Allergic Airway Inflammation Decreases Lung Bacterial Burden following Acute Klebsiella pneumoniae Infection in a Neutrophil- and CCL8-Dependent Manner

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Klebsiella pneumoniae is a significant cause of pneumonia and invasive bacterial disease in both healthy and immunocompromised patients (1–4). Prior mouse studies have demonstrated the critical role of neutrophils and the Th17 cytokines—interleukin-17A (IL-17A), IL-17F, and IL-22—in the successful lung immune response to acute infection with K. pneumoniae (5–9). Moreover, impaired development of Th17 cells and expression of IL-17A are associated with immune-deficient states and invasive bacterial infection in humans (9). IL-17A, IL-17F, and IL-22 are expressed by several cell types and stimulate neutrophil recruitment, production of antibacterial peptides, and maintenance of epithelial barriers (10). Following acute infection with K. pneumoniae, IL-17A is expressed primarily by both γδ T cells and CD4+ T cells (11, 12).

Th2 cytokines—IL-4, IL-5, and IL-13—are responsible for the development of allergic airway inflammation in mouse models of asthma (13). These cytokines induce a phenotype in mice that mimics the airway changes seen in human allergic asthma (13). Characteristic features of this allergic asthma phenotype include goblet cell metaplasia and mucus hypersecretion, airway hyperactivity, smooth muscle hyperplasia, and airway eosinophilia (13). We and others have previously shown that the Th2 cytokines IL-4 and IL-13 negatively regulate IL-17A expression by Th17 cells (14–18). However, the ability of IL-4 and IL-13 to impair IL-17-dependent antibacterial immunity in the lung is not known. Therefore, we undertook the present study to determine the ability of IL-4 and IL-13, expressed in the context of preexisting allergic airway inflammation, to impair IL-17-dependent lung antibacterial immunity to acute K. pneumoniae infection. We hypothesized that (i) preexisting allergic airway inflammation decreases lung IL-17A expression in response to acute K. pneumoniae infection and thereby decreases the lung K. pneumoniae burden. As hypothesized, we found that allergic airway inflammation decreases the number of K. pneumoniae-induced airway neutrophils and lung IL-17A, IL-17F, and IL-22 expression. Despite the marked reduction in postinfection airway neutrophilia and lung expression of Th17 cytokines, allergic airway inflammation significantly decreased the lung K. pneumoniae burden and postinfection mortality. We showed that the decreased lung K. pneumoniae burden was independent of IL-4, IL-5, and IL-17A and partially dependent on IL-13 and STAT6. Additionally, we demonstrated that the decreased lung K. pneumoniae burden associated with allergic airway inflammation was both neutrophil and CCL8 dependent. These findings suggest a novel role for CCL8 in lung antibacterial immunity against K. pneumoniae and suggest new mechanisms of orchestrating lung antibacterial immunity.
orchestrating the clearance of K. pneumoniae (KP) lung infection. Mice underwent intraperitoneal injection of OVA adsorbed to ALUM or ALUM alone at day −18. On days −4 to −1, mice previously injected with OVA-ALUM were exposed to 1% OVA aerosol daily. Control mice did not undergo OVA aerosol challenge. Mice were then infected with either K. pneumoniae or mock infected with PBS and harvested on days 1 and 2 postinfection.

way neutrophilia and an increased lung K. pneumoniae burden and postinfection mortality (6). OVA sensitization and challenge is a well-described model of allergic airway inflammation in which the lung expression of IL-4 and IL-13 is readily induced (13, 20, 21). Thus, the use of each of these models together was designed to specifically address our hypothesis regarding the effect of IL-4 and IL-13 on IL-17-dependent lung antibacterial immunity.

Using this combined model we determined that preexisting allergic airway inflammation decreased K. pneumoniae-induced lung IL-17A expression. Unexpectedly, we found that mice with allergic airway inflammation had a decreased lung K. pneumoniae burden and decreased mortality following acute infection compared to the burden and mortality for mice without allergic airway inflammation. Using KO mice and antibody-mediated neutralization, we demonstrate that the decreased lung K. pneumoniae burden associated with allergic airway inflammation is independent of IL-4, IL-5, and IL-17A and partially dependent on IL-13 and STAT6. In addition, we show that allergic airway inflammation decreases the lung K. pneumoniae burden in a manner dependent on both neutrophils and CCL8. Taken together, these results reveal a novel role for CCL8 in promoting lung antibacterial immunity against K. pneumoniae and suggest novel mechanisms for orchestrating the clearance of K. pneumoniae from the lung.

MATERIALS AND METHODS

Mice. Pathogen-free 8- to 10-week-old female BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). IL-13-KO mice on a BALB/c background were generated as previously described (22). IL-4-KO and STAT6-KO BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-17A-KO BALB/c mice were obtained from Jay Kolls (University of Pittsburgh). In caring for the animals, investigators adhered to the revised 1996 Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (23). All experiments were approved by the Van derbilt Institutional Animal Care and Use Committee.

Induction of allergic airway inflammation. The experimental design is demonstrated in Fig. 1. Mice were sensitized with an intraperitoneal (i.p.) injection of 0.1 ml (10 μg) of ovalbumin (OVA; chicken OVA, grade V; Sigma-Aldrich) adsorbed to 20 mg of Al(OH)₃ (ALUM) on day −18. On days −4, −3, −2, and −1, these mice were placed in an acrylic box and exposed to 1% OVA diluted in sterile phosphate-buffered saline (PBS) using an ultrasonic nebulizer (Ultraneb 99; DeVilbiss) for 40 min. As a control for allergic sensitization, mice were given an i.p. injection of 20 mg of ALUM on day −18 and did not undergo OVA aerosol exposure.

Klebsiella infection. All experimental infections were performed with a serotype 2 K. pneumoniae strain (ATCC 43816). Bacterial stocks of K. pneumoniae were inoculated into 100 ml of tryptic soy broth (TSB) and incubated for 18 h at 37°C and 225 rpm. One milliliter of this culture was transferred to 100 ml of TSB, and bacteria were grown to mid-log phase for 2 h at 37°C. Bacteria were pelleted, washed in sterile PBS, and diluted to the appropriate concentration as previously described (8, 24). To induce pneumonia, mice were anesthetized on day 0 with ketamine-xylazine and were administered 1 × 10⁶ CFU in 50 μl PBS by retropharyngeal instillation. As a control for K. pneumoniae infection, mice were mock infected with 50 μl sterile PBS by retropharyngeal instillation. The inoculum concentration was verified by serial dilution in PBS, plating on tryptic soy agar (TSA), and visual determination of colony counts. In survival experiments, mice were evaluated for clinical illness and mortality. Moribund mice were euthanized according to predetermined criteria.

Combined model of allergic airway inflammation and K. pneumoniae lung infection. The above-described challenges resulted in four experimental groups of mice: (i) ALUM-sensitized nonallergic mice mock infected with PBS (ALUM-PBS mice), (ii) OVA-ALUM-sensitized, OVA-exposed allergic mice mock infected with PBS (OVA-PBS mice), (iii) ALUM-sensitized nonallergic mice infected with K. pneumoniae (ALUM-KP mice), and (iv) OVA-ALUM-sensitized, OVA-exposed allergic mice infected with K. pneumoniae (OVA-KP mice).

Harvest and bacterial quantitation. Mice were euthanized by intraperitoneal injection of pentobarbital sodium (Vortech Pharmaceuticals). Bronchoalveolar lavage (BAL) was performed as described below. The right lung was harvested, placed into 1 ml of minimal essential medium (MEM), and frozen at −70°C for later analysis. The left lung and spleen were harvested separately and placed into 1 ml of sterile PBS, and the organs were ground using sterile glass tissue grinders (Glas-Col). Serial dilutions in PBS were plated onto TSA and grown overnight at 37°C, and colonies were counted.

Cytokine measurements. Cytokine expression was measured from the right lung homogenate with available Duoset (IL-17F, CCL8, myelo-peroxidae [MPO], lipocalcin 2 [LCN2], S100a8) or Quantikine (IL-17A, IL-22) enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) following the manufacturer’s instructions. Additional cytokines (IL-4, IL-5, IL-13, tumor necrosis factor alpha [TNF-α], IL-1β, IL-6, granulocyte colony-stimulating factor [G-CSF], granulocyte-macrophage colony-stimulating factor [GM-CSF], CXCL1, CCL3, CCL4) were measured using a BD cytokine bead array platform (BD Biosciences). Values below the lower limit of detection were assigned a value that was equivalent to half the limit of detection.

Analysis of inflammatory cell infiltration into BAL fluid. BAL was performed by instilling 800 μl of saline through a tracheotomy tube and then withdrawing the fluid with gentle suction via a syringe. White blood cells were counted on a hemocytometer. Cytologic examination was performed on cytospin preparations (Thermo Shandon). Cytospin slides were fixed and stained using DiffQuik strain (American Scientific Products). Differential counts were based on counts of 200 cells using standard morphologic criteria to classify the cells as neutrophils, eosinophils, lymphocytes, or other mononuclear leukocytes (alveolar macrophages and monocytes).

Measurement of peripheral blood neutrophils. Peripheral blood was collected into EDTA-coated tubes, and samples were analyzed using a forcyte veterinary hematology analyzer (Oxford Science Inc., Oxford, CT).
Antibody neutralization. Antibody neutralization of IL-5 was performed by intraperitoneal injection of 20 μg of functional-grade anti-mouse IL-5 (clone TRFK5; ebioscience) or isotype control (rat IgG1 κ chain, clone eBGR1; ebioscience) antibody on days −7 and −4 by the experimental protocol outlined in Fig. 1. Antibody neutralization of CCL8 was performed by i.p. injection of 200 μg on day −4, 100 μg on day −2, and 100 μg on day 0 of either anti-mouse CCL8 (clone 146123; R&D Systems) or isotype control (rat IgG2a, clone 54447; R&D Systems). Antibody-mediated depletion of neutrophils was achieved by i.p. injection and intranasal instillation of either anti-mouse Ly6G (clone 1A8; BioXcell) or isotype control (rat IgG2a, clone 2A3; BioXcell). Dosing for these antibodies was as follows: administration of 1 mg i.p. on day −2, administration of 0.5 mg i.p. on days 0 and +1, and intranasal instillation of 0.2 mg on day 0 (2 h preinfection).

Lung fixation and histological examination. Mice were sacrificed on day 2 postinfection. After perfusion of the lungs with PBS, the trachea was exposed and cannulated and the lungs were inflated with 10% neutral buffered formalin. The lungs were removed and fixed in 10% neutral buffered formalin for 24 h prior to processing and embedding. Sections of 5 μm were cut and stained with hematoxylin-eosin (H&E). To quantify lung eosinophils, lung sections were stained histochemically with Sirius red (as described previously [25]). To detect neutrophils, immunohistochemistry was performed using a Bond Max autostainer (Leica) with rat antineutrophil elastase clone 6A608 (catalog no. sc-71674; Santa Cruz Biotechnology) as previously described (26, 27). To detect and quantify airway mucus expression, lung sections were stained with periodic acid-Schiff (PAS). Lungs were evaluated by light microscopy by an experienced veterinary pathologist (K.L.B.) blinded to group assignment.

A semiquantitative scoring system was performed for five parameters: total lung granulocytes, lung bacteria, lung neutrophils, lung eosinophils, and airway mucus. Total granulocytes were scored as follows from H&E-stained sections: 0, no granulocytes; 1, a few granulocytes; 2, moderate numbers of granulocytes in the perivascular/peribronchiolar location; and 3, many granulocytes in both the perivascular/peribronchiolar and parenchymal locations; and 4, many granulocytes in the perivascular/peribronchiolar location with extensive involvement of the pulmonary parenchyma, including necrosis. The presence of bacteria was scored in H&E-stained sections as follows: 0, no bacteria; 1, rare intracellular bacteria; 2, a few bacteria, mainly intracellular; 3, moderate numbers of intracellular and extracellular bacteria; and 4, large numbers of bacteria, primarily extracellular. To generate neutrophil and eosinophil scores, the percentage of neutrophils and eosinophils in the total granulocyte population was determined and then multiplied by the total granulocyte score. Airway mucus was scored in PAS sections as follows: 0, no PAS-positive cells; 1, less than 10% PAS-positive cells; 2, 10 to 30% PAS-positive cells; and 3, greater than 30% PAS-positive cells.

Statistical analysis. Statistical analyses between two groups were conducted using a two-tailed Student’s t test. Comparisons between multiple groups were performed using one-way analysis of variance with Bonferroni’s posttests for multiple comparisons between all groups. Survival data were analyzed using both the log-rank test and the Wilcoxon test. Analyses were performed using GraphPad Prism, version 5.00, software for Windows (GraphPad Software, San Diego, CA).

RNA sequencing. Specific methods regarding RNA isolation, sequencing, and analysis are included in the supplemental material.

Microarray data accession number. Transcriptome sequencing (RNA-seq) data were submitted to the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) under GEO accession number GSE57602.

RESULTS

Preexisting allergic airway inflammation decreases K. pneumoniae-induced airway neutrophilia and lung expression of IL-17A, IL-17F, and IL-22. Because in vitro and in vivo studies have shown that the Th2 cytokines IL-4 and IL-13 downregulate Th17 cellular expression of IL-17A (14, 16–18) and because IL-17 signaling is critical for airway neutrophilia and bacterial clearance in response to K. pneumoniae (6), we hypothesized that (i) preexisting allergic airway inflammation decreases lung IL-17A expression in response to acute bacterial infection and (ii) decreased lung IL-17A expression results in impaired neutrophil recruitment to the airways, decreased lung bacterial clearance, and an increased lung bacterial burden. To test these hypotheses, we developed a model of preexisting allergic airway inflammation, induced by OVA sensitization and challenge, followed by acute lung bacterial infection with K. pneumoniae (Fig. 1). This combined model allowed us to directly test our hypotheses, as OVA sensitization and challenge readily induce lung expression of IL-4 and IL-13 (13, 20, 21), lung K. pneumoniae infection readily induces lung IL-17A expression, and IL-17 signaling is required for K. pneumoniae-induced airway neutrophilia and clearance of K. pneumoniae from the lung (6, 8, 19).

We first determined the effect of allergic airway inflammation on K. pneumoniae-induced lung IL-17A expression and total airway neutrophils. We hypothesized that allergic airway inflammation impairs the lung expression of IL-17A and airway neutrophilia following challenge with K. pneumoniae. At 1 and 2 days postinfection, ALUM-KP mice had significantly increased lung IL-17A expression and total airway neutrophils compared to ALUM-PBS mice (Fig. 2A and B). In contrast, OVA-KP mice had significantly decreased lung expression of IL-17A and airway neutrophilia compared to ALUM-K. pneumoniae mice at both day 1 and day 2 postinfection. Both lung IL-17A expression and total airway neutrophils in OVA-KP mice were not statistically significantly different from those in ALUM-PBS and OVA-PBS mice. In addition, lung IL-17A expression and total airway neutrophils in OVA-PBS mice were not statistically significantly different from those in ALUM-PBS mice. Notably, no significant differences in total airway macrophages between the groups were noted (see Fig. S1 in the supplemental material). Additionally, ALUM-KP and OVA-KP mice did not have significantly different peripheral blood neutrophilia (see Fig. S1 in the supplemental material).

As lung IL-17A expression was significantly decreased in OVA-KP mice, we next determined the lung expression of IL-17F and IL-22. IL-17F is structurally related to IL-17A, is capable of forming IL-17A–IL-17F heterodimers, shares the IL-17A receptor with IL-17A, and is capable of regulating airway neutrophilia (28). IL-22 is an IL-10 family cytokine that is protective in the acute K. pneumoniae lung infection model (8). ALUM-KP mice had significantly increased IL-17F and IL-22 expression compared to ALUM-PBS and OVA-PBS mice (Fig. 2C and D). In contrast, OVA-KP mice had significantly decreased expression of IL-17F and IL-22 compared to ALUM-KP mice. Thus, these findings supported our hypothesis that allergic airway inflammation significantly impairs the lung IL-17A and airway neutrophil response to acute K. pneumoniae infection.

Preexisting allergic airway inflammation decreases the lung and spleen K. pneumoniae burden following acute infection. We next tested the second aspect of our hypothesis, that allergic airway inflammation impairs lung bacterial clearance. Because neutrophils (7), IL-17 signaling (6), and IL-22 (8) are required for clearance of K. pneumoniae from the lung and we found that allergic airway inflammation significantly reduced each of these factors following K. pneumoniae infection (Fig. 2A to D), we antici-
pated a significant increase in the lung K. pneumoniae burden in OVA-KP mice compared with ALUM-KP mice. In contrast to this anticipated result, OVA-KP mice had a significantly decreased lung K. pneumoniae burden compared to ALUM-KP mice at both day 1 and day 2 postinfection (Fig. 2E). Whereas ALUM-KP mice had a statistically significant 2.2-log_{10} unit increase in the lung K. pneumoniae burden from day 1 to day 2, OVA-KP mice had virtually no change in the lung K. pneumoniae burden from day 1 to day 2 (Fig. 2E).

To determine the effect of allergic airway inflammation on bacterial dissemination and peripheral bacterial clearance, we also quantified the spleen bacterial burden. OVA-KP mice had a significantly decreased spleen K. pneumoniae burden compared to ALUM-KP mice at both day 1 and day 2 (Fig. 2F). In contrast to the lung K. pneumoniae burden, the spleen K. pneumoniae burden in both ALUM-KP and OVA-KP mice significantly increased from day 1 to day 2 (Fig. 2E and F). No bacterial colonies were detected from the lungs or spleens of either ALUM-PBS or OVA-PBS mice (lower limit of detection, 100 CFU/ml). Culture data were not determined beyond day 2, as the onset of mortality began in both ALUM-KP and OVA-KP mice by day 3 postinfection.

To determine whether the decreased lung K. pneumoniae burden in mice with allergic airway inflammation was due to either the altered entry of the K. pneumoniae inoculum into the lower airways or the altered distribution of the inoculum between the right and left lungs, we quantified the lung K. pneumoniae burden at 1 h postinfection from both the right and the left lungs (see Fig. S2 in the supplemental material). No differences in the lung K. pneumoniae burden were found between the right and left lungs. These results demonstrate that allergic airway inflammation decreases the lung K. pneumoniae burden and that this decreased lung K. pneumoniae burden is not due to altered inoculum entry into the lungs.

Allergic airway inflammation decreases the numbers of lung neutrophils and bacteria following acute infection with K. pneumoniae. To determine whether the numbers of lung neutrophils throughout the lung parenchyma were decreased in OVA-KP mice compared to ALUM-KP mice, we next quantified lung granulocytes and distinguished lung eosinophils and neutrophils using histopathology. Lung sections obtained from ALUM-PBS, OVA-PBS, ALUM-KP, and OVA-KP mice on day 2 postinfection were stained with H&E to determine the inflammatory pattern and detect lung granulocytes, Sirius red to quantify lung eosinophils, and antineutrophil elastase to quantify lung neutrophils. Histopathology scores were assigned for total lung granulocytes, and lung eosinophils and neutrophils were scored relative to total granulocytes. Extracellular bacteria were visible in H&E-stained sections at ×100 magnification, and the degree of lung bacteria was therefore scored as well. Sections from ALUM-PBS mice had no inflammatory infiltrate and few to no granulocytes (neutrophils or eosinophils) present in lung sections (see Fig. S3A to E in the supplemental material). Sections from OVA-PBS mice had significantly greater lung eosinophils than those from ALUM-PBS mice (see Fig. S3C and E in the supplemental material). Sections from ALUM-PBS and OVA-PBS mice did not have bacteria visible by H&E staining at a ×100 magnification. Sections from

FIG 2 Preexisting allergic airway inflammation decreases airway neutrophils, lung Th17 cytokine expression, and the lung and spleen K. pneumoniae burden following acute K. pneumoniae lung infection. (A) Lung IL-17A expression measured by ELISA. (B) Airway neutrophils were quantified from airway lavage fluid. (C) Lung IL-17F expression measured by use of a cytokine bead array. (D) Lung IL-22 expression measured by ELISA. Lung (E) and spleen (F) quantitative bacterial burdens were determined by serial dilution. *, P < 0.001; **, P < 0.01; †, P < 0.05. n = 10 mice per group combined from two representative experiments (A to C, E, and F) and n = 5 mice per group from one experiment (D).
ALUM-KP mice had significantly greater lung neutrophils and bacteria than those from both ALUM-PBS and OVA-PBS mice (see Fig. S3B, D, and E in the supplemental material). In marked contrast to the sections from ALUM-KP mice, sections from OVA-KP mice had significantly fewer lung bacteria and neutrophils than sections from ALUM-KP mice (see Fig. S3B, D, and E in the supplemental material). The majority of lung granulocytes in OVA-KP mice were eosinophils, as detected by Sirius red (see Fig. S3C and E in the supplemental material). These results are consistent with our findings of a decreased quantitative lung *K. pneumoniae* burden, despite the decreased numbers of airway neutrophils, in OVA-KP mice, as shown in Fig. 2.

The decreased lung *K. pneumoniae* burden associated with allergic airway inflammation requires both OVA sensitization and OVA aerosol challenge. The development of allergic airway inflammation using OVA requires both a sensitization phase and an airway challenge phase with OVA (Fig. 1). It was therefore possible that either sensitization or aerosol challenge individually was responsible for the decreased lung *K. pneumoniae* burden in OVA-KP mice compared to ALUM-KP mice. To determine whether one or both phases were required for the decreased lung *K. pneumoniae* burden, we modified the protocol described in Fig. 1 with ALUM-KP and OVA-KP mice by including two additional experimental groups (Fig. 3). To determine an effect of OVA aerosol in the absence of prior OVA sensitization, mice were injected with ALUM alone and subsequently exposed to OVA aerosol for four consecutive days. To determine an independent effect of OVA sensitization in the absence of OVA aerosol, mice were injected with OVA-ALUM and not exposed to OVA aerosol. As we previously found, OVA-KP mice had a significantly decreased lung *K. pneumoniae* burden compared to ALUM-KP mice at day 1 and day 2 postinfection (Fig. 3). In contrast, OVA aerosol in the absence of prior sensitization and OVA sensitization in the absence of subsequent OVA aerosol did not decrease the lung *K. pneumoniae* burden compared to that in ALUM-KP mice (Fig. 3). Thus, the decreased lung *K. pneumoniae* burden associated with allergic airway inflammation in our model requires both OVA-ALUM sensitization and OVA aerosol challenge.

In a separate experiment, mice were exposed to PBS aerosol (the vehicle for OVA aerosol) or OVA aerosol for four consecutive days prior to *K. pneumoniae* infection. The lung *K. pneumoniae* burden was compared between these groups and mice that did not undergo any aerosol protocol (see Fig. S4 in the supplemental material). Neither PBS aerosol exposure nor OVA aerosol exposure decreased the lung *K. pneumoniae* burden compared to that in the unexposed mice (see Fig. S4 in the supplemental material). Taken together, these experiments demonstrate that a decreased lung *K. pneumoniae* burden in our model requires both OVA sensitization and OVA airway challenge, does not develop in the absence of either of these phases of our protocol, and is not recapitulated by aerosol exposure to PBS.

Allergic airway inflammation decreases *K. pneumoniae*-induced lung expression of acute-phase cytokines and chemokines. We next investigated the influence of allergic airway inflammation on acute-phase cytokine and chemokine expression in the lung following *K. pneumoniae* infection. Numerous cytokines and chemokines have been implicated in the protective response to lung *K. pneumoniae* infection (6, 19, 29–40). At both day 1 and day 2 following infection, ALUM-KP mice had significantly increased lung expression of acute-phase cytokines (TNF-α, IL-6, IL-1β), chemokines (CCL2, CCL3, CCL4), G-CSF, and GM-CSF compared to both ALUM-PBS and OVA-PBS mice (Fig. 4A to H). Notably, OVA-KP mice had significantly decreased expression of each of these factors compared to ALUM-KP mice on both days analyzed. Thus, as with airway neutrophils and lung Th17 cytokine expression, the decreased lung *K. pneumoniae* burden associated with allergic airway inflammation correlates with decreases in the amounts of antibacterial cytokines, chemokines, and colony-stimulating factors.

Allergic airway inflammation decreases *K. pneumoniae*-induced lung LCN2 expression. Lipocalin 2 (LCN2) is produced by a variety of cell types, including mesenchymal and epithelial cells. Both IL-17A and IL-22 induce the lung expression of LCN2 (8, 24, 41), and LCN2-deficient mice have impaired lung clearance of *K. pneumoniae* (24). Therefore, we next determined the effect of allergic airway inflammation on the lung expression of LCN2. ALUM-KP mice had significantly increased LCN2 expression compared to ALUM-PBS and OVA-PBS mice on both day 1 and day 2 postinfection (Fig. 5). Similar to the results of prior experiments, OVA-KP mice had significantly decreased LCN2 expression compared to ALUM-KP mice on both days (Fig. 5). Thus, preexisting allergic airway inflammation decreased *K. pneumoniae*-induced lung LCN2 expression concurrently with the decreased lung *K. pneumoniae* burden.

*K. pneumoniae* infection alters the postinfection kinetics of preexisting allergic airway inflammation. Lung bacterial infection or exposure to bacterial products can enhance or impair allergic airway inflammation (42–45). We therefore determined the effect of *K. pneumoniae* infection on allergic airway inflammation. Airway eosinophils and lung IL-5 and IL-13 expression were not detected in ALUM-PBS or ALUM-KP mice on day 1 and day 2 postinfection, indicating that *K. pneumoniae* infection alone does not induce allergic airway inflammation (see Fig. S5A to C in the supplemental material). OVA-PBS mice had significantly increased numbers of airway eosinophils as well as lung IL-5 and IL-13 expression compared to alum-PBS mice (see Fig. S5A to C in the supplemental material). OVA-KP mice had significantly increased airway eosinophilia and a trend toward greater lung IL-5

FIG 3 The decreased lung *K. pneumoniae* burden requires both OVA sensitization and OVA aerosol challenge. The lung *K. pneumoniae* burden in mice that underwent the protocol described in Fig. 1 was determined (white bars, ALUM-KP group; black bars, OVA-KP group). In addition, the lung *K. pneumoniae* burden in ALUM-injected mice that underwent OVA challenge (bars with vertical lines) and OVA-ALUM-injected mice that did not undergo OVA aerosol challenge (bars with diagonal lines) was determined. *, *P < 0.001 versus all other day 1 groups; **, *P < 0.001 versus all other day 2 groups. n = 10 mice per group combined from two representative experiments.
and IL-13 expression compared to OVA-PBS mice on day 1 postinfection (see Fig. S5A to C in the supplemental material). In contrast, on day 2 postinfection, OVA-KP mice had significantly decreased lung IL-5 and IL-13 expression compared to OVA-PBS mice (see Fig. S5B and C in the supplemental material). No IL-4 was detectable in lung homogenates on day 1 or day 2 postinfection (data not shown), as expected on the basis of prior data (20, 21). Thus, *K. pneumoniae* infection in the context of preexisting allergic airway inflammation alters the postinfection kinetics of allergic airway inflammation. Allergic airway inflammation decreases *K. pneumoniae*-induced mortality following acute infection. Since allergic airway inflammation significantly decreased both the lung and spleen *Klebsiella* burden at 1 and 2 days postinfection, we next determined whether this decrease in lung *K. pneumoniae* burden was sufficient to decrease *K. pneumoniae*-induced mortality following acute infection.

**FIG 5** Allergic airway inflammation decreases *K. pneumoniae*-induced LCN2 expression in the lung on day 1 and day 2 postinfection. *, $P < 0.001$; **, $P < 0.01$. $n = 5$ mice per group.
Allergic airway inflammation enhances survival following acute *K. pneumoniae* lung infection. Mice that underwent the protocol described in Fig. 1 were infected with *K. pneumoniae* on day 0. Survival was determined twice daily for 14 days postinfection. *, *P* = 0.002 (log-rank test) and *P* = 0.004 (Wilcoxon test) versus ALUM-KP mice. n = 30 for ALUM-KP mice and n = 33 for OVA-KP mice. Data from two representative experiments were combined.

The decreased lung *K. pneumoniae* burden associated with allergic airway inflammation is independent of IL-4. IL-4, IL-5, and IL-13 are Th2 cytokines critical for the development of allergic airway inflammation in the OVA sensitization and challenge model (13). These cytokines contribute to both specific and overlapping effects during the initiation and maintenance of allergic airway inflammation. Because of the unexpected decrease in the lung *K. pneumoniae* burden associated with preexisting allergic airway inflammation, we next determined the contribution of each of these Th2 cytokines individually to the decreased lung *K. pneumoniae* burden phenotype. Understanding the Th2 cytokine(s) critical for this phenotype could suggest the mechanism by which allergic airway inflammation decreased the lung *K. pneumoniae* burden in our model.

IL-4 induces CD4*^+^* Th2 differentiation and IgE antibody class switching (13). In addition, IL-4 induces features of allergic airway inflammation that are also shared by other cytokines, including eosinophilia, goblet cell metaplasia and mucus expression, alternative macrophage activation, and airway remodeling (46). We therefore determined the role of IL-4 in decreasing the lung *K. pneumoniae* burden in our model. Wild-type (WT) and IL-4-KO mice underwent OVA sensitization and challenge and were infected with *K. pneumoniae*, as described in Fig. 1. As previously demonstrated, WT OVA-KP mice had a significantly decreased lung *K. pneumoniae* burden compared to WT ALUM-KP mice at both day 1 and day 2 postinfection (Fig. 7A). However, IL-4-KO OVA-KP mice also had a significantly decreased lung *K. pneumoniae* burden compared to both WT and IL-4-KO ALUM-KP mice on both day 1 and day 2 postinfection (Fig. 7A). IL-4-KO OVA-KP mice also had a significantly decreased lung *K. pneumoniae* burden compared to WT OVA-KP mice on day 1, but not day 2, postinfection. We previously showed that the decreased lung *K. pneumoniae* burden in WT OVA-KP mice occurred despite the significantly decreased number of airway neutrophils compared to that in WT ALUM-KP mice (Fig. 2). Similarly, IL-4-KO OVA-KP mice had significantly decreased numbers of airway neutrophils compared to IL-4-KO alum-*K. pneumoniae* mice, with the degree of neutrophil suppression in IL-4-KO OVA-KP mice being statistically similar to that seen in WT OVA-KP mice (Fig. 7B). As previously shown, WT OVA-KP mice had significantly increased numbers of airway eosinophils compared to WT ALUM-KP mice (Fig. 7C). Notably, IL-4-KO OVA-K mice had significantly decreased numbers of airway eosinophils compared to WT OVA-KP mice. Thus, the decrease in the lung *K. pneumoniae* burden and the numbers of airway neutrophils associated with allergic airway inflammation persisted despite both the absence of IL-4 and the significantly decreased numbers of airway eosinophils in IL-4-KO OVA-KP mice.

The decreased lung *K. pneumoniae* burden associated with allergic airway inflammation is independent of IL-5 and IL-5-induced airway eosinophils. IL-5 promotes the proliferation, migration, and survival of eosinophils during allergic airway inflammation (47). To determine whether IL-5 and IL-5-induced airway eosinophils were responsible for the decreased lung *K. pneumoniae* burden, we neutralized IL-5 during the course of our allergic airway inflammation-infection model using monoclonal anti-IL-5. OVA-KP mice treated with monoclonal antibody against IL-5 (anti-IL-5-OVA-KP mice) had a significantly decreased lung *K. pneumoniae* burden than ALUM-KP mice receiving IL-5 antibody (anti-IL-5-ALUM-KP mice) (Fig. 7D). This difference was similar in degree to that seen between ALUM-KP mice and OVA-KP mice treated with isotype control antibody (iso-ALUM-KP and iso-OVA-KP mice, respectively). No significant differences in KP burden were observed between iso-OVA-KP and anti-IL-5-OVA-KP mice (Fig. 7D). Anti-IL-5-OVA-KP mice had significantly decreased numbers of airway neutrophils compared to anti-IL-5-ALUM-KP mice (Fig. 7E). As expected, anti-IL-5-OVA-KP mice had significantly fewer airway eosinophils than iso-OVA-KP mice (Fig. 7F). Thus, the decreased lung *K. pneumoniae* burden associated with allergic airway inflammation is independent of IL-5 and IL-5-induced airway eosinophilia.

The decreased lung *K. pneumoniae* burden associated with allergic airway inflammation is partially dependent on IL-13. IL-13 plays a central role in inducing mucus hypersecretion in mouse models of allergic airway inflammation (13, 48). It is possible that the decreased pulmonary bacterial burden that we observed was due to a mucus-specific effect. Mice in which IL-13 signaling is blocked are significantly impaired in their development of goblet cell hyperplasia and mucus secretion in the OVA allergic airway inflammation model (48). Therefore, to determine whether IL-13-induced airway mucus expression or IL-13-specific inflammation was responsible for the decreased lung *K. pneumoniae* burden in OVA-KP mice, we performed our model in WT and IL-13-KO mice. IL-13-KO OVA-KP mice had a significantly decreased lung *K. pneumoniae* burden than IL-13-KO ALUM-KP mice on days 1 and 2 postinfection (Fig. 7G). As shown previously in WT and IL-4-KO mice, airway neutrophil recruitment was significantly decreased in IL-13-KO OVA-KP mice compared to IL-13-KO ALUM-KP mice (Fig. 7H). IL-13-KO OVA-KP mice also had significantly fewer airway eosinophils than WT OVA-KP mice (Fig. 7I). Notably, IL-13-KO OVA-KP mice had a greater lung *K. pneumoniae* burden on day 2 than WT OVA-KP mice, suggesting a partial dependence of the decreased lung *K. pneumoniae* burden phenotype on IL-13 (Fig. 7G).

Next we investigated airway mucus expression in WT and IL-13-KO mice in our model. Lung sections obtained from mice on day
2 postinfection were PAS stained and scored for airway mucus expression. WT OVA-PBS mice had significantly increased airway mucus expression compared to WT ALUM-PBS mice (see Fig. S6A and B in the supplemental material). WT ALUM-KP mice had detectable mucus expression, though it was significantly less than that found in WT OVA-KP mice. Both IL-13-KO ALUM-KP and IL-13-KO OVA-KP mice had significantly decreased amounts of airway mucus compared to WT OVA-PBS mice (see Fig. S6A and B in the supplemental material). IL-13-KO OVA-KP mice had significantly decreased amounts of airway mucus compared to WT OVA-KP mice (see Fig. S6A and B in the supplemental material). These findings confirm that the amount of airway mucus was decreased in IL-13-KO mice in our model. Thus, the decreased lung KP burden associated with allergic airway inflammation in our model was not dependent on IL-13 or IL-13-induced airway mucus.

The decreased lung _K. pneumoniae_ burden associated with allergic airway inflammation is partially dependent on STAT6. IL-4 and IL-13 signal through the type 1 IL-4 receptor (IL-4Rα/γc for IL-4) and the type II IL-4 receptor (IL-4Rα/IL-13Rα1 for IL-4 and IL-13) (49, 50). Cytokine engagement of either receptor results in the
phosphorylation and activation of STAT6 (51). We therefore determined the role of STAT6 in our model using STAT6-KO mice. STAT6-KO OVA-KP mice had a significantly decreased lung *K. pneumoniae* burden at days 1 and 2 postinfection compared to STAT6-KO ALUM-KP mice (Fig. 8A). The decrease in the lung *K. pneumoniae* burden between STAT6-KO OVA-KP mice and STAT6-KO ALUM-KP mice was similar to that seen between WT OVA-KP mice and WT ALUM-KP mice on day 1 postinfection. STAT6-KO OVA-KP mice had significantly decreased numbers of airway neutrophils compared to STAT6-KO ALUM-KP mice, as was previously shown for WT, IL-4-KO, IL-13-KO, and IL-5-neutralized mice (Fig. 8B). STAT6-KO OVA-KP mice had minimal to no airway eosinophils detected at day 1 and day 2 postinfection (Fig. 8C). Similar to the lung *K. pneumoniae* burden results for IL-13-KO mice, on day 2 postinfection STAT6-KO OVA-KP mice had a significantly greater lung *K. pneumoniae* burden than WT OVA-KP mice (Fig. 8A). Thus, the decreased lung *K. pneumoniae* burden associated with allergic airway inflammation was partially dependent on STAT6, similar to the partial dependence on IL-13.

The decreased lung *K. pneumoniae* burden associated with allergic airway inflammation occurs in the absence of IL-17A. In addition to the role of IL-17A in antibacterial immunity, there is an increasing appreciation of a role for IL-17A in the development and maintenance of asthma and allergic airway inflammation (52). Thus, it was possible that IL-17A expression during allergic airway inflammation in our model contributed to the decreased lung *K. pneumoniae* burden in OVA-KP mice. We therefore used IL-17A-KO mice to determine whether IL-17A was necessary for the decreased lung *K. pneumoniae* burden in OVA-KP mice. WT and IL-17A-KO mice underwent the protocol described in Fig. 1. Because of the reported role of IL-17A in lung antibacterial immunity, in this experiment WT and IL-17A-KO mice were infected with $10^3$ CFU *K. pneumoniae* rather than $10^4$ CFU *K. pneumoniae*, as in all other experiments. Both WT OVA-KP and IL-17A-KO OVA-KP mice had a decreased lung *K. pneumoniae* burden compared to their respective WT ALUM-KP and IL-17A-KO ALUM-KP controls (Fig. 9A). No difference in lung *K. pneumoniae* burden between IL-17A-KO ALUM-KP and WT ALUM-KP mice was noted (Fig. 9A). Also, there was a trend for decreased numbers of airway neutrophils in IL-17A-KO OVA-KP mice compared to IL-17A-KO ALUM-KP mice, though this difference was not statistically significant (Fig. 9B). No significant differences in total airway neutrophils ALUM-KP and IL-17A-KO ALUM-KP mice were present. Thus, the decreased lung *K. pneumoniae* burden associated with allergic airway inflammation was independent of IL-17A.

The decreased lung *K. pneumoniae* burden associated with allergic airway inflammation is neutrophil dependent. Although we demonstrated significantly reduced numbers of lung and airway neutrophils on days 1 and 2 following *K. pneumoniae* infection of mice with preexisting allergic airway inflammation, it remained possible that neutrophils were recruited to the lower airways following allergic airway inflammation but prior to *K. pneumoniae* infection. We therefore quantified both airway and blood neutrophils from mice that underwent the OVA sensitization and challenge (OVA mice) portion of our protocol and compared the amounts to those from mice that were only alum sensitized (alum mice). These mice were harvested on day 0 and were not *K. pneumoniae* or PBS infected. Notably, OVA mice had significantly increased numbers of airway and peripheral blood neutrophils compared to alum mice (Fig. 9C). No difference in blood eosinophils was noted between groups (Fig. 9C).

**Fig 8** Allergic airway inflammation decreases the lung *K. pneumoniae* burden in the absence of STAT6. WT and STAT6-KO mice underwent the protocol described in Fig. 1. (A) Lung *K. pneumoniae* burden; (B) numbers of airway neutrophils; (C) numbers of airway eosinophils. *, $P < 0.001$; **, $P < 0.01$; †, $P < 0.05$. $n = 9$ to 14 mice per group combined from two representative experiments.

**Fig 9** The decreased lung *K. pneumoniae* burden associated with allergic airway inflammation occurs in the absence of IL-17A. WT and IL-17A-KO mice underwent the protocol described in Fig. 1 and were infected with $10^3$ CFU *K. pneumoniae*. (A) Lung *K. pneumoniae* burden; (B) numbers of airway neutrophils from WT and IL-17A-KO mice. *, $P < 0.001$; **, $P < 0.01$. $n = 8$ to 17 mice per group combined from three representative experiments.
neutrophils compared with alum mice (Fig. 10A and B). Neutrophils possess a variety of effector functions by which bacterial killing is induced. S100a8, a calcium-binding protein that is a constituent of calprotectin, is highly expressed by neutrophils and contributes to host defense against K. pneumoniae (53). Myeloperoxidase contributes to neutrophil killing of pathogens by catalyzing hypochlorous acid production, which leads to direct bacterial killing, and is also critical for host defense against K. pneumoniae (54). We therefore measured S100a8 and myeloperoxidase expression in the lungs of OVA mice on day 0. OVA mice had significantly increased lung protein expression of both S100a8 and myeloperoxidase on day 0 compared to alum mice (Fig. 10C and D). We were not able to detect neutrophil myeloperoxidase activity (myeloperoxidase chlorination assay kit; Cayman Chemical) in fresh lung homogenates of either ALUM or OVA mice (data not shown).

The presence of neutrophils in the airways of OVA mice and the greater expression of S100a8 and MPO in the lungs of OVA mice than in the lungs of ALUM mice suggested that early recruitment of neutrophils to the lungs of OVA mice may be critical for the decreased lung K. pneumoniae burden that we found in OVA-KP mice compared to ALUM-KP mice. To determine the contribution of neutrophils to this phenotype, we used Ly6G antibody (clone 1A8) to deplete neutrophils in our model. Ly6G antibody has been well described to specifically deplete neutrophils (55) and to decrease neutrophil migration to sites of inflammation (56). We treated ALUM-KP and OVA-KP mice with either isotype control antibody (iso-ALUM-KP and iso-OVA-KP mice) or anti-Ly6G (anti-Ly6G-ALUM-KP and anti-Ly6G-OVA-KP mice) and measured peripheral blood neutrophils and airway neutrophils and the lung K. pneumoniae burden on day 2 postinfection. Given the previously described role of neutrophils in K. pneumoniae clearance from the lung, we infected each mouse with 10^3 CFU K. pneumoniae in these experiments.

To quantify neutrophil depletion in our model, we measured peripheral blood and airway neutrophils on day 2 post-K. pneumoniae infection. Anti-Ly6G-ALUM-KP and anti-Ly6G-OVA-KP mice had significantly decreased numbers of peripheral blood neutrophils compared to the respective isotype-treated ALUM-KP and OVA-KP mice (Fig. 10E). Anti-Ly6G treatment significantly decreased the numbers of airway neutrophils in anti-Ly6G-ALUM-KP mice compared to iso-ALUM-KP mice (Fig. 10F), and the degree of airway neutrophilia seen in anti-Ly6G-ALUM-KP mice was similar to that seen in both iso-OVA-KP and anti-Ly6G-OVA-KP mice. As anti-Ly6G treatment was effective in depleting neutrophils, we next quantified the lung K. pneumoniae burden. As before, iso-OVA-KP mice had a significantly decreased lung K. pneumoniae burden compared to iso-ALUM-KP mice (Fig. 10G). Both anti-Ly6G-ALUM-KP and anti-Ly6G-OVA-KP mice had a significantly increased lung K. pneumoniae burden compared to the respective iso-ALUM-KP and iso-OVA-KP groups (Fig. 10G). Notably, there was no difference in the lung K. pneumoniae burden between anti-Ly6G-OVA-KP.
mice and anti-Ly6G-ALUM-KP mice (Fig. 10G). Thus, antibody-mediated neutrophil depletion abrogated the ability of allergic airway inflammation to decrease the lung K. pneumoniae burden in our model.

CCL8 neutralization abrogates the decreased lung K. pneumoniae burden associated with allergic airway inflammation. To elucidate potential mechanisms for the decreased lung K. pneumoniae burden in OVA-KP mice, we compared the lung gene expression profiles between ALUM-KP and OVA-KP mice using an RNA-seq platform. As the decreased lung K. pneumoniae burden phenotype was partially dependent on STAT6, we used both WT and STAT6-KO mice in this experiment to focus on targets that were STAT6 independent. As described previously, WT and STAT6-KO mice were sensitized with either ALUM or OVA-ALUM, and OVA-ALUM-sensitized mice underwent subsequent OVA aerosol, giving four groups (WT ALUM, WT OVA, STAT6-KO ALUM, and STAT6-KO OVA mice). One set of each of these groups was harvested on day 0 prior to K. pneumoniae infection (0 h time point), and another set underwent lung K. pneumoniae infection and was harvested at 6 h postinfection. Total lung RNA was isolated (see the methods in the supplemental material), and whole-transcriptome sequencing and analysis were performed. Figure S7A in the supplemental material shows genes with significantly different expression levels (q value, ≤0.01; log2 fold change, greater than 1.5) between alumin and OVA mice (0 h) or ALUM-KP and OVA-KP mice (6 h) with the WT or STAT6-KO genotype. We identified 30 transcripts at the 0-h time point and 20 transcripts at the 6-h time point that were significantly increased in both WT OVA mice, compared to their levels of expression in WT ALUM mice, and STAT6-KO OVA mice, compared to their levels of expression in STAT6-KO ALUM mice (see Fig. S7B and Table S1 in the supplemental material).

One gene, Ccl8, had a 12- to 14-fold increased expression in WT OVA mice versus WT ALUM mice at 0 h and 6 h (see Fig. S6 and Table S1 in the supplemental material). Ccl8 encodes the CCL8 chemokine, a CC chemokine member of the monocYTE cheMOTACic protein family (57). As CCL8 has been implicated in bacterial immunity (58–60), we measured lung CCL8 protein expression in our model. Prior to K. pneumoniae infection (day 0), OVA mice had significantly increased lung CCL8 protein expression compared to ALUM mice (Fig. 11A). Similarly, OVA-PBS and OVA-KP mice had significantly greater lung CCL8 expression than ALUM-PBS and ALUM-KP mice, respectively (Fig. 11B). Of note, although ALUM-KP mice did not have increased CCL8 expression compared to ALUM-PBS mice, OVA-KP mice did have significantly increased lung CCL8 expression compared to OVA-PBS mice on day 2 postinfection (Fig. 11B). As our RNA-seq analysis suggested that increased lung CCL8 expression in OVA mice might occur in a STAT6-independent manner, we next measured CCL8 expression in both WT and STAT6-KO mice. While WT OVA and WT OVA-KP mice had significantly increased lung CCL8 expression at 0 h, 6 h, day 1, and day 2 in these experiments, STAT6-KO OVA and STAT6-KO OVA-KP mice had significantly decreased lung CCL8 expression compared to their respective WT controls at these time points (Fig. 11C and D). Notably, at the 0-h time point, there was a trend for increased CCL8 expression in the lungs of STAT6-KO OVA compared with the level of expression in STAT6-KO ALUM mice (Fig. 11C). Additionally, at day 1 postinfection, STAT6-KO OVA-KP mice had significantly increased CCL8 expression compared to STAT6-KO ALUM-KP mice (Fig. 11D). Thus, allergic airway inflammation induced by ovalbumin sensitization and challenge promotes lung CCL8 expression in a manner partially dependent on STAT6.

Because CCL8 expression was significantly increased in OVA and OVA-KP mice in our model, we next determined whether CCL8 was responsible for the decreased lung bacterial burden associated with allergic airway inflammation. We used intraperitoneal injection of monoclonal CCL8 antibody to neutralize CCL8 in our model. Notably, while iso-OVA-KP mice had a significantly decreased lung K. pneumoniae burden compared to iso-ALUM-KP mice, no significant difference in the lung K. pneumoniae burden was present between anti-CCL8-OVA-KP and anti-CCL8-ALUM-KP mice (Fig. 12A). Anti-CCL8-ALUM-KP mice had significantly increased numbers of airway neutrophils compared to both iso-ALUM-KP mice and anti-CCL8-OVA-KP mice (Fig. 12C). Airway neutrophils were not significantly different between anti-CCL8-OVA- and iso-OVA-KP mice (Fig. 12C). No differences in either airway eosinophils or airway macrophages were noted between isotype control- and anti-CCL8-injected mice (Fig. 12D and F). Thus, CCL8 neutralization abrogated the decreased lung K. pneumoniae burden associated with allergic airway inflammation.

DISCUSSION

K. pneumoniae is a Gram-negative pathogen that causes pneumonia and invasive disease in healthy and immunocompromised adults and children (1–4). The continued emergence and spread of multidrug-resistant strains of K. pneumoniae highlight the importance of developing innovative therapeutic approaches to these infections (61, 62). With this study, we demonstrate that preexisting allergic airway inflammation induced by OVA sensitization and challenge significantly reduces the lung K. pneumoniae burden and postinfection mortality in a mouse model of acute lung infection with K. pneumoniae. The reduced lung K. pneumoniae burden in this model occurred despite markedly impaired lung expression of IL-17A, IL-17F, and IL-22 on days 1 and 2 postinfection and was dependent on both neutrophils and CCL8. Although, to the best of our knowledge, there is no previously demonstrated relationship between K. pneumoniae and asthma or allergic airway inflammation in humans, our findings have important implications for developing novel mechanisms of orchestrating lung immunity against K. pneumoniae.

Our report is the first to determine the effect of allergic airway inflammation on lung K. pneumoniae infection. As asthma and allergic airway inflammation are generally thought to increase risk for invasive bacterial infection and pneumonia in humans (63–65), several groups have determined the effect of allergic airway inflammation induced by OVA sensitization and challenge in mouse models of lung bacterial infection (66–69). IL-4 and IL-13 expressed during OVA-induced allergic airway inflammation impaired the lung clearance of Mycoplasma pneumoniae by impairing lung IL-6 and Toll-like receptor 2 expression (67). OVA sensitization and challenge decreased the numbers of airway neutrophils, decreased lung IL-6 and IL-1β expression, and increased the Pseudomonas aeruginosa lung burden at 24 h postinfection (66). Two groups induced Streptococcus pneumoniae lung infection in the context of antecedent OVA sensitization and challenge (68, 69). In experiments with a serotype 4 strain of S. pneumoniae, OVA-induced allergic airway inflammation neither enhanced nor impaired lung bacterial clearance or postinfection mortality (69).
Alternately, using a bioluminescent serotype 3 S. pneumoniae strain, a different group showed that OVA-sensitized and -challenged mice had a decreased frequency of development of pneumonia and a decreased lung bacterial burden at the time of pneumonia onset compared to non-OVA-sensitized mice (68). Thus, OVA sensitization and challenge have different influences on lung bacterial infection depending on the model pathogen used.

Mouse models demonstrate that the clearance of K. pneumoniae from the lung and recovery of the host from acute infection are highly dependent on the Th17 cytokines IL-17A, IL-17F, and IL-22 (6, 8). The decreased lung K. pneumoniae burden and mortality, despite decreased lung Th17 cytokine expression, in our model strikingly contrast with the central role of each of these factors in lung K. pneumoniae clearance defined by prior publications (6, 8). We showed that the decreased lung K. pneumoniae burden in OVA-KP mice was independent of IL-17A. IL-17A is expressed in several models of allergic airway inflammation (52). The decreased lung K. pneumoniae burden associated with allergic airway inflammation in IL-17A-KO mice demonstrates that this phenotype was not due to IL-17A expression caused by OVA sensitization and challenge. Notably, IL-17A-KO ALUM-KP mice did not have decreased numbers of airway neutrophils or an increased lung K. pneumoniae burden compared to WT ALUM-KP mice. As IL-17A and IL-17F share a receptor and share many pro-inflammatory functions (10, 28), the lack of an effect of IL-17A’s absence on airway neutrophils and the lung K. pneumoniae burden may reflect compensation by IL-17F expression in our model. Increased IL-17F expression in IL-17A-KO mice compared to WT mice has been described in several models (70, 71). Future work using additional knockout mice will specifically address the dependence of the decreased lung K. pneumoniae burden in our model on other Th17 cytokines.

Neutrophils are critical for the clearance of a wide variety of bacterial pathogens (72). In the mouse model of lung K. pneumoniae infection, antibody-mediated depletion of neutrophils resulted in a significant increase in mortality from 20% at 14 days postinfection in isotype control-injected mice to 100% at 4 days postinfection in anti-Gr-1-injected mice (7). These differences in
mortality correlated to 2-log-unit and 3.5-log-unit increases in the lung and spleen K. pneumoniae burden, respectively, at day 2 postinfection. Although lung and airway neutrophilia was impaired in OVA-KP mice on days 1 and 2 postinfection in our model, we showed that neutrophils were present in the airways of OVA-sensitized and -challenged mice prior to K. pneumoniae infection. In addition to increased numbers of airway neutrophils, these mice also had increased lung expression of S100a8 and MPO. Using antibody-mediated neutrophil depletion, we supported the previously published role of neutrophils in lung K. pneumoniae clearance (7) and showed that the decreased lung bacterial burden associated with allergic airway inflammation was neutrophil dependent. This result suggests the possibility that preexisting allergic airway inflammation results in the localization of highly active neutrophils to the airways.

An additional notable feature of our model was the marked reduction in lung inflammation in OVA-KP mice at days 1 and 2 postinfection, as quantified by neutrophil detection and lung cytokine expression. As noted above, the numbers of airway and lung neutrophils and the lung expression of Th17 cytokines were markedly decreased in OVA-KP mice compared to ALUM-KP mice (Fig. 1 and 2). In addition, the lung expression of numerous proinflammatory cytokines (IL-1β, TNF-α, IL-6), chemokines (CXCL1, CCL3, CCL4), and colony-stimulating factors (G-CSF, GM-CSF) was decreased in OVA-KP compared with that in ALUM-KP mice (Fig. 4). This global reduction in K. pneumoniae-induced lung inflammation suggests either that allergic airway inflammation broadly suppresses lung inflammation after exposure to K. pneumoniae or that allergic airway inflammation prevents the ability of K. pneumoniae to activate proinflammatory pathways. Prevention of proinflammatory pathway expression by K. pneumoniae in OVA-KP mice could be mediated by the phagocytosis and the clearance of K. pneumoniae at early time points postinfection or by expression of an epithelial factor that prevents accessibility of K. pneumoniae to the innate immune system.

Allergic airway inflammation is associated with numerous changes to the lung immune environment that could plausibly have resulted in a decreased lung K. pneumoniae burden in our model. Defining features of the allergic airway inflammation phenotype include goblet cell metaplasia and mucus hypersecretion,
airway eosinophilia, and alternative macrophage activation (13, 73). Airway mucus and mucociliary clearance are critical factors in the innate immune response to bacteria in the lung (74). Eosinophils are demonstrated to possess antibacterial activity through the release of granule proteins and extracellular DNA (75, 76). Matrix metalloproteinase 12, which can be expressed by alternatively activated macrophages, mediates direct antibacterial effects (77, 78). The Th2 cytokines IL-4, IL-5, and IL-13 and the transcription factor STAT6 are critical mediators of allergic sensitization, airway mucus expression, airway eosinophilia, and alternative macrophage activation. In the absence of each of these cytokines and STAT6 individually, the allergic airway inflammation induced by OVA sensitization and challenge significantly decreased the lung K. pneumoniae burden and the numbers of airway neutrophils compared to those in nonallergic mice. This phenotype occurred despite the significantly decreased numbers of airway eosinophils in the absence of each cytokine. Further, as confirmed with PAS staining, a decreased lung K. pneumoniae burden occurred in IL-13-KO mice with preexisting allergic airway inflammation, despite significantly attenuated airway mucus expression. Thus, allergic airway inflammation decreased the lung K. pneumoniae burden in the absence of IL-4, IL-5, IL-13, and STAT6 individually, suggesting that many of the phenotypic features of allergic airway inflammation are not responsible for the decreased lung K. pneumoniae burden in this model.

Of note, while the decreased lung K. pneumoniae burden associated with allergic airway inflammation persisted in IL-13-KO and STAT6-KO mice, our results suggest that the decrease in lung K. pneumoniae burden may be partially dependent on IL-13 and STAT6. IL-13-KO OVA-KP and STAT6-KO OVA-KP mice had decreased lung K. pneumoniae burdens compared to their respective WT ALUM-KP controls on days 1 and 2 postinfection. However, both groups (IL-13-KO OVA-KP and STAT6-KO OVA-KP mice) also had significantly greater lung K. pneumoniae burdens than their respective WT OVA-KP comparator groups on day 2 (Fig. 7G and 8A). Thus, the decreased lung K. pneumoniae burden associated with allergic airway inflammation may be partially dependent on IL-13 and STAT6.

While STAT6 is critical for the development of allergic airway inflammation, investigators have shown that airway inflammation does develop in STAT6-KO mice that undergo OVA sensitization and challenge (79–81). In a prior study, microarray analysis of lung tissue from WT and STAT6-KO mice that underwent OVA sensitization and challenge in a STAT6-independent manner (81). Several of these STAT6-independent transcripts included both CCL and CXCL family chemokines (82). We used WT and STAT6-KO mice in our model to perform lung transcriptome sequencing. This analysis identified increased Ccl8 expression in the lungs of both WT and STAT6-KO OVA-sensitized and challenged mice compared to the lungs of their respective alum controls. We also measured lung CCL8 protein expression in our model and showed that allergic airway inflammation increased lung CCL8 expression in a manner partially dependent on STAT6. Two prior publications have demonstrated CCL8 lung expression in the OVA model of allergic airway inflammation (82, 83). In one study, measurement of Ccl8 transcript expression by Northern blotting suggested that Ccl8 expression was STAT6 independent, but CCL8 lung protein expression was not measured (82). A specific role for CCL8 in allergic airway inflammation has not been defined.

We demonstrate a novel role for CCL8 in decreasing the lung K. pneumoniae burden in our model. CCL8 is a member of the monocyte chemotactic protein family of CC chemokines and promotes the chemotaxis of several cell types (84). Recently, CCL8 was shown to contribute to antibacterial resistance against intravenous infection with Listeria monocytogenes, a Gram-positive facultatively intracellular pathogen (59). Intravenous CCL8 administration increased peripheral blood neutrophilia and decreased the spleen and liver L. monocytogenes burden in a manner dependent on γδ T cells. While a direct role for neutrophils in mediating the effect of CCL8 in this model was not shown, CCL8 contributed to recruitment of γδ T cells that preferentially expressed IL-17F (59). Our study extends these findings by demonstrating that CCL8 is expressed in the lungs during allergic airway inflammation and contributes to lung antibacterial immunity against a Gram-negative extracellular bacterial pathogen. While RNA-seq data suggested that lung Ccl8 expression is STAT6 independent, measurement of lung CCL8 protein expression showed that CCL8 protein expression is partially STAT6 dependent. Thus, it is possible that CCL8 accounts for the lung K. pneumoniae clearance that was partially STAT6 dependent in our model.

The dependence of the decreased lung K. pneumoniae burden in our model on both neutrophils and CCL8 suggests a possible direct link between these two factors in enhancing lung antibacterial immunity against K. pneumoniae. In the previously noted study, CCL8 was not chemotactic for neutrophils (59). In contrast, in a prior study assessing the chemotaxis of human neutrophils, CCL8 synergistically enhanced neutrophil chemotaxis in the setting of low doses of IL-8 (85). Using a model of human γδ T cell–monocyte coculture to study the effect of γδ T cell activation on granulocyte function, another group showed that CCL8 promotes neutrophil degranulation (86). Future studies will assess the ability of CCL8 to promote neutrophil recruitment, degranulation, and phagocytosis.

Two possible mechanisms for the decreased lung K. pneumoniae burden in our model merit further discussion. First, mast cells are innate immune cells that have been shown to have beneficial effects in the clearance of bacteria and other pathogens from numerous sites of infection (87). Mast cells contributed to the clearance of K. pneumoniae from sites of infection, including the peritoneal fluid (29, 88) and the lungs (89). Additionally, mast cell tryptases promote K. pneumoniae clearance, possibly through enhanced neutrophil phagocytosis (90, 91). As mast cell progenitors were recruited to the lungs of OVA-sensitized and -challenged mice in a STAT6-independent manner (92), it is possible that mast cells play a role in the decreased lung K. pneumoniae burden in OVA-KP mice in our model. Second, leukotrienes are immunomodulatory arachidonic acid products that play a critical role in the regulation of allergic airway inflammation (93). Both 5-lipoxygenase, a catalytic enzyme in leukotriene synthesis, and leukotriene B4 were critical for the development of allergic airway inflammation (94, 95). As 5-lipoxygenase and leukotriene B4 enhance the clearance of K. pneumoniae from the lung through effects on neutrophils and alveolar macrophages (96–99), it is possible that leukotrienes contribute to the decreased lung K. pneumoniae burden in OVA-KP mice in our model. Importantly, no prior work has specifically addressed the role of allergic airway inflammation induced by OVA sensitization and challenge in pro-
moting lung bacterial immunity through effects on either mast cells or leukotrienes.

In summary, we demonstrate that preexisting allergic airway inflammation decreases the lung bacterial burden in a model of acute bacterial pneumonia due to K. pneumoniae. The decreased lung K. pneumoniae burden in this model persisted in IL-4-KO, IL-13-KO, STAT6-KO, IL-17A-KO, and IL-5-neutralized mice and was dependent on neutrophils and CCL8. Further investigation into the mechanism of the decreased lung K. pneumoniae burden associated with OVA sensitization and challenge in our model may provide intriguing insights into novel mechanisms of orchestrating lung immunity against K. pneumoniae.

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