Earliest Innate Immune Responses Require Macrophage RelA during Pneumococcal Pneumonia

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NF- κ B regulates cytokine expression to initiate and control the innate immune response to lung infections. The NF- κ B protein RelA is critical for pulmonary host defense during *Streptococcus pneumoniae* pneumonia, but the cell-specific roles of this transcription factor remain to be determined. We hypothesized that RelA in alveolar macrophages contributes to cytokine expression and host defense during pneumococcal pneumonia. To test this hypothesis, we compared mice lacking RelA exclusively in myeloid cells (RelA $^{\Delta/\Delta}$) with littermate controls (RelA $^{F/F}$). Alveolar macrophages from RelA $^{\Delta/\Delta}$ mice expressed no full-length RelA, demonstrating effective targeting. Alveolar macrophages from RelA $^{\Delta/\Delta}$ mice exhibited reduced, albeit detectable, proinflammatory cytokine responses to *S. pneumoniae*, compared with alveolar macrophages from RelA $^{F/F}$ mice. Concentrations of these cytokines in lung homogenates were diminished early after infection, indicating a significant contribution of macrophage RelA to the initial expression of cytokines in the lungs. However, the cytokine content in infected lungs was equivalent by 15 hours. Neutrophil recruitment during *S. pneumoniae* pneumonia reflected a delayed onset in RelA $^{\Delta/\Delta}$ mice, followed by similar rates of accumulation. Bacterial clearance was eventually effective in both genotypes, but began later in RelA $^{\Delta/\Delta}$ mice. Thus, during pneumococcal pneumonia, only the earliest induction of the cytokines measured depended on transcription by RelA in myeloid cells, and this transcriptional activity contributed to effective immunity.

Keywords: RelA; pneumonia; NF- κ B; macrophage; cytokine

Acute lung infections cause more disease worldwide than neoplasms, ischemic heart disease, or HIV/AIDS. However, their importance to public health is relatively underrepresented as a research priority (1). Bacterial pneumonias, a common cause of morbidity and mortality in the United States and worldwide, are estimated to cost the United States approximately \$20 billion per year (2). The most common cause of community-acquired pneumonia is the Gram-positive bacterium *Streptococcus pneumoniae* (3), commonly called pneumococcus.

The recruitment of neutrophils into infected airspaces is crucial for the clearance of invading pneumococci (4, 5). This process is largely made possible by chemokines in which 2 amino-terminal cysteines are separated by a single amino acid (CXC) and preceded by a glutamic acid-leucine-arginine (ELR) motif including KC (CXCL1) and macrophage inflammatory (MIP)-2 (CXCL2) in mice, which can be expressed by alveolar

macrophages and alveolar epithelial cells (6, 7). During pneumococcal pneumonia, the recruitment of neutrophils and the expression of ELR⁺ CXC chemokines require upstream signaling from the early response cytokines, TNF- α and IL-1 (specifically, IL-1 α and IL-1 β) (8).

The induction of TNF- α , IL-1, ELR⁺ CXC chemokines, and other inflammatory cytokines is mediated by multiple transcription factors, including NF- κ B. Of the five NF- κ B proteins, only p50 and RelA (also known as p65) are readily detectable in lung nuclear fractions during acute pulmonary inflammation (8–10). p50 limits the expression of inflammatory cytokines and prevents inflammatory lung injury (11, 12). In contrast, RelA drives proinflammatory responses by promoting the expression of inflammatory cytokines, and the deletion of RelA from all cells severely compromises antibacterial host defense (13, 14). Distinct roles of RelA in different lung cell types during pneumococcal pneumonia remain to be discriminated.

During bacterial pneumonias, alveolar macrophages are the first leukocytes to encounter pathogens (15, 16). Based on studies using cytotoxic agents to diminish the number of alveolar macrophages, these cells were observed to play roles in eliminating bacteria (17–19), facilitating neutrophil migration (20), and resolving inflammation (21, 22). Macrophage-derived cytokines were inferred to be critical in lung defense, and these cytokines are dependent on NF- κ B (23), but this inference has not been rigorously tested. The deletion of RelA in myeloid cells leads to decreased levels of cytokines in the lungs after the instillation of a bolus of heat-killed *Pseudomonas aeruginosa* (24), suggesting that the activity of RelA in macrophages may be involved in net cytokine elaboration during lung infection. In contrast, the deletion of the NF- κ B-activating enzyme I κ B Kinase b (IKKb) from myeloid cells leads to the excess elaboration of cytokines from macrophages in response to LPS or Gram-positive bacteria (25, 26), suggesting that IKK β and perhaps NF- κ B are important in macrophages for limiting rather than promoting the expression of cytokines and inflammatory responses. Because alveolar macrophages are sentinel cells recognizing pneumococcus in the lungs (27), and because RelA is necessary for the expression of cytokines mediating the recruitment of neutrophils (13, 14), we hypothesized that alveolar-macrophage NF- κ B RelA is critical for the initiation of the innate immune response during pneumococcal pneumonia.

MATERIALS AND METHODS

Pneumonia

Mice lacking RelA in myeloid cells were generated by crossing LysM-Cre mice (28) with RelA-floxed mice (29), generating a colony producing LysM-Cre^{tg}/RelA^{loxP/loxP} (RelA $^{\Delta/\Delta}$) and LysM-Cre^{tg}/RelA^{loxP/loxP} (RelA $^{F/F}$) littermates. Fifty microliters of saline containing *S. pneumoniae* serotype 19 (SP19; strain EF3030, kindly provided by Dr. Marc Lipsitch, Boston, MA) or serotype 3 (SP3; strain 6303; ATCC, Manassas, VA) were instilled into the left bronchus of 6–12-week-old mice, as described elsewhere (13). Our protocols were

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TABLE 1. QUANTITATIVE RT-PCR PRIMER AND PROBE SEQUENCES

	Forward	Reverse	Probe
IL-1 α	ATCTCAGATTCACAACGTTCGTG	TTTGGTGTCTTCTGGCAACTCC	CGCTCAAGGAGAAGACCAGCCCC
TLR2	CAACTTACCGAAACCTCAGACAA	GAGGACTGTTATGGCCACCAA	ATCCAGAAGAGCCAAAGAGCTCGTAGCA
TLR4	AGAACTTCAGTGGCTGGATTTA	TGGATAGGGTTCTCTGTCACTA	CATGGCATGGCTTACACCACCTCTCAAAC
MARCO	ACAAAGGGGACATGGGCATGAA	TGACTCCTGGAGATCCAGCCAA	TTCCAGCATCACCTTTACCTCCCTGAGCT

Definition of abbreviations: TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4; MARCO, macrophage receptor with collagenous structure.

Primer and probe sequences were designed to quantify mRNA induction of several transcripts, using quantitative RT-PCR.

approved by the Institutional Animal Care and Use Committees at Harvard University and Boston University.

Collection of Alveolar Macrophages

Lungs were lavaged 10 times, as described previously (30), with 1 ml of ice-cold buffer (20 mM Hepes, 145 mM NaCl, 5.5 mM dextrose, 2.7 mM EDTA, and 100 U/ml Pen-Strep; Invitrogen Life Technologies, Carlsbad, CA). Cells were washed twice with saline. Protein lysates were collected as described elsewhere (13). DNA or RNA was isolated using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) or TRIzol reagent (Invitrogen Life Technologies, Valencia, CA), respectively.

RelA Gene Rearrangement in Alveolar Macrophages

Recombination of the *Rela* locus in alveolar macrophages from RelA Δ/Δ mice was confirmed, using PCR with the following three primers: 5'-CCAGCGAATCCAGACCAACAATAAC-3', 5'-CGACCACTCTTCACTGATTGTTTTTC-3', and 5'-AAGGCTCAGAGGCAGTGA GAGACATAC-3'. Amplification of full-length or rearranged *Rela* was indicated by PCR products of approximately 2,500 or 1,600 bp in length, respectively.

RelA protein was assessed in cell lysates by immunoblotting (10, 13), using polyclonal antibodies raised against RelA (Cell Signaling Technology, Danvers, MA, or Santa Cruz Biotechnology, Santa Cruz, CA) or β -actin (Cell Signaling Technology).

Culturing of Alveolar Macrophages

Lavaged cells were cultured overnight in serum-free RPMI-1640 (Invitrogen Life Technologies) with penicillin and streptomycin. After 1 hour, nonadherent cells were removed by washing. Cells were washed twice, and bacteria were added in media without antibiotics. After 2 hours, the supernatants were collected, and the cells were washed twice and cultured in media containing antibiotics. Media were replaced and supernatants were collected every 2 hours.

Cytokine and Receptor Measurements

mRNA induction for TNF- α , IL-1 α , IL-1 β , KC, MIP-2, Toll-like receptor 2 (TLR2), TLR4, scavenger receptor A (SR-A), and macrophage receptor with collagenous structure (MARCO) were measured in left lobes or bronchoalveolar lavage (BAL) cells, using quantitative RT-PCR (13). Primer and probe sequences are listed in Table 1 or published elsewhere (13, 31, 32). Cytokine protein concentrations were measured in culture supernatants or whole-lung tissue homogenates (30), using Quantikine ELISA kits (R&D Systems, Minneapolis, MN).

Lung Histology

Alveolar neutrophils were quantified by morphometric analyses of hematoxylin-and-eosin-stained sections of left (infected) lung lobes, as described previously (30, 33, 34). Peripheral blood from the vena cava was used for total and differential cell counts.

Bacterial Clearance

Lungs were homogenized in sterile, distilled H₂O containing protease inhibitors (35). Homogenates were serially diluted, plated on 5% sheep blood agar plates using the drop plate method (36), and incubated overnight at 37°C. Colonies were then counted to quantify CFUs/lung.

Statistical Analysis

Statistics were calculated using GraphPad Prism (GraphPad Software, La Jolla, CA) and/or Statistica (StatSoft, Tulsa, OK). Data were analyzed

using unpaired Student *t* tests for comparisons between two individual groups or two-way ANOVA, followed by *post hoc* tests, for individual differences between multiple groups, as indicated. *P* < 0.05 was considered significant.

RESULTS

Disruption of RelA in Alveolar Macrophages

Although *Rela* $^{-/-}$ mice die during gestation (37), RelA Δ/Δ and RelA $^{F/F}$ mice were born in numbers reflecting Hardy-Weinberg equilibrium, indicating that the deletion of RelA in myeloid cells alone does not confer a selective disadvantage during gestation. *Rela* $^{-/-}$ mice survive through birth if they lack tumor necrosis factor receptor 1 (TNFR1) signaling, but display severely shortened life spans because of bacterial infections (14). In contrast, mice with RelA mutated in myeloid cells alone survived to weaning, and demonstrated no gross signs of morbidity through at least 30 weeks of age.

To determine whether RelA was effectively targeted in alveolar macrophages, we collected alveolar macrophages from RelA Δ/Δ and RelA $^{F/F}$ mice, and isolated their DNA and proteins for RelA analyses. No significant differences were evident in the numbers (data not shown) or morphology (Figure 1A) of alveolar macrophages collected from RelA Δ/Δ and RelA $^{F/F}$ mice. PCR with primers specific for the wild-type *Rela* gene or the rearranged gene product (missing exons 7–10) demonstrated that *Rela* was rearranged in DNA from the alveolar macrophages of RelA Δ/Δ mice, but not from those of RelA $^{F/F}$ mice (Figure 1B). We used two different RelA-specific antibodies to determine whether truncation of the RelA protein occurred in alveolar macrophages from RelA Δ/Δ mice. One antibody (catalogue number 3034; Cell Signaling Technology) recognizes the amino acids surrounding Ser276 of human NF- κ B RelA, encoded by exons excised by Cre recombinase if targeting is effective as designed. Alveolar macrophages from RelA Δ/Δ mice had no detectable RelA according to this antibody (Figure 1C, *top*). The other RelA antibody (catalogue number sc-372; Santa Cruz Biotechnology) recognizes the carboxy terminus of RelA, encoded by exons outside the loxP targeting strategy. This epitope should be present in both wild-type (full-length) and mutant (truncated) forms of RelA. The antibody demonstrated that alveolar macrophages from RelA Δ/Δ mice expressed only the truncated version of RelA, whereas those from RelA $^{F/F}$ mice expressed only the full-length RelA (Figure 1C, *middle*). Thus, alveolar macrophages are effectively targeted by the LysM-Cre transgene. Alveolar macrophages in RelA Δ/Δ mice express no full-length RelA.

Cytokine Expression from Isolated Alveolar Macrophages

To determine whether alveolar macrophages produce cytokines in response to *S. pneumoniae* and whether RelA is necessary for this production, we isolated alveolar macrophages from RelA Δ/Δ and RelA $^{F/F}$ mice and cultured them *in vitro* with SP19 for 2 hours. Supernatants were removed, and the medium was replaced every 2 hours. Alveolar macrophages from control RelA $^{F/F}$ mice produced TNF- α , KC, and MIP-2 in response to

Figure 1. Alveolar macrophages from RelA^{Δ/Δ} mice were present in comparable numbers, appeared similar in morphology, and expressed a nonfunctional form of RelA (missing exons 7–10) compared with alveolar macrophages from RelA^{F/F} littermates. (A) Morphology of macrophages recovered from mice did not differ between RelA^{F/F} (left) and RelA^{Δ/Δ} (right) mice, as indicated by Diff-Quik–stained cytocentrifuge preparations. (B) PCR amplification of normal and rearranged RelA (top) in DNA from alveolar macrophages of RelA^{F/F} and RelA^{Δ/Δ} mice demonstrated the gene to be rearranged in cells from the latter (Cre-positive) mice. PCR amplification of signal transducer and activator of transcription 3 (STAT3) (below) was used as a negative control to demonstrate the specificity of targeting. (C) Mutant but not wild-type RelA protein was detected in alveolar macrophages from Cre-positive RelA^{Δ/Δ} mice. Antibodies recognizing an epitope located within exons 7–10 (top) identified a product in the alveolar macrophages from RelA^{F/F} but not RelA^{Δ/Δ} mice. Antibodies recognizing a C-terminal epitope (middle) identified full-length and truncated forms of the RelA protein in RelA^{F/F} and RelA^{Δ/Δ} mice, respectively. β-actin antibodies (below) were used as a loading control.

SP19 infection *in vitro* (Figure 2). This cytokine expression was transient, with the majority of expression occurring within 4 hours after initial exposure of macrophages to bacteria. Between 4–6 hours, little to no TNF-α, KC, or MIP-2 was expressed (Figure 2), and none was detectable after 6 hours of culture (data not shown). Neither IL-1α nor IL-1β was detected in these supernatants from macrophages infected *in vitro* with SP19 (data not shown), which may reflect their distinctive mechanisms of secretion (38, 39). Alveolar macrophages from RelA^{Δ/Δ} mice expressed significantly less TNF-α and MIP-2 in response to infection with SP19 than alveolar macrophages from RelA^{F/F} mice after infection with SP19 (Figure 2), and KC showed a similar trend that did not reach statistical significance. Alveolar macrophages from RelA^{Δ/Δ} mice still expressed some TNF-α, KC, and MIP-2 (Figure 2),

Figure 2. RelA was necessary for early-response cytokine production by alveolar macrophages stimulated with *Streptococcus pneumoniae* *in vitro*. Alveolar macrophages were collected from uninfected RelA^{F/F} or RelA^{Δ/Δ} mice and stimulated with *S. pneumoniae* for 2 hours. Medium was replaced every 2 hours after stimulation with *S. pneumoniae*, and concentrations of (A) TNF-α, (B) KC, and (C) macrophage inflammatory protein-2 (MIP-2) were measured in the supernatant. Data were expressed as mean ± SEM from three independent experiments. Data were analyzed using two-way ANOVA, followed by the Student Newman-Keuls *post hoc* test. **P* < 0.05, compared with RelA^{F/F} mice.

suggesting that additional pathways mediating the RelA-independent expression of these cytokines exist. However, the data suggest that the predominant pathway for the expression of these proinflammatory cytokines from isolated alveolar macrophages stimulated with pneumococcus requires functional RelA.

Cytokine Expression from Alveolar Macrophages in Infected Lungs

To determine whether the expression of alveolar macrophage cytokines is RelA-dependent during pneumococcal pneumonia, we instilled SP19 into the lungs of RelA^{Δ/Δ} and RelA^{F/F} mice and collected alveolar macrophages by BAL 6 hours later. At this early time point of infection, which likely included cells from both infected and uninfected lung regions, over 95% of the collected cells were macrophages. During these *in vivo* infections, alveolar macrophages from RelA^{Δ/Δ} mice had significantly less mRNA for TNF-α, IL-1α, IL-1β, KC, and MIP-2 compared with alveolar macrophages from RelA^{F/F} mice, indicating that the induction of these cytokines in alveolar

macrophages is largely RelA-dependent during pneumococcal pneumonia. However, some TNF- α , IL-1 α , IL-1 β , KC, and MIP-2 mRNA was produced by these cells (Figure 3). Together with the *in vitro* analyses, these data from *in vivo* infections indicate that pneumococcus induces the expression of inflammatory cytokines and chemokines by alveolar macrophages via primarily RelA-dependent pathways.

Receptor Expression by Alveolar Macrophages

To determine whether RelA influenced the expression of macrophage receptors for pneumococcus, we measured relevant transcripts in alveolar macrophages lavaged from control and mutant lungs with and without pneumonia. Pneumococcus can be recognized by TLR2 (40), TLR4 (41), SR-A (42), and MARCO (27). In control RelA^{F/F} mice, pneumococcus increased the expression of TLR2, SR-A, and MARCO, but not TLR4 (Figure 4). In contrast, none of these receptors were induced by pneumococcal exposure in RelA $\Delta\Delta$ mice (Figure 4), demonstrating that increased responsiveness to pneumococcus is facilitated by this transcription factor in these cells. Significantly less MARCO mRNA was evident in RelA $\Delta\Delta$ macrophages compared with RelA^{F/F} macrophages even before infection, and less TLR2 and MARCO mRNA during infection, indicating that the initial recognition of pneumococcus by alveolar macrophages may be compromised by the targeting of RelA. These data suggest that the recognition by alveolar macrophages of pneumococcus is partly dependent on a positive feedback loop involving pattern recognition receptors and RelA.

Cytokine Expression in Pneumonic Lungs

To determine the degree to which macrophage RelA contributes to the total lung content of these cytokines during pneumococcal pneumonia, we measured protein concentrations

of TNF- α , IL-1 α , IL-1 β , KC, and MIP-2 in lung homogenates from RelA $\Delta\Delta$ and RelA^{F/F} mice at 0, 6, 15, and 48 hours after infection with SP19. Before infection with *S. pneumoniae*, no significant differences were evident in lung cytokine concentrations between RelA $\Delta\Delta$ and RelA^{F/F} mice (Figures 5A–5E). Infection increased the lung content of all cytokines measured (Figure 5), likely because of induction in the infected sites, although both infected and uninfected lung regions were included in these analyses. Early after infection (6 hours), the expression of cytokines was significantly reduced in whole lungs of RelA $\Delta\Delta$ mice because of a macrophage RelA deficiency. At later times after infection (up to 48 hours), total lung cytokine concentrations were no longer decreased in the lungs of mice lacking RelA in their macrophages (Figures 5A–5E). Altogether, these data indicate that macrophage RelA is particularly important for the early elaboration of these cytokines during pneumococcal pneumonia.

Neutrophil Recruitment

Because the early-response cytokines and ELR⁺ CXC chemokines that depend on macrophage RelA contribute to neutrophil recruitment in the lungs (23), we hypothesized that RelA $\Delta\Delta$ mice would have fewer emigrated neutrophils compared with RelA^{F/F} littermates during pneumonia. By 9 hours after infection with SP19, neutrophils were evident in the airspaces of RelA^{F/F} mice (Figures 6A and 6B). At this time, RelA $\Delta\Delta$ mice had significantly fewer neutrophils in their alveoli than did the RelA^{F/F} mice (Figures 6A and B), at 32% of control levels. Although neutrophils in the alveolar airspaces increased over time in both genotypes, neutrophil recruitment in the RelA $\Delta\Delta$ mice remained lower than in RelA^{F/F} mice throughout the entire 48-hour time course (Figure 6B). Reduced numbers of neutrophils in the alveoli counts were not secondary to neu-

Figure 3. Production of inflammatory cytokines in alveolar macrophages depended on RelA during infection with *S. pneumoniae*. Quantitative RT-PCR was used to measure fold induction of (A) TNF- α , (B) IL-1 α , (C) IL-1 β , (D) KC, and (E) MIP-2 mRNA in alveolar macrophages collected by bronchoalveolar lavage from RelA^{F/F} and RelA $\Delta\Delta$ mice infected with 10^6 CFUs of *S. pneumoniae* for 6 hours. Data were expressed as geometric mean $\pm$ geometric SEM ($n = 2$ –4 uninfected mice/group, or 4–9 infected mice/group). Data were analyzed using two-way ANOVA, followed by Bonferroni *post hoc* test. * $P < 0.05$, compared with RelA^{F/F} mice.

Figure 4. Pattern recognition receptor expression in alveolar macrophages is partly dependent on RelA during infection with *S. pneumoniae*. Quantitative RT-PCR was used to measure fold induction of (A) Toll-like receptor 2 (TLR2), (B) Toll-like receptor 4 (TLR4), (C) scavenger receptor A (SR-A), and (D) macrophage receptor with collagenous structure (MARCO) mRNA in alveolar macrophages collected by bronchoalveolar lavage from RelA^{F/F} and RelA^{Δ/Δ} mice infected with 10⁶ CFUs of *S. pneumoniae* for 0 or 6 hours. Data were expressed as geometric mean $\pm$ geometric SEM ($n = 6-9$ uninfected mice/group, or 4-6 infected mice/group). Data were analyzed using two-way ANOVA, followed by Bonferroni *post hoc* test. * $P < 0.05$, compared with 0 hours for the same genotype. † $P < 0.05$, compared with RelA^{F/F} mice at same time point.

tropenia, because RelA^{Δ/Δ} mice had as many or more circulating neutrophils as RelA^{F/F} mice throughout their pneumonia (Figure 6C). These data suggest that, in addition to its influence on cytokine and receptor synthesis (Figures 2-5), macrophage RelA contributes to maximal neutrophil recruitment into the alveolar airspaces during pneumococcal pneumonia.

Bacterial Clearance

Because neutrophil recruitment mediates bacterial clearance during pneumococcal pneumonia (4, 5) and was impaired in RelA^{Δ/Δ} mice, we measured viable bacteria in the lung to determine whether myeloid RelA plays roles in integrated host defense. All lung lobes were included together in these analyses;

Figure 5. Initial inflammatory cytokine protein production in the lungs during *S. pneumoniae* pneumonia was dependent on macrophage RelA. Concentrations of (A) TNF- α , (B) IL-1 α , (C) IL-1 β , (D) KC, and (E) MIP-2 in homogenized whole lungs from RelA^{F/F} and RelA^{Δ/Δ} mice infected with 10⁶ CFUs of *S. pneumoniae* for 0, 6, 15, or 48 hours were measured using ELISA. Data were expressed as mean $\pm$ SEM ($n = 5-7$ mice/group) and analyzed using two-way ANOVA, followed by Bonferroni *post hoc* test. * $P < 0.05$, compared with RelA^{F/F} mice at same time point after infection.

Figure 6. Targeted deletion of RelA in myeloid cells impaired recruitment of neutrophils and compromised clearance of *S. pneumoniae*. (A) Representative sections of hematoxylin-and-eosin-stained lungs from uninfected or *S. pneumoniae*-infected (9 hours; 10⁶ CFUs) RelA^{F/F} and RelA^{Δ/Δ} mice, used for lung morphometry. (B) RelA^{Δ/Δ} mice had decreased neutrophils in their alveolar airspaces, measured using morphometry. Data were expressed as mean ± SEM (*n* = 9–11 mice/group). (C) Numbers of circulating neutrophils were not diminished in blood of RelA^{Δ/Δ} mice. (D) Bacterial clearance was compromised in RelA^{Δ/Δ} mice. Viable bacteria per lung were quantified using CFU assays, and data were expressed as mean ± SEM (*n* = 5–11 mice/group). Statistical analyses were performed using two-way ANOVA, followed by Student Newman-Keuls *post hoc* test. **P* < 0.05, compared with RelA^{F/F} mice at the same time point after infection.

hence the numbers of CFUs recovered represent all bacteria throughout the lower respiratory tract. At 6 hours, before the substantive recruitment of neutrophils into the airspaces, lung bacterial burdens were increasing, with no difference attributable to genotype (Figure 6D). Coinciding with neutrophil recruitment in RelA^{F/F} mice, bacterial burdens plateaued, followed by a decline. By 48 hours after pneumococcal infection, RelA^{F/F} mice had cleared bacteria to levels below the initial inoculum of 10⁶ CFUs (Figure 6D). By 15 hours after pneumococcal infection, the lungs of RelA^{F/F} mice contained significantly fewer living bacteria than those of RelA^{Δ/Δ} mice, indicating an important role for myeloid RelA in this bacterial clearance (Figure 6D). RelA^{F/F} mice continued to contain fewer *S. pneumoniae* in their lungs through 48 hours after infection compared with RelA^{Δ/Δ} mice (Figure 6D). Altogether, these data suggest that alveolar macrophage RelA is necessary for optimal bacterial clearance during pneumococcal pneumonia.

Severe Infections

As previously reported (43), the infections performed with serotype 19 pneumococcus were self-limiting. Both genotypes of mice cleared the organisms and survived. To determine whether the early defect in macrophage-derived cytokines influences the outcome of a more severe infection, mice were challenged with higher doses of this bacteria (3 × 10⁷ instead of 1 × 10⁶ CFUs per mouse), sufficient to cause bacteremia and death (43). During high-dose infection, no differences were evident between genotypes in terms of bacteremia (two of six in each genotype at 48 hours) or mortality (four of six in each genotype at 7 days). We also addressed a model of severe infection that provided a means of extending observations to a different bacterium, a serotype 3 pneumococcus, which is extremely virulent at even low levels of infection (8, 43). As

expected, based on previous studies of this organism in mice with compromised innate immunity (8), the growth of this virulent bacterium in the lungs was unaltered by murine genotype (Table 2). However, when cytokines and neutrophils were measured in the lungs of mice with serotype 3 infection, the myeloid RelA mutation showed phenotypes similar to those in the less virulent serotype 19 infection (Table 2), including the effects of myeloid RelA targeting on both the expression of TNF-α and the accumulation of neutrophils. These data suggest that myeloid RelA contributes to early innate immune responses during diverse infections, but that RelA-dependent macrophage responses may be especially important for infections that are less severe because of either the dose or virulence of the microbe.

DISCUSSION

These studies identify roles of alveolar macrophage RelA in cytokine and receptor expression initiating innate immune responses during pneumococcal pneumonia. RelA is necessary in alveolar macrophages for the induction of cytokines and chemokines elicited by pneumococcus. During pneumonia, macrophage RelA contributes greatly to the early burst of cytokines, with long-term consequences for neutrophil recruitment and bacterial clearance. Thus, RelA-mediated cytokine expression by alveolar macrophages has important proinflammatory functions during pneumococcal pneumonia.

The alveolar macrophage expression of cytokines is largely but not completely dependent on RelA during pneumococcal infection. In *in vitro* studies with purified cultures of alveolar macrophages, the vast majority of cytokine elaboration stimulated by pneumococcus required RelA. When alveolar macrophages were collected from murine lungs infected *in vivo* with

TABLE 2. MYELOID RelA TARGETING AND PNEUMONIA CAUSED BY SEROTYPE 3 PNEUMOCOCCUS

	RelA ^{F/F}	RelA ^{Δ/Δ}	P
CFUs (× 10 ⁶) at 24 hours	2.7 ± 1.1	3.7 ± 2.3	0.65
TNF-α (fold uninfected) at 6 hours	52 ± 12	14 ± 1.8	0.01
Neutrophils (percentage of alveolar airspace volume) at 24 hours	29 ± 2.4	18 ± 4.3	0.04

RelA^{Δ/Δ} and littermate control RelA^{F/F} mice were infected by intratracheal instillation of 10⁶ CFUs of serotype 3 pneumococcus. Bacterial burden was measured in whole-lung homogenates, using CFU analyses. TNF-α was measured in mRNA samples collected from the infected left lung lobe using quantitative RT-PCR, and expressed relative to transcript levels in uninfected mice. Emigrated neutrophils were quantified in the infected left lung lobe using morphometric analyses, and were expressed as volume percentage of the alveolar air space. Descriptive statistics were expressed as mean ± SEM, and P values were determined using unpaired Student *t* test.

pneumococcus, their cytokine expression was nearly abrogated by the deletion of functional RelA. Consistent observations across *in vitro* experiments (in which cultured cells are not exposed to potentially relevant host factors) and *in vivo* experiments (in which macrophages are in a dynamic environment, beyond the control of investigators) inspire confidence that these results effectively depict the role of RelA in alveolar macrophages. Because we observed a decreased expression of TLR2 and MARCO in RelA mutant macrophages during pneumonia, signaling other than that downstream of RelA may conceivably be compromised because of the RelA mutation. Hence, the total effect of RelA mutation on cytokine expression may include both direct and indirect effects. However, at least some expression of cytokines is clearly independent of alveolar macrophage RelA. The low-level expression of cytokines remaining in alveolar macrophages without RelA may be mediated by transcription factors such as Ets, Elk-1, Egr-1, ATF-2, c-Jun, Sp1, PU.1, and IRF-4, each of which can function in the transcription of TNF-α and IL-1β (44, 45). The present study, however, demonstrates that in response to pneumococcus, RelA is particularly important to the expression of these cytokines by alveolar macrophages.

When whole-lung lysates were examined, all cytokines were significantly reduced in RelA^{Δ/Δ} mice at the 6-hour time point. The model of LysM-targeted deletion does not allow for discrimination between RelA function in alveolar macrophages and other cells of the myeloid lineage such as neutrophils and dendritic cells, all of which are represented at some concentration in whole-lung lysates. However, alveolar macrophages collected from the airspaces of infected mice expressed cytokines, and these cytokines were diminished by RelA disruption. Thus, the earliest cytokine elaboration during pneumococcal pneumonia is dependent on RelA in myeloid cells (likely alveolar macrophages). We conclude that during pneumococcal pneumonia, the initial, early burst of cytokine expression in the lungs requires RelA in alveolar macrophages.

Interestingly, by 15 hours of infection, no significant differences attributable to genotype were evident in the lung homogenate concentrations of most cytokines. Because alveolar macrophage cytokines are so dependent on RelA, cells other than macrophages are likely sources of these cytokines during later stages of pneumococcal pneumonia. Alveolar epithelial cells, airway epithelial cells, airway smooth muscle cells, interstitial fibroblasts, microvascular endothelial cells, and lung lymphocytes can all generate cytokines that may contribute to inflammatory signaling in infected lungs (23). The delayed time for other cells to produce cytokines may result from changes in bacterial physiology during infection. For example, over time,

pneumococci in the lungs shed their capsules and physically interact with structural cells in the lung (46), and the NF-κB responses of epithelial cells to pneumococcus positively correlate with bacterial adherence (47). Such changes in interaction between lung cells and pneumococcus may stimulate the expression of cytokines from cells other than macrophages during *in vivo* infections. Moreover, RelA-independent signaling may promote the expression of cytokines by macrophages in RelA^{Δ/Δ} mice at later stages of pneumonia. Our results indicate that purified alveolar macrophages express some cytokines even in the absence of functional RelA, albeit to a much lower extent. The inflammatory milieu of a pneumonic lung by 15 hours may be sufficiently different to promote significant synthesis of cytokines downstream from alternative signaling pathways in cells lacking RelA. Lastly, the effect of increased lung bacterial burden evident after 15 hours of infection in RelA^{Δ/Δ} mice may provide a more robust stimulus for the expression of cytokines in the lungs, superseding the observed negative effect of macrophage RelA deletion on this response.

The defect in cytokine expression from RelA-deficient macrophages significantly compromised the integrated host defense of the lung, diminishing the recruitment of neutrophils and exacerbating pneumococcal infection. The clearance of bacteria appeared to be delayed rather than prevented in mutant mice. Similarly, the recruitment of neutrophils appeared to be staggered. It was slower to start in the mutant mice, and then neutrophils accumulated at a comparable rate in both genotypes, resulting in lower numbers of neutrophils in the mutants than in control mice throughout the time course measured. The kinetics of both these host defense functions are consistent with a lag effect, in which a transient defect in innate immunity in mutant mice early during infection is followed by competent responses. This interpretation again emphasizes the concept that macrophage RelA is particularly important in the initial responses to microbes in the lungs.

Defects in host defense were also evident in mice with LysM-targeted RelA deletion during *P. aeruginosa* pneumonia (24), but the host defense phenotype during Group B *Streptococcus* (GBS, *S. agalactiae*) pneumonia in mice with a LysM-targeted deletion of IKKβ was dramatically different (25). The deletion of IKKβ in macrophages resulted in an increased recruitment of neutrophils and decreased bacterial burdens during GBS pneumonia, attributed to enhanced expression of IL-12 (25). We did not observe any increase in lung IL-12 because of RelA targeting in macrophages during pneumococcal pneumonia (data not shown). The deletion of IKKβ in macrophages also led to death during endotoxemia, attributable to increased cytokines driven primarily by an elevated expression of IL-1β (26). In contrast, our studies with LysM-targeted RelA deletion demonstrated decreased cytokines, including IL-1β, at early time points of pneumococcal pneumonia, and at no time did we observe increased IL-1β or increased inflammatory responses. LysM-targeted RelA deletion also decreased rather than increased BAL fluid IL-1β after the instillation of heat-killed *P. aeruginosa* (24). IKKβ is a kinase with many substrates that may influence many signaling pathways, apart from or in parallel with the activation of RelA (48). Although differences in study design may contribute to the disparate results already described, we suggest that IKKβ in macrophages may limit the inflammatory responses elicited by bacterial products through its influence on pathways other than the activation of NF-κB RelA.

Although our studies clearly indicate roles for RelA in mediating the expression of cytokines from alveolar macrophages, integrated host responses such as bacterial clearance and neutrophil recruitment may also result from additional effects of RelA mutations in cells other than alveolar macro-

phages. The LysM-targeting strategy used here targeted neutrophils and some dendritic cells (49). Because RelA mutations in alveolar macrophages clearly decrease the expression of TNF, IL-1, KC, and MIP-2 from alveolar macrophages, and interruptions of these signaling pathways diminish the recruitment of neutrophils and bacterial clearance in the lungs (8, 50), we favor the interpretation that the early loss of alveolar macrophage-derived cytokines is responsible for the early defects in neutrophil recruitment and bacterial clearance.

Lastly, these studies focused exclusively on innate immunity, and the cytokines implicated here as dependent on myeloid RelA early during infection have clear roles in orchestrating the innate immune defenses of the lung (23). In addition, myeloid-derived cytokines such as IL-1 β can influence adaptive immune responses (51), and adaptive immunity can provide effective protection against pneumococcal infections (52). The influences of myeloid RelA on adaptive immune defenses against pneumococcus, such as antibody production or Th17 skewing, remain to be determined.

Our studies suggest that alveolar macrophage RelA plays a crucial role in the innate immune response during pneumococcal pneumonia by initiating the expression of important inflammatory cytokines and chemokines. Inhibiting this early cytokine expression by targeting RelA in macrophages exerts long-lasting effects on the recruitment of neutrophils and bacterial clearance during pneumococcal pneumonia. Based on these observations, we anticipate that natural variations or pharmacologic manipulations that decrease macrophage RelA activity may predispose individuals to pneumococcal pneumonia. The results also offer clear evidence for the expression of cytokines occurring independent of macrophage RelA during pneumococcal pneumonia. The cellular sources, molecular mechanisms, and physiologic significance of this macrophage RelA-independent cytokine elaboration constitute important avenues for future research.

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References

- Mizgerd JP. Lung infection: a public health priority. *PLoS Med* 2006;3:e76.
- Guest JF, Morris A. Community-acquired pneumonia: the annual cost to the National Health Service in the UK. *Eur Respir J* 1997;10:1530–1534.
- Ruiz M, Ewig S, Torres A, Arancibia F, Marco F, Mensa J, Sanchez M, Martinez JA. Severe community-acquired pneumonia: risk factors and follow-up epidemiology. *Am J Respir Crit Care Med* 1999;160:923–929.
- McNamee LA, Harmsen AG. Both influenza-induced neutrophil dysfunction and neutrophil-independent mechanisms contribute to increased susceptibility to a secondary *Streptococcus pneumoniae* infection. *Infect Immun* 2006;74:6707–6721.
- Garvy BA, Harmsen AG. The importance of neutrophils in resistance to pneumococcal pneumonia in adult and neonatal mice. *Inflammation* 1996;20:499–512.
- Standiford TJ, Kunkel SL, Greenberger MJ, Laichalk LL, Strieter RM. Expression and regulation of chemokines in bacterial pneumonia. *J Leukoc Biol* 1996;59:24–28.
- Tsai WC, Strieter RM, Mehrad B, Newstead MW, Zeng X, Standiford TJ. CXC chemokine receptor CXCR2 is essential for protective innate host response in murine *Pseudomonas aeruginosa* pneumonia. *Infect Immun* 2000;68:4289–4296.
- Jones MR, Simms BT, Lupa MM, Kogan MS, Mizgerd JP. Lung NF-kappaB activation and neutrophil recruitment require IL-1 and TNF receptor signaling during pneumococcal pneumonia. *J Immunol* 2005;175:7530–7535.
- Blackwell TS, Lancaster LH, Blackwell TR, Venkatakrishnan A, Christman JW. Differential NF-kappaB activation after intratracheal endotoxin. *Am J Physiol* 1999;277:L823–L830.
- Mizgerd JP, Scott ML, Spieker MR, Doerschuk CM. Functions of IkappaB proteins in inflammatory responses to *Escherichia coli* LPS in mouse lungs. *Am J Respir Cell Mol Biol* 2002;27:575–582.
- Saccani A, Schioppa T, Porta C, Biswas SK, Nebuloni M, Vago L, Bottazzi B, Colombo MP, Mantovani A, Sica A. p50 nuclear factor-kappaB overexpression in tumor-associated macrophages inhibits M1 inflammatory responses and antitumor resistance. *Cancer Res* 2006;66:11432–11440.
- Mizgerd JP, Lupa MM, Kogan MS, Warren HB, Kobzik L, Topulos GP. Nuclear factor-kappaB p50 limits inflammation and prevents lung injury during *Escherichia coli* pneumonia. *Am J Respir Crit Care Med* 2003;168:810–817.
- Quinton LJ, Jones MR, Simms BT, Kogan MS, Robson BE, Skerrett SJ, Mizgerd JP. Functions and regulation of NF-kappaB RelA during pneumococcal pneumonia. *J Immunol* 2007;178:1896–1903.
- Alcamo E, Mizgerd JP, Horwitz BH, Bronson R, Beg AA, Scott M, Doerschuk CM, Hynes RO, Baltimore D. Targeted mutation of TNF receptor I rescues the RelA-deficient mouse and reveals a critical role for NF-kappa B in leukocyte recruitment. *J Immunol* 2001;167:1592–1600.
- Franke-Ullmann G, Pfortner C, Walter P, Steinmuller C, Lohmann-Matthes ML, Kobzik L. Characterization of murine lung interstitial macrophages in comparison with alveolar macrophages *in vitro*. *J Immunol* 1996;157:3097–3104.
- Sibille Y, Reynolds HY. Macrophages and polymorphonuclear neutrophils in lung defense and injury. *Am Rev Respir Dis* 1990;141:471–501.
- Kooguchi K, Hashimoto S, Kobayashi A, Kitamura Y, Kudoh I, Wiener-Kronish J, Sawa T. Role of alveolar macrophages in initiation and regulation of inflammation in *Pseudomonas aeruginosa* pneumonia. *Infect Immun* 1998;66:3164–3169.
- Broug-Holub E, Toews GB, van Iwaarden JF, Strieter RM, Kunkel SL, Paine R III, Standiford TJ. Alveolar macrophages are required for protective pulmonary defenses in murine *Klebsiella pneumoniae*: elimination of alveolar macrophages increases neutrophil recruitment but decreases bacterial clearance and survival. *Infect Immun* 1997;65:1139–1146.
- Dockrell DH, Marriott HM, Prince LR, Ridger VC, Ince PG, Hellewell PG, Whyte MK. Alveolar macrophage apoptosis contributes to pneumococcal clearance in a resolving model of pulmonary infection. *J Immunol* 2003;171:5380–5388.
- Hashimoto S, Pittet JF, Hong K, Folkesson H, Bagby G, Kobzik L, Frevert C, Watanabe K, Tsurufuji S, Wiener-Kronish J. Depletion of alveolar macrophages decreases neutrophil chemotaxis to *Pseudomonas* airspace infections. *Am J Physiol* 1996;270:L819–L828.
- Haslett C. Granulocyte apoptosis and its role in the resolution and control of lung inflammation. *Am J Respir Crit Care Med* 1999;160:S5–S11.
- Knapp S, Leemans JC, Florquin S, Branger J, Maris NA, Pater J, van Rooijen N, van der Poll T. Alveolar macrophages have a protective antiinflammatory role during murine pneumococcal pneumonia. *Am J Respir Crit Care Med* 2003;167:171–179.
- Mizgerd JP. Acute lower respiratory tract infection. *N Engl J Med* 2008;358:716–727.
- Hess C, Herr C, Beisswenger C, Zakharkina T, Schmid RM, Bals R. Myeloid RelA regulates pulmonary host defense networks. *Eur Respir J* 2009;35:343–352.
- Fong CH, Bebie M, Didierlaurent A, Nebauer R, Hussell T, Broide D, Karin M, Lawrence T. An antiinflammatory role for IKKbeta through the inhibition of “classical” macrophage activation. *J Exp Med* 2008;205:1269–1276.
- Greten FR, Arkan MC, Bollrath J, Hsu LC, Goode J, Miething C, Goktuna SI, Neuenhahn M, Fierer J, Paxian S, et al. NF-kappaB is a negative regulator of IL-1beta secretion as revealed by genetic and pharmacological inhibition of IKKbeta. *Cell* 2007;130:918–931.
- Arredouani M, Yang Z, Ning Y, Qin G, Soininen R, Tryggvason K, Kobzik L. The scavenger receptor MARCO is required for lung defense against pneumococcal pneumonia and inhaled particles. *J Exp Med* 2004;200:267–272.
- Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I. Conditional gene targeting in macrophages and granulocytes using LysMCre mice. *Transgenic Res* 1999;8:265–277.

29. Algul H, Treiber M, Lesina M, Nakhai H, Saur D, Geisler F, Pfeifer A, Paxian S, Schmid RM. Pancreas-specific RelA/p65 truncation increases susceptibility of acini to inflammation-associated cell death following cerulein pancreatitis. *J Clin Invest* 2007;117:1490–1501.
30. Quinton LJ, Jones MR, Robson BE, Simms BT, Whitsett JA, Mizgerd JP. Alveolar epithelial STAT3, IL-6 family cytokines, and host defense during *Escherichia coli* pneumonia. *Am J Respir Cell Mol Biol* 2008;38:699–706.
31. Quinton LJ, Jones MR, Robson BE, Mizgerd JP. Mechanisms of the hepatic acute-phase response during bacterial pneumonia. *Infect Immun* 2009;77:2417–2426.
32. Tian G, Wilcockson D, Perry VH, Rudd PM, Dwek RA, Platt FM, Platt N. Inhibition of alpha-glucosidases I and II increases the cell surface expression of functional class A macrophage scavenger receptor (Sr-A) by extending its half-life. *J Biol Chem* 2004;279:39303–39309.
33. Mizgerd JP, Kubo H, Kutkoski GJ, Bhagwan SD, Scharffetter-Kochanek K, Beaudet AL, Doerschuk CM. Neutrophil emigration in the skin, lungs, and peritoneum: different requirements for CD11/CD18 revealed by CD18-deficient mice. *J Exp Med* 1997;186:1357–1364.
34. Weibel ER. Morphometry: stereological theory and practical methods. In: Gil J, editor. Models of lung disease: microscopy and structural methods. New York: Marcel Dekker; 1990. pp. 199–252.
35. Greenberger MJ, Strieter RM, Kunkel SL, Danforth JM, Goodman RE, Standiford TJ. Neutralization of IL-10 increases survival in a murine model of *Klebsiella pneumoniae*. *J Immunol* 1995;155:722–729.
36. Herigstad B, Hamilton M, Heersink J. How to optimize the drop plate method for enumerating bacteria. *J Microbiol Methods* 2001;44:121–129.
37. Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature* 1995;376:167–170.
38. Eder C. Mechanisms of interleukin-1beta release. *Immunobiology* 2009;214:543–553.
39. Prudovsky I, Tarantini F, Landriscina M, Neivandt D, Soldi R, Kirov A, Small D, Kathir KM, Rajalingam D, Kumar TK. Secretion without Golgi. *J Cell Biochem* 2008;103:1327–1343.
40. Knapp S, Wieland CW, van't Veer C, Takeuchi O, Akira S, Florquin S, van der Poll T. 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* 2004;172:3132–3138.
41. Malley R, Henneke P, Morse SC, Cieslewicz MJ, Lipsitch M, Thompson CM, Kurt-Jones E, Paton JC, Wessels MR, Golenbock DT. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc Natl Acad Sci USA* 2003;100:1966–1971.
42. Arredouani MS, Yang Z, Imrich A, Ning Y, Qin G, Kobzik L. The macrophage scavenger receptor Sr-A/II and lung defense against pneumococci and particles. *Am J Respir Cell Mol Biol* 2006;35:474–478.
43. Briles DE, Hollingshead SK, Paton JC, Ades EW, Novak L, van Ginkel FW, Benjamin WH Jr. 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* 2003;188:339–348.
44. Tsai EY, Falvo JV, Tsytsykova AV, Barczak AK, Reimold AM, Glimcher LH, Fenton MJ, Gordon DC, Dunn IF, Goldfeld AE. A lipopolysaccharide-specific enhancer complex involving Ets, Elk-1, Sp1, and CREB binding protein and p300 is recruited to the tumor necrosis factor alpha promoter *in vivo*. *Mol Cell Biol* 2000;20:6084–6094.
45. Zhang Y, Saccani S, Shin H, Nikolajczyk BS. Dynamic protein associations define two phases of IL-1beta transcriptional activation. *J Immunol* 2008;181:503–512.
46. Hammerschmidt S, Wolff S, Hocke A, Rosseau S, Muller E, Rohde M. Illustration of pneumococcal polysaccharide capsule during adherence and invasion of epithelial cells. *Infect Immun* 2005;73:4653–4667.
47. Robson RL, Reed NA, Horvat RT. Differential activation of inflammatory pathways in A549 Type II pneumocytes by *Streptococcus pneumoniae* strains with different adherence properties. *BMC Infect Dis* 2006;6:71.
48. Perkins ND. Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat Rev Mol Cell Biol* 2007;8:49–62.
49. Jakubzick C, Bogunovic M, Bonito AJ, Kuan EL, Merad M, Randolph GJ. Lymph-migrating, tissue-derived dendritic cells are minor constituents within steady-state lymph nodes. *J Exp Med* 2008;205:2839–2850.
50. Herbold W, Maus R, Hahn I, Ding N, Srivastava M, Christman JW, Mack M, Reutershan J, Briles DE, Paton JC, *et al.* Importance of CXC chemokine receptor 2 in alveolar neutrophil and exudate macrophage recruitment in response to pneumococcal lung infection. *Infect Immun* 2010;78:2620–2630.
51. Miossec P, Korn T, Kuchroo VK. Interleukin-17 and Type 17 helper T cells. *N Engl J Med* 2009;361:888–898.
52. Malley R. Antibody and cell-mediated immunity to *Streptococcus pneumoniae*: implications for vaccine development. *J Mol Med* 2010;88:135–142.