macrophage phagocytosis (14, 30). To determine whether obvious immune response defects were associated with delayed Pneumocystis clearance in IKK2<sup>ΔLEC</sup> mice, BAL cells recovered from infected WT and IKK2<sup>ΔLEC</sup> mice were enumerated and analyzed by flow cytometry and differential staining. The only cellular deficiency noted in Pneumocystis-infected IKK2<sup>ΔLEC</sup> mice was a delay in B lymphocyte recruitment to the lung relative to infected WT mice. B cells appeared in the lungs of infected WT mice at day 14, peaked at day 18, and decreased by day 21 (Fig. 3A). In contrast, very low numbers of B cells were present in the lungs of infected IKK2<sup>ΔLEC</sup> mice until day 18, and they peaked at day 21 and were decreased by day 25. Despite the differences in B cell numbers there was no significant effect on the Pneumocystis-specific Ab response in the sera of the two strains of mice (Fig. 3B).

Interestingly, IKK2<sup>ΔLEC</sup> mice had a 5-fold greater number of BAL cells than did WT mice at day 21 postinfection (Table I), which may be related to the persistence of Pneumocystis infection in the deficient mice (Fig. 2). Differential staining analyses of immune cell phenotype revealed that the main contributors to the high numbers of BAL cells in the IKK2<sup>ΔLEC</sup> mice were eosinophils, macrophages, and CD4<sup>+</sup> T cells. There was no significant difference in numbers of CD8<sup>+</sup> T cells or neutrophils between the groups at any time point.

Lung cytokine profiles indicate altered Th polarization in Pc-infected IKK2<sup>ΔLEC</sup> mice

To further investigate the nature of the pulmonary immune response in IKK2<sup>ΔLEC</sup> mice, cytokine levels were measured in lung samples taken from experimentally infected mice. Compared to infected WT mice, IFN-γ levels were lower in the infected IKK2<sup>ΔLEC</sup> group at day 11 (Fig. 4A). However, at later time points levels were comparable among the groups, with the exception of day 21, when IKK2<sup>ΔLEC</sup> mice had higher lung IFN-γ levels. TNF-α levels trended higher in the IKK2<sup>ΔLEC</sup> group at days 14–21 (Fig. 4B), which may be related to higher lung Pneumocystis burden in these mice. Levels of the Th2-associated cytokines IL-4 and IL-10 were also measured. Pneumocystis-infected IKK2<sup>ΔLEC</sup> mice had 2-fold higher IL-4 and IL-10 protein in the lungs than did infected WT mice at days 14–25 (Fig. 4C, 4D). These data demonstrate that mice with LEC-specific IKK2 deficiency have a higher concentration of cytokines that are associated with a Th2 response and are consistent with the lung eosinophilia observed in these mice.

IL-1β and IL-17 cytokine levels were also analyzed. IL-1β is important for promoting IL-17 production in CD4<sup>+</sup> T cells (31, 32). IL-17 is produced mainly by Th17 cells, which are involved in protective immunity against fungal infections, including those caused by Pneumocystis (33). Interestingly, IL-17 levels in the WT mice were elevated at day 7 postinfection and later went down to nearly basal levels. In contrast, IL-1β production was delayed in IKK2<sup>ΔLEC</sup> mice, with peak levels observed at day 14 postinfection (Fig. 4E). IL-17 levels were significantly lower in the lungs of IKK2<sup>ΔLEC</sup> mice at days 14 and 21 postinfection compared with WT mice (Fig. 4F). These data suggest that the loss of IKK2 in LECs alters the nature of the normal pulmonary immune response to Pneumocystis infection.

Chemokine levels were also measured in lung homogenate samples of experimental mice. CCL2 (MCP-1) attracts mainly T lymphocytes and monocytes and is elevated in the lung during the immune response to Pneumocystis infection. Infected IKK2<sup>ΔLEC</sup> mice had higher lung levels of CCL2 than did WT mice at days 14–21 postinfection (Fig. 4G, 4H). This increase in chemokine secretion correlates with the higher number of cells present in the lungs of IKK2<sup>ΔLEC</sup> mice. CCL5 (RANTES) is chemotactic for T cells and is another chemokine that has been associated with the host response to Pneumocystis infection. Despite the fact that we saw higher numbers of T cells in Pneumocystis-infected IKK2<sup>ΔLEC</sup> mice, RANTES lung
levels were similar in both strains of mice (Fig. 4I), indicating that CCL5 levels are not affected by the IKK2 deletion. Overall, these data demonstrate that IKK2-dependent LEC responses regulate the normal immune response to Pneumocystis infection.

**IKK2^ALEC^ mice exhibit delayed pulmonary Th17 responses**

To further investigate how loss of epithelial IKK2 signaling alters the normal immune response to Pneumocystis infection, the phenotype of Th cells recruited to the lung was determined using intracellular cytokine staining. Lymphocytes were isolated from the lavage fluid and lungs of Pneumocystis-infected WT and IKK2^ALEC^ mice. Following restimulation, lung lymphocytes were stained for CD4, CD8, IFN-γ, IL-4, and IL-17. Interestingly, both the ratio and number of IL-17-producing CD4^+^ T cells were significantly lower in the IKK2^ALEC^ mice in both BAL and lung homogenate samples at all of the time points assessed (Fig. 5). Importantly, by day 25 postinfection the percentage and number of IL-17^+^ cells increased in the lungs of infected IKK2^ALEC^ mice, suggesting that this phenomenon is one of delay rather than impairment of Th17 differentiation. To further confirm the ability of CD4^+^ T cells from IKK2^ALEC^ mice to adopt the Th17 phenotype, an in vitro culture system was used to induce differentiation of naive CD4^+^ cells into Th17. Naive CD4^+^ T cells isolated from the spleens of WT or IKK2^ALEC^ mice were equally able to adopt a Th17 phenotype following in vitro differentiation (data not shown). These data show that although the pulmonary Th17 response is delayed in Pneumocystis-infected IKK2^ALEC^ mice, there is no intrinsic deficiency in the CD4^+^ T cells of IKK2^ALEC^ mice that prevents Th17 differentiation.

To detect the presence of Th1 and CD4^+^ T regulatory cells, IFN-γ–producing cells and Foxp3^+^CD4^+^ T cells were analyzed in BAL fluid and lung homogenates of Pneumocystis-infected WT and IKK2^ALEC^ mice. There was no difference in ratios or cell numbers of Th1 cells in the BAL or lung homogenate of infected WT or IKK2^ALEC^ mice (Fig. 6 and data not shown). There was also no difference in the numbers of Foxp3^+^ or IL-4^+^ Th2 cells in infected mice of either strain (data not shown), despite the fact that IL-4 protein was increased in lung homogenates of infected IKK2^ALEC^ mice.

**Altered pulmonary physiology in Pneumocystis-infected IKK2^ALEC^ mice**

To determine whether loss of IKK2 signaling in LECs affects pulmonary function following Pneumocystis infection, dynamic lung compliance and lung resistance measurements were taken on infected WT and IKK2^ALEC^ mice using a whole body plethysmograph. As expected, WT mice cleared the Pneumocystis infection with little evidence of respiratory distress or impaired pulmonary function (Fig. 7A, 7B). In contrast, a nearly 30% reduction in lung compliance as well as a significant increase in lung resistance were observed in IKK2^ALEC^ mice at days 18 and 21 postinfection. These observations of pulmonary dysfunction in IKK2^ALEC^ mice coincide temporally with the elevated lung cytokine levels and higher BAL cellularity relative to WT mice, suggesting that an exaggerated host response contributes to the impaired pulmonary function in IKK2^ALEC^ mice.

To visualize the architecture of the lungs in Pneumocystis-infected WT and IKK2^ALEC^ mice, inflation-fixed sections were stained with H&E. Wild-type lungs were cleared of most cell infiltrates by day 21 (Fig. 7C), coincident with the clearance of organisms from the lung. In contrast, cell infiltrates remained evident in the lungs of IKK2^ALEC^ mice at this time, and perivascular and septal thickening were also observed (Fig. 7C). To determine whether the lungs of IKK2^ALEC^ mice show fibrotic changes, tissue sections were stained for α-smooth muscle actin (α-SMA). Whereas α-SMA staining in infected WT mice varied little from uninfected control mice throughout the course of infection and clearance, the lungs of IKK2^ALEC^ mice displayed transiently elevated perivascular α-SMA.

Table I. Differential analyses of BAL cells recovered from Pneumocystis–infected IKK2^ALEC^ mice and WT mice

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>WT</th>
<th>IKK2^ALEC^</th>
<th>WT</th>
<th>IKK2^ALEC^</th>
<th>WT</th>
<th>IKK2^ALEC^</th>
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<tr>
<td>Total cells (10^7)</td>
<td>4.65 ± 0.14</td>
<td>6.20 ± 0.08</td>
<td>5.23 ± 2.61</td>
<td>23.3 ± 1.75*</td>
<td>2.45 ± 0.60</td>
<td>4.25 ± 1.03</td>
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<tr>
<td>Macrophages (10^3)</td>
<td>0.99 ± 0.37</td>
<td>0.87 ± 0.24</td>
<td>0.63 ± 0.27</td>
<td>3.11 ± 0.75*</td>
<td>1.07 ± 0.41</td>
<td>0.94 ± 0.52</td>
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<td>B cells (10^3)</td>
<td>0.21 ± 0.11</td>
<td>0.01 ± 0.00</td>
<td>0.07 ± 0.02</td>
<td>0.31 ± 0.01*</td>
<td>0.03 ± 0.01</td>
<td>0.12 ± 0.07</td>
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<tr>
<td>CD4^+^ T cells (10^3)</td>
<td>0.61 ± 0.19</td>
<td>0.42 ± 0.04</td>
<td>0.21 ± 0.08</td>
<td>1.28 ± 0.26*</td>
<td>0.24 ± 0.05</td>
<td>0.24 ± 0.90</td>
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<tr>
<td>CD8^+^ T cells (10^3)</td>
<td>0.57 ± 0.14</td>
<td>0.51 ± 0.05</td>
<td>0.19 ± 0.07</td>
<td>0.41 ± 0.05</td>
<td>0.26 ± 0.03</td>
<td>0.11 ± 0.04</td>
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<tr>
<td>PMNs (10^3)</td>
<td>0.76 ± 0.18</td>
<td>0.93 ± 0.14</td>
<td>0.32 ± 0.29</td>
<td>0.22 ± 0.22</td>
<td>0.22 ± 0.11</td>
<td>0.16 ± 0.16</td>
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<tr>
<td>Eosinophils (10^3)</td>
<td>0.31 ± 0.18</td>
<td>2.11 ± 0.16</td>
<td>0.80 ± 0.67</td>
<td>14.37 ± 1.70*</td>
<td>1.01 ± 0.10</td>
<td>1.34 ± 0.89</td>
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Analyses of cells recovered in BAL fluid of experimental mice are shown. Total cell numbers were counted in a hemocytometer. Cell suspensions were fluorescently labeled with Abs specific for CD4, CD8, and CD11c and analyzed by flow cytometry. Cytospin slides were stained to count neutrophils (PMNs) and eosinophils based on their morphologic features. Data show means ± 1 SEM for each group (n = 3–5 mice/time point). Data shown are from a representative experiment of at least three independent experiments.

*p < 0.05 compared with WT.
staining compared with the WT (Fig. 8). This was especially evident at days 14, 18, and 21 postinfection. By day 25 the level of α-SMA staining in IKK2ΔLEC mice had decreased significantly and was similar to infected WT mice. These data suggest that loss of IKK2 signaling in LECs not only affects the immune response to *Pneumocystis* infection, but may also alter the normal resolution of infection.

**Discussion**

A transgenic mouse line with LEC-specific deletion of IKK2 was successfully generated and characterized. Whereas several studies have demonstrated the ability of LECs to respond to various infectious and noninfectious stimuli in vitro, the physiological relevance of the in vivo epithelial response has been more difficult to ascertain because of the many cell types, including alveolar macrophages, which are present in the lung. Likewise, the cell type–specific role of the NF-κB pathway has also been difficult to elucidate because NF-κB regulates a great number of cell processes in most cell types. Global knockout of either the NF-κB p65 subunit or IKK2 results in embryonic lethality caused by TNF-mediated hepatocyte apoptosis (20, 34). Thus, the IKK2ΔLEC mice are a valuable tool for not only studying the in vivo role of LECs during lung infection and disease, but also for gaining insight into the cell type–specific contributions of inducible NF-κB signaling. These mice will not only be useful for our studies of PcP, but will also be valuable for understanding the roles of LECs and NF-κB in other models of acute and chronic lung disease.

The IKK2ΔLEC mouse model was used to study the contribution of IKK2-dependent LEC responses to the normal protective immune response against a respiratory fungal infection. We found that IKK2 is involved in the early host response to *Pneumocystis* infection and likely sets the threshold for immune activation and host defense, which lead to organism clearance. IKK2-dependent LEC responses were not absolutely required for the clearance of this fungal pathogen from the lung, but mice with IKK2-deficient LECs exhibited delayed clearance kinetics. The most notable immune deficit associated with impaired *Pneumocystis* clearance in IKK2ΔLEC mice was a delayed lung Th17 response. Th17

![FIGURE 4. IKK2ΔLEC mice have altered lung cytokine and chemokine production during *Pneumocystis* infection. Protein levels of cytokines and chemokines were measured in the lung homogenates of experimental mice by ELISA: (A) IFN-γ, (B) TNF-α, (C) IL-4, (D) IL-10, (E) IL-1β, (F) IL-17, (G) MCP-1, (H) MIP-2, and (I) RANTES. Values shown are means ± 1 SEM for a representative of three independent experiments (n ≥ 3 mice/group/time point). *p < 0.05 compared with WT.](http://www.jimmunol.org/)

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responses are important for host defense against fungal pathogens (35), including Pneumocystis (33, 36), and mice treated with anti–IL-17 Ab also exhibit delayed Pneumocystis clearance that was similar to the results found in this study (33, 36). Therefore, it is likely that the impaired onset of pulmonary Th17 responses contributed to the delayed fungal clearance observed in IKK2<sup>ΔLEC</sup> mice. Past studies have focused on the mechanisms by which lymphocyte and dendritic cell interactions promote a cytokine environment that is important for the Th17 arm of adaptive host defense. The present study demonstrates that LECs are also key players in the initiation of the Th17 response. We speculate that their contributions could be through cytokine secretion and possibly direct interactions with dendritic cells or T cells. Epithelial cells produce chemokines that recruit IL-23–producing dendritic cells to promote the generation of

**FIGURE 5.** Accumulation of pulmonary IL-17<sup>+</sup>CD4<sup>+</sup> T cells is compromised in IKK2<sup>ΔLEC</sup> mice. Cells were isolated from BAL (A–C) or freshly homogenized lungs (D–F) of Pneumocystis-infected mice and stimulated with PMA and ionomycin as described. Cells were incubated with fluorescently labeled anti-CD4 and anti-IL-17 mAbs. (A and D) Representative dot plot panels showing the percentage of CD4<sup>+</sup> cells that are also IL-17<sup>+</sup>. Each plot is pooled data from three to four mice per time point from a representative experiment. Bar graphs show means ± 1 SEM for percentage of CD4<sup>+</sup> cells that are IL-17<sup>+</sup> (B, E) and absolute number of CD4<sup>+</sup>IL-17<sup>+</sup> cells recovered (C, F) for three combined experiments (n = 3–14 mice/time point). *p < 0.05 compared with WT.
Th17 responses (37). IL-17 promotes maturation and class switching in B cells, and it is also important for neutrophil recruitment and activation (38). Further studies are required to determine the mechanism by which Th17 cells and associated cytokines contribute to *Pneumocystis* clearance.

Another notable defect associated with delayed *Pneumocystis* clearance in IKK2<sup>ΔLEC</sup> mice was a delayed lung recruitment of B lymphocytes. B cells appeared in the lungs of *Pneumocystis*-infected WT mice by 2 wk postinfection and declined in number as the *Pneumocystis* was cleared. In contrast, the appearance of B cells in the lung was delayed several days in the infected IKK2<sup>ΔLEC</sup> mice. The overall Ab response was not greatly affected in the IKK2<sup>ΔLEC</sup> mice and was similar to responses observed in WT mice in the present study and in prior reports (8, 39). B cells as well as CD4<sup>+</sup> T cells are critical for *Pneumocystis* clearance and host defense, and B cells may have Ab-independent functions that...
modulate CD4+ T cell activation or expansion during Pneumocystis infection (27). Some of these functions include Ag presentation, cytokine secretion, and regulation of T cell activity (40). The significance of the delayed B cell response in Pneumocystis-infected IKK2ΔLEC mice is not known, but these studies suggest that IKK2-dependent epithelial responses regulate Th17 and B cell recruitment to the lung during Pneumocystis infection, and that epithelial cells are important regulators of pulmonary adaptive immune responses.

In addition to the finding of delayed Pneumocystis clearance in IKK2ΔLEC mice, it was also notable that these mice mounted an exaggerated pulmonary immune response leading to pulmonary dysfunction. A nearly 30% reduction in lung compliance as well as a significant increase in lung resistance were observed in IKK2ΔLEC mice at days 18 and 21 postinfection. These observations of pulmonary dysfunction in IKK2ΔLEC mice coincided temporally with the elevated lung cytokine levels and higher BAL cellularity relative to WT mice. The higher number of immune cells recruited to the lungs of IKK2ΔLEC mice clearly did not enhance fungal clearance, but instead their presence correlated with impaired lung function in these mice. Many of these cells were eosinophils, which have been found in higher numbers in lungs of AIDS patients (41), and may contribute to lung damage and the observed fibrotic changes. In a study by Swain et al. (42), an increase in eosinophils was associated with enhanced lung injury in a mouse model of PCp. The thickening of the perivascular regions in the lungs of infected IKKΔLEC mice resembles a phenomenon known as transitory pulmonary hypertension, which has been described in mouse models of PCp (43). We speculate that Pneumocystis grew longer and to higher numbers in IKKΔLEC mice before the protective immune response was initiated, which consequently led to the exacerbated response. The immune impairment in IKKΔLEC mice is not permanent, likely because other resident immune cells, such as alveolar macrophages, produce strong cytokine and chemokine responses to activate the immune response in the absence of epithelial IKK2-dependent responses.

The Sftpc-Cre mice used in this study have been previously described and characterized (23, 25), and detailed studies have determined that Cre recombinase is expressed and active in the precursor cells that give rise to nearly all airway and alveolar LECs. Thus, the IKK2ΔLEC mice we generated lack IKK2 in all LECs. However, we suspect that the critical loss of IKK2 that creates the observed consequences on host defense is within the AECs. Pneumocystis resides within the alveolar space and is most often found tightly attached to AECs. The interaction of Pneumocystis with AECs is required for fungal growth and the progression of disease, and in vitro studies have demonstrated that the Pneumocystis/AEC interaction leads to NF-κB–dependent AEC chemokine responses. Therefore, despite the fact that IKK2 is deleted from all LECs, it is likely that the observed effects are directly related to IKK2 deficiency in AECs. We have shown that AECs of IKK2ΔLEC mice are IKK2-deficient, whereas IKK2 expression is preserved in alveolar macrophages. Although Pneumocystis colonization of airway epithelial cells is not typically reported, it is possible that loss of IKK2 in this cell population also contributes to the impaired host defense.

Although Th1 and Th2 phenotypes have both been reported to play a role in host defense against Pneumocystis (44, 45), neither population is solely required because both IL-4−/− and IFN-γ−/− mice are able to clear this infection (46). In the present study the IL-4 and IL-10 cytokine profiles found in lung homogenate samples suggest a Th2-biased response in Pneumocystis-infected IKK2ΔLEC mice. Studies have shown that a Th2 lung environment induces alternative macrophage activation that results in increased phagocytosis of Pneumocystis (14, 47, 48). To the contrary, we did not see faster clearance of Pneumocystis in the IKK2ΔLEC mice. One explanation for this observation is the finding of elevated IL-10 levels in IKK2ΔLEC mice. The presence of IL-10 delays Pneumocystis clearance in immunocompetent mice, and IL-10-deficient mice exhibit accelerated clearance (49). Another possibility is that macrophage phenotype is altered in IKK2ΔLEC mice. Distinct subpopulations of alternatively activated or M2 macrophages exist, and they have distinct functional capabilities (50). Some macrophages, such as those associated with tumors and those found in burn patients, adopt an M2b phenotype that is more potently immunosuppressive than the M2a macrophages that are reported to promote anti-Pneumocystis host defense (48, 51). Our finding of elevated lung IL-10 and MCP-1 levels in IKK2ΔLEC mice suggest that a pro-M2b environment does exist in these mice, which display delayed Pneumocystis clearance. Further studies are required to determine the role...
of LECs in modulation of adaptive immunity and macrophage phenotype.

This study demonstrates that IKK2-dependent LEC responses modulate the pulmonary immune response to respiratory fungal infection. Although this work focused on the normal immune response, the conditional knockout mice will also be useful for studies of lung disease models in which inflammation and the immune response are major components of lung injury. The NF-κB signaling pathway is a popular therapeutic target for blocking inflammatory-mediated disease, and many inhibitors are currently under evaluation. Our findings suggest that NF-κB blockade could have both positive and negative effects on disease depending upon the nature of the disease and which cell types are primarily affected by the inhibitor. Although prior studies have demonstrated a prominent role for NF-κB-dependent macrophage responses in the generation of lung inflammation, a recent study has reported that IKK2-dependent LEC responses do not play a major role in the generation of airway inflammation following cigarette smoke exposure (52). However, this study focused on innate pulmonary responses to the toxicants present in cigarette smoke, and it did not assess the ability of IKK2-dependent epithelial cell responses to regulate adaptive immunity. Furthermore, these investigators were primarily concerned with airway disease, whereas PCP is mainly a disease of the alveolus. Therefore, it is conceivable that IKK2-dependent epithelial cell responses play an active role in the regulation of adaptive immune responses but are dispensable for the generation of nonmice alveolar inflammation. Further investigation into the cell type-specific role of NF-κB during lung disease is critical to the design of optimal therapeutic interventions.

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Disclosures

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References


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