Roles of Lung Epithelium in Neutrophil Recruitment during Pneumococcal Pneumonia

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Abstract

Epithelial cells line the respiratory tract and interface with the external world. Epithelial cells contribute to pulmonary inflammation, but specific epithelial roles have proven difficult to define. To discover unique epithelial activities that influence immunity during infection, we generated mice with nuclear factor- κ B RelA mutated throughout all epithelial cells of the lung and coupled this approach with epithelial cell isolation from infected and uninfected lungs for cell-specific analyses of gene induction. The RelA mutant mice appeared normal basally, but in response to pneumococcus in the lungs they were unable to rapidly recruit neutrophils to the air spaces. Epithelial cells expressed multiple neutrophil-stimulating cytokines during pneumonia, all of which depended on RelA. Cytokine expression by nonepithelial cells was unaltered by the epithelial mutation of RelA. Epithelial cells were the predominant sources of CXCL5 and granulocyte-macrophage colony-stimulating factor (GM-CSF), whereas nonepithelial cells were major sources for other neutrophil-activating cytokines. Epithelial RelA mutation decreased whole lung levels of CXCL5 and GM-CSF during pneumococcal pneumonia, whereas lung levels of

other neutrophil-recruiting factors were unaffected. Defective neutrophil recruitment in epithelial mutant mice could be rescued by administration of CXCL5 or GM-CSF. These results reveal a specialized immune function for the pulmonary epithelium, the induction of CXCL5 and GM-CSF, to accelerate neutrophil recruitment in the infected lung.

Keywords: lung epithelium; NF- κ B RelA; cytokines; neutrophils; pneumococcal pneumonia

Clinical Relevance

This work reveals significant but modest roles of epithelial cells during respiratory infection. Novel transgenic mice and effective isolation of lung epithelial cells were combined to demonstrate that the surface of the lower respiratory tract makes unique contributions to pulmonary immunity during pneumococcal pneumonia, including epithelial-specific cytokine expression, which accelerates neutrophil recruitment.

Lung infections cause a tremendous burden of disease worldwide (1), with *Streptococcus pneumoniae* (pneumococcus) being the leading cause of community-acquired pneumonia (2–4). An improved understanding of lung defense is critical for developing new approaches to treating and preventing pneumococcal pneumonia (5). The deficiency of nuclear factor- κ B (NF- κ B) RelA is severely immunosuppressive

(6) and increases susceptibility to pneumococcal pneumonia (7), demonstrating that NF- κ B is essential to integrated immunity.

The surface of the respiratory tract is composed of epithelial cells, making it likely that the respiratory epithelium has roles in immunity. NF- κ B interruption in epithelial cells due to SPC- or club cell (Clara cell) (CC)10-driven overexpression

of a dominant negative I κ B α transgene decreases neutrophil recruitment and lung-wide expression of every inflammatory mediator measured (7–9). In contrast, an SPC-rtTA developmental strategy for RelA mutagenesis in alveolar epithelial cells diminished lung-wide expression of only a very small subset of genes (2 of 84 measured), and there was little evidence of any impact on integrated immunology (10).

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In addition to presenting contrasting perspectives of epithelial biology in the inflamed lung, these studies had considerable limitations. The SPC-rtTA developmental system of RelA mutagenesis results in serious off-target effects that compromise pulmonary health and homeostasis and confound studies of integrated immunity (10). The dominant negative I κ B α approaches target incomplete fractions of epithelial cells of the lung and rely on overexpression of an inhibitor with imperfect efficacy and specificity (7–9). Furthermore, the bulk of prior studies focus on whole lung or BAL measurements rather than on cell-specific analyses, leaving it unclear which, if any, of the measured signals were derived from epithelial cells directly (7–10). These studies suggest that epithelial cells participate in pulmonary inflammatory responses, but their exact roles and specific products remain speculative.

To identify unique, specialized, epithelial-specific activities during pneumonia by deconstructing the infected lung, we generated a new mouse model for precisely, effectively, and safely mutating RelA throughout all epithelial cells and only epithelial cells of the lung, and we coupled this approach with flow-assisted cell sorting (FACS) strategies that reliably isolate epithelial cells from infected lungs to allow cell-specific interrogations. Our results reveal that epithelial cells lining the lung are singular sources of select cytokines, two of which function to elicit the rapid recruitment of neutrophils to the air spaces during pneumonia.

Materials and Methods

Mice

Mice lacking RelA in epithelial cells were generated by crossing Nkx2-1-Cre mice (11) with *Rela*-floxed mice (12) to generate a colony producing Nkx2-1-Cre^{tg+}/*Rela*^{loxP/loxP} (RelA ^{Δ/Δ}) and Nkx2-1-Cre^{tg+}/*Rela*^{loxP/loxP} (RelA^{F/F}; WT) littermates. Because Nkx2-1 is expressed during gestation in all pulmonary epithelial precursors, Nkx2-1-Cre permanently mutates all epithelial cells throughout the alveoli and conducting airways (11, 13). Cre (+) mutant mice (RelA ^{Δ/Δ} mice) were compared with wild-type (WT) littermates that also contain floxed RelA alleles but were Cre(–). Experiments were performed

at 8 to 19 weeks of age. Animal protocols were approved by the IACUC of Boston University.

Pneumonia

Mice were anesthetized by intraperitoneal administration of ketamine (50 mg/kg) and xylazine (5 mg/kg). For intratracheal instillations, a 24-gauge angiocatheter was placed into surgically exposed tracheas and guided into the left bronchus. A 50- μ l bolus of saline containing 10⁶ CFU of *S. pneumoniae* serotype 19F EF3030 (14) was instilled into the left lung lobe. In select experiments, 500 ng of recombinant murine CXCL5, granulocyte-macrophage colony-stimulating factor (GM-CSF) (both from R&D Systems, Minneapolis, MN), CCL20 (PeproTech, Rocky Hill, NJ), or vehicle (PBS with 1% BSA) were included with the bacteria that were administered into the left lung lobe of RelA ^{Δ/Δ} mice. At the indicated time points, mice were killed by isoflurane overdose.

Emigrated and Circulating Neutrophils

Bronchoalveolar lavage fluid (BALF) was collected for analyses of cells and proteins as previously described (7, 15). The recovery rates of BALF were 87 $\pm$ 5% for WT and 89 $\pm$ 7% for RelA ^{Δ/Δ} . Peripheral blood was drawn from the inferior vena cava for leukocyte counts using a hemacytometer and stained blood smears.

Alveolar Edema

The total protein concentration of the first milliliter of BALF was quantified using a BCA assay kit with BSA (both from SIGMA) as standard according to the manufacturer's instructions.

Bacterial Burdens

Whole lungs were homogenized using a Bullet Blender (Next Advance, Averill Park, NY) in 5 ml of sterile H₂O containing protease inhibitor tablet (Roche), serially diluted, plated, and grown overnight at 37°C on 5% sheep blood agar plates. Viable bacteria were enumerated by colony counts and expressed as total CFU per lung.

Quantitative RT-PCR

Cell lysate or lung mRNA was quantified for cytokines (CXCL1, CXCL2, CXCL5, G-CSF, GM-CSF, and CCL20) and cell markers (SP-C, aquaporin [Aqp]-5, CC10, Foxj1, E-selectin, platelet endothelial cell adhesion

molecule [PECAM]-1, Ly6G, and macrophage receptor with collagenous structure [MARCO]) using quantitative RT-PCR. Left (infected) lung lobes were homogenized in RLT buffer (Qiagen, Valencia, CA) using a Bullet Blender (Next Advance), and total RNA was purified using the RNeasy kit (Qiagen). Isolated cell lysates by FACS were collected using TRIzol reagent (Life Technologies, Grand Island, NY), and total RNA was purified following the manufacturer's protocol. Quantitative RT-PCR was performed using the StepOnePlus real-time PCR systems and the TaqMan RNA-to-CT 1-step kit (both from Applied Biosystems, Foster City, CA). The sequences of the primers and probes for mouse CXCL1, CXCL2, CXCL5, G-CSF, CCL20, MARCO, and Ly6G were previously published (7, 10, 16). The primers and TaqMan probe set were designed for mouse GM-CSF using the CLC DNA Workbench software (CLC bio, Cambridge, MA) with the following sequences: forward, 5'-ATTACTTTTCC-TGGGCATTGTGG-3', reverse, 5'-CAG-GAGGTTTCAGGGCTTCTTTG-3', probe, 5'-TACAGCCTCTCAGACCCACCCGC-3'. Taqman gene expression assays were used for Aqp5 (Mm00437579_m1), Sftpc (Mm00488144_m1), CC10 (Mm00442046_m1), Foxj1 (Mm01267279_m1), E-selectin (Mm00441278_m1), and PECAM-1 (Mm01242584_m1). Expression values were normalized to 18 s rRNA content (17) and expressed as fold induction. To discriminate full-length *Rela* and rearranged *Rela*, RT-PCR was performed as previously described (10).

Elisa

ELISA-paired antibody sets (R&D Systems) for murine CXCL1, CXCL2, CXCL5, G-CSF, GM-CSF, and CCL20 levels were used to quantify their respective concentrations in lung homogenate (from bacterial burden studies) or BAL samples.

Sorting of Lung Epithelial Cells

Left lung lobes from control and pneumonic mice infected 9 hours with *S. pneumoniae* were digested to single-cell suspension using the Elastase (Roche) digestion method as previously described (10). Lung single-cell suspensions were subjected to FACS. Dead cells were excluded using

7-aminoactinomycin D (BD Pharmingen, San Jose, CA). FITC-conjugated rat anti-mouse CD45 (BD Pharmingen) and APC-conjugated rat anti-mouse CD326 (eBioscience, San Diego, CA) were used to separate leukocyte and epithelial cells. Cell isolation was performed using FACSaria III (BD Biosciences). The purity of the isolated populations after sorting was greater than 96% for CD45⁺CD326⁺ (epithelial) cells and greater than 98% for CD45⁺ cells (leukocytes). Collected cells were stored in TRIzol (Life Technologies) at -80°C for further RNA isolation.

Statistics

Data were presented as means $\pm$ SE. Statistical comparisons among groups for continuous variables were performed using Student's *t* test (see Table E1 in the online supplement), a one-way ANOVA followed by a Bonferroni's post test (Figures 3 and 6), or a two-way ANOVA followed by a Bonferroni's *post hoc* analysis (Figures 1, 2, 4, 5, and 7, and Figure E1). The

Bonferroni tests allowed for all possible comparisons. Values were log transformed before ANOVA in cases of correlation between group means and standard deviations. A value of $P < 0.05$ was considered significant.

Results

Epithelial RelA Mediates the Initial Neutrophil Recruitment during Pneumonia

This newly established colony of RelA-targeted mice exhibited Hardy-Weinberg equilibrium, normal lung histology without air space enlargements or other abnormalities, and no signs of spontaneous phenotype of any kind (data not shown). These mice did not exhibit the spontaneous infections observed in mice lacking RelA in all cells (6) or the pulmonary derangements of models mutating RelA in lung epithelial cells using a different targeting system (10). This transgenic

approach appears well suited to experimental use and analyses of integrated innate immune responses during infection.

Pneumococci were instilled into the left lobe, which models the most common cause of community-acquired pneumonia (i.e., lobar pneumococcal pneumonia). The instillation of pneumococci elicited neutrophil recruitment to the air spaces within 9 hours (Figure 1A). Emigrated neutrophil numbers were significantly decreased in mice with epithelial RelA mutation compared with WT cohorts (Figure 1A). Numbers of neutrophils heading toward the lungs in the venous blood were unchanged by mutation (Figure 1B). Total lung neutrophil content, including those in the blood, tissue, and air space compartments, was estimated using mRNA quantity for the neutrophil-specific protein Ly6G (10, 18). Total lung neutrophils increased with pneumonia but were not significantly affected by genotype in healthy or pneumonic mice (Figure 1C). Together, these results suggest

Figure 1. Air space neutrophil emigration and air space edema requires lung epithelial nuclear factor- κ B (NF- κ B) RelA. Neutrophils in (A) bronchoalveolar lavage fluid (BALF) and (B) blood were counted in the presence or absence of lung epithelial RelA. ($n = 7$ mice/group in uninfected; $n = 8$ –9 mice in the pneumonia group, collected over three independent experiments). * $P < 0.05$ compared with uninfected mice of same genotype. $\dagger P < 0.05$ compared with wild type (WT) mice. EpRelA $\Delta\Delta$ = lung epithelial RelA mutant. (C) Ly6G mRNA in left lung lobe was measured by quantitative RT-PCR. Data were expressed as fold-induction compared with uninfected WT mice. Means $\pm$ SEM were calculated by combining data from two independent experiments ($n = 4$ –5 mice in the uninfected; $n = 6$ –8 mice in the pneumonia group). Data were collected after 0 hours (uninfected) and 9 hours (pneumonia) of pneumococcal pneumonia. * $P < 0.05$ compared with uninfected mice of same genotype. (D) Airspace neutrophils were counted ($n = 5$ –9 mice/group, collected over five independent experiments). $\dagger P < 0.05$ compared with WT mice. (E) Airspace albumin was measured by ELISA with BALF ($n = 4$ –8 mice/group, collected over five independent experiments). (F) Lung bacterial burden was measured by whole lung CFU ($n = 5$ –7 mice/group, collected over three independent experiments). $\dagger P < 0.05$ compared with WT mice. Data were statistically analyzed by using a two-way ANOVA followed by a Bonferroni's *post hoc* test.

Figure 2. Lung epithelial NF- κ B RelA is necessary for elevated concentrations of CXCL5, granulocyte-macrophage colony-stimulating factor (GM-CSF), and CCL20 protein during pneumococcal pneumonia. Concentrations of CXCL1 (A), CXCL2 (B), CXCL5 (C), G-CSF (D), GM-CSF (E), and CCL20 (F) were measured in whole lung homogenate using ELISA at 0 (uninfected) and 9 hours (pneumonia) after intratracheal pneumococcus ($n = 6$ mice in the uninfected; $n = 6$ –7 mice in the pneumonia group, collected over two independent experiments). * $P < 0.05$ compared with uninfected mice of same genotype. † $P < 0.05$ compared with WT mice. Data were statistically analyzed by using a two-way ANOVA followed by a Bonferroni's *post hoc* test.

that neutrophils were in the blood and/or interstitium but had migrated less well into the air spaces. Neutrophil migration from the blood into the air spaces was compromised by mutation of RelA in epithelial cells during pneumonia.

Although the initial inflammatory responses were decreased by epithelial RelA mutation, these defects were transient. By 24 hours after infection, neutrophil recruitment further increased, and the elevated neutrophils at this time point were not diminished by epithelial mutation of RelA (Figure 1D). Pulmonary edema, as inferred from concentrations of albumin in BALF, was not dependent on epithelial RelA throughout the time course (Figure 1E). Bacterial clearance was very effective in both genotypes, with approximately 90% elimination within the first day (Figure 1F); modest effects of genotype were observed at 48 hours, but most of the bacteria were eliminated in both cases. Thus, epithelial RelA is particularly involved in the initial neutrophil recruitment after pneumococcus infection, raising the question of what RelA in epithelial cells does to elicit early neutrophil responses.

Select Cytokines Depend on Epithelial RelA during Pneumonia

To determine local concentrations of neutrophil-activating cytokines, we

measured the chemokines CXCL1, CXCL2, and CXCL5 (Figure 2A–2C) and the colony-stimulating factors G-CSF and GM-CSF (Figures 2D and 2E), all of which contribute to neutrophil recruitment during pneumonia (19–22) and all of which can be regulated by NF- κ B (7, 9, 10, 16, 20, 23).

CXCL1, CXCL2, and G-CSF increased in lung homogenates due to pneumonia, but concentrations were unaffected by epithelial mutation of RelA (Figures 2A, 2B, and 2D). In contrast, the pneumonia-induced increases in lung CXCL5 and lung GM-CSF were significantly decreased by epithelial

Figure 3. Neutrophil recruitment could be rescued by CXCL5 or GM-CSF but not by CCL20 in the lungs of epithelial RelA mutant mice with pneumococcal pneumonia. Neutrophil counts in BALF (A) and blood (B) were assessed in samples collected 9 hours after intratracheal instillation of pneumococcus plus recombinant murine (rm) CXCL5, GM-CSF, CCL20, or vehicle (PBS + 1% BSA) ($n = 6$ mice in the vehicle group; $n = 4$ mice in the recombinant protein group, collected over two independent experiments). * $P < 0.05$ compared with WT mice with vehicle. † $P < 0.05$ compared with RelA mutant mice with vehicle. Data were statistically analyzed by using a one-way ANOVA, followed by a Bonferroni *post hoc* test.

Figure 4. Induction of CXCL5, GM-CSF, and CCL20 mRNA requires lung epithelial RelA during pneumococcal pneumonia. mRNA levels of CXCL1 (A), CXCL2 (B), G-CSF (C), CXCL5 (D), GM-CSF (E), and CCL20 (F) were measured using quantitative RT-PCR in left lung lobes collected 0, 6, or 9 hours after intratracheal pneumococcus. Data were expressed as fold-induction compared with WT uninfected (0 h) mice. Means $\pm$ SEM were calculated by combining data from two independent experiments ($n = 4-5$ mice in the uninfected group; $n = 5-10$ mice in the pneumonia group, collected over four independent experiments). * $P < 0.05$ compared with uninfected mice of same genotype. † $P < 0.05$ compared with WT mice. Data were statistically analyzed by using a two-way ANOVA, followed by a Bonferroni's *post hoc* test.

RelA mutation (Figures 2C and 2E). Because air space neutrophils were diminished by epithelial RelA mutation, we also measured neutrophil chemokines in BALF (Figure E1), and these showed similar degrees of epithelial dependence as observed in lung homogenates. Although not previously implicated in neutrophil recruitment, we also measured CCL20 because it had been identified as epithelial-derived during pneumonia (10). The increase in CCL20 concentration during pneumonia was significantly diminished by epithelial RelA mutation (Figure 2F). Again, timing influenced the roles of epithelial RelA; concentrations of CXCL5 or GM-CSF were no longer dependent on epithelial RelA by 24 hours of infection, whereas CCL20 remained significantly decreased in mutant lungs even at this later time point (Table E1). These results suggest that epithelial RelA contributes directly or indirectly to the early increases in CXCL5, GM-CSF, and CCL20 concentrations during pneumococcal pneumonia.

CXCL5 and GM-CSF Enhance Neutrophil Recruitment

Because of the decreased neutrophils associated with decreased CXCL5, GM-CSF, and CCL20, these were leading candidates

for mediating epithelial contributions to neutrophil recruitment. We tested the abilities of these cytokines to restore neutrophil recruitment in the mutant mice using recombinant murine CXCL5, GM-CSF, or CCL20 administered with the intratracheal bacteria. As anticipated, after coinstillation of vehicle with pneumococcus, mutant mice had significantly diminished BAL neutrophils compared with control mice (Figure 3A). CXCL5 and GM-CSF were capable of increasing neutrophil numbers in the mutant lungs, whereas CCL20 was not (Figure 3A). Furthermore, circulating neutrophils were decreased in groups receiving CXCL5 or GM-CSF (Figure 3B), suggesting that these epithelial-derived cytokines specifically enhance neutrophil recruitment from the blood into the air spaces.

Epithelial-Dependent Products Have Distinct Kinetics

To determine whether there were distinctive kinetics for induction of epithelial-dependent and epithelial-independent genes during pneumonia, we compared expression levels in the lungs from early time periods after infection. The epithelial-independent cytokines CXCL1, CXCL2, and G-CSF mRNA levels were all significantly induced

by 6 hours, with maintained but not increased levels observed at 9 hours (Figures 4A–4C). There were no differences due to epithelial RelA mutation at any time point (Figures 4A–4C). The epithelial-dependent cytokines CXCL5, GM-CSF, and CCL20 mRNA were induced more slowly in the lungs, with no significant induction of any by 6 hours but significant induction of all by 9 hours (Figures 4D–4F). Transcripts for CXCL5, GM-CSF, and CCL20 were reduced by RelA mutation in the epithelium at that later time point (Figures 4D–4F). These kinetic data support our previously proposed model that the initial responses to pneumococcus in the air spaces are generated by alveolar macrophages, whereas epithelial cells become activated at later time points due to stimulation by macrophage-derived TNF and IL-1 (16, 24, 25). Epithelial RelA is essential for transcript increases in CXCL5, GM-CSF, and CCL20, which occurs later than the induction of epithelial-independent transcripts.

Epithelial and Myeloid Cytokines Are Not Interdependent

The fact that only a small fraction of cytokines was altered by epithelial mutation suggested that epithelial-derived cytokines

Figure 5. Epithelial and myeloid cytokines are not interdependent. Concentrations of TNF- α (A), IL-1 α (B), and IL-1 β (C) were measured in whole lung homogenate by ELISA at 0 (uninfected) and 9 hours (pneumonia) after intratracheal pneumococcus infection, using WT and EpiRelA Δ/Δ mice ($n = 4$ mice in the uninfected group; $n = 7$ mice in the pneumonia group, collected over two independent experiments). Concentrations of TNF- α (D), IL-1 α (E), IL-1 β (F), CXCL5 (G), GM-CSF (H), and CCL20 (I) were measured in whole lung homogenate by ELISA at 0 (uninfected) and 9 hours (pneumonia) after intratracheal pneumococcus, using WT and AM RelA Δ/Δ (myeloid RelA mutant, with targeted alveolar macrophages) mice ($n = 4$ mice in the uninfected group; $n = 10$ mice in the pneumonia group, collected over four independent experiments). * $P < 0.05$ compared with uninfected mice of same genotype. † $P < 0.05$ compared with WT mice. Data were statistically analyzed by using a two-way ANOVA followed by a Bonferroni's *post hoc* test.

were not essential for the expression of cytokines by other cells in the infected lung. TNF- α , IL-1 α , and IL-1 β are derived from myeloid cells (16), and testing whether these cytokines were altered by epithelial RelA mutation revealed that none was (Figures 5A–5C). The reciprocal was also true. Using mice in which RelA was effectively mutated in alveolar macrophages (16), we found that myeloid-derived but not epithelial-derived cytokines were diminished (Figures 5D–5I). These results suggest that these cytokine sources behave largely independent from each other in the early responses to pneumococcus in the air spaces.

Collecting Epithelial Cells from Infected Mouse Lungs

To evaluate cytokine expression by epithelial cells specifically, we developed a FACS

strategy for their isolation. Based on relative expression of CD45 and CD326, three distinct populations were readily distinguished in live singleton-gated lung cell suspensions (Figure 6A). These surface characteristics are consistent with the separation of leukocytes (CD45 $^{+}$), epithelial cells (CD45 $^{-}$ CD326 $^{+}$), and others (CD45 $^{-}$ CD326 $^{-}$). We examined the relative expression of independent cell markers in sorted cells from mice with pneumonia. All markers examined for epithelial cells, including SP-C for alveolar epithelial type II cells (26), Aqp-5 for alveolar epithelial type I cells (27), CC10 for conducting airway Clara cells (28), and Foxj1 for conducting airway ciliated cells (29), were abundant in the CD45 $^{-}$ CD326 $^{+}$ cells but not in either of the other two cell populations (Figure 6B). In contrast, the

leukocyte markers, Ly6G for neutrophils and MARCO for macrophages (30), were expressed by CD45 $^{+}$ cells but not by the other cell populations (Figures 6C). Finally, the endothelial markers PECAM-1 and E-selectin were only prominent in CD45 $^{-}$ CD326 $^{-}$ cells (Figures 6D). Taken together, these data demonstrate effective separation of lung cells into three distinct groups, with all lung epithelial cells collected together in one cell population that contains neither leukocytes nor endothelial cells.

Epithelial-Specific Cytokines in the Infected Lung

The FACS strategy allows the examination of gene expression from epithelial cells and the comparison to other cells in the infected lung. To evaluate the efficacy and

Figure 6. Flow-assisted cell sorting collection of epithelial cells from infected lungs. (A) Single-cell suspensions from the elastase-digested left lung lobe separate into three distinct populations on the basis of relative expression of CD45 and CD326. Results are representative of $n = 6$ mice collected over two independent experiments after 9 hours of pneumococcal pneumonia. Sorted lung cells from each of the three populations were measured for mRNA expression of SP-C, aquaporin 5 Aqp-5, Clara cell CC10, and Foxj1 (B); Ly6G and MARCO (macrophage receptor with collagenous structure) (C); and E-selectin and PECAM-1 (D) by quantitative RT-PCR. Data were expressed as fold-induction compared with the cell population expressing the least of that marker. Means $\pm$ SEM were calculated by combining data from two independent experiments ($n = 3$ mice/group). * $P < 0.05$ compared with cells expressed least. Data were statistically analyzed by using a one-way ANOVA followed by a Bonferroni's *post hoc* test. WBCs = white blood cells.

specificity of our gene targeting strategy, we applied this cell sorting approach to the lungs of mice in the transgenic colony, and we used RT-PCR to differentiate the recombined and mutant *Rela* gene product from the floxed-but-not-recombined version encoding WT *Rela* (10). We found that lung epithelial cells (defined as $CD45^-CD326^+$) expressed the mutant allele in the $Cre^+RelA^{\Delta/\Delta}$ mice, whereas epithelial cells of Cre^- WT littermates did not (Figure 7A). Other cells in the lung, including $CD45^+$ cells and $CD45^-CD326^-$ cells, expressed exclusively WT *Rela* in all cases (Figure 7A). These results confirm effective and specific targeting of *Rela* across all epithelial cells but not other cells in the lung.

The combination of this novel transgenic mouse colony and this novel cell-sorting strategy allowed insight into which

cells were special sources of neutrophil-recruiting cytokines that may be dependent on epithelial *RelA*. CXCL5, GM-CSF, and CCL20 were predominantly induced in epithelial cells (Figures 7B–7D). CXCL5 also showed some induction (to lesser levels) in the nonepithelial, nonleukocyte population (Figure 7B). In contrast, CXCL2 was predominantly from leukocytes, CXCL1 was predominantly from cells other than epithelial cells or leukocytes (endothelial cells or fibroblasts may be leading candidates), and G-CSF was similarly induced across all three different cell populations (Figures 7E–7G). Thus, all three of the cytokines that were decreased in whole lung homogenates by epithelial *RelA* mutation were strongly induced in epithelial cells especially, whereas the other cytokines showed different cell-specific expression patterns.

The epithelial *RelA* mutation significantly decreased epithelial expression of all six cytokines, including CXCL1, CXCL2, and G-CSF, in addition to the epithelial-specific cytokines (Figures 7A–7G). This highlights the importance of cell-specific analyses, elucidating *RelA*-dependent gene induction in epithelial cells that was not appreciable in whole lung extracts. Epithelial *RelA* mutation had no discernible effects on the expression of these cytokines from leukocytes or other nonepithelial cells (Figure 7). Although *RelA* is essential to the induction of a wide range of innate immunity genes in epithelial cells, only a subset of cytokines requires epithelial cells as unique sources. Epithelial cells are special sources of CXCL5, GM-CSF, and CCL20 during pneumococcal pneumonia.

Figure 7. Epithelial cells are the major sources of CXCL5, GM-CSF, and CCL20 during pneumococcal pneumonia, and all cytokine induction in epithelial cells depends on RelA. (A) RelA is effectively targeted by transgenesis. RT-PCR of WT RelA and mutant RelA allele products in sorted CD45⁺CD326⁺ (Epithelial), CD45⁺ (WBCs), and CD45⁺CD326⁺ (Others) cells from left lung lobe. Results are representative of $n = 6$ mice collected over two independent experiments. These cell populations were sorted from the uninfected (Ctrl) and 9 hours pneumonic (PN) lungs of WT and mutant mice, and cytokines were measured using quantitative RT-PCR for CXCL5 (B), GM-CSF (C), CCL20 (D), CXCL1 (E), CXCL2 (F), and G-CSF (G). Data were expressed as fold-induction compared with epithelial cells from uninfected WT mice. Means $\pm$ SEM were calculated by combining data from two independent experiments ($n = 3$ mice/group in each uninfected and pneumonia). * $P < 0.05$ compared with uninfected mice of the same genotype. † $P < 0.05$ compared with WT mice. Data were statistically analyzed by using a two-way ANOVA followed by a Bonferroni's *post hoc* test.

Discussion

These results demonstrate that lung epithelial NF- κ B RelA is necessary for the initial neutrophil recruitment during pneumococcal pneumonia, using novel mice lacking this transcription factor in all epithelial cells in the lung. Select cytokines, including CXCL5, GM-CSF, and CCL20, were diminished in the absence of lung epithelial cell RelA, all of which were predominantly epithelial in origin during pneumonia. Recombinant CXCL5 or GM-CSF, but not CCL20, could restore neutrophil recruitment in mice lacking lung epithelial RelA. We conclude that lung epithelial cells are critical sources of CXCL5 and GM-CSF to coordinate early neutrophil recruitment.

The transient nature of the immunity defect in the epithelial mutant mice was unexpected and intriguing. Although the first neutrophil recruitment was strongly dependent on RelA activity in epithelial cells, there was no effect of genotype on this outcome by 24 hours of infection. Similarly, CXCL5 and GM-CSF in the lungs were not dependent on RelA in epithelial cells at this later time point. Our prior study of epithelial RelA mutation revealed no defects in neutrophil recruitment or CXCL5 expression at 15 hours of pneumococcal pneumonia, using a different strategy for targeting RelA as well as a different serotype of pneumococcus (10), suggesting that the transition from epithelial RelA dependence to independence may occur by

15 hours of infection. Further studies are required to determine which cells and pathways mediate cytokine expression and innate immunity during these later hours of pneumonia. Transcription factors other than RelA may mediate immunity gene expression by epithelial cells at later times. Alternatively, cells other than the epithelium may compensate for the loss of epithelial-derived factors. We favor the latter interpretation. Most importantly, compared with our previous studies, which demonstrate persistent life-threatening loss of host defense capabilities due to the deficiency of RelA in all cells (6, 7), we have consistently found much more limited effects of mutating RelA in distinct cell types (10, 16, 31). Although RelA is critical

for host defense, it seems that many different cell types elaborate immunity products using NF- κ B, and no individual cell-type has proven to be an essential source of NF- κ B activity during pneumonia.

The present studies reveal that the earliest neutrophil recruitment during pneumococcal pneumonia requires RelA in epithelial cells. RelA in macrophages is also required for this early recruitment (16). Macrophage RelA products (e.g., TNF- α , IL-1 α , and IL-1 β) were unaffected by epithelial mutation of RelA, and epithelial RelA products (e.g., CXCL5, GM-CSF, and CCL20) were unaffected by macrophage mutation of RelA. Furthermore, the sorting studies revealed that the induction of cytokines such as CXCL1, CXCL2, and G-CSF by nonepithelial cells in the lungs was unaffected by RelA mutation in epithelial cells and the resulting diminution of epithelial cell cytokines. These data reveal a surprising degree of cellular independence in these cytokine responses to pneumococcal infection and suggest that effective early neutrophil recruitment into the infected air spaces requires independent concerted RelA transcriptional activities in macrophages and epithelial cells.

The fact that recombinant CXCL5 restored air space neutrophils in the epithelial RelA mutant mice suggests that this chemokine was present in limiting concentrations at early time points, when it was expressed primarily by epithelial cells. These results complement prior evidence that CXCL5 blockade diminishes neutrophil recruitment elicited by LPS or cytokines in the lungs (19, 32, 33). We conclude that CXCL5 is induced by RelA in epithelial cells to enhance rapid neutrophil recruitment to the lung during infection. An additional new finding here is that the cell sources and/or molecular regulation of CXCL5 expression is dynamic during the time course of pneumonia. By 24 hours,

CXCL5 in the lungs was no longer dependent on epithelial RelA. The cells expressing CXCL5, the transcription factors mediating this reaction, and the functional significance of late CXCL5 expression are current avenues of active exploration.

Basal GM-CSF is essential to the maturation and host defense functions of macrophages (34, 35), but basal GM-CSF was not altered by mutations of RelA in epithelial cells, and the mutant mice did not exhibit pulmonary alveolar proteinosis or other signs of constitutive GM-CSF deficiency. However, we show here that the pneumococcus-induced increase in lung GM-CSF is from epithelial cells and dependent on RelA, providing to our knowledge the first cellular and molecular insights into the regulation of this important cytokine during pneumococcal pneumonia. Acute blockade of GM-CSF decreases neutrophil recruitment elicited by LPS or cytokines in the lungs (36–38). This complements our present gain-of-function study, suggesting conserved roles for GM-CSF in enhancing alveolar neutrophil recruitment across several settings. In addition, lung GM-CSF can promote macrophage-mediated microbial killing, epithelial cytoprotection, and antimicrobial adaptive immunity during diverse severe lung infections (39–42), all of which may thus be downstream of epithelial activation during pneumonia. The results reported here highlight RelA activity in epithelial cells as an essential source of early GM-CSF expression during pneumonia.

CCL20 was induced only in lung epithelial cells and was consistently dependent on RelA throughout pneumococcal pneumonia. Our previous study suggested that T1 α -negative alveolar epithelial cells, likely type II cells, were the source of CCL20 during pneumococcal pneumonia (10). This chemokine was not able to restore neutrophil recruitment in

the present study, which is consistent with the observation that its receptor CCR6 is predominantly expressed by subsets of T cells, B cells, and dendritic cells rather than by neutrophils (43). The functional significance of CCL20 is unclear but is an interesting question because of its consistent expression from epithelial cells selectively in the infected lung. The cellular distribution of CCR6 suggests that CCL20, and hence epithelial RelA, are involved in adaptive immune responses in the lungs, which were not components of the model of acute pneumonia here studied but which will be avenues for future investigation using different models of pulmonary disease.

In conclusion, RelA is required for cytokine expression by lung epithelial cells, and these cells are special sources for select cytokines during pneumonia. The rapid recruitment of neutrophils during pneumonia requires the epithelial production of CXCL5 and GM-CSF. As the interface between the external environment and the internal milieu, it is likely that epithelial cells perform additional functions, such as immune modulation by CCL20 or cytokine-independent activities such as cytoprotection. This new colony of transgenic mice will be useful in dissecting such roles. During the setting of acute pneumococcal pneumonia, epithelial cells are vital sources of distinct mediators, including CXCL5, CCL20, and GM-CSF, and RelA-mediated transcription in epithelial cells enhances the brisk recruitment of neutrophils to the infected lung. ■

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