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Lung CD4+ resident memory T cells remodel epithelial responses to accelerate neutrophil recruitment during pneumonia

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Abstract

Previous pneumococcal experience establishes lung-resident IL-17A-producing $CD4^+$ memory T_{RM} cells that accelerate neutrophil recruitment against heterotypic pneumococci. Herein, we unravel a novel crosstalk between $CD4^+$ T_{RM} cells and lung epithelial cells underlying this protective immunity. Depletion of $CD4^+$ 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 like GM-CSF or CCL20, suggesting a skewing by $CD4^+$ T_{RM} cells. Genome-wide expression analyses revealed a significant remodeling of the epithelial transcriptome of infected lungs due to infection history, ~80% of which was $CD4^+$ cell-dependent. The $CD4^+$ T_{RM} cell product IL-17A stabilized CXCL5 but not GM-CSF or CCL20 mRNA in cultured lung epithelial cells, implicating post-transcriptional 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_{RM} 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 which may apply broadly to mucosal immunology.

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AUTHOR CONTRIBUTIONS

GAW and ATS executed experiments. EIA, AKW, NMSS and IMCM provided experimental assistance. GAW, ATS and JPM designed experiments and analyzed data. MRJ, LJQ and JPM provided reagents and resources. ATS and JPM wrote original draft of the manuscript and all co-authors reviewed and edited the manuscript.

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Keywords

CD4$^+$ T cells; resident memory T cell; IL-17A; lung epithelial cells; *Streptococcus pneumoniae*; pneumonia

INTRODUCTION

Acute lower respiratory tract infections represent a great burden of disease in the US and worldwide[1–3]. The gram-positive pathobiont *Streptococcus pneumoniae* (pneumococcus) is the most common bacterial cause of community-acquired pneumonia[4–9]. Conjugate vaccines against pneumococcus elicit serotype-specific antibodies that are effective in preventing pneumonia from specified serotypes[10]. 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[11]. The limitations of 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[12]. 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[1].

In addition to systemic immunity changes, exposure to inspired microbes results in significant remodeling of local lung immunity as well[13–15]. Diverse pathogens have been demonstrated to induce T_{RM} cells in multiple tissues, and a hallmark of T_{RM}-mediated protection is accelerated immune responses[16–20]. This applies to pneumococcal pneumonia, as resolution of prior respiratory infections seeds the lungs with pneumococcus-responsive CD4$^+$ T_{RM} cells, and CD4$^+$ cell depletion exacerbates infection in such mice[16]. The pneumococcus-recovered lungs have accelerated neutrophil recruitment and bacterial clearance during subsequent heterotypic infections with pneumococcus[16], 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$^+$ T cells[13]. Such remodeling of AMs to accelerate neutrophil recruitment could be involved in improved defenses after pneumococcal infections, although neither CD8$^+$ T_{RM} cells nor trained AMs have been described in this setting. In addition, resolution of influenza infection seeds the lungs with B_{RM} cells[21], but lung B_{RM} 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$^+$ T_{RM} cells are ultimately

responsible for accelerating neutrophil recruitment[22–24], and we aimed to define the cells and molecules mediating this pivotal immune response.

RESULTS

CD4+ T cells amplify CXCL5 and neutrophil accumulation in lungs with heterotypic immunity.

In order to model naturally acquired adaptive immunity[1, 16], 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 fashion with a lethal serotype 3 strain (Sp3). In order to determine if the $CD4^+$ T_{RM} cells established in these lungs after prior infection[16] are critical for orchestrating this rapid neutrophil infiltration, we devised a strategy to deplete $CD4^+$ 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 $CD4^+$ T cells (Fig 1B, C) and ~99.5% of all lung-resident $CD4^+$ $CD69^+CD11\ a^{high}$ T_{RM} cells (Fig 1B, C).

We quantified the recruitment of leukocytes in the bronchoalveolar lavage (BAL) fluid 7 hours post Sp3 infection when neutrophil recruitment is increased due to heterotypic immunity[16] within the GK1.5- and control IgG-treated heterotypic immune mice (Fig 2A). GK1.5 treatment in heterotypic immune mice significantly reduced 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 $CD4^+$ cell depletion, we quantified ELR^+-CXC chemokines in the BAL fluids collected 7 hours 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 $CD4^+$ cells significantly diminished CXCL5 levels in the airways of heterotypic immune mice (Fig 2G). These data suggest that lung $CD4^+$ T_{RM} 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 if the elevated airway levels of CXCL5 downstream of $CD4^+$ 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 $CD4^+$ cells depleted by GK1.5.

Exogenous CXCL5 significantly increased neutrophil recruitment (Fig 3E), confirming that airway CXCL5 levels was present in limiting concentrations and directly influenced neutrophil recruitment in these lungs. Collectively, our observations demonstrate that CXCL5 is downstream of $CD4^+$ T cells and upstream of neutrophil recruitment in lungs with heterotypic immunity.

$CD4^+$ T cells remodel lung epithelial cell responses during heterotypic immunity.

CXCL5 is typically produced by epithelial cells in the infected lung, while CXCL1 and CXCL2 are derived from other cell sources[16, 25]. Thus, $CD4^+$ 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 ($CD45^-EpCAM^+$) cells from infected lungs that were naïve or experienced by FACS sorting, 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 if 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[25]. Unlike CXCL5, epithelial cells from naïve and experienced mice expressed comparable levels of 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 fashion due to heterotypic immunity.

To achieve a more comprehensive view of epithelial remodeling, and to identify the extent to which this event is $CD4^+$ T cell-dependent, we performed transcriptomic analyses on sorted $CD45^-EpCAM^+$ cells from lungs of heterotypic immune or naïve mice treated with GK1.5 or control IgG (Fig 5A). All mice had 7 hours of Sp3 pneumonia in these analyses (Fig 5A). Sp3 pneumonia profoundly changes the lung epithelial transcriptome [26], 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 if this depended on $CD4^+$ T cells. Genome-wide expression profiling revealed that a subset of the epithelial cell transcriptome in pneumonic lungs was altered due to heterotypic immunity and/or $CD4^+$ cell depletion (Fig 5B). Unsupervised clustering of genes differing at the significance level of $p<0.05$ revealed 6 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 $CD4^+$ cell depletion in lungs with heterotypic immunity compared to 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 $CD4^+$ cells. CXCL5 was included in module 5, increased by heterotypic immunity and dependent on $CD4^+$ 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 $CD4^+$ cells. This, to our knowledge, is the first report of previous microbial experience and tissue resident $CD4^+$ 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 pair-wise comparisons of the saline versus Het IgG groups. A total of 144 transcripts were differentially expressed at an FDR $q<0.05$ in experienced lung epithelial cells (Fig 5C, and Supplemental 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, pIgR, S100A9, and Pglyrp1, potentially enhancing immune resistance. Intracellular enzymes with known defense and inflammatory roles, like Oas1a, Oas1g, Oas2, Oas3, caspase 4, and SOCS1, were also more highly expressed in experienced lung 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 versus Het GK1.5 groups revealed that, in the absence of $CD4^+$ 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 $CD4^+$ T cells. CXCL5 was among the transcripts increased by heterotypic immunity only if $CD4^+$ cells were present, corroborating and expanding upon results from independent experiments (Figures 1 and 3). Taken together, heterotypic immunity and $CD4^+$ T_{RM} 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-κB RelA.

We probed the genes altered by heterotypic immunity and dependent on $CD4^+$ cells for potential regulatory elements. Using Enrichr analyses to connect transcriptome changes observed here with prior ChIP-Seq analyses of transcription factor activities suggested that NF-κB RelA might regulate the altered transcription of genes in lung epithelial cells from experienced lungs (Fig 5D)[27]. To determine whether the remodeling of lung epithelial cells to enhance CXCL5 expression and neutrophil recruitment depended on NF-κB 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[25] (Fig 6A). Cytokines and cells in BAL fluids were quantified to determine 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 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 $CD4^+$ T_{RM} cells produce multiple cytokines upon antigen presentation, the most abundant of which is IL-17A[16]. They also produce TNF-α after antigen presentation[16], which can synergize with IL-17A in orchestrating epithelial cell responses[28–31]. Under conditions tested, recombinant TNF-α was sufficient to drive production and secretion of CXCL5, GM-CSF, and CCL20 by 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-α-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[30, 32]. 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-α alone or by TNF-α supplemented with IL-17A were treated with Actinomycin D to halt continued transcription before measuring CXCL5, GM-CSF, and CCL20 transcript content by 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 $CD4^+$ T_{RM} cells in heterotypic immune lungs may be mediated at the level of post-transcriptional regulation. As a whole, our results suggest that the rapid neutrophil recruitment in experienced lungs requires $CD4^+$ 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 $CD4^+$ T_{RM} 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 $CD4^+$ T_{RM} 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 alveolar macrophage phenotype and responses to later stimuli, including elevated macrophage expression of CXCL2 which accelerates neutrophil recruitment during subsequent pneumococcal infections[13]. In this setting of virus-induced trained immunity, $CD8^+$ T cell -derived IFN-γ produced during the initial infection pushes the alveolar macrophages 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 $CD8^+$ T cell involvement during those challenges. While accelerated chemokine-mediated neutrophil recruitment is a common result, we observe that the resolution of pneumococcal infections influences lung immunity by different mechanisms. First, while 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, while resolution of adenoviral infections improves lung defense against unrelated challenges (like pneumococcus), the resolution of pneumococcal infections improves lung defense against related (diverse serotypes of pneumococcus) but not unrelated (e.g., *Klebsiella pneumoniae*) challenges[16]. 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 ($CD4^+$ T cells) during the later serotype-mismatched challenges. Thus, while 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_{RM} 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.

$CD4^+$ T_{RM} cells and accelerated neutrophil recruitment are each recognized as critical mediators of anti-microbial protection against pneumococcal pneumonia[14, 16, 18], 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 $CD4^+$ T cells at the time of infection. Blockade of CXCL5 revealed that it was independently essential to neutrophil accumulation in the air spaces early after infection of experienced lungs. CXCL5 is consistently derived from epithelial cells during respiratory infection[24, 25, 33, 34], and a preferential basolateral secretion[31] suggests it may be pivotal to neutrophil migration from the interstitial connective tissue across the basal lamina and into air spaces of the lung. We observed that CXCL5 transcripts were increased in epithelial cells due to prior experience, which depended on CD4+ T cells. In addition to defining a mechanism by which neutrophil recruitment is enhanced in lungs with prior relevant experience, the observation that $CD4^+$ cells boosted CXCL5 accumulation in the lung led to

the discovery of a crosstalk of T_{RM} 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 naïve mice, $CD4^+$ T cell-sufficient experienced mice, and $CD4^+$ 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 $CD4^+$ T cells was not globally exaggerated but instead very selective and specific testifies to the discrete and optimized tuning abilities of T_{RM} 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[35, 36]. Their host defense effector functions include mucociliary clearance and the production of surfactant proteins and other antimicrobial molecules. They also perform critical affector functions involving production of immunomodulatory cytokines like CXCL5, GM-CSF, and CCL20 [1, 24, 25]. Both effector and affector host defense roles of the epithelium are enhanced by $CD4^+$ T cells in experienced lungs, as revealed by increased production of Pglyrpl, pIgR, chemokines like CXCL9, CXCL11, CCL2, and other factors itemized in Supplemental 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 $CD4^+$ 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[24], revealing that this cytokine directs the activity of these cells in preventing bacterial infection of the lung. IL-17A is produced by lung memory $CD4^+$ T cells stimulated by presentation of pneumococcal antigen, more than any other cytokine measured[16]. IL-17A can trigger transcription, but has especially profound effects on post-transcriptional gene regulation[30, 32]. Consistent with this, we found that the effects of heterotypic immunity on lung epithelial cells were independent of the transcription factor NF-κB 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[32]. Many AUBPs may regulate CXCL5 stability, because it has a large 3'-UTR (1112 bases long, compared to 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 stability of transcripts for CXCL5 as well as G-CSF but not for lipocalin-2[28–30, 37–39]. Consistent with this, our microarray analyses revealed that transcripts for G-CSF (p=0.0185), like CXCL5, but not Lipocalin 2 (p=0.6572) were diminished due to CD4+ 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 $CD4^+$ T_{RM} 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.

While our study adds a novel perspective to the conventional roles of lung epithelial cells owing to their crosstalk with pneumococcus-specific $CD4^+$ T_{RM} cells, we recognize limitations constraining interpretation. The administration of GK1.5 is an effective means of depleting $CD4^+$ cells, but this is not specific to lung T_{RM} cells. Some effects of GK1.5 in this study could conceivably reflect roles for other $CD4^+$ 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 $CD4^+$ T_{RM} cells more specifically, based on suggested cause and effect relationships[40]. Our study design grouped $CD45^-EpCAM^+$ 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 $CD4^+$ T_{RM} cells remains an important question and topic of investigation. Although we focused our studies on roles of lung $CD4^+$ T_{RM} 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, $CD8^+$ T_{RM} cells, and innate lymphocytes such as γδ5-T cells, MAIT cells, iNKT cells, NK cells, and other ILCs[1, 21]. If and how epithelial cells may be altered by lymphocytes other than $CD4^+$ 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 $CD4^+$ T cells and CXCL5, the expression of the latter being selectively and post-transcriptionally enhanced in lung epithelial cells by $CD4^+$ T_{RM} cell-derived IL-17A. Our study sheds light on a role for $CD4^+$ T_{RM} 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^{tg+}/Rela$^{loxP/loxP}$ (Epi RelA$^{\Delta/\Delta}$) and Nkx2–1-Cre^{tg-}/Rela$^{loxP/loxP}$ (WT) littermates were used for studies elucidating requirement of epithelial NFκB RelA in generating heterotypic immunity[25]. Studies involved both sexes and mice at 6–14 weeks of age. Mice were housed in a specific pathogen-free environment, on a 12-hour 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, *Streptococcus pneumoniae* suspended in sterile saline was instilled via an angiocatheter directed to the left lobe after surgical exposure of the trachea[25]. Heterotypic protection was generated as previously described[16]. Mice were infected intratracheally (i.t.) with 1–5×10^6 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 1×10^6 CFU of serotype 3 pneumococcus (Strain ATCC6303). Where indicated, 500ng 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$^+$ cells were depleted using 500μg and 100μg of GK1.5 [BioXcell, West Lebanon, NH] i.p. and i.n. respectively, both 72 and 24 hours prior to the i.t. Sp3 challenge. CXCL5 was depleted using 100μg of anti-mouse CXCL5 antibody (Clone 61905) [Leinco Technologies, Inc., MO] i.t. simultaneous to the i.t. Sp3 challenge.

Bronchoalveolar lavage.

After euthanasia with isoflurane, the trachea was cannulated and lungs were lavaged with serial 1 mL aliquots of PBS (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$^+$ T cells were generated by digestion of lungs with Type 2 collagenase (Worthington Biochemicals, Lakewood, NJ) as previously described[16]. To exclude circulating leukocytes during enumeration of lung-resident CD4$^+$ T cells, anesthetized mice were intra-venously administered 2μg anti-CD45 antibody 3 minutes prior to euthanasia. Mice were euthanized 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[25]. 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 RNAeasy 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[25].

Microarray.

Mouse Gene 2.0 ST CEL files were normalized to produce gene-level expression values using the implementation of the Robust Multiarray Average (RMA)[41] 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[42]. Array quality was assessed by computing Relative Log Expression (RLE) and Normalized Unscaled Standard Error (NUSE) 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 false discovery rate (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 (25ng/mL) or TNF-α (25ng/mL) + IL-17A (50ng/mL) for 4 hours before Actinomycin D treatment (5μg/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_T 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 time points were calculated by normalizing to 18S rRNA and expressed as fraction of the transcripts present at 0-hours.

ELISA.

CXCL5, GM-CSF AND CCL20 levels secreted by mouse lung epithelial (MLE12) cells treated with TNF-α (25ng/mL), IL-17A (50ng/mL) or TNF-α (25ng/mL) + IL-17A (50ng/mL) were measure using respective kits from R&D systems 8 hours 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 False Discovery Rate to correct for multiple comparisons, as indicated above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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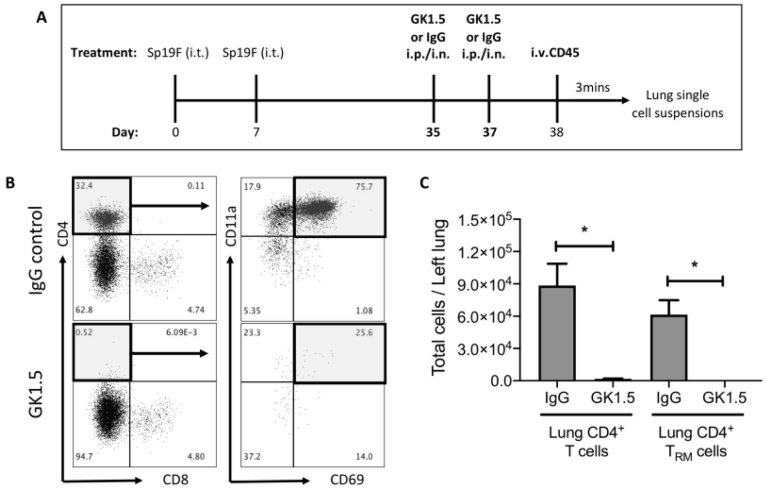

Figure 1: Strategy for depletion of lung resident CD4$^+$ T cells.

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 minutes prior to euthanasia to exclude circulating leukocytes and identify lung resident CD4$^+$ T cells by flow cytometry. **B)** Representative dot plots gated on extravascular CD45$^+$CD3$^+$ T cells and on extravascular CD45$^+$CD3$^+$CD4$^+$ T cells isolated from IgG control treated or GK1.5 treated heterotypic immune mice. **C)** Total lung CD4+ T cell numbers (identified as extravascular CD45$^+$CD3$^+$CD4$^+$ cells) and lung CD4+ T_{RM} cells numbers (identified as extravascular CD45$^+$CD3$^+$CD4$^+$ CD69$^+$CD11 a^{high} cells) in left lungs of IgG or GK1.5 treated heterotypic immune mice. All extravascular CD45$^+$CD3$^+$CD4$^+$CD69$^+$CD11a^{high} cells were CD62L^{low}CD44^{high} (data not shown). Two independent experiments with n≥7 total mice. Statistical analyses: Student's *t*- Test. *p*-value: * ≤ 0.05.

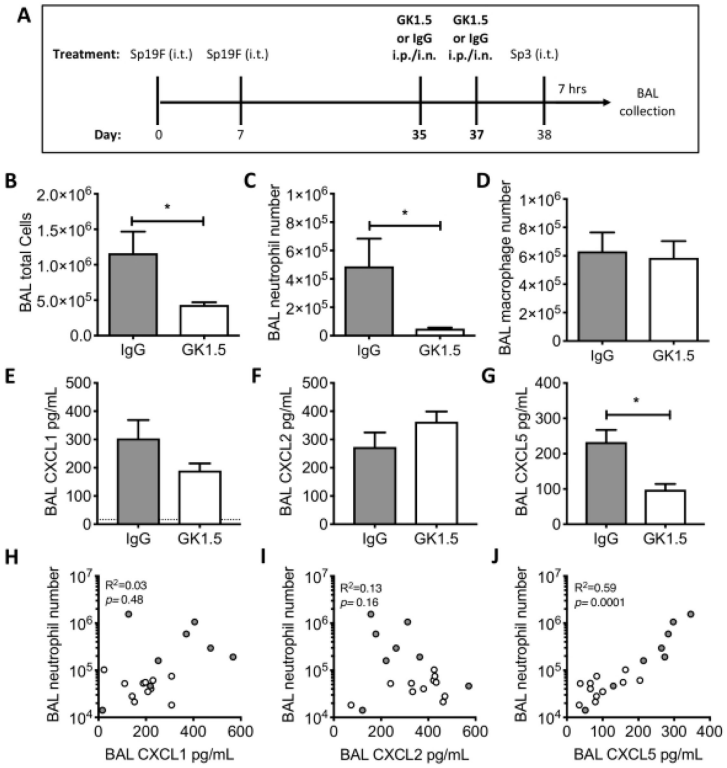

Figure 2: Depletion of CD4$^+$ cells from heterotypic immune mice diminishes CXCL5 and neutrophil accumulation in the lungs.

A) Het. Imm. mice were treated with CD4 depleting GK1.5 or control antibody 72hrs and 24hrs before final Sp3 challenge. BAL from CD4 depleted vs control treated het imm mice were collected for determining cellularity and cytokine levels 7hrs post SP3 challenge. **B)** Total cellularity **C)** macrophage and **D)** neutrophil numbers in the GK1.5 treated vs control IgG treated het imm. mice 7hrs 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 7hrs post Sp3 challenge. **H-J)** Correlation analyses of BALF CXCL1, CXCL2 and CXCL5 levels with neutrophil numbers from the GK1.5 treated (*blank dots*) vs control IgG treated (*filled dots*) het imm. mice 7hrs post Sp3 challenge. Two independent experiments with n≥3 mice each. Statistical analyses: Student's *t*- Test. *p*- value: * ≤ 0.05.

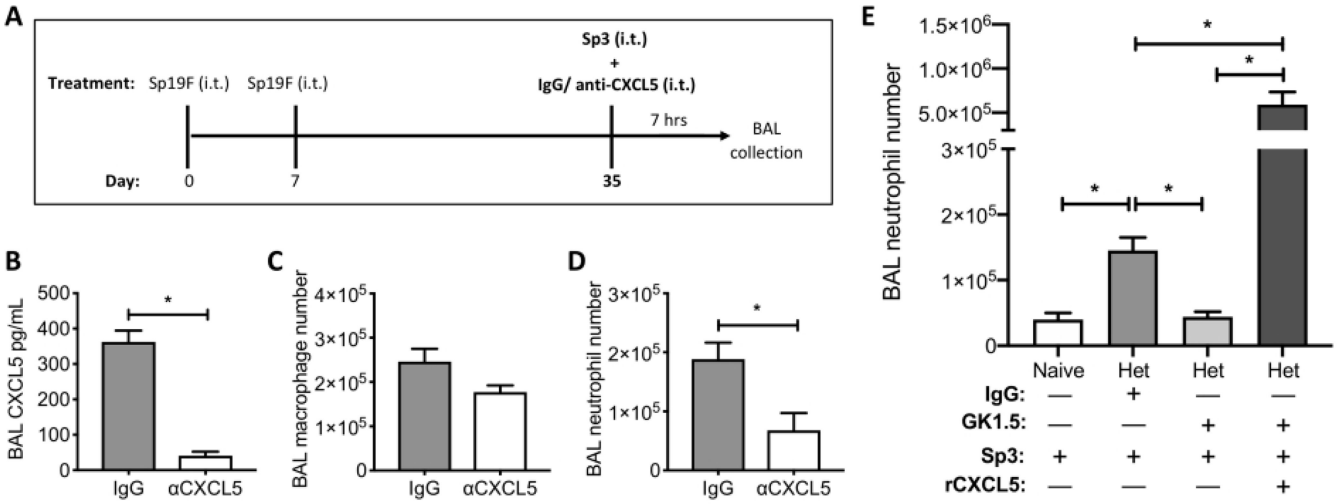

Figure 3: CXCL5 neutralization inhibits neutrophil recruitment to heterotypic immune murine lungs post Sp3 challenge.

A) CXCL5 was depleted using anti-CXCL5 antibody treatment simultaneous to Sp3 challenge. **B)** Total BAL cells collected from CXCL5 depleted vs control treated het imm. mice 7hrs post Sp3 challenge. **C)** Total macrophage and **D)** neutrophil numbers in the CXCL5 depleted vs control treated het imm. mice 7hrs post Sp3 challenge. Two independent experiments with n≥3 mice each. **E)** Total BAL neutrophil numbers in naïve, IgG- or GK1.5- treated Het. Imm. mice 7hrs post Sp3 challenge. One group of GK1.5 treated het. imm. mice received 500ng recombinant CXCL5 (rCXCL5) simultaneous to Sp3 challenge. Two independent experiments with n≥ 6 total mice. Statistical analyses: Student's t- Test. *p*-value: * ≤ 0.05.

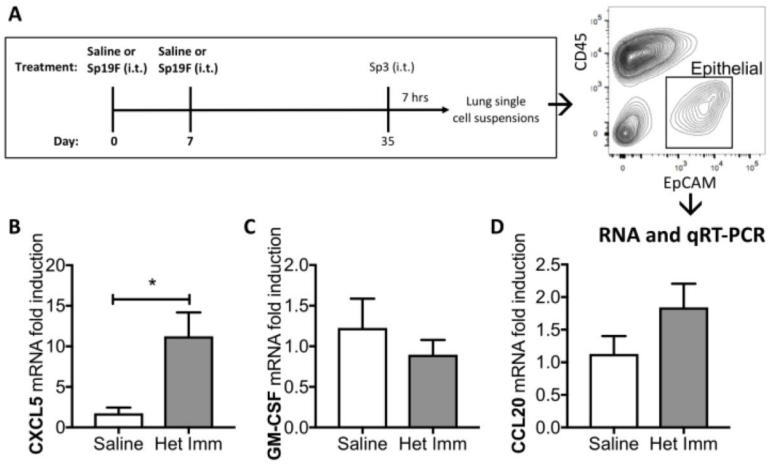

Figure 4: Heterotypic immunity increases lung epithelial induction of CXCL5 during infection.

A) $CD45^{-}EpCAM^{+}$ epithelial cells from heterotypic immune or saline treated murine lungs were flow sorted 7hrs post Sp3 challenge. **B)** CXCL5, **C)** GM-CSF and **D)** CCL20 expression in flow sorted lung epithelial cells of het. imm. vs naive mice 7hrs post Sp3 challenge was determined by RT-PCR. Two independent experiments with n≥3 mice each. Statistical analyses: Student's *t*- Test. *p*- value: * ≤ 0.05.

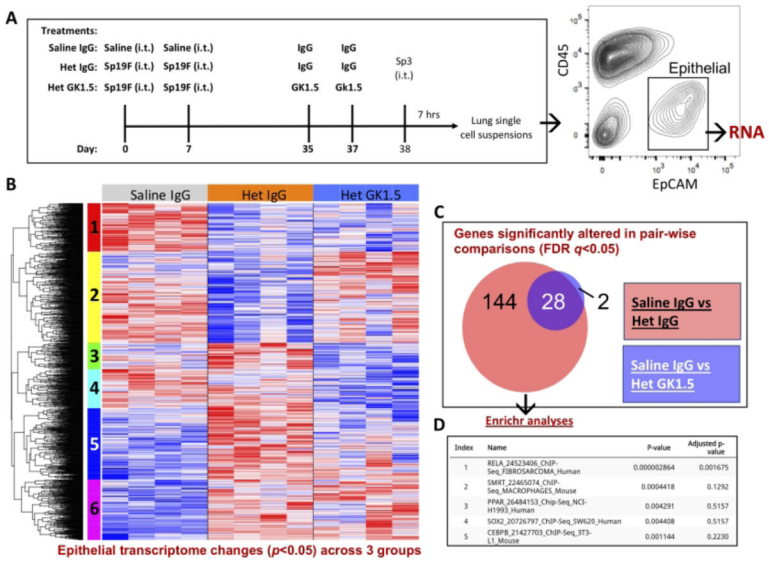

Figure 5: Lung CD4^{+} T_{RM} cells remodel epithelial responses during infection.

A) CD45^{-} EpCAM^{+} epithelial cells from het. imm. mice pretreated with GK1.5 or control antibody 72hrs and 24hrs before final Sp3 challenge were flow sorted 7hrs 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 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^{+} dependent epithelial gene signature is depicted.

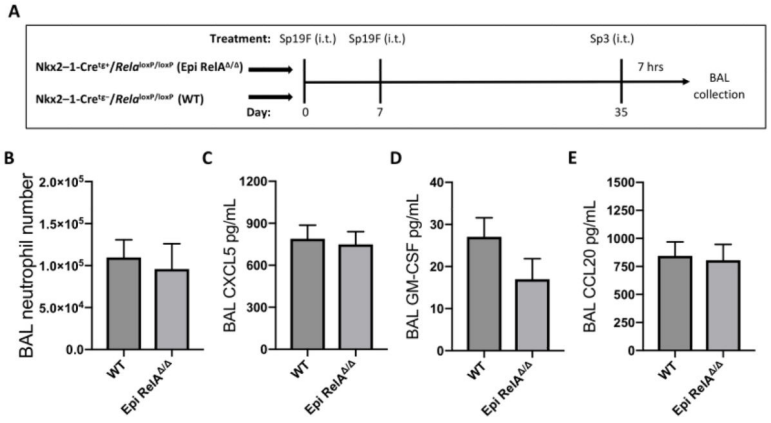

Figure 6: Heterotypic immunity remodels lung responses by mechanisms independent of NF-κB RelA in epithelial cells.

A) BALs from het. imm. wildtype and Epi RelA$^{\Delta/\Delta}$ mice were analyzed 7hrs post Sp3 challenge. **B)** Total cells, **C)** Neutrophil and **D)** macrophage numbers in the BALFs of Wildtype and Epi RelA$^{\Delta/\Delta}$ heterotypic immune or saline treated mice 7hrs post Sp3 challenge. Three independent experiments with n≥3 mice each. **E)** CXCL5, **F)** GM-CSF and **G)** CCL20 levels in the BALs of Wildtype and Epi-RelA$^{\Delta/\Delta}$ heterotypic immune or saline treated mice 7hrs post Sp3 challenge. Two independent experiments with n≥3 mice each. Statistical analyses: Student's *t*- Test. No statistical significance observed.

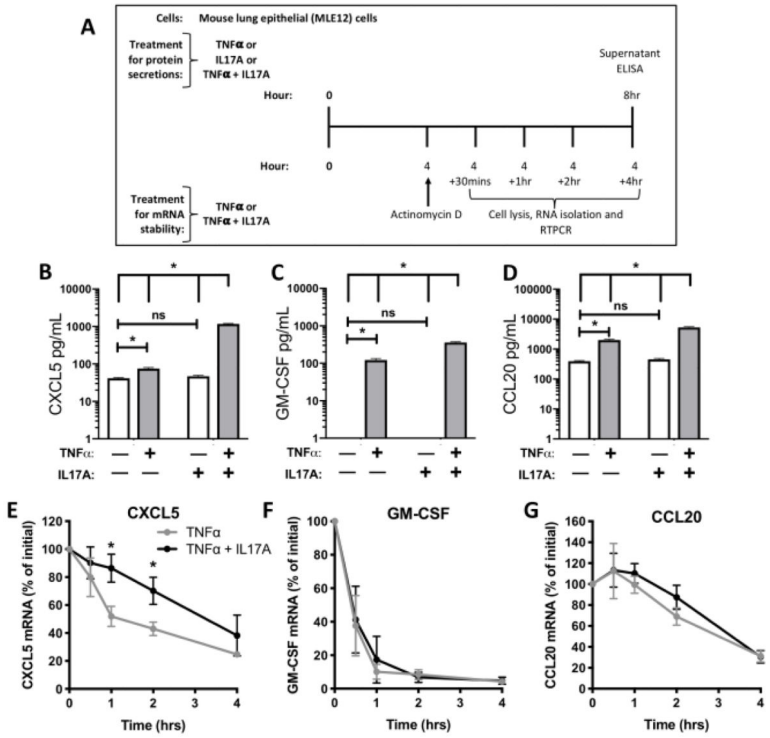

Figure 7: IL-17A selectively stabilizes and enhances CXCL5 from lung epithelial cells.

A) For protein analyses, MLE12 cells were treated with TNF-α or IL-17A or TNF-α + IL-17A for 8hrs before collection of supernatants for ELISA. For mRNA stability analyses MLE12 cells were pretreated with TNF-α or TNF-α + IL-17A for 4hrs before Actinomycin D treatment. **B)** CXCL5, **C)** GM-CSF and **D)** CCL20 levels in the supernatants of MLE12 cells treated with TNF-α or TNF-α + IL-17A at 8hrs. Three independent experiments with 3 technical replicates each. Statistical analyses: One Way ANOVA. mRNA levels of **E)** CXCL5, **F)** GM-CSF, and **G)** CCL20 transcripts expressed as percentage of initial amounts at 0hr were measured at designated timepoints. Three independent experiments with 2 technical replicates each. Statistical analyses: Student's *t*- Test. *p*- value: * ≤ 0.05.