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

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

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

39 **Keywords:** Papain allergy, type 3 innate lymphoid cell (ILC3), RoR γ t, Acetylcholine.

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41 **To the Editor**

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

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

57

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

69

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

72 concentrations of IL-13, IL-17A, IL-22 and IL-23 in comparison to saline challenged mice (**Fig**
73 **1A**). IL-17 and IL-23 promoted pathology suggests a RoRyt driven inflammation. Papain
74 challenge of RoRyt-GFP reporter mice demonstrated a significant expansion of ILC3s (Lin^-
75 $\text{CD45}^+\text{CD127}^+\text{ICOS}^-\text{RoRyt-GFP}^+$), ILC2s ($\text{Lin}^- \text{CD45}^+\text{CD127}^+\text{ICOS}^+\text{RoRyt-GFP}^-$) and $\text{CD3}^+\text{CD4}^+$
76 RoRyt-GFP^+ T cells relative to saline treated controls (**Fig 1B and Supplementary Fig S1**). Re-
77 stimulation and intracellular cytokine capture (ICC) of lung CD4^+ T cells from papain
78 challenged mice detected increased IL-5 and IL-13 but not IL-17 when compared to saline
79 treated controls (**Fig 1C**). However, in $\text{CD45}^+\text{CD3}^-\text{Lineage}^-$ cells, in addition to increased IL-5
80 and IL-13, raised IL-17 was detected in papain challenged mice when compared to saline
81 treated controls (**Fig 1C**). Moreover, anti-CD3 depletion of T cells did not protect against and
82 in fact promoted papain driven pathology (**Supplementary Fig S2**). These findings support
83 IL-17-driven pathology as being independent of RoRyt^+ Th_{17} T cell IL-17 production.

84

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

94 To further characterise the input of ILC3s to allergic airway pathology, we compared
95 pulmonary responses to papain in WT and $\text{Rorc}^{-/-}$ mice (**Fig 1E**). $\text{Rorc}^{-/-}$ mice do not show

96 significant baseline differences to C57Bl/6 in BAL cell composition (**Supplementary Fig S3**),
97 but histological analysis of lung sections revealed decreased inflammation and detection of
98 EB leakage into BAL in *Rorc*^{-/-} mice challenged with papain when compared to WT mice (**Fig**
99 **1E**). Total BAL immune cell infiltration was also reduced for all immune cell populations (**Fig**
100 **1E**). Moreover, detection of both IL-13 and IL-17A, as well as IL-22 was reduced in *Rorc*^{-/-}
101 mice (**Fig 1E**). In agreement with findings by others (6) *Rorc*^{-/-} mice had expanded ILC2s (Lin⁻
102 CD45⁺CD127⁺ICOS⁺) when compared to WT mice (**Fig 1E**). However, as expected, detection
103 of ILC3 subsets, such as NCR⁺ ILC3s (Lin⁻CD45⁺CD127⁺ICOS⁻NKp46⁺) in *Rorc*^{-/-} mice was
104 acutely reduced when compared to WT mice; the small number of cells detected were most
105 likely to be non-NK, non-RoRγt expressing type-1 ILCs (ILC1s). This body of work identifies a
106 previously unappreciated, T cell-independent role for IL-23-responsive ILC3s in contributing
107 to the onset of papain driven lung pathology.

108

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

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

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

141 An equivalent phenotype was demonstrated following *Alternaria alternata* extract-driven
142 acute allergic inflammation (**Fig 2C**). As with papain, *Alternaria* extract challenge of
143 RoRyt^{Cre}ChAT^{loxP} mice resulted in decreased histological detection of inflammation and

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

147

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

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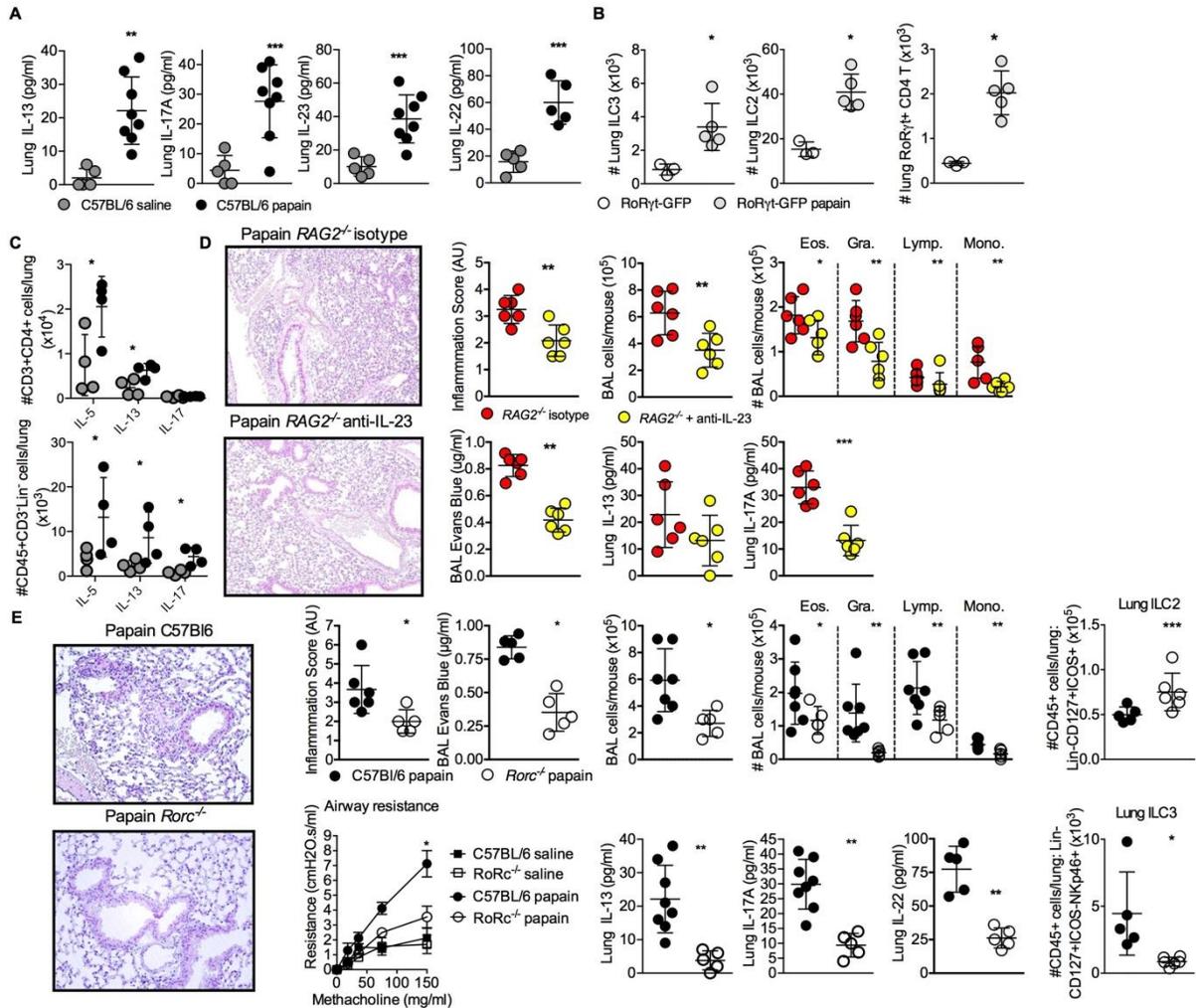
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Figure 1. RoRyt-expressing cells drive protease-induced airway inflammation. A. Detection

230

of pulmonary IL-13, IL-17A and IL-23 by ELISA following acute papain challenge. **B.** ILC3

231

(Lineage⁻CD45⁺CD127⁺ICOS⁻RoRyt-GFP⁺), ILC2 (Lineage⁻CD45⁺CD127⁺ICOS⁺RoRyt-GFP⁺) and

232

Th17 T Cells (CD3⁺CD4⁺RoRyt-GFP⁺) were detected in the lung by flow cytometry using

233

RoRyt-GFP reporter mice following acute papain challenge. **C.** PMA restimulation of lung

234

cells potentiated CD4⁺ T IL-13 but not IL-17 in papain treated mice, measured by flow

235

cytometry. But CD45⁺CD3⁻Lineage⁻ cells did show IL-17 production following PMA

236

restimulation **D.** Representative H&E staining of lung sections, pathology scoring and

237

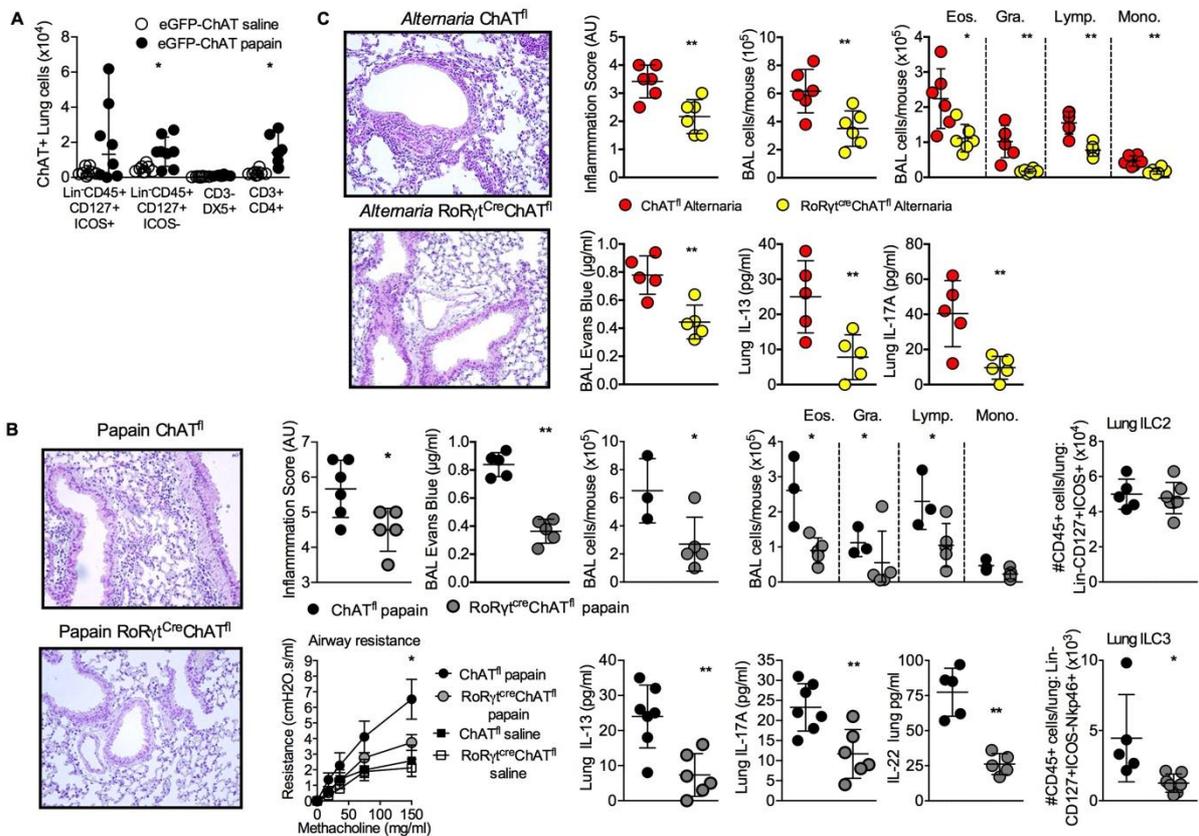
quantification of airway epithelial integrity using Evans Blue following papain challenge of

238

RAG2^{-/-} and IL-23 depleted *RAG2*^{-/-} mice. Airway cellular infiltration and detection of

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

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250

251

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

252 **protease-induced lung-inflammation. A.** ChAT expression in pulmonary innate lymphoid
253 cells (Lineage⁻CD45⁺CD127⁺ICOS⁺, Lineage⁻CD45⁺CD127⁺ICOS⁻ and CD3⁻DX5⁺) or CD4 T cells
254 (CD3⁺CD4⁺) in saline or papain challenged ChAT(BAC)-eGFP reporter mice by flow cytometry.
255 **B.** Representative H&E staining of lung sections following papain challenge of ChAT^{fl} and
256 RoRyt^{Cre}ChAT^{fl} (disrupted ChAT expression driven by RoRyt-induced Cre expression) mice
257 and quantification of epithelial integrity by Evans Blue. Airway resistance to methacholine
258 challenge, quantification of airway cellular infiltration, detection of pulmonary IL-13 and IL-
259 17A by ELISA and detection of ILC2 (Lin⁻CD45⁺CD127⁺ICOS⁺) and NCR⁺ ILC3 (Lineage⁻
260 CD45⁺CD127⁺ICOS⁻NKp46⁺) in the lung by flow cytometry in papain challenged ChAT^{fl} and
261 RoRyt^{Cre}ChAT^{fl} mice. **C.** Representative H&E staining of lung sections, quantification of
262 epithelial integrity by Evans Blue, quantification of airway cellular infiltration and detection
263 of pulmonary IL-13 and IL-17A by ELISA in *Alternaria alternata* challenged ChAT^{fl} and
264 RoRyt^{Cre}ChAT^{fl} mice. Data is representative of 2-3 equivalent experiments, n=3-9
265 mice/group. All data is a comparison of unchallenged/saline-treated or papain challenged
266 C57BL/6-background mice. Values represent mean +/- SD; *P<0.05, **P<0.01, ***P<0.001.

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