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Type I Alveolar Epithelial Cells Mount Innate Immune Responses during Pneumococcal Pneumonia

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Abstract

Pneumonia results from bacteria in the alveoli. The alveolar epithelium consists of type II cells, which secrete surfactant and associated proteins, and type I cells, which constitute 95% of the surface area and met anatomic and structural needs. Other than constitutively expressed surfactant proteins, it is unknown whether alveolar epithelial cells have distinct roles in innate immunity. Since innate immunity gene induction depends on NF- κ B RelA (also known as p65) during pneumonia, we generated a murine model of RelA mutated throughout the alveolar epithelium. In response to LPS, only 2 of 84 cytokine transcripts (CCL20 and CXCL5) were blunted in lungs of mutants, suggesting that a very limited subset of immune mediators is selectively elaborated by the alveolar epithelium. Lung CCL20 induction required epithelial RelA regardless of stimulus, whereas lung CXCL5 expression depended on RelA after instillation of LPS but not pneumococcus. RelA knockdown *in vitro* suggested that CXCL5 induction required RelA in type II cells but not type I cells. Sorted cell populations from mouse lungs revealed that CXCL5 was induced during pneumonia in type I cells, which did not require RelA. TLR2 and STING were also induced in type I cells, with RelA essential for TLR2 but not STING. To our knowledge, these data are the first direct demonstration that type I cells, which constitute the majority of the alveolar surface, mount innate immune responses during bacterial infection. These are also the first evidence for entirely RelA-independent pathways of innate immunity gene induction in any cell during pneumonia.

Introduction

The epithelium represents the first line of defense against pathogens in the lung. Epithelial cells provide a mechanical barrier to prevent infection, and they can produce chemokines and cytokines which recruit and activate phagocytic cells to eradicate organisms and infected cells (1–2). The alveolar epithelium consists of two main populations: alveolar type I (AT1) and type II (AT2) epithelial cells. AT2 cells synthesize and secrete pulmonary surfactant, express chemokines and cytokines, and participate in the innate immune response of the lung (3). Despite being the predominant cell in the alveolar space by number, AT2 cells cover only 5% of the surface. The remaining 95% is covered by large attenuated AT1 cells (4–6). Although constituting so much of the surface area of the lung, very little is known about any potential contribution of AT1 cells to pulmonary innate immunity.

Proinflammatory cytokines orchestrate innate immunity and are 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 (7–9). p50 limits the expression of inflammatory cytokines and prevents lung injury during pneumonia (10–11). In contrast, RelA drives inflammatory responses by promoting the expression of many cytokines, and the deletion of RelA from all cells severely compromises antibacterial host defense (12–13). Mice with a surfactant protein C (SPC)-driven dominant-negative I κ B α (dnI κ B α) inhibitor of NF- κ B have increased bacterial burdens during pneumococcal pneumonia (12), suggesting that NF- κ B in AT2 cells contributes to host defense. These mice also have decreased neutrophil recruitment and inflammatory cytokines after LPS inhalation (14), indicating that NF- κ B in AT2 cells participates in acute inflammatory responses. However, the dnI κ B α protein is not specific to distinct NF- κ B proteins, and the efficacy of NF- κ B inhibition by this approach is based on dynamic stoichiometry which has not been analyzed in these lung cells. Importantly, neither these nor other studies to date have examined roles of AT1 cells. The goal of the present study was to evaluate unique roles of alveolar epithelial cells in innate immunity mediator expression elicited by bacterial stimuli in the lungs, and to assess their dependence on NF- κ B RelA.

Materials and Methods

Mice

Rela^{flx/flx} mice (15) (kindly provided by Roland Schmid, Technical University of Munich) were bred with SPC-rtTA^{tg/-}/(tetO)₇CMV-Cre^{tg/tg} mice (16) (kindly provided by Jeffrey Whitsett, Cincinnati Children's Hospital Medical Center) to generate colonies of wild-type control mice (SP-CrtTA^{-/-}/(tetO)₇CMV-Cre^{tg/tg}/*Rela*^{flx/flx}) or *Rela* ^{Δ/Δ} mice (SP-CrtTA^{tg/-}/(tetO)₇CMV-Cre^{tg/tg}/*Rela*^{flx/flx}) in which the *Rela* gene is selectively mutated in alveolar epithelial cells. Results obtained from *Rela* ^{Δ/Δ} mice were compared with sex-matched littermate controls. Doxycycline was provided in the chow (625 mg/kg, S-5086, Bio-Serv) to all mice throughout gestation and nursing to induce Cre-recombinase-mediated *Rela* mutation in the alveolar epithelium of the rtTA-transgenics, including both AT1 cells and AT2 cells (17). Mice were not exposed to the doxycycline diet after weaning from their mothers at 3 weeks of age, preventing effects of confounding by doxycycline during experiments. At the time of experimentation, mice were 7 to 11 weeks of age. Experiments with nontransgenic mice were performed using C57BL/6 mice. All experimental protocols were approved by the Boston University Institutional Animal Care and Use Committee.

Pneumonia

Mice were anesthetized by i.p. injection of ketamine (50 mg/kg)/xylazine (5 mg/kg). An angiocatheter was placed down the left bronchus, and mice received intratracheal (i.t.) instillations of 50 μ l of saline containing 10⁶ CFU of *Streptococcus pneumoniae* serotype 3 (*Sp*; 6303; American Type Culture Collection) or 50 μ g LPS (*Escherichia coli* O111:B4, Sigma-Aldrich) into the left lung lobe. These stimuli were chosen because they are relevant to human health, are each well-characterized in murine models of pulmonary inflammation, and are to our knowledge the only 2 bacterial stimuli that have been studied in the context of epithelial NF- κ B roles (12, 14, 18). Mice were euthanized by an aerosolized isoflurane overdose 15 hours after *Sp* instillation or 6 hours after LPS instillation.

Bacterial Clearance

Lungs were homogenized in sterile distilled H₂O containing protease inhibitors (19). Homogenates were serially diluted, plated on 5% sheep blood agar plates using the drop

plate method (20), and incubated overnight at 37°C. Colonies were counted to quantify CFU/lung.

Lung histology

Alveolar neutrophils were quantified by morphometric analyses of hematoxylin-and-eosin-stained sections of left (instilled) lung lobes, as described previously (17, 21–22).

RelA interference in murine lung epithelial cell lines

The murine AT1-like cell line E10 ((23), obtained from Dr. Alvin Malkinson, University of Colorado) was cultured in CMRL1066 medium containing 10% FBS, 2mM L-glutamine, 100 units/ml penicillin, and 100µg/ml streptomycin. The murine AT2 cell-derived MLE15 cell line ((24), obtained from Dr. Jeffrey A. Whitsett, University of Cincinnati) was cultured in a 1:1 mixture of Dulbecco's modified Eagle's Medium and F12 medium supplemented with 2% FBS, 100 units/ml penicillin, 100µg/ml streptomycin, 5µg/ml insulin, 10µg/ml transferrin, 30nM sodium selenite, 10nM hydrocortisone, 10nM β-estradiol, and 10mM HEPES. Cells were studied at approximately 80% confluence. Cells were transfected by using 0.1µM of RelA-targeting siRNA (Sense 5'–GCUCAAGAUCUGCCGAGUAUU–3', Antisense 5'–PUACUCGGCAGAUCUUGAGCUU–3') or Control non-targeting siRNA (D-001810-01-20) which did not complement known sequences in the mouse transcriptome (both from Dharmacon). After 24h, cells were washed once with PBS and were stimulated after another 24h culture with fresh media. Cells were stimulated for 6 hours with 10ng/ml recombinant murine TNF-α, 10µg/ml LPS, or 5µg/ml BSA (all from Sigma-Aldrich). The cell pellets were collected after washing with PBS and resuspended in 1ml Trizol (Invitrogen) for RNA isolation.

Immunoblot assay

Western blots were performed to determine siRNA-mediated knockdown of RelA in E10 and in MLE15 cells, as previously described (12). Abs for RelA (#3034) and pan actin (#4968S) were purchased from Cell Signaling Technology.

RNA purification and analyses

Total RNA was isolated from mouse lung, alveolar epithelial cell lines, and isolated cells with RNeasy Mini kit (QIAGEN) or TRIZOL reagent (Invitrogen) and RNase-free DNase (QIAGEN) set. Quantitative RT-PCR (qRT-PCR) was performed using the Taqman RNA-to-C_T 1-Step Kit and the StepOnePlus Real-Time PCR Systems (both from Applied Biosystems). The primers and Taqman probes for mouse CXCL5, TNF-α, IL-6, IL-1α, IL-1β, CXCL1, CXCL2, TLR2, and TLR4 were previously published (12, 25–26). The primers and TaqMan probe set were designed for mouse CCL20, G-CSF, and Ly6G using the CLC DNA Workbench software (CLC bio) with the following sequences: CCL20, 5'–CCTCAGCCTAAGAGTCAAGAAGA–3' and 5'–ACAAGTCCACTGGGACACAAA–3'; probe 5'–ACACAGCCCCAAGGAGGAAATGATCACAGC–3'; G-CSF, 5'–TTCCCCTGGTCACTGTCAGC–3' and 5'–CACAGCTTGTAGGTGGCACAC–3'; probe 5'–ACCATCCCCTGCCTCTGCCCCGAAG–3'; Ly6G, 5'–TCCTGTGTGCTCATCCTTCTT–3' and 5'–TCCAGAGCAACGCAAAATCCAT–3'; probe 5'–TTCCTGCAACACAACCTGCCCCCTT–3'. TaqMan gene expression assays were used for podoplanin (T1α) (Mm00494716_m1), caveolin-1 (Mm00483057_m1), Nkx2-1 (Mm00447558_m1), Sftpc (Mm00488144_m1), Sftpb (Mm00455681_m1), CC10 (00442046_m1), PECAM-1 (Mm01242584_m1), p75 (Mm01309638_m1), and STING (Mm01158117_m1, all from Applied Biosystems) mRNA analyses. For each sample, values were normalized to the content of 18S rRNA (27–28). RT² Profiler™ Mouse Inflammatory Cytokines and Receptors PCR Array (PAMM-011, SABiosciences) was used for the multi-

analyte qRT-PCR analysis of mouse inflammatory cytokines. Results of the PCR Arrays were deposited at NCBI Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>), retrievable with accession numbers GSE38398 and GSE38399. Efficacy of *Rela* gene rearrangement was determined by examining sizes of amplification products using electrophoresis. For *Rela* RT-PCR, isolated RNA (100µg) was reverse-transcribed using SuperScript VILO cDNA Synthesis Kit (Applied Biosystems), and PCR reactions were performed with GoTaq DNA polymerase (Promega). Primers sequences for *Rela* were: forward, 5'-ACAATAACCCCTTTCACGTTCTTA-3'; reverse, 5'-CCCAAGTCTTCATCAGCATCAA-3'. Full-length or rearranged *Rela* products were indicated by amplification products of approximately 939 or 490 bp in length, respectively.

ELISA

ELISA kits (R&D Systems) for murine CXCL5 and CCL20 levels were used to measure their respective concentrations in lung homogenate or in supernatants of murine lung cell lines.

Isolation of AT1 cells from lung digests

After euthanasia, lungs were perfused with 10 ml of HBSS via the right ventricle through the pulmonary artery. 1 ml of porcine elastase (4.5U, Roche diagnostics) was instilled through a tracheal cannula followed by 0.5 ml of low melting agarose solution warmed to 45°C. Lungs were immediately covered with ice for 2 min to gel the agarose, and incubated in 2 ml of elastase for 45 min at 37°C. After this incubation, lung lobes were gently separated from the bronchi and minced in RPMI 1640 medium (GIBCO) containing 50% FBS and 100 units/ml DNase I (QIAGEN). Cells in suspension were subsequently filtered through 100-, 70-, and 40-µm nylon mesh. Cell suspensions from these lung digests were subjected to FACS. The following Abs and isotype controls were used: PE-conjugated hamster anti-mouse Podoplanin (T1α), PE-conjugated hamster IgG (both hamster products from Biolegend), FITC-conjugated mouse anti-mouse CD45, FITC-conjugated mouse IgG2aκ, Rat anti-mouse CD16/CD32, and 7-AAD (all non-hamster products from BD Pharmingen). Cell isolation was performed using a MoFlo cell sorter. Collected cells were stored in Trizol (Invitrogen).

Immunofluorescence staining of sorted cells

Cells were stained for AT1 and AT2 markers using immunofluorescence. Cytocentrifuged cell preparations sorted from lungs of C57BL/6 mice were fixed with 4% paraformaldehyde, blocked, and permeabilized. Slides were labeled with caveolin-1 or pro-SP-C primary antibodies followed by Alexa Fluor 488-conjugated donkey anti-rabbit IgG secondary antibodies. Immunofluorescence images were taken using the AxioVision system (Zeiss).

Statistics

Statistical analyses were performed using GraphPad Prism. Data were presented as means ± SE. Real-time RT-PCR data were expressed as fold induction and therefore used geometric means ± geometric SE used as descriptive statistics. Statistical comparisons among groups for continuous variables were performed using a student's t test or a two-way ANOVA followed by a Bonferroni's *post hoc* analysis. A value of $P < 0.05$ was considered significant.

Results

Phenotype of the alveolar epithelial RelA-mutant mouse model

We generated a mouse model devoid of functional RelA in alveolar epithelial cells. Breeding was designed so that all offspring should be homozygous for tetO-Cre and floxed RelA, with half being negative and half positive for SPC-rtTA. When no doxycycline chow was included in the breeding cages, that ratio was observed. However, the inclusion of doxycycline chow during gestation and nursing resulted in a skewing from Hardy-Weinberg equilibrium (29–30), with only 10% instead of 50% of the offspring being positive for the rtTA transgene and hence mutant for RelA in alveolar epithelial cells when genotyped at weaning (3 weeks of age). Furthermore, some but not all adult mice that survived RelA mutation via such a strategy displayed emphysematous-like enlarged alveolar air spaces. These results could indicate that RelA in epithelial cells is essential for preventing lethality during embryonic and neonatal periods and for lung development postnatally. However, these phenotypes more likely represent an artifact of the strategy, as evidenced by similar findings using the SPC-rtTA/tetO-Cre system in the absence of floxed genes (31–32), with variable penetrance that depends on genetic background. The interpretation that these phenotypes resulted from the rtTA-based strategy rather than the RelA deletion is also supported by the fact that we observe neither phenotype in mice lacking RelA in all cells (12–13). Although these phenotypes precluded extensive analyses of integrated responses in these mice, we were able to use them for a limited set of studies to interrogate cell-specific gene expression during acute pulmonary inflammation.

Alveolar epithelial cell RelA is required for induction of select cytokines

The expression of lung inflammatory cytokines was evaluated using PCR array. To our surprise, during LPS-induced pulmonary inflammation, only 2 out of 84 cytokines in the PCR array were decreased in the lungs of mice with RelA mutated in alveolar epithelial cells, CCL20 and CXCL5 (Figure 1A). One gene, C-reactive protein, appeared to be increased in mutant mice in this array, but this was not followed up as the mRNA levels were very low even in mutants (Figure 1A). qRT-PCR confirmed that CCL20 and CXCL5 were significantly induced by LPS in the lungs, CXCL5 peaking at 6–15 hours and CCL20 peaking at 4–6 hours (Figure 1B). Induction of each was almost entirely abrogated by the mutation of RelA in alveolar epithelial cells (Figure 1C). Seven other cytokines represented on the array; CXCL1, CXCL2, IL-1 α , IL-1 β , TNF- α , IL-6, and G-CSF, were measured by qRT-PCR, which confirmed that they were induced by LPS but were not significantly affected by the mutation of RelA in alveolar epithelial cells (Figure 1C). The fact that the majority of cytokines examined were comparable in WT and mutant lungs suggests that the strategy to target alveolar epithelial RelA did not have global or widespread immunity consequences, but rather very selective effects on a small subset of mediators. The strategy in these mice was designed to selectively target alveolar epithelial cells (16), suggesting AT2 or AT1 cells as potentially critical sources of the cytokines CCL20 and CXCL5 during LPS-induced pulmonary inflammation.

To determine whether cytokines showed similar dependence on RelA in alveolar epithelial cells in response to pneumococcus, qRT-PCR was performed on lung mRNA extracts from mice with pneumococcal pneumonia. While neither CCL20 nor CXCL5 were induced by 6 hours, both were strongly induced by 15 hours after infection (Figure 2A), consistent with prior publications demonstrating innate immunity gene induction responses to pneumococci to involve slower kinetics compared to Gram-negative bacterial products (7, 12, 26, 33). As with LPS-induced inflammation, many genes induced in the lungs did not require RelA specifically in alveolar epithelial cells (Figure 2B). Also similar to LPS-induced inflammation, CCL20 induction did depend on RelA in alveolar epithelial cells, being over

90% diminished by the mutation of RelA in alveolar epithelial cells (Figure 2B). Interestingly, CXCL5 was induced despite RelA mutation in the alveolar epithelial cells 15 h after pneumococcal infection (Figure 2B), which sharply contrasted with the results from lungs 6 h after LPS instillation. A PCR array suggested similar cytokine expression patterns in the lungs of mutant mice compared to wild type after pneumococcus infection (Figure 2C). CXCL5 was unaffected by RelA mutation, CCL20 was diminished, and 2 additional transcripts (for CXCR3 and IL-10R β) were potentially blunted by epithelial RelA mutation during pneumococcal pneumonia. While these results suggest additional new lines of inquiry, we chose to maintain focus on the epithelial-derived chemokines and pursue the suggestion that CCL20 is consistently dependent upon epithelial RelA while CXCL5 dependency appears to differ after LPS or pneumococcus. Importantly, the disparate roles for alveolar epithelial RelA in pneumococcal induction of CXCL5 and CCL20 were similarly observed when examined at the protein level using ELISA (Figure 2D). These results suggest a heretofore unappreciated stimulus specificity relating to the role of alveolar epithelial RelA in CXCL5 induction, which could result from distinct cell sources (e.g., AT2 vs. AT1 cells) and/or distinct molecular regulation (RelA-dependent vs. independent pathways) within the same cells.

Integrated immune responses

Limitations of the animal model, discussed above, precluded extensive analyses of integrated immune responses, but exploratory studies were performed in an effort to reveal roles of epithelial RelA. Bacterial burdens were measured 24 hours after pneumococcal infection. Neither WT nor mutant mice showed effective host defense during this infection, and there were no significant differences between genotypes (Figure 3A). In this transgenic model, there were no significant effects of epithelial RelA mutation on neutrophil recruitment in the lungs, measured by quantifying neutrophils in the alveolar air spaces using morphometry after LPS instillation (Figure 3B) and by quantifying neutrophil-specific Ly6G mRNA in the lung using qRT-PCR after either LPS or pneumococcus instillation (Figure 3C). Thus, these data do not reveal roles epithelial RelA in integrated innate immune responses. However, the limitations of this transgenic mouse model system, particularly the perinatal lethality phenotype, restrict the numbers and types of experiments that can be done using this model. A better understanding of the functional significance of epithelial RelA in integrated pulmonary immunity will require alternative and improved models for cell-specific targeting of this transcription factor.

Distinct cytokine expression patterns in alveolar epithelial cell lines *in vitro*

To begin teasing apart specific roles of AT1 and AT2 cells in the expression of CCL20 and CXCL5, we turned to E10 and MLE15 cells, which are immortalized epithelial cell lines derived from mouse lungs. The E10 and MLE15 cell lines model many characteristics of AT1 and AT2 cells, respectively (12, 17, 24, 34). Consistent with prior studies suggesting that pneumococcus does not activate NF- κ B in alveolar epithelial cells (12), this stimulus failed to induce expression of CXCL5 or CCL20 from either cell line (data not shown). After LPS or TNF- α stimulation, however, CXCL5 and CCL20 mRNA and protein levels were induced in both cell lines (Figures 4C–4F). Protein expression data suggest greater elaboration of CXCL5 by E10 and greater CCL20 by MLE15 (Figure 4), supporting cell-specific cytokine profiles at the alveolar surface.

We used an siRNA approach to identify the influence of RelA on chemokine expression by these cell lines. RelA was successfully knocked down in both cell lines (Figures 4A and 4B). Induction of CCL20 required RelA following either stimulation and in both cell lines (Figure 4C and 4E). In contrast, RelA knockdown decreased CXCL5 induction only in MLE15 cells (Figure 4D and 4F). These data suggest the unexpected hypothesis that AT1

cells may be important sources of CXCL5, and they may be able to produce this chemokine in a RelA-independent manner.

***In vivo* responses of primary AT1 cells during pneumonia**

To elucidate distinct cytokine expression patterns from different epithelial cell types *in vivo*, AT1 cells were isolated from the lung using Ab against T1 α , also known as podoplanin or RT140, a surface protein expressed by AT1 cells (35–39). In lung single cell suspensions, the T1 α -bright cell population was clearly separated from T1 α -negative cells (Figure 5A). T1 α -positive cells express high podoplanin and caveolin-1, but not markers of other epithelial cells (SP-C, SP-B, Nkx2-1, or CC10), suggesting successful separation of AT1 cells from AT2 cells and Clara cells (Figure 5B). Immunofluorescence of sorted cells confirmed that caveolin-1 staining, characteristic of AT1 cells, was observed in the T1 α ⁺ population but not the T1 α [−] population, while pro-SP-C staining, characteristic of AT2 cells, showed the opposite pattern (Figure 5C). Complete purity was not achieved, as a fraction of cells in the T1 α ⁺ population did not stain for caveolin-1 (Figure 5C). Potential T1 α ⁺ cells in addition to AT1 cells may include lymphatic endothelial cells, basal cells, and pleural cells (40–41). PECAM-1 and p75, characteristic of lymphatic endothelial cells and basal cells, respectively, were less prominent in the T1 α ⁺ population compared to the non-selected cells (Figure 5B). Muc16, characteristic of pleural cells but not other cells from the lung (42), was higher in the T1 α ⁺ population (data not shown), suggesting cells from the visceral pleura as the other cells included with AT1 cells in these preparations. We interpret these T1 α ⁺ cell pools as greatly enriched for AT1 cells and devoid of their most closely related and apposed AT2 cells, although we recognize that they are not pure AT1 cell populations.

During LPS-induced pulmonary inflammation, both CXCL5 and CCL20 were induced in cells other than AT1 cells and not in the T1 α -bright cells (Figures 5D and 5E). During pneumococcal pneumonia, as after LPS, CCL20 was only induced in cells other than AT1 cells (Figure 5F). In contrast, CXCL5 was strongly induced in the T1 α -bright AT1 cells during pneumococcal pneumonia (Figure 5G). These results indicate that CXCL5 is induced in AT1 cells during pneumococcal pneumonia, which differs from LPS-induced inflammation and which is to our knowledge the first direct evidence of an innate immune response from type I alveolar epithelial cells.

AT1 cells induce CXCL5 independent of RelA during pneumococcal pneumonia

Altogether, the above data suggest the hypothesis that AT1 cells may be activated to express CXCL5 in a RelA-independent fashion during pneumococcal pneumonia. To clarify RelA dependency of CXCL5 induction in AT1 cells, we applied our cell sorting approach to the lungs of mice with alveolar epithelial mutations in RelA. Using an RT-PCR strategy to differentiate the recombined *Rela* gene product from the floxed-but-not-recombined version encoding wild type RelA, we found that AT1 cells of RelA Δ/Δ mice expressed the mutant allele whereas the AT1 cells of WT littermates did not (Figure 6A). Leukocytes expressed wild type RelA in both cases (Figure 6A). These results confirm effective transgene-specific targeting of the *Rela* locus in alveolar epithelial cells, and they also add confidence to our interpretation of the T1 α -bright population as consisting of primarily AT1 cells with few non-epithelial contaminants. To determine whether or not the AT1 cell induction of CXCL5 during pneumococcal pneumonia required RelA, we compared CXCL5 expression from isolated T1 α -bright cells of WT and RelA Δ/Δ mice. CXCL5 was significantly induced in T1 α -bright cells from both WT and mutant mice during pneumococcal pneumonia, with no significant differences due to genotype (Figure 6B). These data demonstrate that the induction of CXCL5 in AT1 cells during pneumococcal pneumonia does not require NF- κ B

RelA, which is to our knowledge the first evidence of truly RelA-independent gene induction by any cell-type during pneumonia.

AT1 cells express STING independent of RelA during pneumococcal pneumonia

The discovery of innate immune responses in ATI cells raises the question of whether these cells express distinct innate immunity-initiating pattern recognition receptors (PRRs). We refined our cell sorting approaches to distinguish responses of T1 α -bright AT1 cells from both CD45-positive leukocytes as well as cells not collected in either of those gates (including AT2 cells plus many others). The qRT-PCR of chemokines in these cell populations demonstrated no induction of CCL20 or CXCL5 in leukocytes, reinforcing the interpretations that CCL20 is induced selectively in AT2 cells while CXCL5 is induced in both AT2 and AT1 cells during pneumococcal pneumonia (Figure 7A). Because they have been implicated in responses to pneumococcal pneumonia (43–46), the PRRs TLR2, TLR4, and stimulator of IFN gene (STING) were measured in these sorted cell populations. TLR4 did not demonstrate much variability between cell-types and revealed little effect of infection (Figure 7B). TLR2 was much more highly expressed in leukocytes than epithelial cells, but it was induced in AT1 cells during infection (Figure 7B). Interestingly, STING was most highly expressed in T1 α ⁺ cells compared to leukocytes or T1 α -negative cells, and STING also was significantly induced in AT1 cells during pneumonia (Figure 7B), supporting the concept that AT1 cells have roles in innate immunity. Because TLR2 and STING were induced in AT1 cells, we endeavored to determine whether these cell-specific innate immune responses were mediated by RelA. The expression of TLR2 in T1 α -positive cells during pneumonia was entirely dependent on RelA (Figure 7C). In contrast, STING was significantly induced in RelA-mutant AT1 cells, and this induction level did not differ from that observed in WT littermates (Figure 7D). These results bolster the conclusion that AT1 cells respond to bacteria in the lungs, and they indicate that these cells use both RelA-dependent and -independent pathways to induce innate immunity genes. Like CXCL5, STING in AT1 cells is another innate immunity gene that can be induced independent of RelA during pneumonia.

Discussion

These results demonstrate that NF- κ B RelA in alveolar epithelial cells is essential for induction of select cytokines in the lung, including CXCL5 and CCL20. The effects of alveolar epithelial mutation of RelA on cytokine expression were more modest than anticipated. Many cytokines were expressed similarly regardless of the epithelial cell mutation, including IL-1 β , CXCL1, and CXCL2, which contrasts with prior publications in which the NF- κ B pathway was targeted by overexpression of dnI κ B α (14). This discrepancy may result from differences in the models, including distinct delivery systems for LPS or off-target effects of the SPC-driven dnI κ B α transgene, or conceivably they may suggest possible roles for factors other than RelA being downstream of pathways inhibited by dnI κ B α overexpression. Cytokines such as TNF- α and IL-1 β more likely result from leukocytes than epithelial cells (47–48), suggesting that decreases observed in the SPC-dnI κ B α transgenic mice may reflect indirect effects rather than direct expression by alveolar epithelial cells. In contrast to other cytokines, two chemokines were strongly diminished by RelA mutation in alveolar epithelial cells during LPS-induced inflammation, CCL20 and CXCL5. CCL20 is induced in human AT2 cells with LPS stimulation (49), but it can also be elaborated by other cells such as T lymphocytes (50). CCL20 has direct antimicrobial activities against gram-negative bacteria (50–51), and it signals to dendritic cells and T cells in adaptive immune responses (52). CXCL5 has been suggested to be an AT2 cell-specific product based on immunohistochemistry (53), and it influences neutrophil influx to the lung after LPS inhalation and during *E.coli* pneumonia (54). Our results from targeted RelA

mutation identify alveolar epithelial cells as essential sources of select innate immunity mediators important to lung host defense.

CCL20 induction was consistently abrogated by mutation of RelA in the alveolar epithelial cells. The AT2-like MLE15 cells make much more CCL20 than do the AT1-like E10 cells, and this induction is consistently dependent upon RelA. The sorted cell studies revealed that CCL20 is induced in cells other than AT1 cells or leukocytes. Altogether, these 3 sets of results suggest that AT2 cells may be unique sources of CCL20 during acute pulmonary inflammation, and this chemokine expression is uniformly dependent upon RelA.

In our study, CXCL5 was induced by LPS and pneumococcus in T1 α ⁺ cells which include AT2 cells, consistent with a prior study demonstrating CXCL5 induction in this cell type following an intrapulmonary LPS challenge (53). Excitingly, our *in vivo* studies revealed AT1 cells as a novel source of CXCL5 during pneumococcal pneumonia. Previous studies have demonstrated that purified bacterial products are capable of inducing chemokines in primary AT1-like cells or cell lines under *in vitro* culture conditions (55–56), which we have here expanded and confirmed with studies of AT1-like E10 cultures. Due to their anatomic localizations and morphological characteristics, AT1 cells have been difficult to study using immunohistochemistry or *in situ* hybridization. There is little or no information about *in vivo* AT1 responses to bacterial infection. The T1 α -based sorting method allows the study of primary AT1 cells collected from living lungs. The present studies reveal that AT1 cells respond *in vivo* during a bacterial infection of the lung. To our knowledge, this is the first demonstration that AT1 cells induce innate immunity genes during pneumonia. The discovery that the cells constituting the majority of the alveolar surface respond to infection with the elaboration of innate immunity mediators broadens our understanding of lung cell biology and pulmonary immunity.

Our conclusion that AT1 cells have roles in innate immunity is further supported by the evidence that the pattern recognition receptors, TLR2 and STING, are induced in AT1 cells during pneumococcal pneumonia, although TLR2 it is more highly expressed by leukocytes. Interestingly, STING is most highly expressed in AT1 cells at baseline and induced to the highest levels during infection in AT1 cells. STING mediates recognition pathways for pneumococcal DNA and drives the expression of innate immunity mediators such as type I interferons (43–44). Thus, our data suggest that AT1 cells during infection increase their expression of both membrane and cytosolic receptors capable of responding to pneumococcus.

The significance of AT1 cell induction of innate immunity genes (including but likely not limited to TLR2, STING, and CXCL5) is unclear. Because the vast majority of the lung surface is AT1 cells, the increased expression of pattern recognition receptors and neutrophil chemokines may limit the spread of microbes throughout alveoli or across epithelial barriers. The fact that we did not see defects in inflammation and host defense is certainly not evidence for a lack of a role; these particular challenges and the limitations of this transgenic mouse model (discussed above) may have been inadequate to reveal functionally significant effects of AT1-derived genes. It is tempting to speculate that AT1 responses may be especially important in settings where microbes subvert macrophage responses, since alveolar macrophages are typically sentinel cells alerting to lung infection (2, 57). The functional significance of innate immunity gene induction by AT1 cells now becomes an important focus for future research.

Not only do the results indicate that CXCL5, TLR2, and STING are induced in AT1 cells during infection, they also illuminate distinct transcriptional regulation in these cells. TLR2 induction in AT1 cells was entirely abrogated by RelA deletion, revealing that AT1 cells can

use RelA to induce the expression of innate immunity mediators, similar to prior observations with other cells (12–13, 25). In contrast, the induction of CXCL5 and STING was unaffected by the mutation of RelA. This was unexpected and represents the first observations of truly NF- κ B RelA-independent innate immunity gene induction in pneumonic lungs. These results highlight novel pathways for innate immunity gene expression yet to be discovered.

Most significantly, these results demonstrate that AT1 cells are under-appreciated players in lung immunity and antibacterial host defense. These cells constitute the vast majority of the surface with which bacteria can interact during lung infection. Which products they elaborate, by what recognition and signaling and transcriptional pathways, and to what effects on integrated immune and physiological processes now become critical next questions. An improved understanding of the mechanisms and the significance of AT1 cell responses to infection may suggest new susceptibility determinants and therapeutic approaches for acute bacterial pneumonia.

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Abbreviations

<i>Sp</i>	<i>Streptococcus pneumoniae</i>
AT1	alveolar epithelial type I
AT2	alveolar epithelial type II
Ly6G	lymphocyte antigen 6 complex, locus G
STING	stimulator of IFN gene

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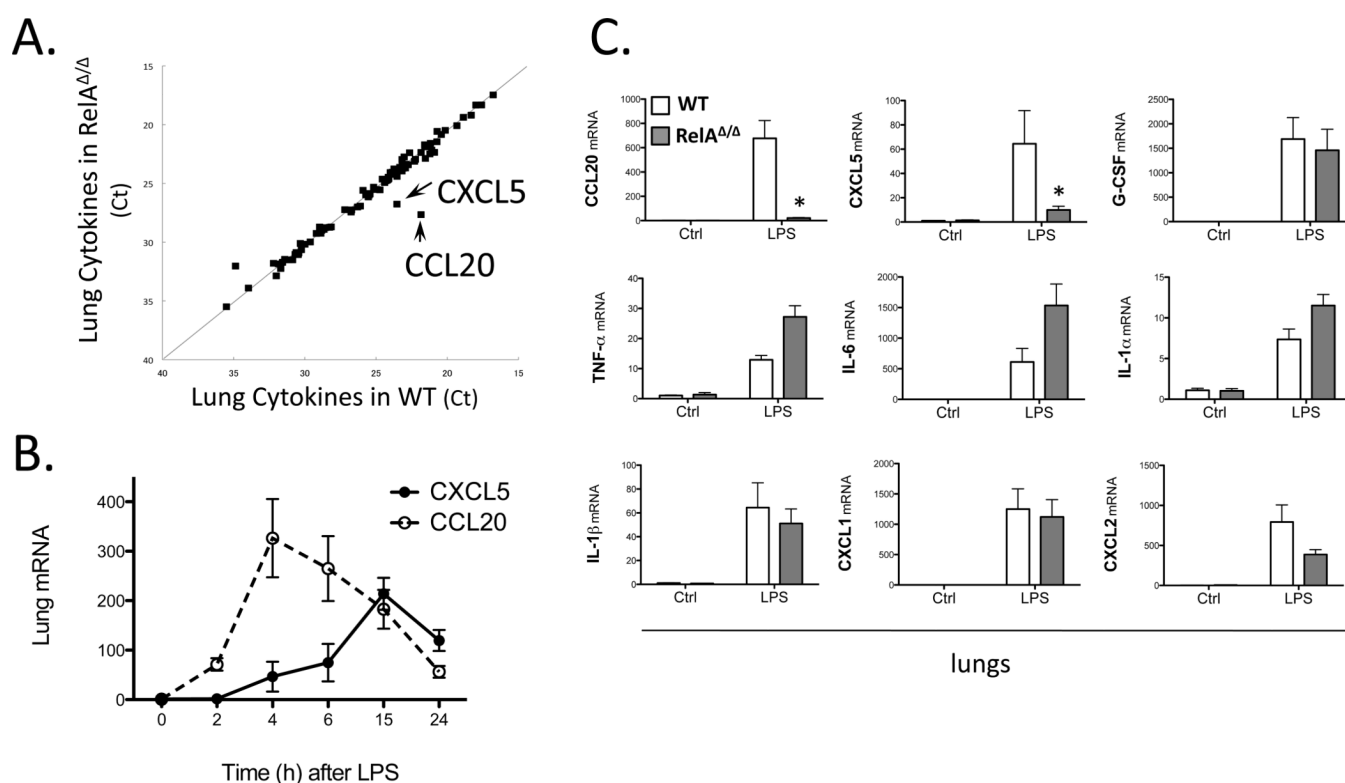


Figure 1. A small subset of cytokines is decreased in the absence of alveolar epithelial RelA during LPS-induced pulmonary inflammation

A. Lung inflammatory cytokine mRNA expression after 6h of intratracheal LPS in presence or absence of functional RelA in alveolar epithelial cells (SPC-rtTA^{wt/tg} tetO-Cre^{tg/tg} RelA^{F/F}). A total of 84 cytokine-related mRNA levels were determined using PCR array and data for the each group were expressed as Ct. Data shown are results from lung RNA pooled from 6 mice in each group.

B. Lung inflammatory cytokine mRNA expression (CXCL5 and CCL20) over a time-course after intratracheal LPS instillation, measured in C57BL/6 mice using qRT-PCR (n=3–5 in each time-point, collected over 2 independent experiments).

C. Lung inflammatory cytokine mRNA expression after 0h or 6h of intratracheal LPS were measured by qRT-PCR in presence and absence of functional RelA in alveolar epithelial cells. WT, wild-type non-targeted mice (tetO-Cre^{tg/tg} RelA^{F/F}, no Cre transgene); RelA Δ/Δ , transgenic mice with mutant RelA in alveolar epithelial cells (SPC-rtTA^{wt/tg} tetO-Cre^{tg/tg} RelA^{F/F}). *, $p < 0.05$ compared with WT mice. n=3–4 mice/group in Ctrl, and n=6 mice/group in LPS, collected over 3 independent experiments.

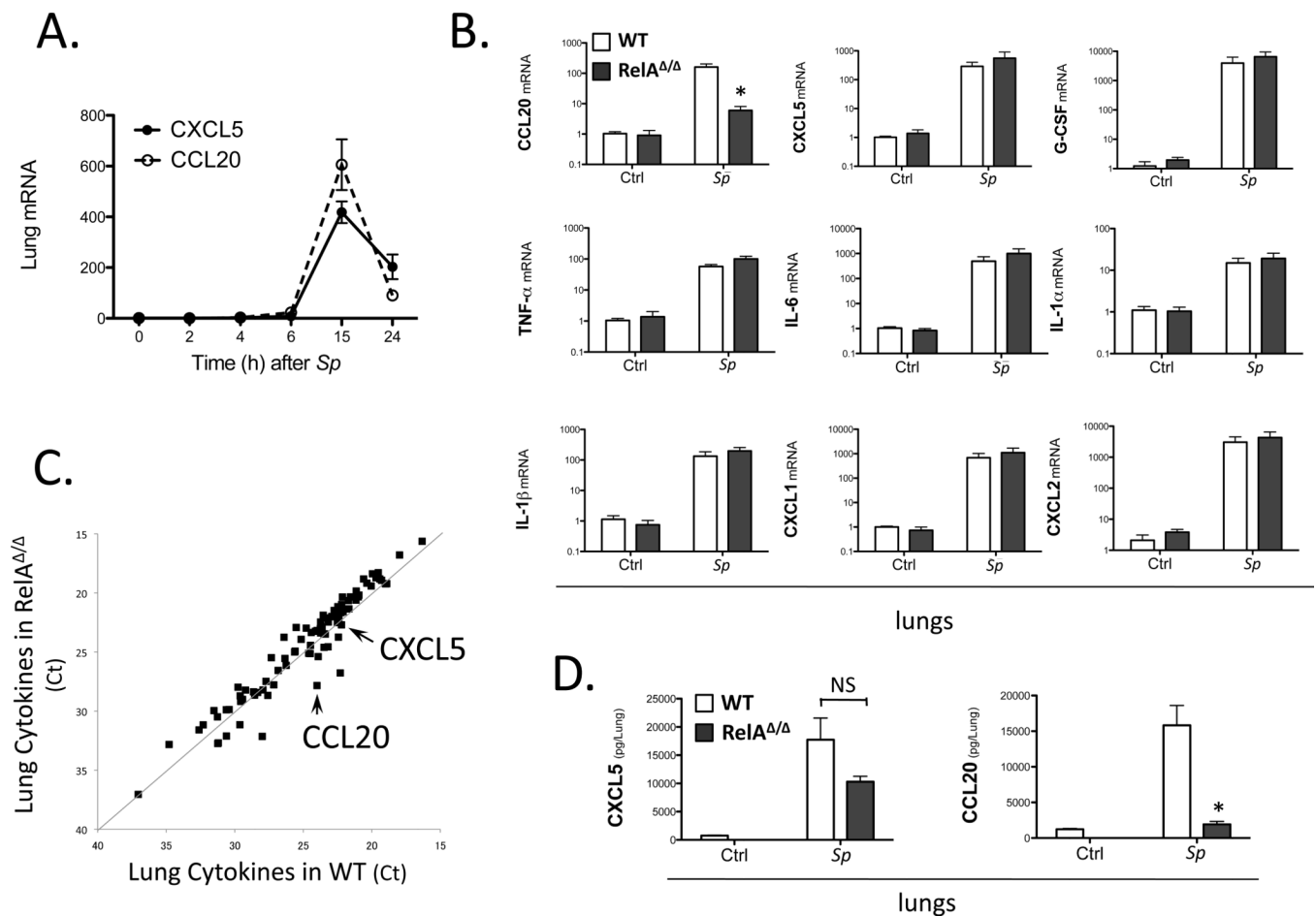


Figure 2. During pneumococcal pneumonia, CCL20 but not CXCL5 requires alveolar epithelial RelA

A. Lung inflammatory cytokine mRNA expression (CXCL5 and CCL20) over a time-course after *S. pneumoniae* serotype 3 (*Sp*) instillation, measured in C57BL/6 mice using qRT-PCR (n=3 in each time-point, collected over 2 independent experiments).

B. Lung inflammatory cytokine mRNA expression after 0h or 15h of intratracheal *Sp* were measured by qRT-PCR in presence (WT) or absence of functional RelA (*RelA*^{Δ/Δ}) in alveolar epithelial cells (n=3–4 uninfected mice/group in Ctrl, and n=5–6 infected mice/group in *Sp*, collected over 3 independent experiments).

C. Lung inflammatory cytokine mRNA expression after 15h of intratracheal *Sp* in presence (WT) or absence of functional RelA (*RelA*^{Δ/Δ}) in alveolar epithelial cells. A total of 84 cytokine-related mRNA levels were determined using PCR array and data for the each group were expressed as Ct. Data shown are results from lung RNA pooled from 5–6 mice in each group.

D. CXCL5 and CCL20 were measured by ELISA in lung homogenates harvested from uninfected mice and mice intratracheally infected with *Sp* for 15h, in presence (WT) or absence (*RelA*^{Δ/Δ}) of functional RelA in alveolar epithelial cells. *, p < 0.05 compared with WT mice (n=3 uninfected/group, and n=3–8 infected/group, collected over 2 independent experiments).

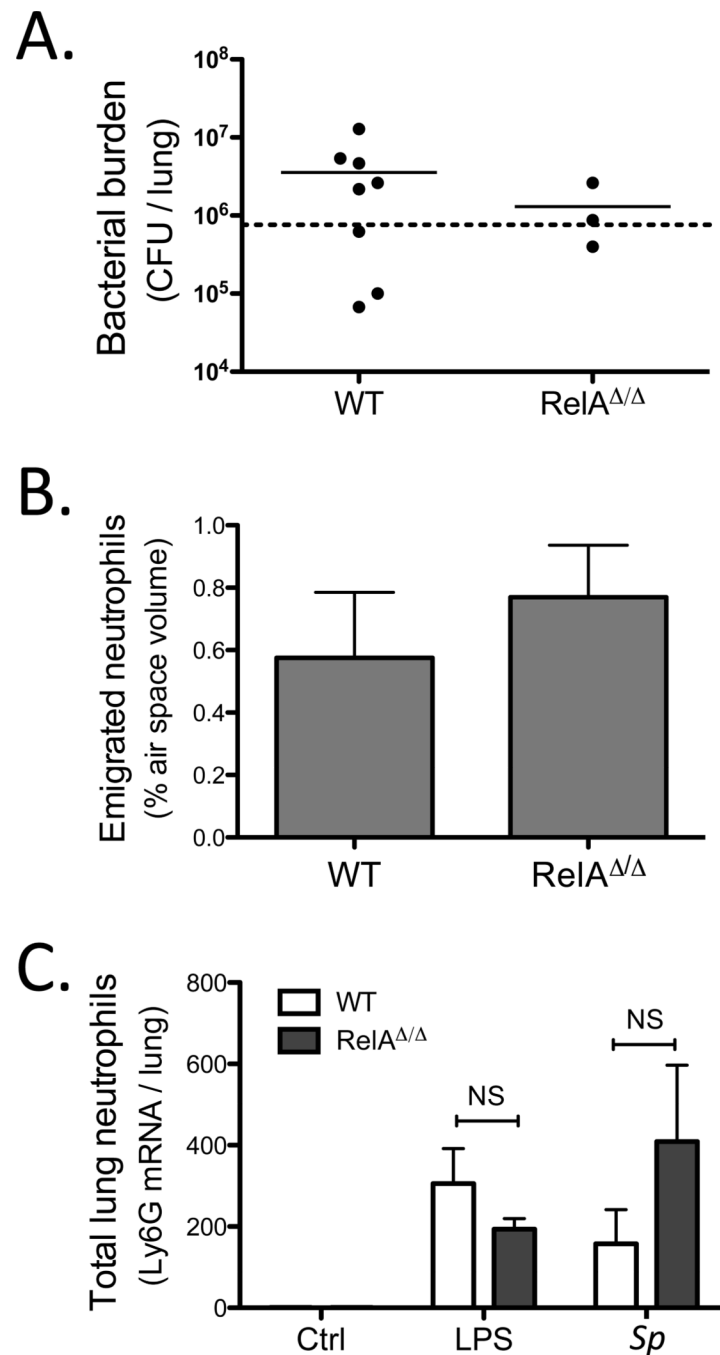


Figure 3. Integrated immunity in mice with alveolar epithelial cell mutations in RelA

A. Viable bacteria in the lung were quantified using CFU assays after 15h pneumococcal infection (n=3–8 mice/group, collected over 2 independent experiments).

B. Emigrated neutrophils were measured by quantifying neutrophils in alveolar airspaces using morphometry (n=5–9 mice/group, collected over 3 independent experiments).

C. Neutrophil accumulation was evaluated by measuring Ly6G mRNA in lung lysates after 6h of LPS challenge or 15h of pneumococcal infection, expressed as fold WT Ctrl (n=3–4 mice/group in Ctrl, and n=5–6 mice/group in each of LPS and in *Sp* groups, collected over 3 independent experiments).

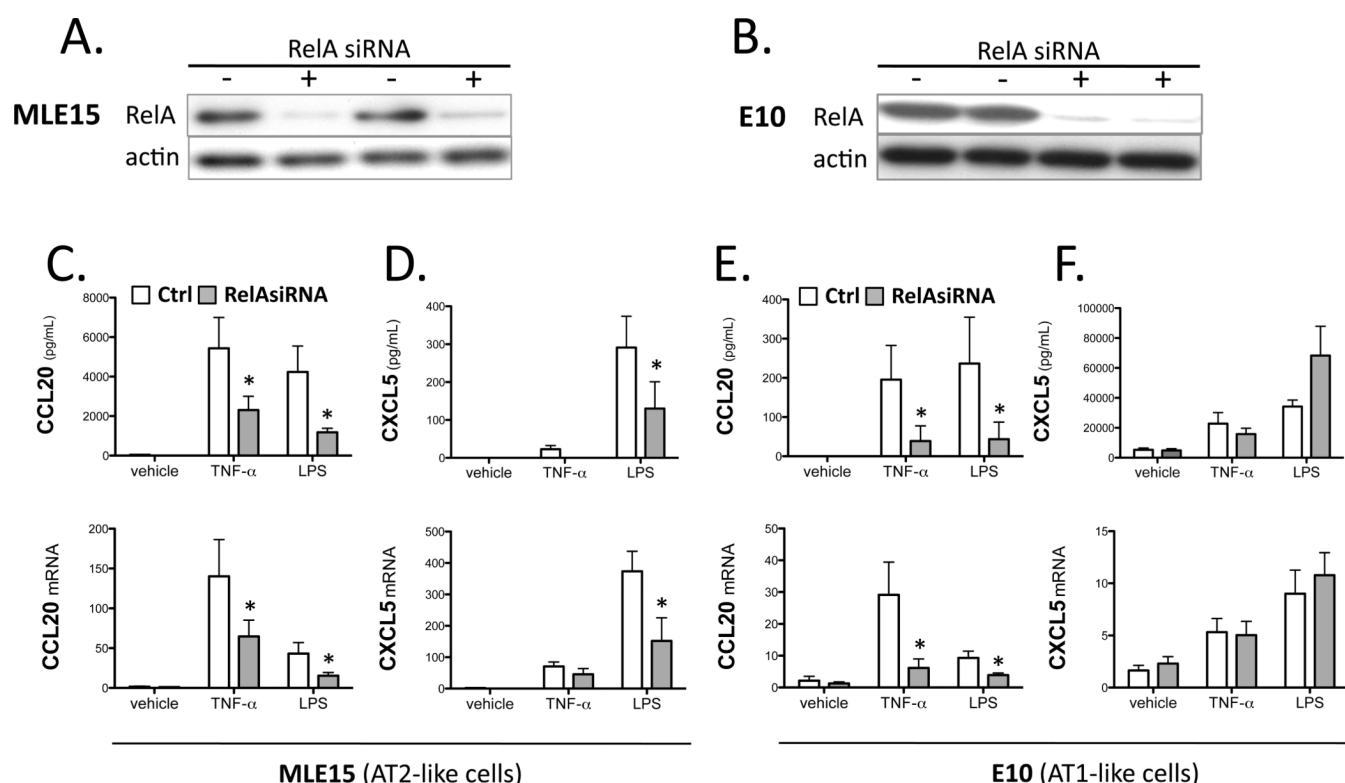


Figure 4. CCL20 induction is consistently dependent on RelA, whereas CXCL5 induction is RelA-dependent in AT2-like cells (MLE15) but RelA-independent in AT1-like cells (E10)

A. Immunoblot shows that RelA was knocked down by siRNA in MLE15 cells. Data are representative of n=5 independent experiments.

B. Immunoblot shows that RelA was knocked down by siRNA in E10 cells. Actin was used for loading control. Data are representative of n=4 independent experiments.

C. CCL20 was measured by qRT-PCR of cell lysates and by ELISA of cell supernatants after 6h of stimulations (TNF- α and LPS) in MLE15 cells in which RelA was knocked down by siRNA. Results reflect data from n=5 independent experiments.

D. CXCL5 was measured by qRT-PCR of cell lysates and by ELISA of cell supernatants after 6h of stimulations (TNF- α and LPS) in MLE15 cells in which RelA was knockdown by siRNA. Results reflect data from n=5 independent experiments.

E. CCL20 was measured by qRT-PCR of cell lysates and by ELISA of cell supernatants after 6h of stimulations (TNF- α and LPS) in E10 cells in which RelA was knockdown by siRNA. Results reflect data from n=4 independent experiments.

F. CXCL5 was measured by qRT-PCR of cell lysates and by ELISA of cell supernatants after 6h of stimulations (TNF- α and LPS) in E10 cells in which RelA was knockdown by siRNA. Results reflect data from n=4 independent experiments. *, p < 0.05 compared with control (non-targeted siRNA).

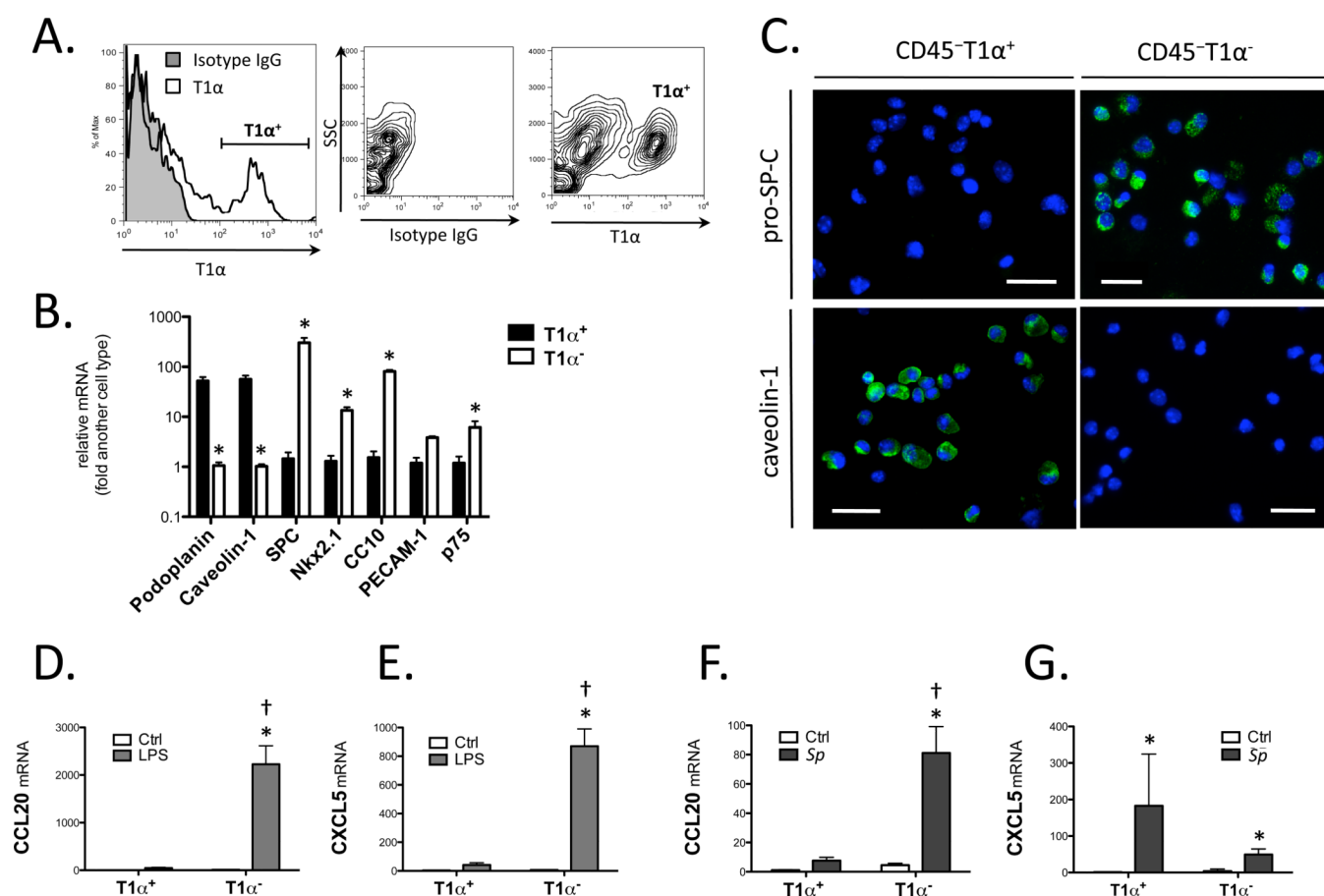


Figure 5. During pneumococcal pneumonia, CXCL5 is induced in AT1 cells

A. AT1 cells were collected by sorting for surface expression of T1α within lung single cell suspensions from C57BL/6 mice using flow cytometry. Results are representative of n=5 mice collected over 3 independent experiments.

B. qRT-PCR of cell-type marker expression amongst T1α⁺ cells and T1α⁻ cells reveals that they former are positive for AT1 markers (podoplanin and caveolin-1) while markers for AT2 (SP-C, Nkx2-1) or other cells (CC10, PECAM-1, p75) were higher in the latter. Data are from n=5 mice/group, collected over 3 independent experiments. *, p < 0.05 compared with T1α⁺ cells.

C. Immunofluorescence images of sorted CD45⁻T1α⁺ cells and CD45⁻T1α⁻ cells demonstrates caveolin-1 staining (characteristic of type I cells) exclusively in the former, and pro-SP-C staining (characteristic of type II cells) exclusively in the latter. pro-SP-C, pro-surfactant protein-C. Scale bars: 100 μm. Results are representative of n=4 mice collected over 2 independent experiments.

D. CCL20 was primarily induced in T1α⁻ cells during LPS-induced pulmonary inflammation in C57BL/6 mice, measured using qRT-PCR (n=3 mice/group in Ctrl, and n=7 mice/group in LPS, collected over 2 independent experiments).

E. CXCL5 was primarily induced in T1α⁻ cells during LPS-induced pulmonary inflammation in C57BL/6 mice, measured using qRT-PCR (n=3 mice/group in Ctrl, and n=7 mice/group in LPS, collected over 2 independent experiments).

F. CCL20 was primarily induced in T1α⁻ cells during pneumococcal pneumonia in C57BL/6 mice, measured using qRT-PCR (n=4 mice/group in Ctrl, and n=9 mice/group in Sp, collected over 4 independent experiments).

G. CXCL5 was primarily induced in T1 α ⁺ cells during pneumococcal pneumonia in C57BL/6 mice, measured using qRT-PCR, evidence that AT1 cells respond with innate immunity gene induction during pneumococcal pneumonia (n=4 uninfected mice/group, and n=9 infected mice/group, collected over 4 independent experiments). *, p < 0.05 compared with uninfected control mice. †, p < 0.05 compared with T1 α ⁺ cells.

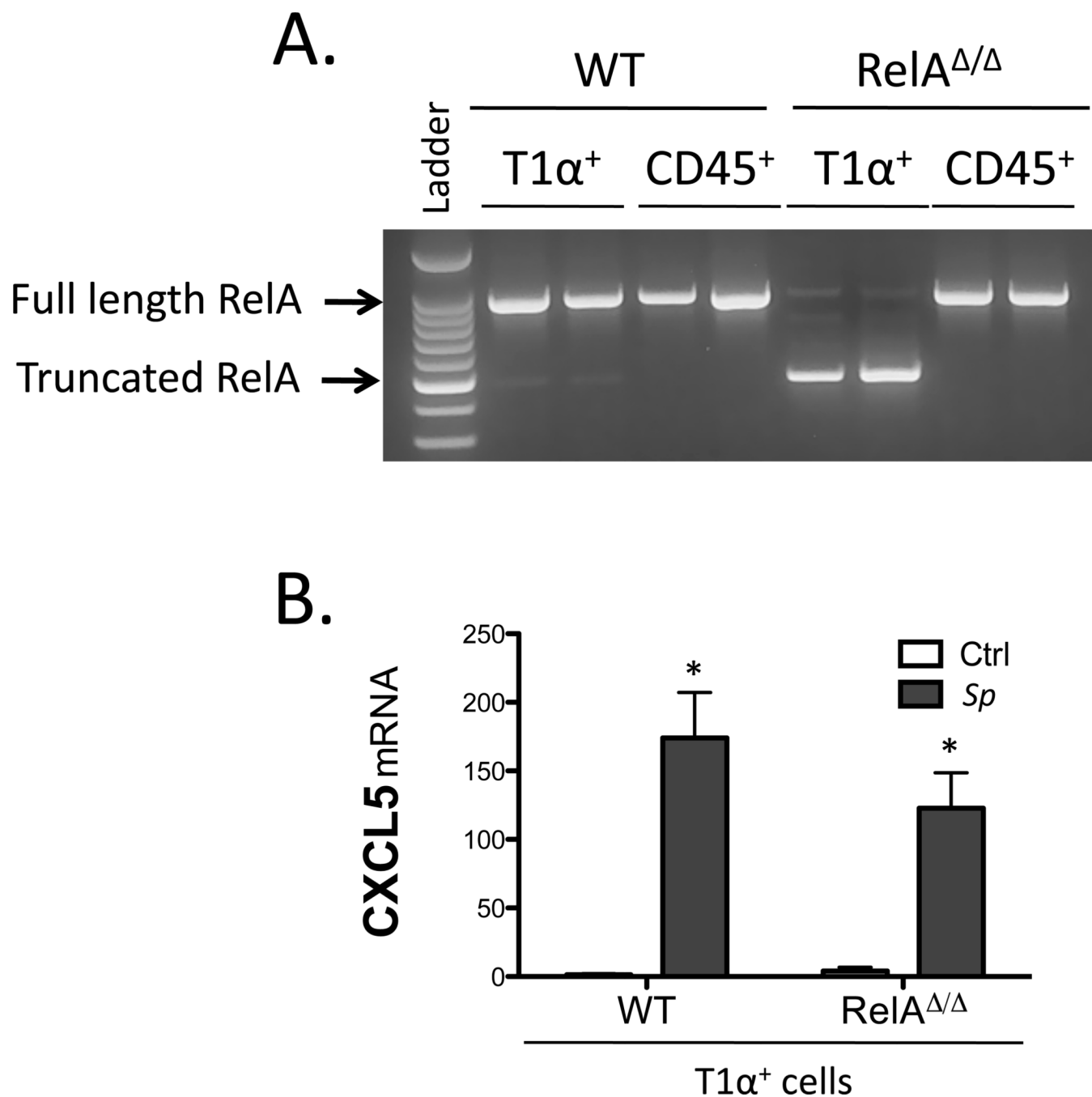


Figure 6. AT1 cell induction of CXCL5 during pneumococcal pneumonia is independent of RelA

A. RelA is effectively targeted in AT1 cells by transgenesis. RT-PCR of wild-type and mutant RelA allele products in T1 α^+ cells and CD45 $^+$ cells isolated from lung single cell suspension of WT (tetO-Cre $^{tg/tg}$ RelA $^{F/F}$, no Cre transgene) or epithelial mutant RelA Δ/Δ (SPC-rtTA $^{wt/tg}$ tetO-Cre $^{tg/tg}$ RelA $^{F/F}$) mice reveals that the T1 α^+ cells express the mutant allele, in targeted mice selectively. Results are representative of n=4 mice collected over 2 independent experiments.

B. The induction of CXCL5 in T1 α^+ cells does not require RelA. CXCL5 was measured by qRT-PCR in sorted T1 α^+ cells from uninfected mice or during *Sp* pneumonia, using WT or epithelial mutant RelA Δ/Δ mice (n=3 mice/group in Ctrl, and n=6 mice/group in *Sp*,

collected over 3 independent experiments). *, $p < 0.05$ compared with uninfected control mice.

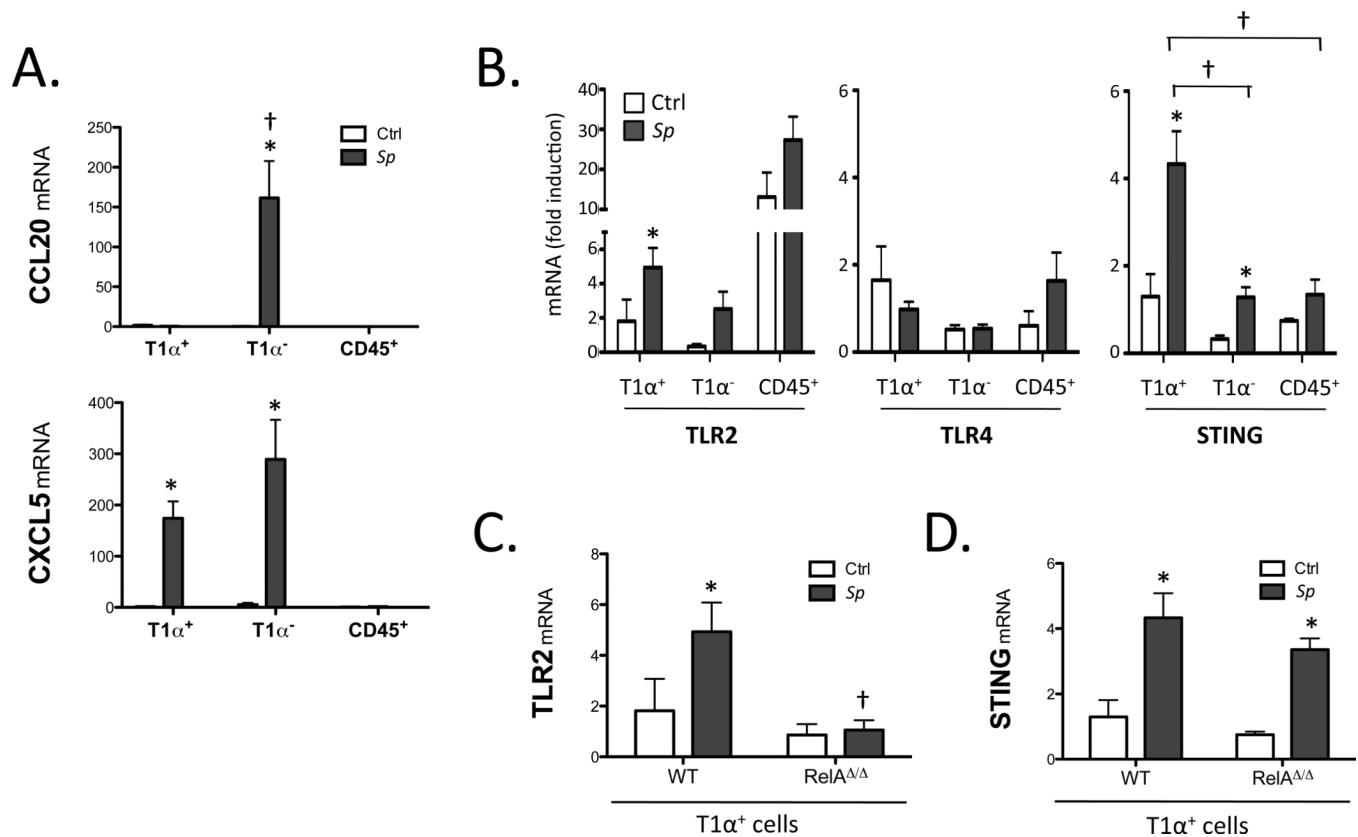


Figure 7. AT1 cell induction of TLR2 is dependent on RelA, whereas STING induction is RelA-independent

A. Sorting strategy revised to differentiate gene expression from CD45⁺ leukocytes supports the concept that CCL20 and CXCL5 are exclusively epithelial in origin. The induction of CCL20 and CXCL5 was measured by qRT-PCR in T1α⁺ cells, T1α⁻ cells, and CD45⁺ cells collected from uninfected lungs and during *Sp* pneumonia (n=3 mice/group in Ctrl, and n=6 mice/group in *Sp*, collected over 3 independent experiments).

B. The expression of PRRs (TLR2, TLR4, and STING) was measured using qRT-PCR in T1α⁺ cells, T1α⁻ cells, and CD45⁺ cells collected from uninfected lungs and during *Sp* pneumonia (n=3 mice/group in Ctrl, and n=6 mice/group in *Sp*, collected over 3 independent experiments). *, p < 0.05 compared with uninfected control mice, †, p < 0.05 compared with T1α⁺ cells.

C. The induction of TLR2 in T1α⁺ cells, measured by qRT-PCR, was abrogated by RelA mutation during *Sp* pneumonia, using lungs from WT (tetO-Cre^{tg/tg} RelA^{F/F}, no Cre transgene) or epithelial mutant RelA^{Δ/Δ} (SPC-rtTA^{wt/tg} tetO-Cre^{tg/tg} RelA^{F/F}) mice, with n=3 mice/group in Ctrl and n=6 mice/group in *Sp*, collected over 3 independent experiments.

D. STING was induced in T1α⁺ cells during *Sp* pneumonia even if epithelial cells were deficient in RelA, using qRT-PCR to measure STING in lungs from WT or epithelial mutant RelA^{Δ/Δ} mice (n=3 mice/group in Ctrl, and n=6 mice/group in *Sp*, collected over 3 independent experiments). *, p < 0.05 compared with uninfected control mice. †, p < 0.05 compared with WT mice.