Functions and Regulation of NF-κB RelA during Pneumococcal Pneumonia[1]

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Eradication of bacteria in the lower respiratory tract depends on the coordinated expression of proinflammatory cytokines and consequent neutrophilic inflammation. To determine the roles of the NF-κB subunit RelA in facilitating these events, we infected RelA-deficient mice (generated on a TNFR1-deficient background) with *Streptococcus pneumoniae.* RelA deficiency decreased cytokine expression, alveolar neutrophil emigration, and lung bacterial killing. *S. pneumoniae* killing was also diminished in the lungs of mice expressing a dominant-negative form of IκBα in airway epithelial cells, implicating this cell type as an important locus of NF-κB activation during pneumonia. To study mechanisms of epithelial RelA activation, we stimulated a murine alveolar epithelial cell line (MLE-15) with bronchoalveolar lavage fluid (BALF) harvested from mice infected with *S. pneumoniae*. Pneumonic BALF, but not *S. pneumoniae*, induced degradation of IκBα and IκBβ and rapid nuclear accumulation of RelA. Moreover, BALF-induced RelA activity was completely abolished following combined but not individual neutralization of TNF and IL-1 signaling, suggesting either cytokine is sufficient and necessary for alveolar epithelial RelA activation during pneumonia. Our results demonstrate that RelA is essential for the host defense response to pneumococcus in the lungs and that RelA in airway epithelial cells is primarily activated by TNF and IL-1. *The Journal of Immunology,* 2007, 178: 1896–1903.

Lower respiratory infections are a leading burden of disease worldwide and the greatest cause of infection-related deaths in the United States (1–3). The most common cause of community-acquired bacterial pneumonia is *Streptococcus pneumoniae* (4). As bacteria colonize the lower respiratory tract, their removal is largely dependent on the emigration of neutrophils into infected alveoli (5, 6), which is made possible by the coordinated expression of cytokines and adhesion molecules (7, 8). A majority of the genes encoding these inflammatory mediators contain consensus sites within their promoter/enhancer regions that bind the transcription factor NF-κB, while many of these molecules can themselves initiate activation of the NF-κB pathway (9).

Upon activation, NF-κB is rapidly liberated from inhibitory IκB proteins and translocates from the cytosol to the nucleus to promote the expression of κB-driven genes (10). Of the five NF-κB family members, only RelA and p50 have been identified in nuclear extracts of lungs exposed to bacterial stimuli (11–13). p50-deficient mice survive to adulthood and have an exaggerated inflammatory response to *Escherichia coli* in the lungs, suggesting that p50 serves as a negative regulator of pulmonary innate immunity (14). RelA deletion, however, results in embryonic lethality due to TNF-α-induced apoptosis (15), historically limiting the ability of researchers to assess its biological function.

To circumvent embryonic lethality caused by homozygous RelA deletion, RelA-deficient mice were crossed with mice lacking either TNF-α (16) or TNFR1 (17, 18). RelA/TNFR1-deficient mice have impaired inflammatory responses to LPS in their air spaces (17). Upstream manipulation of the NF-κB pathway has also been used as an alternative strategy to RelA deletion. Overexpression of IκB kinases in the lungs is sufficient to induce pulmonary inflammation (19), whereas inhibition of NF-κB activity in airway epithelium with a dominant-negative (dn)[3] form of IκB prevents inflammatory responses to Gram-negative stimuli (20–22).

These studies suggest that RelA, particularly in epithelial cells, may be necessary for promoting pulmonary inflammation in response to Gram-negative stimuli. RelA nuclear translocation is also induced in the lungs during pneumococcal pneumonia (12, 23), yet its functional significance is unknown. Furthermore, neither the consequence of RelA deletion nor its mechanisms of activation in alveolar epithelial cells have been determined within the context of any form of bacterial pneumonia.

Materials and Methods

Mice

Tnfrsf1a$^{-/-}$/*Rela*$^{+/-}$ mice (129/Sv × C57BL/6 background) were bred to generate TNFR1-deficient mice that were *Rela*$^{+/+}$, *Rela*$^{+/-}$, or *Rela*$^{-/-}$ (17). TNFR1/RelA-deficient mice (homozygous mutant) were compared with littermates with one or both alleles of *Rela* remaining functional. Since TNFR1/RelA-deficient mice are susceptible to bacterial infections (17, 18), antibiotics were included in the drinking water of all progeny until 2–3 days before experimentation as described previously (24). Mice expressing a dn form of IκB-α in airway epithelial cells (surfactant protein C

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[3] Abbreviations used in this paper: dn, dominant negative; AM, alveolar macrophage; BALF, bronchoalveolar lavage fluid; i.t., intratracheal; LIX, LPS-induced CXC chemokine; MLE, murine lung epithelial; rm, recombinant murine; sIL-IR, IL-1 signaling; SP19, type 19 *Streptococcus pneumoniae*; SP3, type 3 *Streptococcus pneumoniae*; SPC, surfactant protein-C; WT, wild type.

Table I. *Primer and probe sequences for real-time RT-PCR*[a]

	Forward Primer	Reverse Primer	TaqMan Probe
IL-6	AGTTGCCTTCTTGGGACTGATG	CAGGTCTGTTGGGAGTGGTATC	AACCACGGCCTTCCCTACTTCACA
KC	ACCCAAACCGAAGTCATAGCC	TGGACAATTTTCTGAACCAAGGG	CTTCAGGGTCAAGGCAAGCCTCGC
MIP2	ATCCAGAGCTTGAGTGTGACG	TTAGCCTTGCCTTTGTTCAGTATC	CCTACTGCGCCCAGACAGAAGTCA
LIX	TGATCGCTAATTTGGAGGTGATCC	TGAACACTGGCCGTTCTTTCC	TGCAGGTCCACAGTGCCCTACGGT

[a] Primer and probes were designed to amplify an 80- to 200-bp region within the open reading frame of the designated transcripts (listed 5′-3′). TaqMan probes were modified with the reporter dye 6-FAM and Black Hole Quencher-1 at the 5′ and 3′ ends, respectively. Probe cleavage during elongation resulted in dissociation of the two dyes, and the increases in fluorescence intensity were used to quantify mRNA levels.

(SPC)-dnIκBα) (22) were backcrossed at least nine generations onto a C57BL/6 background, and hemizygotes were compared with wild-type (WT) littermate control mice. Other experiments used mice on a mixed 129/Sv × C57BL/6 background as indicated in the corresponding figure legends. At the time of experimentation, mice were 5–8 wk of age. All experimental protocols were approved by the Harvard Medical Area Standing Committee on Animals.

Pneumonia

Mice were anesthetized by an i.p. injection of ketamine (50 mg/kg)/xylazine (5 mg/kg). An angiocatheter was placed down the left bronchus, and mice were intratracheally (i.t.) administered 50 μl of saline containing 10^6 CFU of *S. pneumoniae* serotype 3 (SP3, 6303; American Type Culture Collection) or *S. pneumoniae* serotype 19 (SP19; provided by Dr. M. Lipsitch, Harvard School of Public Health, Boston, MA). The concentration of living bacteria was estimated by OD and verified by quantifying serial dilutions on 5% sheep blood agar plates. For histological experiments, 1% colloidal carbon was included in the instillate to visualize pulmonary deposition.

Cytokine mRNA expression

IL-6, KC, MIP-2, and LPS-induced CXC chemokine (LIX) mRNA content was determined in lung tissue using real-time RT-PCR. Left lung lobes were removed from mice 15 h after i.t. SP3 and stored in 1 ml of RNAlater solution (Qiagen) at 4°C overnight or at −80°C for archival storage. Total RNA was extracted and purified using the RNeasy Mini kit and optional RNase-free DNase set as per the manufacturer's protocol. Real-time RT-PCR was performed using the iScript One-Step RT-PCR Kit for Probes (Bio-Rad) and the iCycler iQ Real-Time PCR detection system (Bio-Rad). Primers and TaqMan probes (Table I) were designed using the Beacon Designer software (Premier Biosoft International). Probes contained the reporter dye 6-FAM at the 5′ end and Black Hole Quencher-1 at the 3′ end. For each sample, values were normalized to the content of 18S rRNA (25, 26) and expressed the fold induction vs TNFR1-deficient/RelA$^{+/+}$ mice.

Alveolar neutrophil recruitment

Mice were euthanized by halothane overdose 24 h after i.t. SP3, and the heart was ligated to maintain pulmonary blood volume. Lungs were removed and fixed with 6% gluteraldehyde at 23-cm H_2O pressure. The percentage of alveolar air space occupied by neutrophils was quantified by morphometric analysis on H&E-stained lung sections as described previously (27, 28).

Lung bacteriology

Lungs were harvested 48 h after i.t. SP19 and homogenized in 10 ml of sterile distilled H_2O. Lung homogenates were serially diluted, plated on 5% sheep blood agar plates, and grown overnight at 37°C in a humidified atmosphere containing 5% CO_2. Colonies were counted on the following day, and data are expressed as total CFU/lung.

Bronchoalveolar lavage

At the indicated times after i.t. SP3, lungs were removed from euthanized WT mice (TNFRI$^{+/+}$ and RelA$^{+/+}$ on a 129/Sv × C57BL/6 background) and cannulated with a 20-gauge, blunted stainless steel needle. One milliliter of DMEM supplemented with 10% FBS (no antibiotics) was instilled and withdrawn. Bronchoalveolar lavage fluid (BALF) from each set of mouse lungs was then exposed to two rounds of centrifugation: 1) 300 × *g* for 5 min at 4°C to remove cells; and 2) 16,100 × *g* for 5 min at 4°C to remove bacteria and other remaining particulate matter. Samples were stored at −20°C for subsequent analyses.

Cytokine protein expression

Quantikine ELISA kits (R&D Systems) for murine TNF-α and IL-1β levels were used to measure their respective concentrations in BALF. Assays were performed according to the protocols provided by the manufacturer.

Immunoblot assay

Murine lung epithelial (MLE)-15 cells (29) were maintained in DMEM (Invitrogen Life Technologies; supplemented with 10% FBS, penicillin, and streptomycin) at 37°C in a humidified atmosphere containing 5% CO_2. MLE cells (4.0×10^5/well) were cultured overnight in 6-well tissue culture plates (Falcon). On the following day, cells were washed once with PBS and stimulated with antibiotic-free medium, antibiotic-free medium containing 10^6 CFU of SP3, 0-h BALF (see above), or 15-h BALF. After the indicated times, cells were washed once with PBS, resuspended in protein extraction buffer (2% Nonidet P-40, 25 mM Tris (pH 7.4), 50 mM NaCl, 0.5% sodium deoxycholate, and 0.2% SDS) and incubated on ice for 15 min. Lysates were centrifuged at 15,000 × *g* for 20 min at 4°C, and supernatants were collected for immunoblots. Protein concentrations were determined using the bicinchoninic acid assay (Sigma-Aldrich), and Western blots were performed as previously described (12) using the NuPAGE Gel System (Invitrogen Life Technologies). Membranes were probed using polyclonal Abs raised against IκB-α, IκB-β (Santa Cruz Biotechnology), and β-actin (Cell Signaling Technology). Primary Abs were detected using a HRP-conjugated anti-rabbit polyclonal Ab, which was visualized using the ECL$^+$ Western Blotting Detection System and Hyperfilm and ECL chemiluminescence film (Amersham Biosciences).

RelA nuclear translocation

RelA nuclear translocation was quantified in MLE-15 cells using scanning cytometry. Before experimentation, MLE-15 cells were cultured at 50,000 cells/well on black, flat/clear-bottomed 96-well tissue culture plates (Greiner) precoated with Cell-Tak adhesive (BD Biosciences). After 24h, cells were washed with prewarmed PBS and exposed to one of the following (all of which lack antibiotics): medium alone (DMEM plus 10% FBS), medium plus SP3, medium plus recombinant murine (rm)IL-1β, medium plus rmTNF-α, or medium that had been instilled and removed from mouse lungs (BALF). Where indicated, TNF-α and/or IL-1 (α and β) activity was neutralized using 5 μg/ml TNF-α-specific IgG and/or a rmIL-1RI/Fc fusion protein, respectively. Neutralization doses were selected after preliminary dose response experiments in which the efficacy of each inhibitor was tested in the presence of their respective cytokine target and pneumonic BALF. All cytokines and cytokine inhibitors were purchased from R&D Systems. For all experiments, cells were incubated for 10 min (37°C; 5% CO_2), washed with PBS, fixed (3.7% paraformaldehyde), and permeabilized (0.1% Triton X-100). RelA was then revealed with a RelA polyclonal Ab (Santa Cruz Biotechnology) and an Alexa Fluor 488-conjugated secondary Ab (Molecular Probes). Nuclei were counterstained with Hoechst 33342 (Molecular Probes) to discriminate between nuclear and cytosolic cellular compartments, and Alexa Fluor 488 fluorescence intensity (representing RelA content) was imaged and quantified using a BD Pathway Bioimager. Values for a particular sample were determined by averaging the individual cytosolic or nuclear Alexa Fluor 488 intensity values collected from all cells within a well. Data analysis was performed using the BD Image Data Explorer, and data were expressed as the average difference between the mean nuclear and cytoplasmic Alexa Fluor 488 fluorescence intensity.

Statistics

Statistical analyses were performed using the Statistica statistical software program (StatSoft). Data are presented as means ± SE for the number of

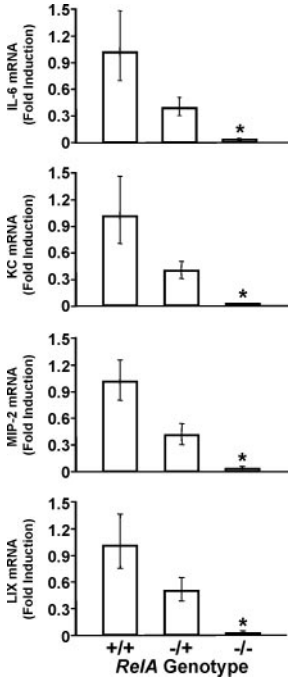

FIGURE 1. Lung cytokine mRNA expression 15 h after intratracheal SP3 (10^6 CFU) in presence and absence of functional RelA. All mice were on a TNFR1-deficient background, and littermates were present in each experiment for all three RelA genotypes. Cytokine mRNA levels were determined using real-time RT-PCR and normalized to the content of 18s rRNA. Data for each group were expressed as geometric means ± geometric SE ($n = 6–12$) of the fold induction of the values determined for $RelA^{+/+}$ mice. *, $p < 0.05$ compared with $RelA^{+/+}$ mice.

samples identified in each figure legend. Real-time RT-PCR data are expressed as fold induction and thus presented as geometric means ± geometric SE. Comparisons were performed with a Student's *t* test or a one-way ANOVA followed by a Bonferroni's post hoc analysis. When data failed Levene's test for homogeneity of variance, they were log-transformed before analyses. Differences were considered statistically significant when $p < 0.05$.

Results

Cytokine expression is dependent on RelA

IL-6, KC, MIP-2, and LIX are expressed in the lungs in response to bacteria and/or bacterial stimuli and are important for promoting alveolar neutrophil recruitment and host defense (25, 30–33). To determine the influence of RelA on inflammatory gene expression during pneumococcal pneumonia, steady-state lung mRNA levels of these cytokines were measured using real-time RT-PCR following i.t. SP3 in mice with zero, one, or two alleles for RelA. All mice used for these studies were bred on a TNFR1-deficient background to prevent the TNF-α-dependent embryonic lethality previously reported in RelA-deficient mice (17, 18). Fifteen hours after the pneumococcal challenge, lung mRNA levels of IL-6, KC, MIP-2, and LIX were significantly reduced in homozygous RelA-deficient mice as compared with littermate control mice containing one or both alleles of the functional *RelA* gene (Fig. 1). In fact, mRNA content for all four cytokines was reduced in $RelA^{-/-}$ mice to <3% of the values observed in $Rela^{+/+}$ mice.

RelA is required for alveolar neutrophil recruitment

Alveolar neutrophil emigration is a hallmark of innate immunity and is required for the effective removal of bacteria from the lower respiratory tract (5, 6). Previously, we showed that pneumococcal pneumonia initiates a neutrophil response that does not require

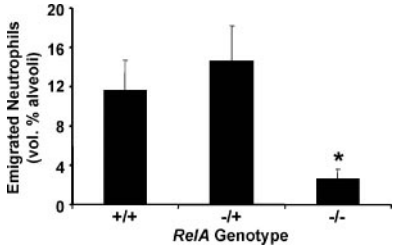

FIGURE 2. Alveolar neutrophil emigration 24 h after intratracheal SP3 (10^6 CFU) in presence and absence of functional RelA. All mice were on a TNFR1-deficient background, and littermates were present in each experiment for all RelA genotypes. Alveolar neutrophil counts were determined by morphometric analysis of histologic lung sections. Data for each group were expressed as means ± SE ($n = 4–6$) of the percentage of alveolar space occupied by neutrophils. *, $p < 0.05$ compared with $RelA^{+/+}$ mice.

TNFR (12). In the present study, i.t. SP3 induced neutrophil recruitment by 24 h in TNFR1-deficient mice with functional RelA. However, neutrophilic inflammation was almost completely ablated in the absence of RelA (Fig. 2). These results demonstrate that RelA is essential for the emigration of neutrophils into the infected alveolar space during pneumococcal pneumonia.

Bacterial killing is impaired by RelA deficiency

SP3 is a virulent serotype of *S. pneumoniae* that readily proliferates in the lungs of WT, immunocompetent mice, resulting in ~100% mortality (12, 34). Due to the uncontrollable growth of SP3 in the lungs, we used a less virulent and more effectively eliminated serotype of pneumococcus, SP19 (12, 34), to determine requirements for RelA in bacterial killing during pneumonia. Lungs harvested from RelA-deficient mice had ~100-fold more viable bacteria than littermate controls expressing one or both copies of *RelA* (Fig. 3).

Bacterial killing is impaired by epithelial-specific NF-κB inhibition

NF-κB can be activated in several lung cell types (21), and previous studies suggest that its activation in airway epithelial cells is particularly important for modifying inflammatory responses (19–22). To determine the importance of airway epithelial NF-κB activation during pneumococcal pneumonia, we administered SP19 intratracheally to WT mice or transgenic mice expressing a dn

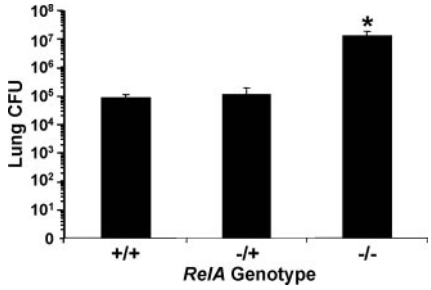

FIGURE 3. Lung bacterial killing 48 h after intratracheal SP19 (10^6 CFU) in the presence and absence of functional RelA. All mice were on a TNFR1-deficient background, and littermates were present in each experiment for all RelA genotypes. Viable SP19 were quantified by colony counts on 5% sheep blood agar plates. Data for each group were expressed as means ± SE ($n = 6–9$) total lung CFU. Statistical analyses were performed on values normalized to the inoculum in a given experiment. *, $p < 0.05$ compared with $RelA^{+/+}$ mice.

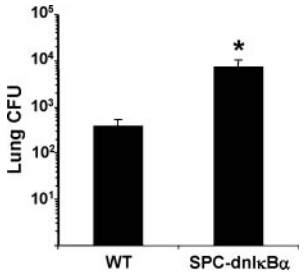

FIGURE 4. Lung bacterial killing 48 h after intratracheal SP19 (10^6 CFU) in the presence and absence of dnIκBα in airway epithelial cells. Transgenic mice overexpressed the NF-κB inhibitor dnIκBα under transcriptional control of the SP-C promoter. Viable SP19 were quantified by colony counts on 5% sheep blood agar plates. Data for each group were expressed as means ± SE ($n = 8–11$) total lung CFU. Statistical analyses were performed on values normalized to the inoculum in a given experiment. *, $p < 0.05$ compared with WT mice.

form of IκB-α under the transcriptional control of the SPC promoter (SPC-dnIκBα). This degradation-resistant form of IκB-α prevents NF-κB nuclear translocation and limits the expression of κB-driven genes in the alveolar and bronchial epithelium (22). Lungs from SPC-dnIκBα mice had significantly more viable bacteria than those harvested from WT mice 48 h after i.t. SP19 (Fig. 4), demonstrating that epithelial NF-κB activation is required for maximal host defense during pneumococcal pneumonia.

RelA activation in MLE-15 cells is initiated by pneumonic BALF

Since our current data support a requirement for RelA and a role for NF-κB in epithelial cells of the lung during pneumococcal pneumonia (see above), we studied IκB degradation and the nuclear translocation of RelA in MLE-15 cells, an immortalized cell line derived from and representative of murine type II alveolar epithelial cells (29). BALF (cleared of cells and bacteria) was used as the primary stimulus in these experiments to represent the complex inflammatory milieu present in pneumonic alveolar lining fluid. As our goal was to determine factors responsible for direct, receptor-mediated NF-κB activation, cells were stimulated for only 10 min in most studies to minimize the potential involvement of secondary, downstream MLE-cell-derived stimuli capable of activating NF-κB.

Since IκB degradation in epithelial cells proved necessary for bacterial killing (Fig. 4), we hypothesized that pneumonic BALF would directly induce IκB degradation in MLE-15 cells in vitro. After a 10-min exposure, pneumonic BALF collected from mice 15 h after i.t. SP3 (15-h BALF) induced degradation of both IκBα and IκBβ (compared with cells treated with medium alone) as

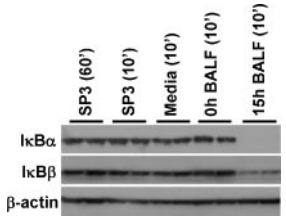

FIGURE 5. IκBα and IκBβ degradation in MLE-15 cells stimulated with SP3 (10^6 CFU/ml) or BALF. BALF was collected from mice (129/Sv × C57BL/6) and pooled 0 or 15 h after intratracheal SP3 (10^6 CFU). MLE-15 cells were treated for 10 or 60 min with SP3 or with BALF for 10 min and then lysed for protein extractions. IκBα and IκBβ levels were visualized by immunoblot, with β-actin serving as a loading control. The data shown represent one of three separate experiments.

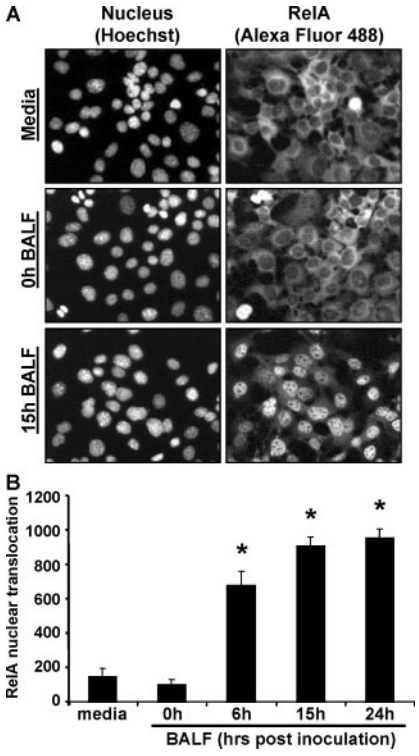

FIGURE 6. RelA nuclear translocation in MLE-15 cells 10 min after stimulation with BALF. BALF was collected from mice (129/Sv × C57BL/6) at the indicated times after intratracheal SP3 (10^6 CFU/ml), and MLE-15 cells were incubated with BALF from individual mice for 10 min. Scanning cytometry was used to measure cytosolic and nuclear RelA content in fixed cells. *A*, Representative images are shown from MLE-15 cells exposed to media, 0-h BALF, or 15-h BALF. Alexa Fluor 488 fluorescence intensity corresponds to RelA content, whereas Hoechst intensity was used as a nuclear counterstain to discriminate between cytosolic and nuclear compartments for each cell. *B*, RelA nuclear translocation in response to pneumonic BALF was calculated as the difference between nuclear and cytosolic Alexa Fluor 488 fluorescence intensity. Data were expressed as means ± SE of the average values obtained from BALF of individual mice ($n = 6–8$). Data for cells treated with media alone represented the average ± SE of data collected from 4 wells. *, $p < 0.05$ compared with media only treatment.

determined by immunoblot (Fig. 5). In contrast, neither BALF from uninfected mice (0-h BALF) nor SP3 itself resulted in IκB degradation. Interestingly, SP3 failed to induce IκB degradation even after an hour of stimulation, suggesting that soluble factors in alveolar lining fluid and not bacteria are responsible for NF-κB activation in alveolar epithelial cells during pneumonia.

To specifically assess the activation of RelA, we used scanning cytometry as a sensitive and quantitative means to measure its nuclear translocation. Following a 10-min incubation, RelA was localized within the nuclei of MLE-15 cells stimulated with 15-h BALF, whereas RelA remained primarily in the cytoplasms of cells treated with medium alone or 0-h BALF (Fig. 6*A*). BALF harvested from mice at all times after SP3 inoculation (6, 15, and 24 h) induced a significant and substantial increase in RelA nuclear translocation compared with that resulting from medium alone or 0-h BALF (Fig. 6*B*).

TNF and IL-1 are essential for BALF-induced RelA activation

Since SP3 failed to induce IκB degradation, we hypothesized that alveolar epithelial RelA activation could be mediated by host-derived factors such as TNF-α and IL-1 in alveolar lining fluids of

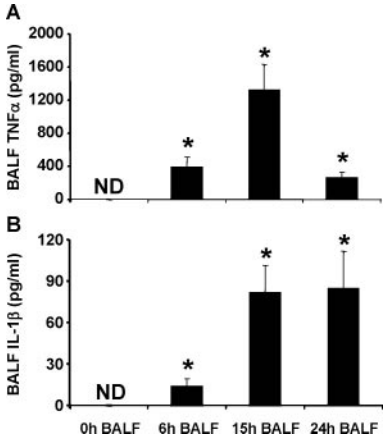

FIGURE 7. TNF-α (*A*) and IL-1β (*B*) levels in BALF in response to i.t. SP3 (10^6 CFU). Cytokine protein levels were determined by ELISA in BALF collected from mice (129/Sv $\times$ C57BL/6) at the indicated times after i.t. SP3. Samples used for cytokine analyses were the same as those used for scanning cytometry in Fig. 6. Data for each group were expressed as means $\pm$ SE ($n = 4-8$) in pg/ml. ND, Not detected (below the limit of detection). For statistical analyses, the lowest standard curve values were substituted for those below the limit of detection. $*$, $p < 0.05$ compared with 0 h.

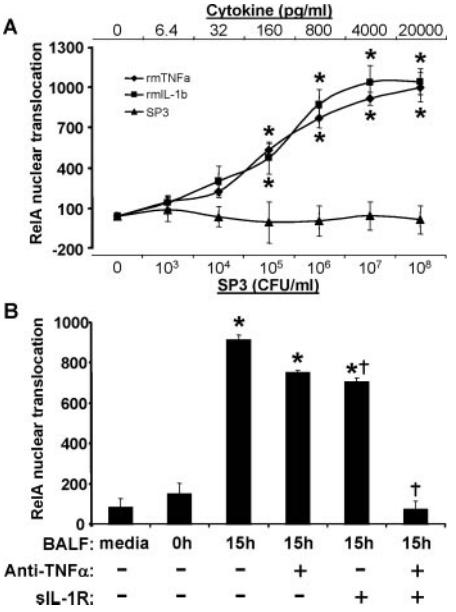

FIGURE 8. TNF-α and IL-1β, but not SP3, are sufficient and necessary for RelA nuclear translocation in MLE-cells. Scanning cytometry was used to determine nuclear RelA content in MLE-15 cells incubated for 10 min with cytokines, SP3, or BALF. RelA nuclear translocation was calculated as the difference between nuclear and cytosolic Alexa Fluor 488 fluorescence intensity. *A*, MLE-15 cells were incubated for 10 min with the indicated concentrations of cytokines or bacteria. *B*, BALF was collected from mice (129/Sv $\times$ C57BL/6) at the indicated times after intratracheal SP3 (10^6 CFU/ml), pooled, and exposed to MLE-15 cells for 10 min. Samples were supplemented with a TNF-α-neutralizing Ab, sIL-1RI/Fc chimera (sIL-IR), and/or the appropriate vehicle(s) for each inhibitor. Data for each group were expressed as means $\pm$ SE ($n = 3$) of the average values obtained from three separate experiments (samples run in triplicate for each experiment). $*$, $p < 0.05$ compared with (*A*) media or (*B*) media plus inhibitor vehicles. †, $p < 0.05$ compared with (*B*) 15-h BALF.

mice with pneumococcal pneumonia. TNF-α and IL-1β levels were measured in the BALF samples used to study BALF-induced RelA activation (Fig. 6*B*). Concentrations of both cytokines were below their limits of detection in 0-h BALF but were significantly elevated at all time points after i.t. SP3 (Fig. 7).

To determine whether these cytokines or SP3 could activate RelA in alveolar epithelial cells, the nuclear translocation of RelA was quantified using scanning cytometry in MLE-15 cells treated with varied doses of each cytokine or bacteria for 10 min. RelA nuclear translocation was significantly increased compared with baseline values at a dose of 160 pg/ml rmTNF-α or rmIL-1β (Fig. 8*A*). Maximum RelA activation was achieved in response to both cytokines at doses $\geq$ 4000 pg/ml. In contrast to the substantial RelA activation observed after treatment with rmTNF-α and rmIL-1β, SP3 consistently failed to induce RelA nuclear translocation in response to all of the doses tested, which ranged from 10^3 to 10^8 CFU/ml (Fig. 8*A*). These data are consistent with the inability of SP3 to induce IκB degradation described above (Fig. 5). Furthermore, this absence of SP3-induced RelA activation was evident over the entire range of SP3 doses at additional time points up through 1 h (data not shown).

Since TNF-α and IL-1β were both present in pneumonic BALF and sufficient to induce RelA activation in MLE-15 cells, we determined the contribution of each cytokine to the RelA-activating capacity of pneumonic BALF. MLE-15 cells were treated for 10 min with medium or pooled BALF in the presence and absence of a neutralizing TNF-α Ab and/or an IL-1RI/Fc fusion protein (sIL-IR) that inhibits all IL-1 signaling. As with the results shown in Fig. 6*A*, 15-h BALF (including the appropriate inhibitor vehicles) significantly triggered RelA activation in MLE-15 cells (Fig. 8*B*). Neutralization of TNF-α alone did not significantly reduce RelA activation vs normal 15-h BALF despite a noticeable trend, whereas IL-1 neutralization had a significant but modest inhibitory effect. However, neutralization of both cytokines in pneumonic BALF completely ablated RelA nuclear translocation, such that RelA activity in the absence of TNF-α and sIL-IR was no different from medium alone or 0-h BALF (Fig. 8*B*). These results indicate that TNF-α and IL-1 are not only present and sufficient for MLE-15 cell RelA activation but also that these cytokines are necessary for these responses.

Discussion

Proinflammatory cytokine expression and the resulting alveolar neutrophil response is critical to the resolution of bacterial pneumonia (5, 6, 35). Our results demonstrate that RelA is required for this process during pneumococcal pneumonia. Furthermore, our results highlight the airway epithelium as an important locus of NF-κB activation during this infection, such that inhibition of epithelial NF-κB activation decreases bacterial killing. Finally, the nuclear translocation of RelA in an alveolar epithelial cell line stimulated with alveolar lining fluid from pneumonic lungs is mediated by the early response cytokines TNF-α and IL-1.

The generation of RelA/TNFR1-deficient mice (17) and their maintenance through adulthood (24) provides the opportunity to directly study RelA lung biology in vivo. Although TNFR1 deficiency was controlled for in these studies (TNFR1/RelA-deficient mice were compared with RelA-expressing littermate control mice that also lacked TNFR1), the absence of TNFR1 must be considered when using these mice. TNFR1-specific roles of RelA (i.e., not induced by TNFR2 or other receptors) cannot be revealed using the current strategy. While mice deficient in TNF-α receptors have no defect in alveolar neutrophil recruitment during pneumococcal pneumonia (12), TNF-α can contribute to host defenses

against some pulmonary pathogens (36–41). Immune consequences of TNFR1 deficiency such as defects in secondary lymphoid development and IgG production (42–45) may also influence results from these studies. Despite these limitations, this approach has resulted in to our knowledge the first direct evidence that RelA is required for host defense during bacterial pneumonia.

During acute pulmonary inflammation, the CXC chemokines KC, MIP-2, and LIX generate the chemotactic gradients that direct neutrophils from the vascular to the alveolar spaces (30, 31, 33, 46). IL-6 is also expressed in the lungs and is required for maximal neutrophil recruitment and bacterial killing (25, 47). In the present study, expression of these cytokines, neutrophilic inflammation, and bacterial killing were dependent on the NF-κB subunit RelA. Other aspects of innate immunity, such as neutrophil and/or macrophage bactericidal function, may also be altered in the absence of functional RelA, thus contributing to the increase in bacterial burden. Our results suggest that RelA-induced pulmonary cytokine production is a principle mechanism through which alveolar neutrophil recruitment and consequently host defense is established during pneumococcal pneumonia. Interestingly, a single functional *Rela* allele was sufficient since none of the parameters measured in this study were significantly altered in heterozygous mice. Although cytokine mRNA expression was seemingly lessened in *Rela* heterozygotes, this apparent decrease was insufficient to influence neutrophil emigration or bacterial killing.

The epithelium of the lung is emerging as a critical site of NF-κB activation in response to diverse pathogens in the air spaces. Previous studies manipulating the NF-κB pathway using tissue-specific transgenes (20, 22), bone marrow chimeras (17, 48, 49), and adenoviral vectors (19, 21) suggest that NF-κB activity in epithelial cells is involved in and/or necessary for initiating inflammatory responses elicited by Gram-negative stimuli. We conducted experiments using alveolar/bronchial epithelial-specific SPC-dnIκBα transgenic mice to complement our findings in mice lacking RelA in all cells. While our results obtained with these mice do not discriminate between the roles of different NF-κB subunits in airway epithelial cells, they demonstrate that NF-κB activation in this cell type is necessary for maximal killing of pneumococcus.

NF-κB may be activated in epithelial cells by a variety of microbial and host-derived factors (50). To more specifically study RelA activation in an alveolar epithelial cell line, we used scanning cytometry, with many advantages including a high-throughput multiwell format, quantitative measurements and analyses, and the ability to discriminate between cytosolic and nuclear localizations. Our results show that RelA rapidly (10 min) and substantially accumulates in nuclei of MLE-15 cells in response to BALF collected during pneumococcal pneumonia. Similarly, Nys et al. (51) showed that NF-κB translocation increases in A549 cells (human alveolar epithelial cell line) after a 1-h exposure to BALF collected from patients with pneumonia. In the current study, BALF was processed in such a way as to minimize the potential effects of lavaged cells and/or remaining SP3. Additional experiments were performed, however, to more carefully differentiate between the effects of bacteria/bacterial products and other soluble factors such as cytokines.

TLR2 is expressed on the surface of type II alveolar epithelial cells (52, 53) and has been shown to permit *S. pneumoniae*-induced NF-κB activation in this cell type (54, 55). Interestingly, SP3 failed to induce RelA nuclear translocation in our current studies, regardless of dose or timing. Robson et al. (56) recently showed that the adhesiveness of *S. pneumoniae* inversely correlates with NF-κB activity in A549 cells, such that the low binding affinity of SP3 resulted in no NF-κB activation. SP3 may have a similarly low binding affinity to MLE-15 cells, possibly contributing to the present lack of SP3-induced RelA activation. Although others have demonstrated SP3-induced NF-κB activation in bronchial epithelial cells (54, 57, 58), our current data argue against this serotype of *S. pneumoniae* as a significant stimulus for RelA activation in alveolar epithelial cells during pneumococcal pneumonia.

In contrast to the effect of SP3, however, we show the presence of either TNF-α or IL-1 is sufficient and necessary for MLE-15 cell RelA activation induced by pneumonic BALF. Since inhibition of TNF-α or IL-1 alone caused only modest reductions in BALF-induced RelA activity, our results also demonstrate a functional redundancy between these two cytokines, such that the presence of one can largely compensate for absence of the other. Similar results have been observed for neutrophil recruitment in vivo; signaling is required from TNF-α receptors or IL-1R but not both (12, 59–61). The remarkable consistency of these in vitro data with previous whole lung in vivo findings (12) strongly endorses the conclusion that these cytokines are the principle mediators of lung RelA activation and neutrophil recruitment during pneumococcal pneumonia. TNF-α and IL-1 neutralization are becoming increasingly commonplace in the treatment of patients with autoimmune and inflammatory disorders (62) and may influence patients' susceptibility to infections (63). Accumulating evidence from in vivo and in vitro studies suggests that the simultaneous inhibition of both pathways may substantially compromise NF-κB activation and host defense against pneumococcus in the lungs.

Depletion studies have identified alveolar macrophages (AM) as major contributors to pulmonary inflammation and cytokine production, including TNF-α and IL-1β (64–67). Experiments using bone marrow chimeras also endorse AM as the key source of TNF-α and IL-1β during pulmonary inflammation, whereas cells other than AM, including alveolar epithelial cells, appear to be the predominant source of CXC chemokines (48, 49). Results from in vitro analyses have identified alveolar epithelial cells, particularly type II cells, as a major source of CXC chemokines in response to several inflammatory stimuli, including TNF-α, IL-1β, LPS, and Gram-negative bacteria (68–70). Based on these observations and our own, we speculate that AM-derived TNF-α and IL-1 may be largely responsible for initiating innate immune responses in alveolar epithelial cells during pneumococcal pneumonia. In support of our hypothesis, conditioned media from activated AM has been shown to induce NF-κB activation in A549 cells in a TNF-α- and IL-1β-dependent fashion (71). Importantly, murine AM have been shown to produce TNF-α in response to type III pneumococcus in a TLR2-dependent fashion (72). The mechanism through which AM and not MLE-15 cells respond to SP3 remains unknown but may be attributable to basal differences in TLR2 expression (52). Alternatively, it is possible that epithelial cells are less responsive to pneumolysin, which signals through TLR4 (73), and/or other pathogen-associated molecular patterns that are recognized by AM.

These results elucidate mechanisms regulating host defense against pneumococcus in the lungs. They demonstrate that NF-κB RelA is essential for cytokine expression, neutrophil recruitment, and bacterial killing; that airway epithelial cells are a critical locus of NF-κB activity; and that RelA activation in epithelial cells stimulated by pneumonic extracts is specifically mediated by TNF-α and IL-1. Synthesizing these observations, we propose that the activation of RelA in alveolar epithelial cells by TNF-α and IL-1 is essential to neutrophil recruitment and host defense against pneumococcus in the lungs.

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Disclosures

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References

1. Michaud, C. M., C. J. Murray, and B. R. Bloom. 2001. Burden of disease—implications for future research. *J. Assoc. Med. Assoc.* 285: 535–539.
2. Mizgerd, J. P. 2006. Lung infection—a public health priority. *PloS. Med.* 3: e76.
3. Armstrong, G. L., L. A. Conn, and R. W. Pinner. 1999. Trends in infectious disease mortality in the United States during the 20th century. *J. Assoc. Med. Assoc.* 281: 61–66.
4. Ruiz, M., S. Ewig, A. Torres, F. Arancibia, F. Marco, J. Mensa, M. Sanchez, and J. A. Martinez. 1999. Severe community-acquired pneumonia: risk factors and follow-up epidemiology. *Am. J. Respir. Crit. Care Med.* 160: 923–929.
5. Garvy, B. A., and A. G. Harmsen. 1996. The importance of neutrophils in resistance to pneumococcal pneumonia in adult and neonatal mice. *Inflammation* 20: 499–512.
6. Toews, G. B. 1986. Determinants of bacterial clearance from the lower respiratory tract. *Semin. Respir. Infect.* 1: 68–78.
7. Mizgerd, J. P. 2002. Molecular mechanisms of neutrophil recruitment elicited by bacteria in the lungs. *Semin. Immunol.* 14: 123–132.
8. Wagner, J. G., and R. A. Roth. 2000. Neutrophil migration mechanisms, with an emphasis on the pulmonary vasculature. *Pharmacol. Rev.* 52: 349–374.
9. Pahl, H. L. 1999. Activators and target genes of Rel/NF-κB transcription factors. *Oncogene* 18: 6853–6866.
10. Li, Q., and I. M. Verma. 2002. NF-κB regulation in the immune system. *Nat. Rev. Immunol.* 2: 725–734.
11. Blackwell, T. S., L. H. Lancaster, T. R. Blackwell, A. Venkatakrishnan, and J. W. Christman. 1999. Differential NF-κB activation after intratracheal endotoxin. *Am. J. Physiol.* 277: L823–L830.
12. Jones, M. R., B. T. Simms, M. M. Lupa, M. S. Kogan, and J. P. Mizgerd. 2005. Lung NF-κB activation and neutrophil recruitment require IL-1 and TNF receptor signaling during pneumococcal pneumonia. *J. Immunol.* 175: 7530–7535.
13. Mizgerd, J. P., M. L. Scott, M. R. Spieker, and C. M. Doerschuk. 2002. Functions of IκB proteins in inflammatory responses to *Escherichia coli* LPS in mouse lungs. *Am. J. Respir. Cell Mol. Biol.* 27: 575–582.
14. Mizgerd, J. P., M. M. Lupa, M. S. Kogan, H. B. Warren, L. Kobzik, and G. P. Topulos. 2003. Nuclear factor κB p50 limits inflammation and prevents lung injury during *Escherichia coli* pneumonia. *Am. J. Respir. Crit. Care Med.* 168: 810–817.
15. Beg, A. A., W. C. Sha, R. T. Bronson, S. Ghosh, and D. Baltimore. 1995. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-κB. *Nature* 376: 167–170.
16. Doi, T. S., M. W. Marino, T. Takahashi, T. Yoshida, T. Sakakura, L. J. Old, and Y. Obata. 1999. Absence of tumor necrosis factor rescues RelA-deficient mice from embryonic lethality. *Proc. Natl. Acad. Sci. USA* 96: 2994–2999.
17. Alcamo, E., J. P. Mizgerd, B. H. Horwitz, R. Bronson, A. A. Beg, M. Scott, C. M. Doerschuk, R. O. Hynes, and D. Baltimore. 2001. Targeted mutation of TNF receptor I rescues the RelA-deficient mouse and reveals a critical role for NF-κB in leukocyte recruitment. *J. Immunol.* 167: 1592–1600.
18. Rosenfeld, M. E., L. Prichard, N. Shiojiri, and N. Fausto. 2000. Prevention of hepatic apoptosis and embryonic lethality in RelA/TNFR-1 double knockout mice. *Am. J. Pathol.* 156: 997–1007.
19. Sadikot, R. T., W. Han, M. B. Everhart, O. Zoia, R. S. Peebles, E. D. Jansen, F. E. Yull, J. W. Christman, and T. S. Blackwell. 2003. Selective IκB kinase expression in airway epithelium generates neutrophilic lung inflammation. *J. Immunol.* 170: 1091–1098.
20. Poynter, M. E., C. G. Irvin, and Y. M. Janssen-Heininger. 2003. A prominent role for airway epithelial NF-κB activation in lipopolysaccharide-induced airway inflammation. *J. Immunol.* 170: 6257–6265.
21. Sadikot, R. T., H. Zeng, M. Joo, M. B. Everhart, T. P. Sherrill, B. Li, D. S. Cheng, F. E. Yull, J. W. Christman, and T. S. Blackwell. 2006. Targeted immunomodulation of the NF-κB pathway in airway epithelium impacts host defense against *Pseudomonas aeruginosa*. *J. Immunol.* 176: 4923–4930.
22. Skerrett, S. J., H. D. Liggitt, A. M. Hajjar, R. K. Ernst, S. I. Miller, and C. B. Wilson. 2004. Respiratory epithelial cells regulate lung inflammation in response to inhaled endotoxin. *Am. J. Physiol.* 287: L143–L152.
23. Amory-Rivier, C. F., J. Mohler, J. P. Bedos, E. Azoulay-Dupuis, D. Henin, M. Muffat-Joly, C. Carbon, and P. Moine. 2000. Nuclear factor κB activation in mouse lung lavage cells in response to *Streptococcus pneumoniae* pulmonary infection. *Crit. Care Med.* 28: 3249–3256.
24. Meffert, M. K., J. M. Chang, B. J. Wiltgen, M. S. Fanselow, and D. Baltimore. 2003. NF-κB functions in synaptic signaling and behavior. *Nat. Neurosci.* 6: 1072–1078.
25. Jones, M. R., L. J. Quinton, B. T. Simms, M. M. Lupa, M. S. Kogan, and J. P. Mizgerd. 2006. Roles of interleukin-6 in activation of STAT proteins and recruitment of neutrophils during *Escherichia coli* pneumonia. *J. Infect. Dis.* 193: 360–369.
26. Schmittgen, T. D., B. A. Zakrajsek, A. G. Mills, V. Gorn, M. J. Singer, and M. W. Reed. 2000. Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. *Anal. Biochem.* 285: 194–204.
27. Mizgerd, J. P., H. Kubo, G. J. Kutkoski, S. D. Bhagwan, K. Scharffetter-Kochanek, A. L. Beaudet, and C. M. Doerschuk. 1997. Neutrophil emigration in the skin, lungs, and peritoneum: different requirements for CD11/CD18 revealed by CD18-deficient mice. *J. Exp. Med.* 186: 1357–1364.
28. Weibel, E. R. 1990. Morphometry: stereological theory and practical methods. In *Models of Lung Disease: Microscopy and Structural Methods.*, Vol. 47. J. Gil, ed. Marcel Dekker, New York, pp. 199–252.
29. Wikenheiser, K. A., D. K. Vorbroker, W. R. Rice, J. C. Clark, C. J. Bachurski, H. K. Oie, and J. A. Whitsett. 1993. Production of immortalized distal respiratory epithelial cell lines from surfactant protein C/simian virus 40 large tumor antigen transgenic mice. *Proc. Natl. Acad. Sci. USA* 90: 11029–11033.
30. Greenberger, M. J., R. M. Strieter, S. L. Kunkel, J. M. Danforth, L. L. Laichalk, D. C. McGillicuddy, and T. J. Standiford. 1996. Neutralization of macrophage inflammatory protein-2 attenuates neutrophil recruitment and bacterial clearance in murine *Klebsiella* pneumonia. *J. Infect. Dis.* 173: 159–165.
31. Jeyaseelan, S., H. W. Chu, S. K. Young, and G. S. Worthen. 2004. Transcriptional profiling of lipopolysaccharide-induced acute lung injury. *Infect. Immun.* 72: 7247–7256.
32. Jeyaseelan, S., R. Manzer, S. K. Young, M. Yamamoto, S. Akira, R. J. Mason, and G. S. Worthen. 2005. Induction of CXCL5 during inflammation in the rodent lung involves activation of alveolar epithelium. *Am. J. Respir. Cell Mol. Biol.* 32: 531–539.
33. Tsai, W. C., R. M. Strieter, J. M. Wilkowski, K. A. Bucknell, M. D. Burdick, S. A. Lira, and T. J. Standiford. 1998. Lung-specific transgenic expression of KC enhances resistance to *Klebsiella pneumoniae* in mice. *J. Immunol.* 161: 2435–2440.
34. Briles, D. E., S. K. Hollingshead, J. C. Paton, E. W. Ades, L. Novak, F. W. van Ginkel, and W. H. Benjamin, Jr. 2003. Immunizations with pneumococcal surface protein A and pneumolysin are protective against pneumonia in a murine model of pulmonary infection with *Streptococcus pneumoniae*. *J. Infect. Dis.* 188: 339–348.
35. Standiford, T. J., S. L. Kunkel, M. J. Greenberger, L. L. Laichalk, and R. M. Strieter. 1996. Expression and regulation of chemokines in bacterial pneumonia. *J. Leukocyte Biol.* 59: 24–28.
36. Laichalk, L. L., S. L. Kunkel, R. M. Strieter, J. M. Danforth, M. B. Bailie, and T. J. Standiford. 1996. Tumor necrosis factor mediates lung antibacterial host defense in murine *Klebsiella* pneumonia. *Infect. Immun.* 64: 5211–5218.
37. van der Poll, T., C. V. Keogh, W. A. Buurman, and S. F. Lowry. 1997. Passive immunization against tumor necrosis factor α impairs host defense during pneumococcal pneumonia in mice. *Am. J. Respir. Crit. Care Med.* 155: 603–608.
38. Kolls, J. K., D. Lei, S. Nelson, W. R. Summer, S. Greenberg, and B. Beutler. 1995. Adenovirus-mediated blockade of tumor necrosis factor in mice protects against endotoxic shock yet impairs pulmonary host defense. *J. Infect. Dis.* 171: 570–575.
39. Kolls, J. K., D. H. Lei, C. Vasquez, G. Odom, W. R. Summer, S. Nelson, and J. Shellito. 1997. Exacerbation of murine *Pneumocystis carinii* infection by adenoviral-mediated gene transfer of a TNF inhibitor. *Am. J. Respir. Cell Mol. Biol.* 16: 112–118.
40. Standiford, T. J., J. M. Wilkowski, T. H. Sisson, N. Hattori, B. Mehrad, K. A. Bucknell, and T. A. Moore. 1999. Intrapulmonary tumor necrosis factor gene therapy increases bacterial clearance and survival in murine gram-negative pneumonia. *Hum. Gene Ther.* 10: 899–909.
41. Ulich, T. R., L. R. Watson, S. M. Yin, K. Z. Guo, P. Wang, H. Thang, and J. del Castillo. 1991. The intratracheal administration of endotoxin and cytokines. I. Characterization of LPS-induced IL-1 and TNF mRNA expression and the LPS-, IL-1-, and TNF-induced inflammatory infiltrate. *Am. J. Pathol.* 138: 1485–1496.
42. Le Hir, M., H. Bluethmann, M. H. Kosco-Vilbois, M. Muller, F. di Padova, M. Moore, B. Ryffel, and H. P. Eugster. 1995. Tumor necrosis factor receptor-1 signaling is required for differentiation of follicular dendritic cells, germinal center formation, and full antibody responses. *J. Inflamm.* 47: 76–80.
43. Le Hir, M., H. Bluethmann, M. H. Kosco-Vilbois, M. Muller, F. di Padova, M. Moore, B. Ryffel, and H. P. Eugster. 1996. Differentiation of follicular dendritic cells and full antibody responses require tumor necrosis factor receptor-1 signaling. *J. Exp. Med.* 183: 2367–2372.
44. Neumann, B., A. Luz, K. Pfeffer, and B. Holzmann. 1996. Defective Peyer's patch organogenesis in mice lacking the 55-kD receptor for tumor necrosis factor. *J. Exp. Med.* 184: 259–264.
45. Pasparakis, M., S. Kousteni, J. Peschon, and G. Kollias. 2000. Tumor necrosis factor and the p55TNF receptor are required for optimal development of the marginal sinus and for migration of follicular dendritic cell precursors into splenic follicles. *Cell. Immunol.* 201: 33–41.
46. Tsai, W. C., R. M. Strieter, B. Mehrad, M. W. Newstead, X. Zeng, and T. J. Standiford. 2000. CXC chemokine receptor CXCR2 is essential for protective innate host response in murine Pseudomonas aeruginosa pneumonia. *Infect. Immun.* 68: 4289–4296.
47. van der Poll, T., C. V. Keogh, X. Guirao, W. A. Buurman, M. Kopf, and S. F. Lowry. 1997. Interleukin-6 gene-deficient mice show impaired defense against pneumococcal pneumonia. *J. Infect. Dis.* 176: 439–444.
48. Hajjar, A. M., H. Harowicz, H. D. Liggitt, P. J. Fink, C. B. Wilson, and S. J. Skerrett. 2005. An essential role for non-bone marrow-derived cells in control of *Pseudomonas aeruginosa* pneumonia. *Am. J. Respir. Cell Mol. Biol.* 33: 470–475.

49. Noulin, N., V. F. Quesniaux, S. Schnyder-Candrian, B. Schnyder, I. Maillet, T. Robert, B. B. Vargaftig, B. Ryffel, and I. Couillin. 2005. Both hemopoietic and resident cells are required for MyD88-dependent pulmonary inflammatory response to inhaled endotoxin. *J. Immunol.* 175: 6861–6869.
50. Diamond, G., D. Legarda, and L. K. Ryan. 2000. The innate immune response of the respiratory epithelium. *Immunol. Rev.* 173: 27–38.
51. Nys, M., G. Deby-Dupont, Y. Habraken, S. Legrand-Poels, D. Ledoux, J. L. Canivet, P. Damas, and M. Lamy. 2002. Bronchoalveolar lavage fluids of patients with lung injury activate the transcription factor nuclear factor κB in an alveolar cell line. *Clin. Sci. Lond.* 103: 577–585.
52. Armstrong, L., A. R. Medford, K. M. Uppington, J. Robertson, I. R. Witherden, T. D. Tetley, and A. B. Millar. 2004. Expression of functional Toll-like receptor-2 and -4 on alveolar epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 31: 241–245.
53. Droemann, D., T. Goldmann, D. Branscheid, R. Clark, K. Dalhoff, P. Zabel, and E. Vollmer. 2003. Toll-like receptor 2 is expressed by alveolar epithelial cells type II and macrophages in the human lung. *Histochem. Cell Biol.* 119: 103–108.
54. Schmeck, B., S. Huber, K. Moog, J. Zahlten, A. C. Hocke, B. Opitz, S. Hammerschmidt, T. J. Mitchell, M. Kracht, S. Rosseau, et al. 2006. Pneumococci induced TLR- and Rac1-dependent NF-κB-recruitment to the IL-8 promoter in lung epithelial cells. *Am. J. Physiol.* 290: L730–L737.
55. Yoshimura, A., E. Lien, R. R. Ingalls, E. Tuomanen, R. Dziarski, and D. Golenbock. 1999. Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J. Immunol.* 163: 1–5.
56. Robson, R. L., N. A. Reed, and R. T. Horvat. 2006. Differential activation of inflammatory pathways in A549 type II pneumocytes by *Streptococcus pneumoniae* strains with different adherence properties. *BMC Infect. Dis.* 6: 71.
57. N′Guessan, P. D., S. Hippenstiel, M. O. Etouem, J. Zahlten, W. Beermann, D. Lindner, B. Opitz, M. Witzenrath, S. Rosseau, N. Suttorp, and B. Schmeck. 2006. *Streptococcus pneumoniae* induced p38 MAPK- and NF-κB-dependent COX-2 expression in human lung epithelium. *Am. J. Physiol.* 290: L1131–L1138.
58. Schmeck, B., J. Zahlten, K. Moog, V. van Laak, S. Huber, A. C. Hocke, B. Opitz, E. Hoffmann, M. Kracht, J. Zerrahn, et al. 2004. Streptococcus pneumoniae-induced p38 MAPK-dependent phosphorylation of RelA at the interleukin-8 promotor. *J. Biol. Chem.* 279: 53241–53247.
59. Mizgerd, J. P., J. J. Peschon, and C. M. Doerschuk. 2000. Roles of tumor necrosis factor receptor signaling during murine *Escherichia coli* pneumonia. *Am. J. Respir. Cell Mol. Biol.* 22: 85–91.
60. Mizgerd, J. P., M. R. Spieker, and C. M. Doerschuk. 2001. Early response cytokines and innate immunity: essential roles for TNF receptor 1 and type I IL-1 receptor during *Escherichia coli* pneumonia in mice. *J. Immunol.* 166: 4042–4048.
61. Mizgerd, J. P., M. M. Lupa, J. Hjoberg, J. C. Vallone, H. B. Warren, J. P. Butler, and E. S. Silverman. 2004. Roles for early response cytokines during Escherichia coli pneumonia revealed by mice with combined deficiencies of all signaling receptors for TNF and IL-1. *Am. J. Physiol.* 286: L1302–L1310.
62. Moreland, L. W. 1999. The role of cytokines in rheumatoid arthritis: inhibition of cytokines in therapeutic trials. *Drugs Today* 35: 309–319.
63. Dinarello, C. A. 2003. Anti-cytokine therapeutics and infections. *Vaccine* 21(Suppl. 2): S24–S34.
64. Beck-Schimmer, B., R. Schwendener, T. Pasch, L. Reyes, C. Booy, and R. C. Schimmer. 2005. Alveolar macrophages regulate neutrophil recruitment in endotoxin-induced lung injury. *Respir. Res.* 6: 61.
65. Broug-Holub, E., G. B. Toews, J. F. van Iwaarden, R. M. Strieter, S. L. Kunkel, R. Paine, III, and T. J. Standiford. 1997. Alveolar macrophages are required for protective pulmonary defenses in murine *Klebsiella* pneumonia: elimination of alveolar macrophages increases neutrophil recruitment but decreases bacterial clearance and survival. *Infect. Immun.* 65: 1139–1146.
66. Hashimoto, S., J. F. Pittet, K. Hong, H. Folkesson, G. Bagby, L. Kobzik, C. Frevert, K. Watanabe, S. Tsurufuji, and J. Wiener-Kronish. 1996. Depletion of alveolar macrophages decreases neutrophil chemotaxis to *Pseudomonas* airspace infections. *Am. J. Physiol.* 270: L819–L828.
67. Lentsch, A. B., B. J. Czermak, N. M. Bless, N. Van Rooijen, and P. A. Ward. 1999. Essential role of alveolar macrophages in intrapulmonary activation of NF-κB. *Am. J. Respir. Cell Mol. Biol.* 20: 692–698.
68. Manzer, R., J. Wang, K. Nishina, G. McConville, and R. J. Mason. 2006. Alveolar epithelial cells secrete chemokines in response to IL-1β and lipopolysaccharide but not to ozone. *Am. J. Respir. Cell Mol. Biol.* 34: 158–166.
69. Pechkovsky, D. V., G. Zissel, M. W. Ziegenhagen, M. Einhaus, C. Taube, K. F. Rabe, H. Magnussen, T. Papadopoulos, M. Schlaak, and J. Muller-Quernheim. 2000. Effect of proinflammatory cytokines on interleukin-8 mRNA expression and protein production by isolated human alveolar epithelial cells type II in primary culture. *Eur. Cytokine Netw.* 11: 618–625.
70. Vanderbilt, J. N., E. M. Mager, L. Allen, T. Sawa, J. Wiener-Kronish, R. Gonzalez, and L. G. Dobbs. 2003. CXC chemokines and their receptors are expressed in type II cells and upregulated following lung injury. *Am. J. Respir. Cell Mol. Biol.* 29: 661–668.
71. Ishii, H., T. Fujii, J. C. Hogg, S. Hayashi, H. Mukae, R. Vincent, and S. F. van Eeden. 2004. Contribution of IL-1β and TNF-α to the initiation of the peripheral lung response to atmospheric particulates (PM10). *Am. J. Physiol.* 287: L176–L183.
72. Knapp, S., C. W. Wieland, C. van't Veer, O. Takeuchi, S. Akira, S. Florquin, and T. van der Poll. 2004. Toll-like receptor 2 plays a role in the early inflammatory response to murine pneumococcal pneumonia but does not contribute to antibacterial defense. *J. Immunol.* 172: 3132–3138.
73. Srivastava, A., P. Henneke, A. Visintin, S. C. Morse, V. Martin, C. Watkins, J. C. Paton, M. R. Wessels, D. T. Golenbock, and R. Malley. 2005. The apoptotic response to pneumolysin is Toll-like receptor 4 dependent and protects against pneumococcal disease. *Infect. Immun.* 73: 6479–6487.