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**ILC3 derived acetylcholine promotes protease driven allergic lung pathology**

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### **Capsule Summary (28 out of 30 words)**

We show synthesis of the neurotransmitter acetylcholine (ACh) by ILC3 is important for protease-allergen-promoted IL-17-dependent lung inflammation. This identifies a central non-neuronal role for ACh in allergic pathology.

**Keywords:** Papain allergy, type 3 innate lymphoid cell (ILC3), RoR $\gamma$ t, Acetylcholine.

### **To the Editor**

Initiation of allergic airway pathology often depends on the protease activity of the inhaled allergen. Previous studies have shown IL-17 driven neutrophil and eosinophil responses can promote allergic pathology (1) and are associated with an initial epithelial release of IL-23. IL-23 is accepted as important in promoting Th17 and type-3 innate lymphoid cell (ILC3) responses. ILC3s are a developmentally and phenotypically diverse ILC subset including natural killer (NK) cell receptor (NCR) positive and negative populations (NCR<sup>+</sup> ILC3, NCR<sup>-</sup>

ILC3) as well as CCR6<sup>+</sup> lymphoid tissue inducer cells (LTi). However, all ILC3 are defined by expression of transcript variant 2 of *RORC*, encoding retinoid-related orphan receptor  $\gamma$ t (RoR $\gamma$ t) and production of IL-17 and IL-22 (1, 2). Work carried out in murine models has resulted in ILC3s emerging as critical regulators of infectious (2) and non-infectious (1) pulmonary diseases despite ILC3s representing only a minor lung immune cell population in mice. In contrast, ILC3s are the major ILC population in the human lung (3). Importantly, ILC3-associated preclinical phenotypes are reflective of observations in humans (2, 4). However, our understanding of the molecular machinery enabling ILC3s to exert their influence on lung immunity is incomplete.

In this study we demonstrate that expansion of lung ILC3s occurs in response to the protease papain and that these cells promote an IL-17 associated lung pathology. Critically, we show induction of papain-driven pathology strongly associates with ILC3 synthesis of acetylcholine (ACh). We have previously identified ACh responsiveness by immune cells in the lung as important for CD4<sup>+</sup> T cell driven adaptive immunity to *Nippostrongylus brasiliensis* infection (5). Here, we extend this insight by demonstrating that ACh from lineage negative CD127<sup>+</sup> lymphocytes expressing RoR $\gamma$ t is instrumental for promoting protease induction of allergic inflammation. This identifies a new paradigm for how ILC3 and ACh contribute to early promotion of allergic pathology which is distinct from our traditional understanding of ACh driven neuro-muscular interactions causing allergic pulmonary airway resistance.

We identified association of ILC3s with protease-induced lung pathology following acute papain challenge of wild type (WT) C57BL/6 mice. Papain challenge increased lung

concentrations of IL-13, IL-17A, IL-22 and IL-23 in comparison to saline challenged mice (**Fig 1A**). IL-17 and IL-23 promoted pathology suggests a RoRyt driven inflammation. Papain challenge of RoRyt-GFP reporter mice demonstrated a significant expansion of ILC3s ( $\text{Lin}^- \text{CD45}^+ \text{CD127}^+ \text{ICOS}^- \text{RoRyt-GFP}^+$ ), ILC2s ( $\text{Lin}^- \text{CD45}^+ \text{CD127}^+ \text{ICOS}^+ \text{RoRyt-GFP}^-$ ) and  $\text{CD3}^+ \text{CD4}^+$  RoRyt-GFP<sup>+</sup> T cells relative to saline treated controls (**Fig 1B and Supplementary Fig S1**). Re-stimulation and intracellular cytokine capture (ICC) of lung  $\text{CD4}^+$  T cells from papain challenged mice detected increased IL-5 and IL-13 but not IL-17 when compared to saline treated controls (**Fig 1C**). However, in  $\text{CD45}^+ \text{CD3}^- \text{Lineage}^-$  cells, in addition to increased IL-5 and IL-13, raised IL-17 was detected in papain challenged mice when compared to saline treated controls (**Fig 1C**). Moreover, anti-CD3 depletion of T cells did not protect against and in fact promoted papain driven pathology (**Supplementary Fig S2**). These findings support IL-17-driven pathology as being independent of RoRyt<sup>+</sup> Th<sub>17</sub> T cell IL-17 production.

To test a requirement for any T (and B) cell contribution to IL-23/IL-17 promoted pathology we challenged *RAG2*<sup>-/-</sup> mice with papain in the presence or absence of an IL-23 neutralising mAb (anti-IL-23) (**Fig 1D**). Decreased lung inflammation in IL-23-depleted *RAG2*<sup>-/-</sup> mice was revealed by histological analysis as well as reduced detection of Evans Blue (EB) leakage into BAL (**Fig 1D**) when compared to isotype treated *RAG2*<sup>-/-</sup> mice. BAL immune cell infiltration was reduced in all immune cell populations and tissue levels of IL-17A (but not IL-13) was lower in the IL-23-depleted *RAG2*<sup>-/-</sup> mice (**Fig 1D**). This abrogation of papain induced allergic inflammation in anti-IL-23 treated *RAG2*<sup>-/-</sup> mice supports ILC3s as a key contributing lymphoid cell population driving protease-mediated lung inflammation.

To further characterise the input of ILC3s to allergic airway pathology, we compared pulmonary responses to papain in WT and *Rorc*<sup>-/-</sup> mice (**Fig 1E**). *Rorc*<sup>-/-</sup> mice do not show

significant baseline differences to C57Bl/6 in BAL cell composition (**Supplementary Fig S3**), but histological analysis of lung sections revealed decreased inflammation and detection of EB leakage into BAL in *Rorc*<sup>-/-</sup> mice challenged with papain when compared to WT mice (**Fig 1E**). Total BAL immune cell infiltration was also reduced for all immune cell populations (**Fig 1E**). Moreover, detection of both IL-13 and IL-17A, as well as IL-22 was reduced in *Rorc*<sup>-/-</sup> mice (**Fig 1E**). In agreement with findings by others (6) *Rorc*<sup>-/-</sup> mice had expanded ILC2s (Lin<sup>-</sup>CD45<sup>+</sup>CD127<sup>+</sup>ICOS<sup>+</sup>) when compared to WT mice (**Fig 1E**). However, as expected, detection of ILC3 subsets, such as NCR<sup>+</sup> ILC3s (Lin<sup>-</sup>CD45<sup>+</sup>CD127<sup>+</sup>ICOS<sup>-</sup>NKp46<sup>+</sup>) in *Rorc*<sup>-/-</sup> mice was acutely reduced when compared to WT mice; the small number of cells detected were most likely to be non-NK, non-RoRγt expressing type-1 ILCs (ILC1s). This body of work identifies a previously unappreciated, T cell-independent role for IL-23-responsive ILC3s in contributing to the onset of papain driven lung pathology.

An additional striking feature of these results was protection from cholinergic promoted airway resistance during papain challenge in the absence of *Rorc* expression (Figure 1E). Lymphocytes are important responders to, and sources of, neurotransmitters. For example, the ILC2 response to the neurotransmitter NMU is critical for inducing type 2 immunity (7), and production of ACh by immune cells following type 2 immune challenge can promote host type 2 immune responses (8). Moreover, ACh producing T cells can contribute to control of chronic viral infection (9) and CD4<sup>+</sup> T cell responses to ACh via the M3 muscarinic receptor is required for optimal adaptive immunity to helminth and bacterial infections (5). In the spleen, lymphocytes are major effectors of the cholinergic anti-inflammatory pathway (CAIP) through their synthesis and release of ACh which downregulates inflammation. ILC3 responses to cholinergic stimulation can also contribute to the CAIP, by regulating

neutrophilia in sepsis models. However, whether ILC3 themselves may be an immune cell-derived source of ACh capable of regulating immunity has not previously been investigated. To identify whether lung ILC3s may produce ACh during acute protease-induced inflammation, we challenged ChAT(BAC)-eGFP reporter mice with papain. In addition to increased ChAT production by CD3<sup>+</sup>CD4<sup>+</sup> cells (and a trend for increased production by Lin<sup>-</sup>CD45<sup>+</sup>CD127<sup>+</sup>ICOS<sup>+</sup> ILC2), we identified increased numbers of a ChAT expressing ILC3-enriched (Lin<sup>-</sup>CD45<sup>+</sup>CD127<sup>+</sup>ICOS<sup>-</sup>) population, but with no effect on NK (CD3<sup>-</sup>DX5<sup>+</sup>) cells in the lungs of papain challenged mice (**Fig 2A**). Therefore, RoRyt expressing ILC3 increase synthesis of ACh following papain challenge.

To test if ILC3 synthesis of ACh contributes to papain driven pathology, we generated RoRyt<sup>Cre</sup>ChAT<sup>loxP</sup> mice. These mice lack the ability to generate ACh following deletion of ChAT in RoRyt expressing cells. Papain challenge of RoRyt<sup>Cre</sup>ChAT<sup>loxP</sup> mice resulted in decreased histological detection of inflammation and vascular leakage of EB in BAL compared to C57BL/6 mice (**Fig 2B**) along with reduced methacholine induced airway resistance and reduced neutrophils and eosinophils in the BAL (**Fig 2B**). Detection of cytokines in lung homogenates identified reduced IL-13 and IL-17A, as well as IL-22 in RoRyt<sup>Cre</sup>ChAT<sup>loxP</sup> mice as compared to controls (**Fig 2B**). Therefore, disruption of ChAT in RoRyt positive cells was sufficient to recapitulate the reduced pathology seen in papain challenged *Rorc*<sup>-/-</sup> mice (**Fig 1**). Quantification of ILCs revealed equivalent ILC2 (Lin<sup>-</sup>CD45<sup>+</sup>CD127<sup>+</sup>ICOS<sup>+</sup>) numbers but decreased NCR<sup>+</sup> ILC3 (Lin<sup>-</sup>CD45<sup>+</sup>CD127<sup>+</sup>ICOS<sup>-</sup>NKp46<sup>+</sup>) between papain challenged WT and RoRyt<sup>Cre</sup>ChAT<sup>loxP</sup> mice (**Fig 2B**).

An equivalent phenotype was demonstrated following *Alternaria alternata* extract-driven acute allergic inflammation (**Fig 2C**). As with papain, *Alternaria* extract challenge of RoRyt<sup>Cre</sup>ChAT<sup>loxP</sup> mice resulted in decreased histological detection of inflammation and

vascular leakage of EB in BAL along with reduced methacholine induced airway resistance, reduced neutrophils and eosinophils in the BAL and reduced detection of IL-13 and IL-17A in lung homogenates compared to C57BL/6 mice (**Fig 2C**).

In summary, upon acute protease challenge we find raised IL-13, IL-23, IL-22 and IL-17A expression in the lung and increased numbers of ILC3. Papain challenge did not induce elevated CD4<sup>+</sup> T cell IL-17 irrespective of raised RoRyt<sup>+</sup>CD4<sup>+</sup> T cell numbers. Moreover, in the absence of T cells, papain-induced lung inflammation was maintained but abrogated when IL-23 or RoRyt function was disrupted. This strongly supports ILC3 expansion in the lung as a driving factor in IL-17-associated inflammation following an acute protease lung challenge. Reduction in airway cholinergic responsiveness led us to investigate if ILC3 were a physiologically relevant source of ACh following papain challenge. We tested this by generating RoRyt<sup>Cre</sup>ChAT<sup>loxP</sup> mice that lack the ability to generate ACh in RoRyt expressing cells. Remarkably, this ILC3 biased disruption of ChAT expression protected against pathology to an equivalent extent to that seen in *Rorc*<sup>-/-</sup> mice. This identifies RoRyt<sup>+</sup> cell expression of ChAT as an important component in the promotion of protease mediated allergic lung pathology. Indeed, these data support ILC3 expressing ACh as playing a central role in initiating the IL-17 promoted allergic inflammatory cascade. These findings place ILC3 synthesis of ACh as a central requirement for allergic lung inflammation adding a critical new paradigm to our understanding of cholinergic responses in driving allergic lung inflammation and pathology.

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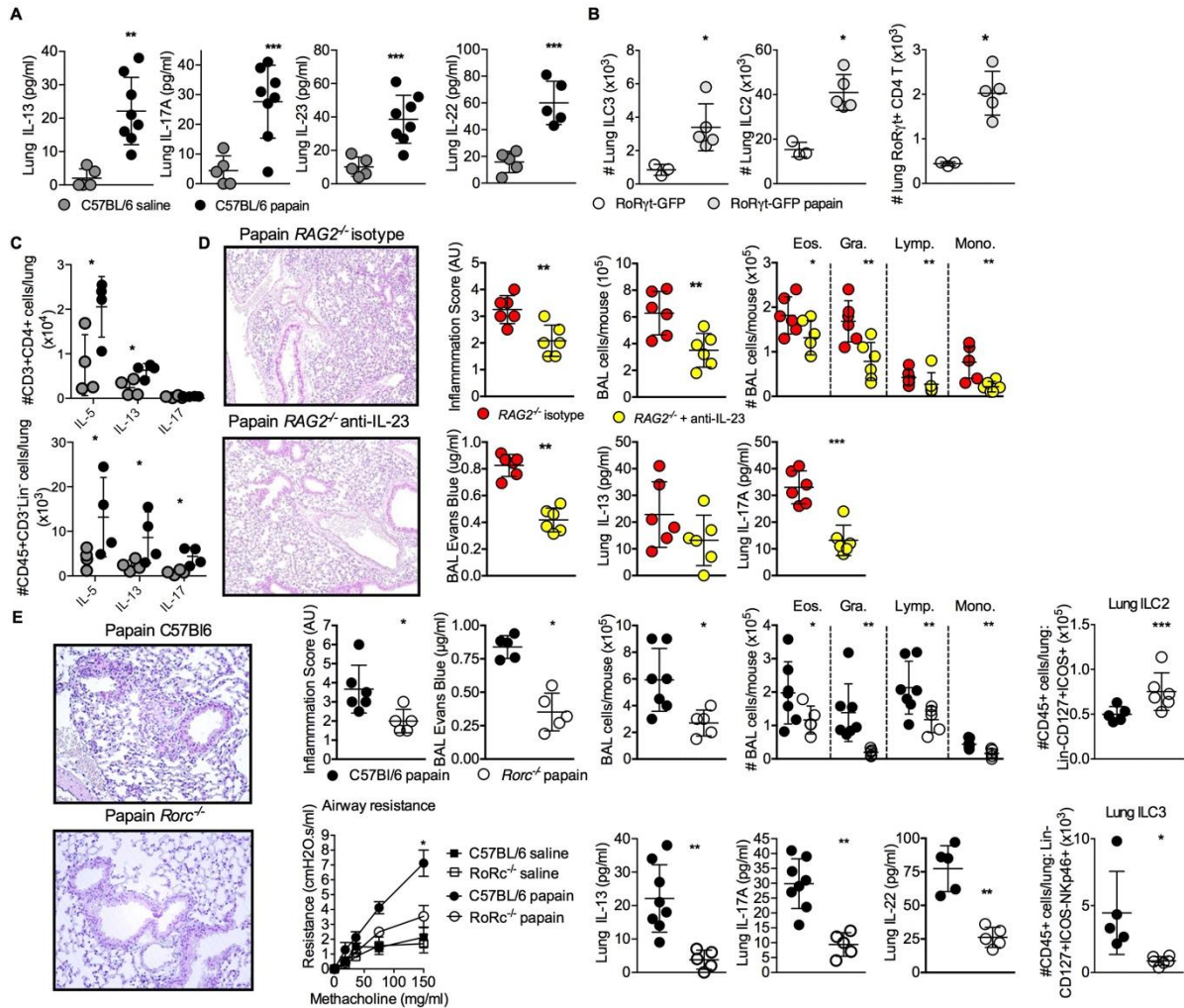
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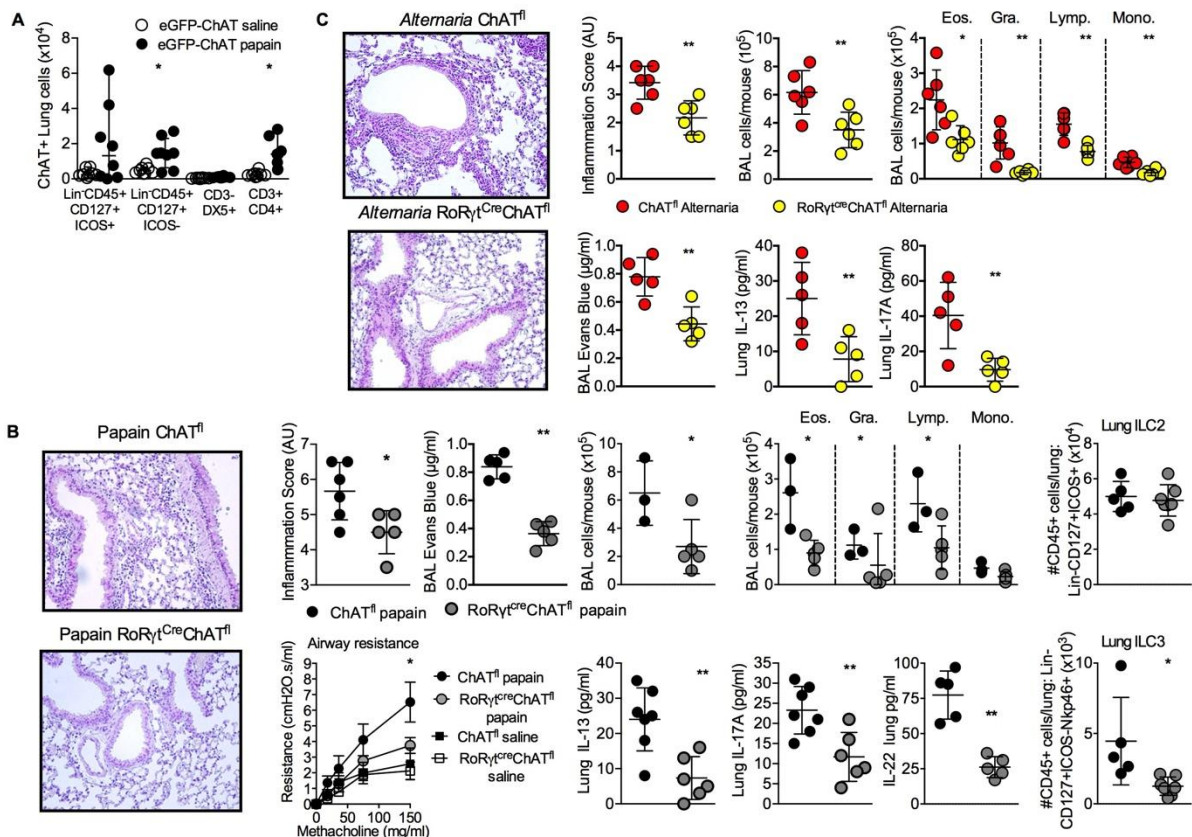
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**Figure 1. RoRyt-expressing cells drive protease-induced airway inflammation.** **A.** Detection of pulmonary IL-13, IL-17A and IL-23 by ELISA following acute papain challenge. **B.** ILC3 (Lineage<sup>-</sup>CD45<sup>+</sup>CD127<sup>+</sup>ICOS<sup>-</sup>RoRyt-GFP<sup>+</sup>), ILC2 (Lineage<sup>-</sup>CD45<sup>+</sup>CD127<sup>+</sup>ICOS<sup>+</sup>RoRyt-GFP<sup>+</sup>) and Th17 T Cells (CD3<sup>+</sup>CD4<sup>+</sup>RoRyt-GFP<sup>+</sup>) were detected in the lung by flow cytometry using RoRyt-GFP reporter mice following acute saline papain challenge. **C.** PMA restimulation of lung cells potentiated CD4<sup>+</sup> T IL-13 but not IL-17 in papain treated mice, measured by flow cytometry. But CD45<sup>+</sup>CD3<sup>-</sup>Lineage<sup>-</sup> cells did show IL-17 production following PMA restimulation. **D.** Representative H&E staining of lung sections, pathology scoring and quantification of airway epithelial integrity using Evans Blue following papain challenge of RAG2<sup>-/-</sup> and IL-23 depleted RAG2<sup>-/-</sup> mice. Airway cellular infiltration and detection of

pulmonary IL-13 and IL-17A by ELISA is also shown. **E.** Representative H&E staining of lung sections, pathology scoring and quantification of airway epithelial integrity using Evans Blue following papain challenge of C57BL/6 and *Rorc*<sup>-/-</sup> mice. Airway resistance to methacholine challenge following papain challenge in *Rorc*<sup>-/-</sup> mice. Airway cellular infiltration in *Rorc*<sup>-/-</sup> mice. Detection of pulmonary IL-13 and IL-17A by ELISA in papain challenged *Rorc*<sup>-/-</sup> mice. Detection of ILC2 (Lin<sup>-</sup>CD45<sup>+</sup>CD127<sup>+</sup>ICOS<sup>+</sup>) and NCR<sup>+</sup> ILC3 (Lineage<sup>-</sup>CD45<sup>+</sup>CD127<sup>+</sup>ICOS<sup>-</sup> NKp46<sup>+</sup>) in the lung by flow cytometry. Data is representative of 2-3 equivalent experiments, n=3-9 mice/group. All data is a comparison of unchallenged/saline-treated or papain challenged C57BL/6 background mice. Values represent mean +/- SD; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.



**Figure 2. Disruption of ChAT in RoRyt expressing cells is sufficient to protect against**

**protease-induced lung-inflammation. A.** ChAT expression in pulmonary innate lymphoid cells (Lineage<sup>-</sup>CD45<sup>+</sup>CD127<sup>+</sup>ICOS<sup>+</sup>, Lineage<sup>-</sup>CD45<sup>+</sup>CD127<sup>+</sup>ICOS<sup>-</sup> and CD3<sup>-</sup>DX5<sup>+</sup>) or CD4 T cells (CD3<sup>+</sup>CD4<sup>+</sup>) in saline or papain challenged ChAT(BAC)-eGFP reporter mice by flow cytometry.

**B.** Representative H&E staining of lung sections following papain challenge of ChAT<sup>fl</sup> and RoRyt<sup>Cre</sup>ChAT<sup>fl</sup> (disrupted ChAT expression driven by RoRyt-induced Cre expression) mice and quantification of epithelial integrity by Evans Blue. Airway resistance to methacholine challenge, quantification of airway cellular infiltration, detection of pulmonary IL-13 and IL-17A by ELISA and detection of ILC2 (Lin<sup>-</sup>CD45<sup>+</sup>CD127<sup>+</sup>ICOS<sup>+</sup>) and NCR<sup>+</sup> ILC3 (Lineage<sup>-</sup>CD45<sup>+</sup>CD127<sup>+</sup>ICOS<sup>-</sup>NKp46<sup>+</sup>) in the lung by flow cytometry in papain challenged ChAT<sup>fl</sup> and RoRyt<sup>Cre</sup>ChAT<sup>fl</sup> mice.

**C.** Representative H&E staining of lung sections, quantification of epithelial integrity by Evans Blue, quantification of airway cellular infiltration and detection of pulmonary IL-13 and IL-17A by ELISA in *Alternaria alternata* challenged ChAT<sup>fl</sup> and RoRyt<sup>Cre</sup>ChAT<sup>fl</sup> mice. Data is representative of 2-3 equivalent experiments, n=3-9 mice/group. All data is a comparison of unchallenged/saline-treated or papain challenged C57BL/6-background mice. Values represent mean +/- SD; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.