



## ARTICLE

Lung CD4<sup>+</sup> resident memory T cells remodel epithelial responses to accelerate neutrophil recruitment during pneumoniaAnukul T. Shenoy<sup>1</sup>, Gregory A. Wasserman<sup>1,2</sup>, Emad I. Arafa<sup>1,3</sup>, Alicia K. Wooten<sup>1,3</sup>, Nicole M. S. Smith<sup>1,4</sup>, Ian M. C. Martin<sup>1</sup>, Matthew R. Jones<sup>1,3</sup>, Lee J. Quinton<sup>1,2,3,4</sup> and Joseph P. Mizgerd<sup>1,2,3,5</sup>

Previous pneumococcal experience establishes lung-resident IL-17A-producing CD4<sup>+</sup> memory T<sub>RM</sub> cells that accelerate neutrophil recruitment against heterotypic pneumococci. Herein, we unravel a novel crosstalk between CD4<sup>+</sup> T<sub>RM</sub> cells and lung epithelial cells underlying this protective immunity. Depletion of CD4<sup>+</sup> cells in pneumococcus-experienced mice diminished CXCL5 (but not CXCL1 or CXCL2) and downstream neutrophil accumulation in the lungs. Epithelial cells from experienced lungs exhibited elevated mRNA for CXCL5 but not other epithelial products such as GM-CSF or CCL20, suggesting a skewing by CD4<sup>+</sup> T<sub>RM</sub> cells. Genome-wide expression analyses revealed a significant remodeling of the epithelial transcriptome of infected lungs due to infection history, ~80% of which was CD4<sup>+</sup> cell-dependent. The CD4<sup>+</sup> T<sub>RM</sub> cell product IL-17A stabilized CXCL5 but not GM-CSF or CCL20 mRNA in cultured lung epithelial cells, implicating posttranscriptional regulation as a mechanism for altered epithelial responses. These results suggest that epithelial cells in experienced lungs are effectively different, owing to their communication with T<sub>RM</sub> cells. Our study highlights the role of tissue-resident adaptive immune cells in fine-tuning epithelial functions to hasten innate immune responses and optimize defense in experienced lungs, a concept that may apply broadly to mucosal immunology.

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## INTRODUCTION

Acute lower respiratory tract infections represent a great burden of disease in the United States and worldwide.<sup>1–3</sup> The Gram-positive pathobiont *Streptococcus pneumoniae* (pneumococcus) is the most common bacterial cause of community-acquired pneumonia.<sup>4–9</sup> Conjugate vaccines against pneumococcus elicit serotype-specific antibodies that are effective in preventing pneumonia from specified serotypes.<sup>10</sup> Despite aggressive and successful vaccination campaigns targeting the 7–13 most concerning of the >97 identified serotypes, these vaccines provide no protection against most pneumococci and serious infections with non-vaccine serotypes are rising in multiple communities.<sup>11</sup> The limitations of the current pneumococcal vaccines emphasize a requirement for more broadly effective immune protection against pneumococcus.

Susceptibility to pneumonia is highest during early childhood and late adulthood.<sup>12</sup> In the intervening period, a multifactorial naturally acquired immunity protects the healthy older child or young adult from most or all respiratory microbes, including pneumococci. This immunity results from recurrent sub-lethal infections from the microbes most responsible for severe pneumonia, improving defense through a combination of training

for innate immunity cells and memory for adaptive immunity cells.<sup>1</sup>

In addition to systemic immunity changes, exposure to inspired microbes results in significant remodeling of local lung immunity as well.<sup>13–15</sup> Diverse pathogens have been demonstrated to induce tissue resident memory T<sub>RM</sub> cells in multiple tissues and a hallmark of T<sub>RM</sub>-mediated protection is accelerated immune responses.<sup>16–20</sup> This applies to pneumococcal pneumonia, as resolution of prior respiratory infections seeds the lungs with pneumococcus-responsive CD4<sup>+</sup> T<sub>RM</sub> cells and CD4<sup>+</sup> cell depletion exacerbates infection in such mice.<sup>16</sup> The pneumococcus-recovered lungs have accelerated neutrophil recruitment and bacterial clearance during subsequent heterotypic infections with pneumococcus,<sup>16</sup> but mechanisms mediating the accelerated neutrophil recruitment are unknown. Respiratory infection by an adenovirus has recently been demonstrated to remodel alveolar macrophages (AMs) so that they accelerate neutrophil recruitment and subsequent bacterial clearance in the lungs, all of which is dependent on CD8<sup>+</sup> T cells.<sup>13</sup> Such remodeling of AMs to accelerate neutrophil recruitment could be involved in improved defenses after pneumococcal infections, although neither CD8<sup>+</sup> T<sub>RM</sub> cells nor trained AMs have been described in this setting. In

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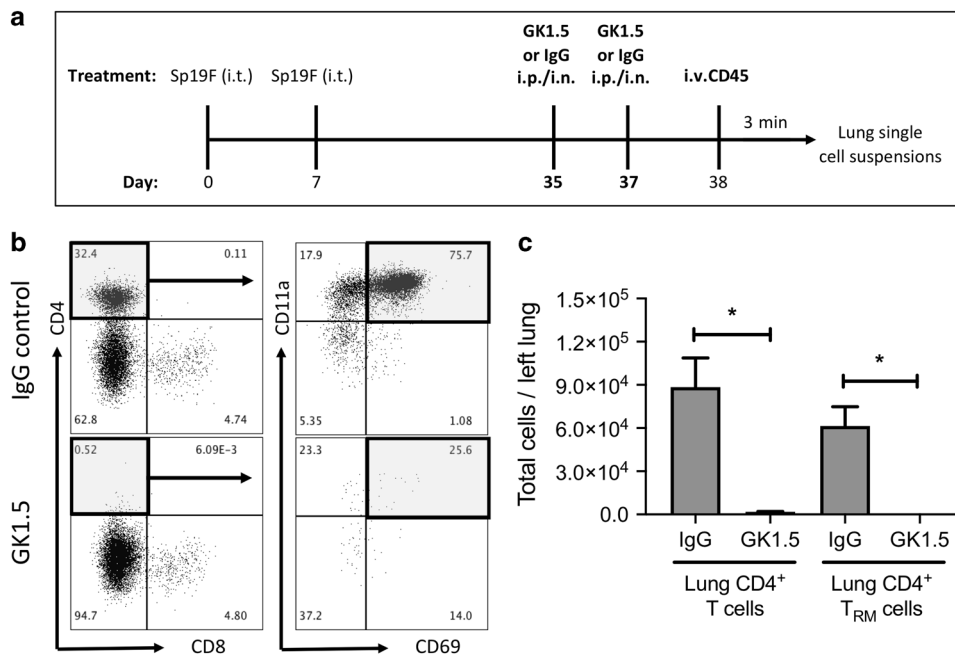
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**Fig. 1** Strategy for depletion of lung-resident CD4<sup>+</sup> T cells. **a** Het. Imm. mice were treated with GK1.5 or IgG intraperitoneally and intranasally on days 35 and 37. On day 38, mice were intravenously administered anti-CD45 antibody 3 min prior to killing, to exclude circulating leukocytes and identify lung-resident CD4<sup>+</sup> T cells by flow cytometry. **b** Representative dot plots gated on extravascular CD45<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup> T cells and on extravascular CD45<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup> T cells isolated from IgG control-treated or GK1.5-treated heterotypic immune mice. **c** Total lung CD4<sup>+</sup> T-cell numbers (identified as extravascular CD45<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup> cells) and lung CD4<sup>+</sup> T<sub>RM</sub> cells numbers (identified as extravascular CD45<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup> CD69<sup>+</sup>CD11a<sup>high</sup> cells) in the left lungs of IgG or GK1.5-treated heterotypic immune mice. All extravascular CD45<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup> CD69<sup>+</sup>CD11a<sup>high</sup> cells were CD62L<sup>low</sup>CD44<sup>high</sup> (data not shown). Two independent experiments with  $n \geq 7$  total mice. Statistical analyses: Student's *t*-test. \**p*-value  $\leq 0.05$ .

dition, resolution of influenza infection seeds the lungs with B<sub>RM</sub> cells,<sup>21</sup> but lung B<sub>RM</sub> cells have not been defined after bacterial infections and whether and how this population of resident memory cells may accelerate subsequent pathways of lung immune defenses are unknown. We aimed to define mechanistic steps in the improved lung defense resulting from prior pneumococcal infections. In particular, we hypothesized that CD4<sup>+</sup> T<sub>RM</sub> cells are ultimately responsible for accelerating neutrophil recruitment<sup>22–24</sup> and we aimed to define the cells and molecules mediating this pivotal immune response.

## RESULTS

### CD4<sup>+</sup> T cells amplify CXCL5 and neutrophil accumulation in lungs with heterotypic immunity

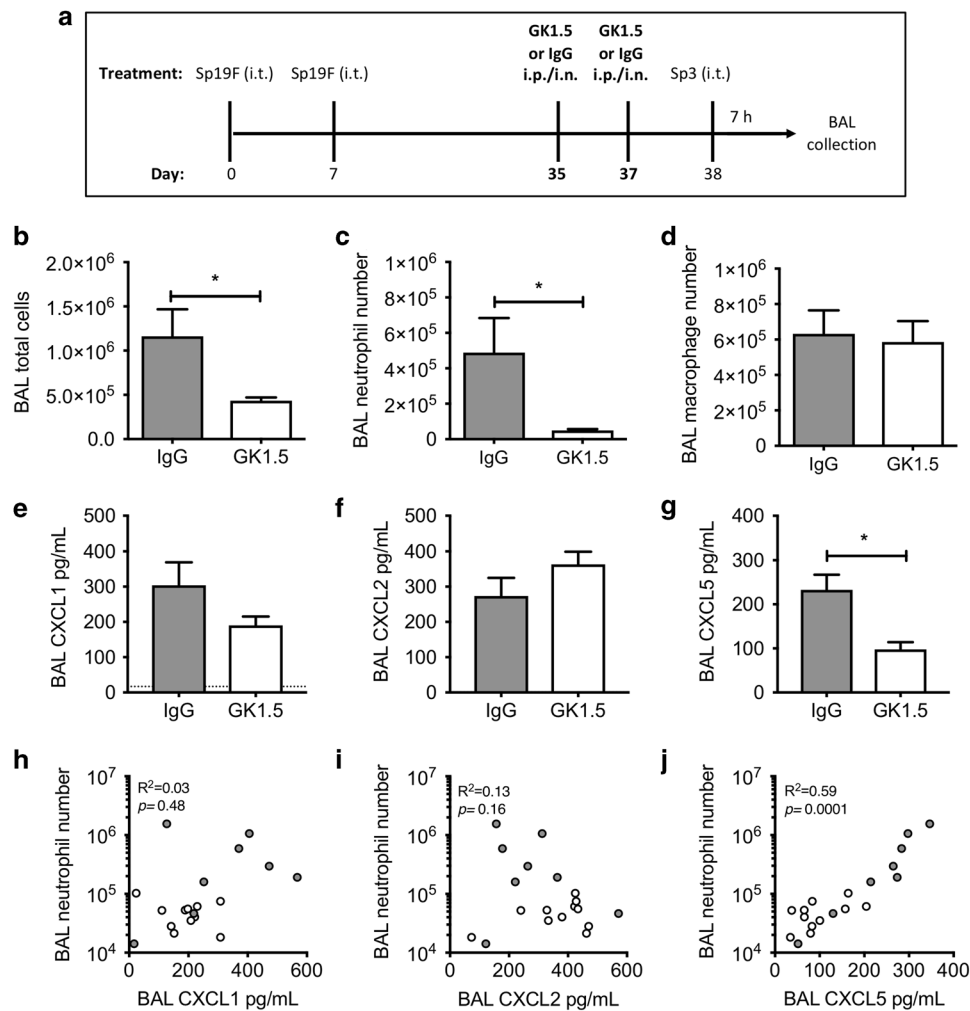
To model naturally acquired adaptive immunity,<sup>1,16</sup> C57BL/6 mice were infected twice with a non-lethal strain of pneumococcus (serotype 19F, Sp19F). After 4 weeks to allow for the full resolution of infection and inflammation, mice were challenged in the same manner with a lethal serotype 3 strain (Sp3). To determine whether the CD4<sup>+</sup> T<sub>RM</sub> cells established in these lungs after prior infection<sup>16</sup> are critical for orchestrating this rapid neutrophil infiltration, we devised a strategy to deplete CD4<sup>+</sup> cells from these mice using the GK1.5 monoclonal antibody (Fig. 1a). Intravascular administration of anti-CD45 antibody was used to exclude circulating leukocytes and identify lung-resident immune cells. Our strategy depleted ~98% of all extravascular CD4<sup>+</sup> T cells (Fig. 1b, c) and ~99.5% of all lung-resident CD4<sup>+</sup> CD69<sup>+</sup>CD11a<sup>high</sup> T<sub>RM</sub> cells (Fig. 1b, c).

We quantified the recruitment of leukocytes in the bronchoalveolar lavage (BAL) fluid 7 h post Sp3 infection when neutrophil recruitment is increased due to heterotypic immunity<sup>16</sup> within the GK1.5- and control IgG-treated heterotypic immune mice (Fig. 2a). GK1.5 treatment in heterotypic immune mice significantly reduced

the total cellularity (Fig. 2b) and neutrophil numbers (Fig. 2c), but did not affect macrophage numbers (Fig. 2d) in the airspaces of heterotypic immune mice. To determine whether and which neutrophil chemo-attractants may be altered by the CD4<sup>+</sup> cell depletion, we quantified ELR<sup>+</sup>-CXC chemokines in the BAL fluids collected 7 h post Sp3 infection. GK1.5 treatment of heterotypic immune mice prior to Sp3 challenge did not alter the airway levels of CXCL1 (Fig. 2e) or CXCL2 (Fig. 2f). However, depletion of CD4<sup>+</sup> cells significantly diminished CXCL5 levels in the airways of heterotypic immune mice (Fig. 2g). These data suggest that lung CD4<sup>+</sup> T<sub>RM</sub> cells in heterotypic immune mice are responsible for the rapid accumulation of CXCL5 and neutrophils in the airways.

### CXCL5 drives neutrophil recruitment in lungs with heterotypic immunity

Mechanisms mediating the accelerated neutrophil recruitment in lungs with heterotypic immunity have not been identified. Correlation analyses of neutrophil numbers with chemokine levels revealed a strong positive correlation of neutrophil infiltration with airway CXCL5 but not with either CXCL1 or CXCL2 (Fig. 2h–j). Thus, CXCL5 may have specific roles in context of heterotypic immunity. To determine whether the elevated airway levels of CXCL5 downstream of CD4<sup>+</sup> cells is required for the enhanced neutrophil recruitment in this setting, we neutralized CXCL5 in the airways of mice using anti-CXCL5 antibody (Fig. 3a). Near-complete neutralization of CXCL5 was achieved by intratracheal administration of the antibody (Fig. 3b). Administration of anti-CXCL5 antibody had minimal effects on macrophage numbers (Fig. 3c) but significantly decreased neutrophil recruitment (Fig. 3d) to the airways. We tested whether CXCL5 addition was sufficient to increase neutrophil recruitment in mice with heterotypic immunity but without CD4<sup>+</sup> cells (depleted by GK1.5). Exogenous CXCL5 significantly increased neutrophil recruitment (Fig. 3e), confirming that airway CXCL5 levels was present in limiting



**Fig. 2** Depletion of CD4<sup>+</sup> cells from heterotypic immune mice diminishes CXCL5 and neutrophil accumulation in the lungs. **a** Het. Imm. mice were treated with CD4 depleting GK1.5 or control antibody 72 h and 24 h before the final Sp3 challenge. BAL from CD4-depleted vs. control-treated het imm mice were collected for determining cellularity and cytokine levels 7 h post Sp3 challenge. **b** Total cellularity, **c** macrophage, and **d** neutrophil numbers in the BALs of GK1.5-treated vs. control IgG-treated het imm. mice 7 h post Sp3 challenge. **e** CXCL1, **f** CXCL2, and **g** CXCL5 levels in the BALs of GK1.5-treated vs. control IgG-treated het imm. mice 7 h post Sp3 challenge. **h–j** Correlation analyses of BAL CXCL1, CXCL2, and CXCL5 levels with neutrophil numbers from the GK1.5-treated (blank dots) vs. control IgG-treated (filled dots) het imm. mice 7 h post Sp3 challenge. Two independent experiments with  $n \geq 3$  mice each. Statistical analyses: Student’s *t*-test. \**p*-value  $\leq 0.05$ .

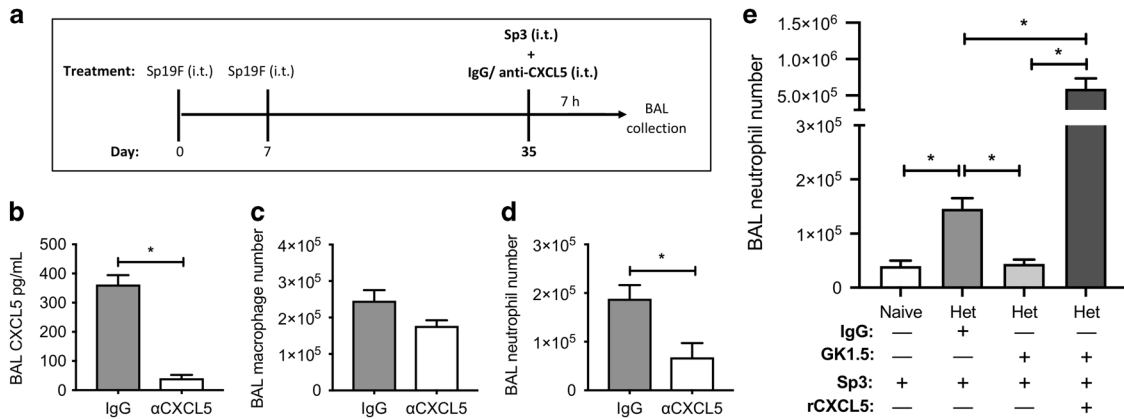
concentrations and directly influenced neutrophil recruitment in these lungs. Collectively, our observations demonstrate that CXCL5 is downstream of CD4<sup>+</sup> T cells and upstream of neutrophil recruitment in lungs with heterotypic immunity.

#### CD4<sup>+</sup> T cells remodel lung epithelial cell responses during heterotypic immunity

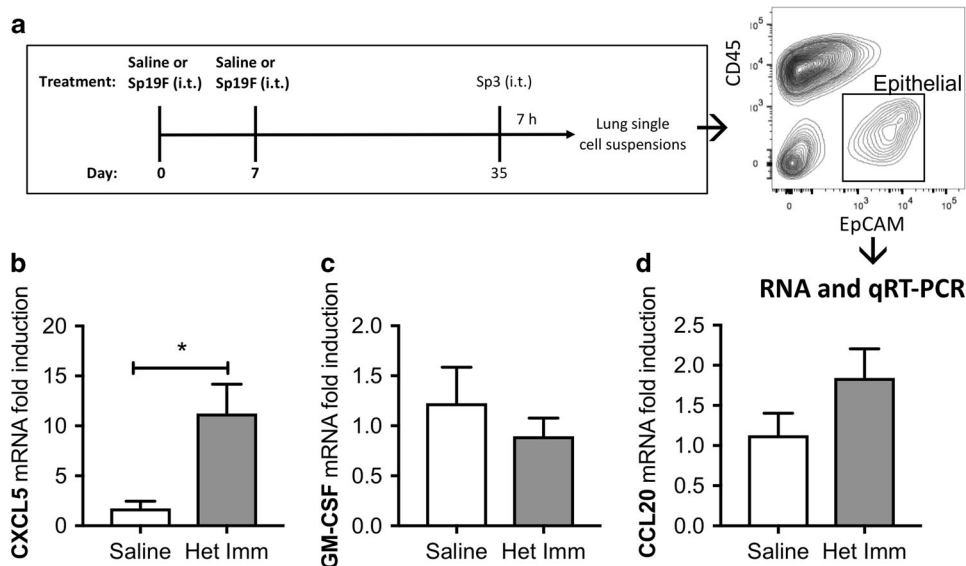
CXCL5 is typically produced by epithelial cells in the infected lung, whereas CXCL1 and CXCL2 are derived from other cell sources.<sup>16,25</sup> Thus, CD4<sup>+</sup> T-cell-dependent increases in CXCL5 during heterotypic immunity implicate the epithelium as a potential intermediate in neutrophil recruitment in these lungs. We isolated epithelial (CD45<sup>-</sup>EpCAM<sup>+</sup>) cells from infected lungs that were naive or experienced by fluorescence-activated cell sorting (FACS) and compared cytokine induction (Fig. 4a). Demonstrating that epithelial cells induce greater CXCL5 expression due to heterotypic immunity, CXCL5 mRNA was significantly increased in lung epithelial cells from mice that had fully recovered from prior pneumococcal infections (Fig. 4b). To determine whether this enhanced CXCL5 response was due to globally exaggerated responses by lung epithelial cells during heterotypic recall infections, we examined expression levels of other cytokines

produced selectively by epithelial cells during pneumococcal pneumonia.<sup>25</sup> Unlike CXCL5, epithelial cells from naive and experienced mice expressed comparable levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) and CCL20 mRNA (Fig. 4c, d). The fact that neither epithelial GM-CSF nor CCL20 were changed in experienced lungs meant that this CXCL5 response is not a uniform hyper-response, but rather a skewing or selective change in epithelial responses due to heterotypic immunity. The data thus reveal that the lung epithelium remodels in a discrete and non-uniform manner due to heterotypic immunity.

To achieve a more comprehensive view of epithelial remodeling and to identify the extent to which this event is CD4<sup>+</sup> T-cell-dependent, we performed transcriptomic analyses on sorted CD45<sup>-</sup>EpCAM<sup>+</sup> cells from lungs of heterotypic immune or naive mice treated with GK1.5 or control IgG (Fig. 5a). All mice had 7 h of Sp3 pneumonia in these analyses (Fig. 5a). Sp3 pneumonia profoundly changes the lung epithelial transcriptome,<sup>26</sup> but that was not the specific focus of the present study. Instead, we aimed to determine whether prior experience with unrelated serotypes altered the epithelial transcriptome during pneumonia and whether this depended on CD4<sup>+</sup> T cells. Genome-wide expression profiling revealed that a subset of the epithelial cell transcriptome



**Fig. 3** CXCL5 neutralization inhibits neutrophil recruitment to heterotypic immune murine lungs post Sp3 challenge. **a** CXCL5 was depleted using anti-CXCL5 antibody treatment simultaneous to Sp3 challenge. **b** BAL CXCL5 levels from CXCL5-depleted vs. control-treated het imm. mice 7 h post Sp3 challenge. **c** Total macrophage and **d** neutrophil numbers in the CXCL5-depleted vs. control-treated het imm. mice 7 h post Sp3 challenge. Two independent experiments with  $n \geq 3$  mice each. **e** Total BAL neutrophil numbers in naive, IgG-, or GK1.5-treated Het. Imm. mice 7 h post Sp3 challenge. One group of GK1.5-treated het. imm. mice received 500 ng recombinant CXCL5 (rCXCL5) simultaneous to Sp3 challenge. Two independent experiments with  $n \geq 6$  total mice. Statistical analyses: Student's *t*-test. \* $p$ -value  $\leq 0.05$ .

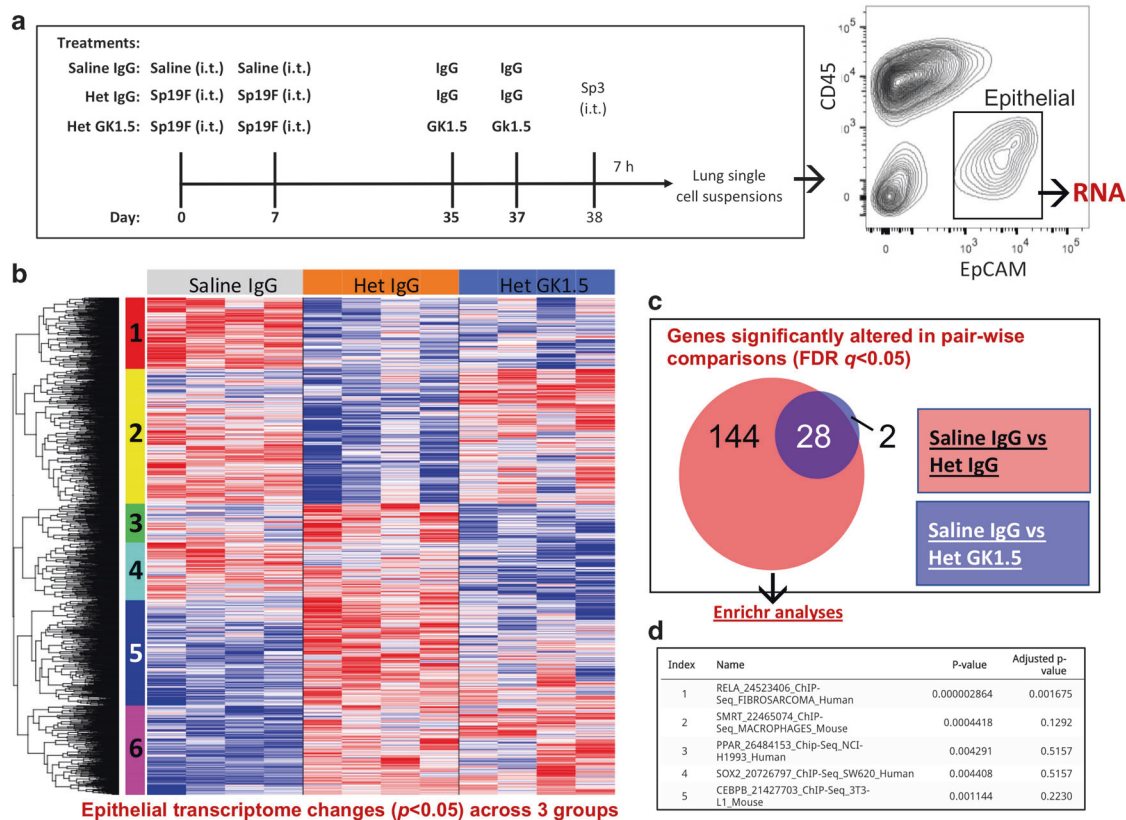


**Fig. 4** Heterotypic immunity increases lung epithelial induction of CXCL5 during infection. **a** CD45<sup>+</sup>EpCAM<sup>+</sup> epithelial cells from heterotypic immune- or saline-treated murine lungs were flow sorted 7 h post Sp3 challenge. **b** CXCL5, **c** GM-CSF, and **d** CCL20 expression in flow-sorted lung epithelial cells of het. imm. vs. naive mice 7 h post Sp3 challenge was determined by RT-PCR. Two independent experiments with  $n \geq 3$  mice each. Statistical analyses: Student's *t*-test. \* $p$ -value  $\leq 0.05$ .

in pneumonic lungs was altered due to heterotypic immunity and/or CD4<sup>+</sup> cell depletion (Fig. 5b). Unsupervised clustering of genes differing at the significance level of  $p < 0.05$  revealed six modules of genes that changed in expression levels in at least one of the three tested conditions. Genes within modules 1, 2, 5, and 6 were altered by heterotypic immunity. Genes within modules 2, 3, 4, and 5 were altered by CD4<sup>+</sup> cell depletion in the lungs with heterotypic immunity compared with IgG control-treated experienced lungs. Modules 2 and 5 reflect changes in epithelial cells from pneumonic lungs that were caused both by heterotypic immunity and dependent on CD4<sup>+</sup> cells. CXCL5 was included in module 5, increased by heterotypic immunity, and dependent on CD4<sup>+</sup> cells. None of the other genes measured previously (CXCL1, CXCL2, GM-CSF, CCL20) differed across groups in these transcriptome analyses. These data demonstrate that the lung epithelium is transcriptionally remodeled by heterotypic immunity and this is partially dependent on the presence of CD4<sup>+</sup> cells. This, to our knowledge, is the first report of previous microbial experience and

tissue-resident CD4<sup>+</sup> cells remodeling the lung epithelial immune response in conjunction with improved host response against infection.

To identify individual genes that were specifically altered during heterotypic immunity in epithelial cells from pneumonic lungs, we performed pairwise comparisons of the saline vs. Het IgG groups. A total of 144 transcripts were differentially expressed at an false discovery rate (FDR)  $q < 0.05$  in experienced lung epithelial cells (Fig. 5c and Supplementary Table 1). Notable among the genes increased in lungs with prior experience were chemokines including CXCL5, CXCL9, CXCL11, CCL2, CCL7, and CCL11, suggesting neutrophils are not the only cells with bolstered recruitment. Other extracellular products with inflammatory or antimicrobial activities included Sectm1a, Sectm1b, complement C2, plgR, S100A9, and Pglyrp1, potentially enhancing immune resistance. Intracellular enzymes with known defense and inflammatory roles, such as Oas1a, Oas1g, Oas2, Oas3, caspase 4, and SOCS1, were also more highly expressed in experienced lung



**Fig. 5** Lung CD4<sup>+</sup> T<sub>RM</sub> cells remodel epithelial responses during infection. **a** CD45<sup>-</sup>EpCAM<sup>+</sup> epithelial cells from het. imm. mice pretreated with GK1.5 or control antibody 72 h and 24 h before the final Sp3 challenge were flow sorted 7 h post Sp3 challenge for microarray analyses.  $n = 4$  samples per condition were sorted for analyses. **b** Unsupervised heat map representing distinct modules of differentially expressed genes across the tested conditions is depicted (FDR  $p < 0.05$ ). **c** Venn diagram representing the number of differentially expressed genes across tested conditions as determined by pairwise comparison (FDR  $q < 0.05$ ). **d** Results of Enrichr analyses of the differentially enriched transcriptional pathways (FDR  $q < 0.05$ ) within the upregulated genes from the CD4<sup>+</sup> dependent epithelial gene signature is depicted.

epithelial cells, suggesting refinement of cellular responses. Finally, the Serpin superfamily of anti-proteases including Serpina3f, Serpina3g, Serpina3n, and Serping1 were upregulated during recall responses, suggesting enhanced tissue resilience pathways.

Pairwise comparison of the saline IgG vs. Het GK1.5 groups revealed that, in the absence of CD4<sup>+</sup> cells, only 30 genes were significantly altered by prior experience (Fig. 5c). Most of these (28 of 30) were within the larger set of 144 genes that were identified as modified by prior experience (Fig. 5c). These results suggest that ~80% of the gene changes caused by heterotypic immunity in pneumonic lung epithelial cells depended on CD4<sup>+</sup> T cells. CXCL5 was among the transcripts increased by heterotypic immunity only if CD4<sup>+</sup> cells were present, corroborating and expanding upon results from independent experiments (Figs. 1 and 3). Taken together, heterotypic immunity and CD4<sup>+</sup> T<sub>RM</sub> cells refine expression of a subset of immunomodulatory, antimicrobial, and tissue-protective factors from epithelial cells, consistent with improved immune resistance and tissue resilience in the infected lung.

Enhanced lung epithelial responses due to heterotypic immunity are independent of NF- $\kappa$ B RelA

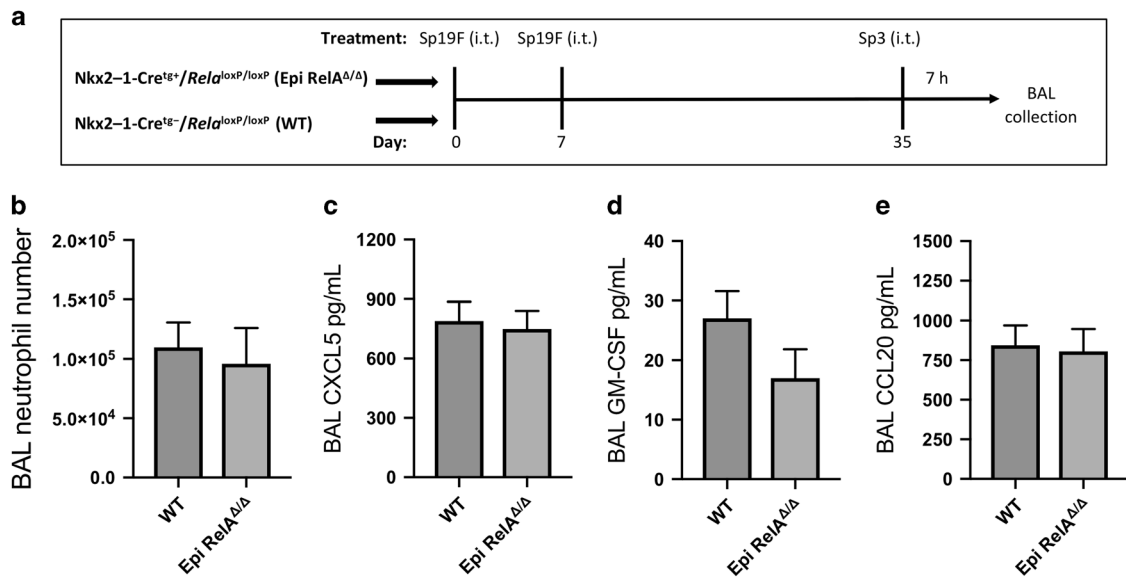
We probed the genes altered by heterotypic immunity and dependent on CD4<sup>+</sup> cells for potential regulatory elements. Using Enrichr analyses to connect transcriptome changes observed here with prior chromatin immunoprecipitation-sequencing analyses of transcription factor activities suggested that nuclear factor- $\kappa$ B (NF- $\kappa$ B) RelA might regulate the altered transcription of genes in lung

epithelial cells from experienced lungs (Fig. 5d).<sup>27</sup> To determine whether the remodeling of lung epithelial cells to enhance CXCL5 expression and neutrophil recruitment depended on NF- $\kappa$ B RelA in lung epithelial cells, we generated heterotypic immunity in mice in which the *Rela* gene was targeted in lung epithelial cells using Nkx2.1-Cre<sup>25</sup> (Fig. 6a). Cytokines and cells in BAL fluids were quantified to determine the roles of epithelial RelA in heterotypic immunity. The early neutrophil recruitment in lungs with heterotypic immunity did not require RelA in lung epithelial cells (Fig. 6b). Similarly, lung epithelial cell RelA deletion did not affect BAL CXCL5 (Fig. 6c), GM-CSF (Fig. 6d), or CCL20 (Fig. 6e) in mice with heterotypic immunity. These data demonstrate that, although many of the genes involved in the improved responses are regulated by RelA in some settings, their expression in the epithelial cells of lungs with heterotypic immunity was mediated by pathways other than this transcription factor.

IL-17A selectively prolongs lung epithelial CXCL5 mRNA stability leading to enhanced CXCL5 secretion

To model components of heterotypic immunity using a more reductionist system, we stimulated lung epithelial cell line cultures with recombinant cytokines relevant to heterotypic immunity (Fig. 7a). Pneumococcus-specific CD4<sup>+</sup> T<sub>RM</sub> cells produce multiple cytokines upon antigen presentation, the most abundant of which is interleukin (IL)-17A.<sup>16</sup> They also produce tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) after antigen presentation,<sup>16</sup> which can synergize with IL-17A in orchestrating epithelial cell responses.<sup>28–31</sup> Under conditions tested, recombinant TNF- $\alpha$  was sufficient to drive the production and secretion of CXCL5, GM-CSF, and CCL20 by





**Fig. 6** Heterotypic immunity remodels lung responses by mechanisms independent of NF- $\kappa$ B RelA in epithelial cells. **a** BALs from het. imm. wild-type and Epi RelA<sup>Δ/Δ</sup> mice were analyzed 7 h post Sp3 challenge. **b** Neutrophil numbers, **c** CXCL5, **d** GM-CSF, and **e** CCL20 levels in the BALs of wild-type and Epi- RelA<sup>Δ/Δ</sup> heterotypic immune- or saline-treated mice 7 h post Sp3 challenge. Two independent experiments with  $n \geq 3$  mice each. Statistical analyses: Student's *t*-test. No statistical significance observed.

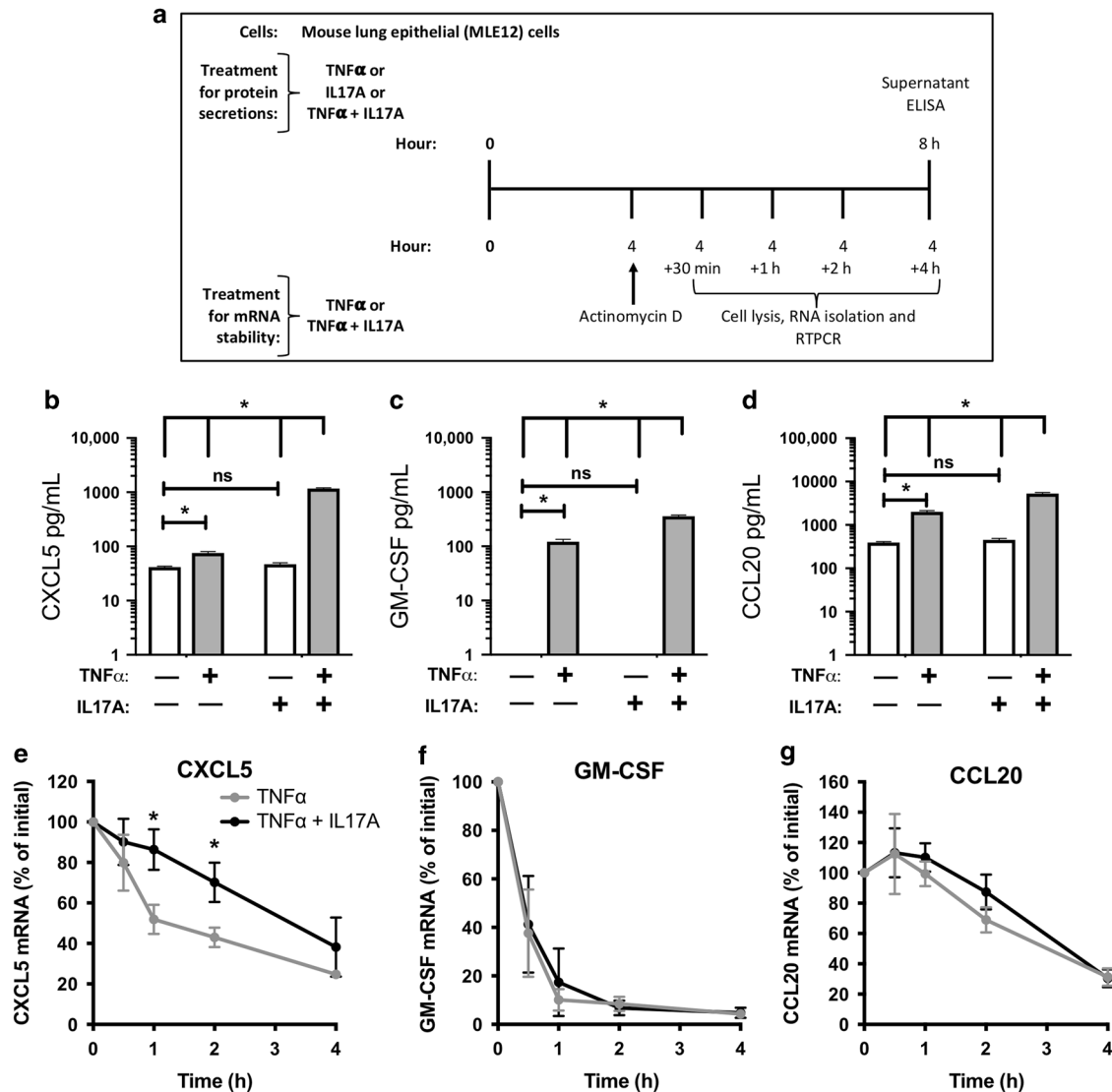
epithelial cells (Fig. 7b-d). In contrast, recombinant IL-17A was not (Fig. 7b-d). However, IL-17A significantly and substantially (~10-fold) magnified the CXCL5 produced by TNF- $\alpha$ -stimulated lung epithelial cells (Fig. 7b). Effects of IL-17A were much more modest (~2.5-fold) for both GM-CSF (Fig. 7c) and CCL20 (Fig. 7d). Thus, IL-17A has a more pronounced effect on CXCL5 than it does on GM-CSF or CCL20, consistent with the pattern of cytokine selectivity observed in vivo with heterotypic immunity.

IL-17A can post-transcriptionally regulate gene expression by multiple mechanisms including increasing mRNA stability.<sup>30,32</sup> We hypothesized that IL-17A signaling may stabilize CXCL5 more than GM-CSF or CCL20 transcripts in lung epithelial cells. Mouse lung epithelial cells stimulated by TNF- $\alpha$  alone or by TNF- $\alpha$  supplemented with IL-17A were treated with Actinomycin D, to halt continued transcription before measuring CXCL5, GM-CSF, and CCL20 transcript content by quantitative reverse transcriptase-PCR (qRT-PCR) (Fig. 7a). IL-17A signaling significantly prolonged CXCL5 mRNA stability (Fig. 7e). In contrast, the stabilities of GM-CSF and CCL20 transcripts were unaffected (Fig. 7f, g). This distinct stabilization effect of IL-17A for CXCL5 exactly mirrors the cytokine selective effects of heterotypic immunity on lung epithelial cells in vivo and suggests that the epithelial remodeling downstream of CD4<sup>+</sup> T<sub>RM</sub> cells in heterotypic immune lungs may be mediated at the level of posttranscriptional regulation. As a whole, our results suggest that the rapid neutrophil recruitment in experienced lungs requires CD4<sup>+</sup> T cells and CXCL5, the expression of the latter being selectively and post-transcriptionally enhanced by IL-17A signaling in epithelial cells.

## DISCUSSION

Our findings illuminate a novel immune axis in the experienced lung. Lung CD4<sup>+</sup> T<sub>RM</sub> cells discretely remodel epithelial cell responses during heterotypic memory-recall infections. This epithelial remodeling results in the enhancement of CXCL5 transcript stability, which increases the early accumulation of CXCL5 in the lungs and hastens neutrophil recruitment. In these ways, a major function of adaptive immunity (including CD4<sup>+</sup> T<sub>RM</sub> cells) during recall respiratory infection is to accelerate activities typical of innate immunity (including epithelial cytokine expression and neutrophil recruitment).

There is a precedent for adaptive immunity remodeling resident lung cells to improve pulmonary defense. Non-lethal infection with an adenovirus alters AM phenotype and responses to later stimuli, including elevated macrophage expression of CXCL2 which accelerates neutrophil recruitment during subsequent pneumococcal infections.<sup>13</sup> In this setting of virus-induced trained immunity, CD8<sup>+</sup> T-cell-derived interferon- $\gamma$  produced during the initial infection pushes the AMs into a different phenotype, which persists for months, long after the virus is cleared. The altered macrophage responses result from cell-intrinsic metabolic and hence presumable epigenetic changes that make them respond differently to every type of subsequent lung challenge tested, independent of further CD8<sup>+</sup> T-cell involvement during those challenges. Although accelerated chemokine-mediated neutrophil recruitment is a common result, we observe that the resolution of pneumococcal infections influences lung immunity by different mechanisms. First, although accelerated neutrophil recruitment in lungs remodeled by prior adenoviral infection is mediated by elevated production of macrophage-derived CXCL2, the accelerated neutrophil recruitment in lungs remodeled by prior pneumococcal infections is mediated by elevated production of epithelium-derived CXCL5. Second, although resolution of adenoviral infections improves lung defense against unrelated challenges (such as pneumococcus), the resolution of pneumococcal infections improves lung defense against related (diverse serotypes of pneumococcus) but not unrelated (e.g., *Klebsiella pneumoniae*) challenges.<sup>16</sup> Third, improved immunity after adenovirus requires antigen-specific adaptive immune cells in the initial adenoviral infections but not in the later unrelated challenges, while improved immunity after pneumococcal infection requires antigen-specific adaptive immune cells (CD4<sup>+</sup> T cells) during the later serotype-mismatched challenges. Thus, although resolution of adenoviral infection results in trained innate immunity in the lung that persists independent of adaptive immune memory, the resolution of pneumococcal infection results in adaptive immune memory that is responsible for rapidly redirecting innate immunity pathways during subsequent infections. Such a conferral of antigen specificity to epithelial cells by T<sub>RM</sub> cells could be a safety feature to avoid nonspecific and excessive activation of lung epithelial cells in response to unrelated and potentially innocuous inspired material.



**Fig. 7** IL-17A selectively stabilizes and enhances CXCL5 from lung epithelial cells. **a** For protein analyses, MLE12 cells were treated with TNF- $\alpha$  or IL-17A, or TNF- $\alpha$  + IL-17A for 8 h before collection of supernatants for ELISA. For mRNA stability analyses, MLE12 cells were pretreated with TNF- $\alpha$  or TNF- $\alpha$  + IL-17A for 4 h before Actinomycin D treatment. **b** CXCL5, **c** GM-CSF, and **d** CCL20 levels in the supernatants of MLE12 cells treated with TNF- $\alpha$  or TNF- $\alpha$  + IL-17A at 8 h. Three independent experiments with three technical replicates each. Statistical analyses: one-way ANOVA. mRNA levels of **e** CXCL5, **f** GM-CSF, and **g** CCL20 transcripts expressed as the percentage of initial amounts at 0 h were measured at designated timepoints. Three independent experiments with two technical replicates each. Statistical analyses: Student's *t*-test. \**p*-value  $\leq$  0.05.

CD4<sup>+</sup> T<sub>RM</sub> cells and accelerated neutrophil recruitment are each recognized as critical mediators of antimicrobial protection against pneumococcal pneumonia,<sup>14,16,18</sup> but the present study is the first in our knowledge to mechanistically link these phenomena. The molecular and cellular link is CXCL5 from epithelial cells. Among neutrophil-attracting chemokines, CXCL5 was unique in being both modified by prior pneumococcal experience and dependent on CD4<sup>+</sup> T cells at the time of infection. Blockade of CXCL5 revealed that it was independently essential to neutrophil accumulation in the airspaces early after infection of experienced lungs. CXCL5 is consistently derived from the epithelial cells during respiratory infection<sup>24,25,33,34</sup> and a preferential basolateral secretion<sup>31</sup> suggests it may be pivotal to neutrophil migration from the interstitial connective tissue across the basal lamina and into airspaces of the lung. We observed that CXCL5 transcripts were increased in epithelial cells due to prior experience, which depended on CD4<sup>+</sup> T cells. In addition to defining a mechanism by which neutrophil recruitment is

enhanced in lungs with prior relevant experience, the observation that CD4<sup>+</sup> cells boosted CXCL5 accumulation in the lung led to the discovery of a crosstalk of T<sub>RM</sub> cells with epithelial cells in the setting of optimal immunity against pneumococcal pneumonia.

The notion that epithelial cells in experienced lungs are effectively different in their responses to pathogenic challenges, owing to their communication with resident T cells, is novel. Our bioinformatic comparisons of sorted lung epithelial cells from naive mice, CD4<sup>+</sup> T-cell-sufficient experienced mice, and CD4<sup>+</sup> T-cell-deficient experienced mice led to that conclusion. Although the transcriptomic modulation was limited in scope, the requirement of T cells in recruiting neutrophils via epithelial CXCL5 argues its biological importance. Our observations that remodeling of epithelial transcriptome by CD4<sup>+</sup> T cells was not globally exaggerated but instead very selective and specific testifies to the discrete and optimized tuning abilities of T<sub>RM</sub> cells in modulating the lung epithelial response. Epithelial cells occupy the entire surface area of the lungs and are major contributors to immune

protection from inspired pathogens.<sup>35,36</sup> Their host defense effector functions include mucociliary clearance and the production of surfactant proteins and other antimicrobial molecules. They also perform critical effector functions involving production of immunomodulatory cytokines like CXCL5, GM-CSF, and CCL20.<sup>1,24,25</sup> Both effector and effector host defense roles of the epithelium are enhanced by CD4<sup>+</sup> T cells in experienced lungs, as revealed by increased production of Pglyrp1, plgR, chemokines such as CXCL9, CXCL11, CCL2, and other factors itemized in Supplementary Table 1, in addition to CXCL5. Thus, our study sheds light into previously unrecognized modulation of innate epithelial responses by tissue-resident adaptive immune cells and offers a new dimension to fine-tuned epithelial functions during optimum immunity in lungs which have prior experience with pneumonia-causing pathogens.

Mechanisms by which CD4<sup>+</sup> T cells dictate acute responses of epithelial cells are beginning to be appreciated, and apply to lungs with prior pneumococcal experience. IL-17R on epithelial cells is essential to lung defense,<sup>24</sup> revealing that this cytokine directs the activity of these cells in preventing bacterial infection of the lung. IL-17A is produced by lung memory CD4<sup>+</sup> T cells stimulated by presentation of pneumococcal antigen, more than any other cytokine measured.<sup>16</sup> IL-17A can trigger transcription, but has especially profound effects on post-transcriptional gene regulation.<sup>30,32</sup> Consistent with this, we found that the effects of heterotypic immunity on lung epithelial cells were independent of the transcription factor NF- $\kappa$ B RelA, and that IL-17A did not stimulate cytokine expression from cultured epithelial cells but did increase cytokine transcript stability. IL-17A signaling stabilizes mRNA of pro-inflammatory mediators by directing appropriate AU-rich element binding proteins (AUBPs) to the 3'-UTR of the target mRNA.<sup>32</sup> Many AUBPs may regulate CXCL5 stability, because it has a large 3'-UTR (1112 bases long, compared with 318 or 500 bases long for GM-CSF and CCL20 messages, respectively). IL-17A significantly prolonged the stability of CXCL5, but the exact mechanism is unknown. The effects of IL-17A were more potent for increasing transcript stability and for the extracellular accumulation of CXCL5 than they were for either GM-CSF or CCL20. In other settings, IL-17A has been demonstrated to increase the stability of transcripts for CXCL5 as well as G-CSF but not for lipocalin-2.<sup>28–30,37–39</sup> Consistent with this, our microarray analyses revealed that transcripts for G-CSF ( $p = 0.0185$ ), similar to CXCL5, but not Lipocalin-2 ( $p = 0.6572$ ) were diminished due to CD4<sup>+</sup> cell depletion in epithelial cells from pneumonic mice with heterotypic immunity. Taken together, we interpret these data as evidence that the post-transcriptional activities of IL-17A mediate the selective remodeling of lung epithelial cells due to prior infection experience and lung CD4<sup>+</sup> T<sub>RM</sub> cells, leading to increased CXCL5 production (among other targets) by lung epithelial cells. It is this epithelial derived CXCL5, which then hastens neutrophil recruitment on memory recall.

Although our study adds a novel perspective to the conventional roles of lung epithelial cells, owing to their crosstalk with pneumococcus-specific CD4<sup>+</sup> T<sub>RM</sub> cells, we recognize limitations constraining interpretation. The administration of GK1.5 is an effective means of depleting CD4<sup>+</sup> cells, but this is not specific to lung T<sub>RM</sub> cells. Some effects of GK1.5 in this study could conceivably reflect roles for other CD4<sup>+</sup> cells in quickly remodeling epithelial responses in the lung. Perhaps inducible mutation of the gene for IL-7 in lymphatic endothelial cells could be an approach for future studies to eliminate lung CD4<sup>+</sup> T<sub>RM</sub> cells more specifically, based on suggested cause and effect relationships.<sup>40</sup> Our study design grouped CD45<sup>-</sup>EpcAM<sup>+</sup> cells together as a general population of lung epithelial cells. We recognize that there are many sub-types of lung epithelial cells within this cell population, and which specific lung epithelial subsets directly interact with and are remodeled due to lung CD4<sup>+</sup> T<sub>RM</sub> cells remains an important question and topic of investigation.

Although we focused our studies on roles of lung CD4<sup>+</sup> T<sub>RM</sub> cells, we acknowledge the possibility of additional influences on the epithelium from other lymphocytes that seed the lungs after resolution of respiratory infection. Pertinent to this, lungs of experienced mice may possess resident memory B cells, CD8<sup>+</sup> T<sub>RM</sub> cells, and innate lymphocytes such as  $\gamma\delta$ -T cells, MAIT cells, iNKT cells, NK cells, and other ILCs.<sup>1,21</sup> If and how epithelial cells may be altered by lymphocytes other than CD4<sup>+</sup> T cells remains to be determined. These limitations present new opportunities for elucidating roles of lung epithelial cell remodeling and crosstalk with professional immune cells in the improved mucosal immunity subsequent to resolution of acute infection.

Taken together, our results suggest that the rapid neutrophil recruitment in experienced lungs requires CD4<sup>+</sup> T cells and CXCL5, the expression of the latter being selectively and post-transcriptionally enhanced in lung epithelial cells by CD4<sup>+</sup> T<sub>RM</sub> cell-derived IL-17A. Our study sheds light on a role for CD4<sup>+</sup> T<sub>RM</sub> cells in reshaping the lung epithelial transcriptome during heterotypic immunity and emphasizes the pivotal role played by lung epithelial cells in translating adaptive immune signals into a rapid protective response during memory-recall infection. Although adaptive immunity is typically perceived to be slow, and pathogen-specific memory is a feature reserved to adaptive immune cells, this study emphasizes the importance of collaboration between immune cell compartments. In lungs which have recovered from prior pneumococcal infections, adaptive immune cells hasten immune responses from tissue cells, a concept we believe may be broadly applicable to mucosal defense.

## MATERIALS AND METHODS

### Mice

All animal studies were approved by the Boston University Institutional Care and Use Committee. C57BL6/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Nkx2-1-Cre<sup>tg+</sup>/Rela<sup>loxP/loxP</sup> (Epi RelA<sup>Δ/Δ</sup>) and Nkx2-1-Cre<sup>tg-</sup>/Rela<sup>loxP/loxP</sup> (WT) littermates were used for studies elucidating requirement of epithelial NF- $\kappa$ B RelA in generating heterotypic immunity.<sup>25</sup> Studies involved both sexes and mice at 6–14 weeks of age. Mice were housed in a specific pathogen-free environment, on a 12 h light–dark cycle, with access to food and water ad libitum.

### Experimental pneumonia

Mice were anesthetized via intraperitoneal (i.p.) injection of ketamine and xylazine. To induce pneumonia, *S. pneumoniae* suspended in sterile saline was instilled via an angiocatheter directed to the left lobe after surgical exposure of the trachea.<sup>25</sup> Heterotypic protection was generated as previously described.<sup>16</sup> Mice were infected intratracheally (i.t.) with 1–5  $\times 10^6$  CFU of serotype 19F pneumococcus (Strain EF3030) or saline. A week later, mice were exposed again to either saline or Sp19F, and then allowed to recover for 28–35 days. After recovery, mice were challenged i.t. with 1  $\times 10^6$  CFU of serotype 3 pneumococcus (Strain ATCC6303). Where indicated, 500 ng of recombinant CXCL5 (R&D systems, Minneapolis, MN) or vehicle (saline) was administered with Sp3 into the left lung of heterotypic immune mice.

### Antibody depletion

CD4<sup>+</sup> cells were depleted using 500  $\mu$ g and 100  $\mu$ g of GK1.5 [BioXcell, West Lebanon, NH] i.p. and i.n., respectively, both 72 and 24 h prior to the i.t. Sp3 challenge. CXCL5 was depleted using 100  $\mu$ g of anti-mouse CXCL5 antibody (Clone 61905) [Leinco Technologies, Inc., MO] i.t. simultaneous to the i.t. Sp3 challenge.

### Bronchoalveolar lavage

After killing with isoflurane, the trachea was cannulated and lungs were lavaged with serial 1 mL aliquots of phosphate-buffered





saline (10 mL total volume). BAL cells were isolated by centrifugation. The supernatant of the first lavage was saved for protein analyses.

#### Lung digest and flow cytometry

Single-cell suspensions for enumeration of lung-resident CD4<sup>+</sup> T cells were generated by digestion of lungs with type 2 collagenase (Worthington Biochemicals, Lakewood, NJ) as previously described.<sup>16</sup> To exclude circulating leukocytes during enumeration of lung-resident CD4<sup>+</sup> T cells, anesthetized mice were i.v. administered 2 µg anti-CD45 antibody 3 min prior to killing. Mice were killed using isoflurane overdose. Cells were stained using antibodies to CD45, CD3, CD4, CD8, CD11a, CD69, CD44, CD62L, and 7AAD (viability dye). Single-cell suspensions for epithelial cell isolation were generated by digestion with elastase, as previously described.<sup>25</sup> Cells were isolated using a FACS Aria II after antibody staining for CD45, EpCAM, and 7AAD.

#### RNA preparation and qRT-PCR

RNA was prepared using TRIzol via the manufacturer's recommendation. Isolated RNA was purified and DNase I-treated using the RNeasy column method (Qiagen). qRT-PCR was performed using the RNA-to-Ct kit (Life Technologies). Primers and probes for Cxcl5, Csf2 (GM-CSF), Ccl20, and 18s have been described previously.<sup>25</sup>

#### Microarray

Mouse Gene 2.0 ST CEL files were normalized to produce gene-level expression values using the implementation of the Robust Multiarray Average<sup>41</sup> in the affy R package (version 1.36.1) and an Entrez Gene-specific probeset mapping (17.0.0) from the Molecular and Behavioral Neuroscience Institute (Brainarray) at the University of Michigan.<sup>42</sup> Array quality was assessed by computing relative log expression and normalized unsealed SE using the affyPLM R package (version 1.34.0). Differential expression was assessed using the moderated (empirical Bayesian) t-test implemented in the limma R package (version 3.14.4) (i.e., creating simple linear models with lmFit, followed by empirical Bayesian adjustment with eBayes). Analyses of variance were performed using the f.pvalue function in the sva R package (version 3.4.0). Correction for multiple hypothesis testing was accomplished using the Benjamini–Hochberg FDR. All microarray analyses were performed using the R environment for statistical computing (version 2.15.1). Complete datasets were deposited with NCBI GEO (Series ID GSE128897).

#### mRNA stability determination

Mouse lung epithelial (MLE12) cells were pretreated with TNF-α (25 ng/mL) or TNF-α (25 ng/mL) + IL-17A (50 ng/mL) for 4 h before Actinomycin D treatment (5 µg/mL). RNA was collected by lysing cells in TRIzol and was isolated using the Direct-zol™ RNA Miniprep kit (Zymo Research, R2052). qRT-PCR was performed using the TaqMan RNA-to-C<sub>T</sub> 1-step Kit (Applied Biosystems, CA). TaqMan gene expression assays were used for Cxcl5, Gm-csf (Csf2), and Ccl20. The quantity of the stable detectable mRNA at designated timepoints was calculated by normalizing to 18S rRNA and was expressed as a fraction of the transcripts present at 0 h.

#### Enzyme-linked immunosorbent assay

CXCL5, GM-CSF, and CCL20 levels secreted by mouse lung epithelial (MLE12) cells treated with TNF-α (25 ng/mL), IL-17A (50 ng/mL), or TNF-α (25 ng/mL) + IL-17A (50 ng/mL) were measured using respective kits from R&D Systems 8 h post treatment.

#### Statistics

Statistical analyses were performed using Prism VII (GraphPad Software). Differences were considered statistically significant if  $p < 0.05$ . The applied tests were communicated in figure legends.

Data are represented as mean ± SEM. Microarray analysis utilized the FDR to correct for multiple comparisons, as indicated above.

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#### AUTHOR CONTRIBUTIONS

G.A.W. and A.T.S. executed experiments. E.I.A., A.K.W., N.M.S.S. and I.M.C.M. provided experimental assistance. G.A.W., A.T.S. and J.P.M. designed experiments and analyzed data. M.R.J., L.J.Q. and J.P.M. provided reagents and resources. A.T.S. and J.P.M. wrote original draft of the manuscript and all co-authors reviewed and edited the manuscript.

#### ADDITIONAL INFORMATION

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