

## Acetylcholine production by group 2 innate lymphoid cells promotes mucosal immunity to helminths

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**Title**

Acetylcholine production by type 2 innate lymphoid cells promotes mucosal immunity to helminths

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## Abstract

Innate lymphoid cells (ILCs) are critical mediators of immunological and physiological responses at mucosal barrier sites. Whereas neurotransmitters can stimulate ILCs, the synthesis of small-molecule neurotransmitters by these cells has only recently been appreciated. Type 2 innate lymphoid cells (ILC2s) are shown here to synthesize and release acetylcholine (ACh) during parasitic nematode infection. The cholinergic phenotype of pulmonary ILC2s was associated with their activation state, could be induced by *in vivo* exposure to extracts of *Alternaria alternata* or the alarmin cytokines interleukin (IL)-33 and IL-25, and was augmented by IL-2 *in vitro*. Genetic disruption of ACh synthesis by murine ILC2s resulted in increased parasite burdens, lower numbers of ILC2s, and reduced lung and gut barrier responses to *Nippostrongylus brasiliensis* infection. These data demonstrate a functional role for ILC2-derived ACh in the expansion of ILC2s for maximal induction of type 2 immunity.

## One-sentence summary

Synthesis of acetylcholine by type 2 innate lymphoid cells is important for optimal immune responses to helminth infection.

## MAIN TEXT

### Introduction

Acetylcholine (ACh) is best known as a small-molecule neurotransmitter, but its role in cholinergic signaling also regulates the immune system. This is best described in the cholinergic anti-inflammatory pathway (CAIP), in which sensory perception of inflammatory stimuli leads to a vagal reflex culminating in  $\alpha 7$  nicotinic receptor (nAChR) subunit-dependent inhibition of TNF- $\alpha$ , IL-1 $\beta$  and IL-18 production by splenic macrophages (1, 2). The identification of cells that synthesize ACh has been facilitated by the use of reporter mice to visualize expression of choline acetyltransferase (ChAT), the enzyme which synthesizes ACh (3). CD4<sup>+</sup> T cells with an effector/memory (CD44<sup>+</sup>CD62L<sup>lo</sup>) phenotype were identified as the source of ACh in the spleen responsible for signaling to macrophages in the CAIP (4), and B cell-derived ACh inhibited neutrophil recruitment during sterile endotoxemia (5). Additionally, CD4<sup>+</sup> and CD8<sup>+</sup> T cell expression of

64 ChAT induced by IL-21 is essential for tissue trafficking required for T cell-mediated control of viral  
65 infection (6). Adaptive immunity is also regulated by ACh, and optimal type 2 effector responses to the  
66 nematode parasite *Nippostrongylus brasiliensis* require signaling through the M3 muscarinic receptor  
67 (mAChR) (7).

68  
69 Group 2 innate lymphoid cells (ILC2s) play an important role in initiating type 2 immune responses,  
70 producing cytokines such as IL-13 and IL-5, which drive allergic inflammation and immunity to helminth  
71 infection (8, 9). ILC2s have been shown recently to be both positively and negatively regulated by  
72 neurotransmitters such as neuromedin U (NMU) (10–12) and noradrenaline (13), whereas group 3 innate  
73 lymphoid cells (ILC3s) upregulate lipid mediator synthesis in response to vagally-derived ACh (14).  
74 Interestingly, ILCs expressing receptors responsive to neurotransmitters colocalize with neurons in mucosal  
75 tissues, forming neuroimmune cell units (NICUs) (15). ILC2s also express the neuropeptide calcitonin gene-  
76 related protein, CGRP (16). ILC2s have been shown to express tryptophan hydroxylase 1 (Tph1), which is  
77 the rate-limiting enzyme for the synthesis of the small-molecule neurotransmitter serotonin and have also  
78 been shown to produce serotonin (17).

79  
80 In this study, we demonstrate that pulmonary ILC2s upregulate their capacity to synthesize and release ACh  
81 during infection with *N. brasiliensis*, and we show that the cholinergic phenotype of ILC2s is induced by  
82 the alarmin cytokines IL-33 and IL-25. *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> transgenic mice, which have ILC2s that do not  
83 synthesize ACh, have impaired immunity to *N. brasiliensis*, reduced expression of type 2 cytokines IL-5 and  
84 IL-13 in the lung, the mucins Muc5b and Muc5ac in the lung, and altered intestinal barrier responses. These  
85 data demonstrate that the production and release of ACh by ILC2s is an important factor in driving type 2  
86 immunity.

87 **Results**

88

89 **ILC2s synthesize and release acetylcholine during type 2 immunity**

90 The cholinergic phenotype of immune cells was monitored across the time course of a primary infection  
91 with *N. brasiliensis* using ChAT-eGFP<sup>BAC</sup> mice (3). From day 4 post infection (D4 p.i., immediately  
92 following the pulmonary migratory phase of parasite larvae) until at least D21 p.i., (long past the peak  
93 of the acute phase of infection-driven inflammation) the proportion and number of CD45<sup>+</sup> cells in lung  
94 tissue that expressed ChAT (ChAT-eGFP<sup>+</sup>) was elevated compared with uninfected (naïve) controls  
95 (**Figure 1A**). Analysis of ChAT-eGFP<sup>+</sup> leukocytes revealed that most of these cells were from lymphoid  
96 rather than myeloid lineages, as previously reported in other models and tissues (5) (**Figure 1B**). Of the  
97 populations screened, expression of ChAT-eGFP was dramatically upregulated only in ILC2s at an early  
98 time point (D4) in infection (**Figure 1B, Figure S1A**).

99

100 ChAT-eGFP expression by ILC2s in lung and bronchoalveolar lavage (BAL) samples increased by D4  
101 p.i., peaked at D7, and remained elevated in both sites at D21. The proportion of ILC2s that were ChAT-  
102 eGFP<sup>+</sup> was consistently greater in BAL than in the lungs (**Figure 1C, 1D**). Real-time (RT)-qPCR  
103 confirmed that *Chat* expression in pulmonary ChAT-eGFP<sup>+</sup> ILC2s from infected ChAT-eGFP<sup>BAC</sup> mice  
104 was upregulated in comparison to ILC2 from uninfected ChAT-eGFP<sup>BAC</sup> animals, as well as to ChAT-  
105 eGFP<sup>neg</sup> ILC2, validating our reporter system (**Figure 1E**). HPLC-mass spectrometry was used to verify  
106 that WT ILC2s synthesize and release ACh and showed that this was greatly enhanced during parasite  
107 infection (**Figure 1F**). In these experiments, cells were isolated from infected animals at D11 p.i. to  
108 maximize the number of ACh-producing ILC2s obtained. We observed that ChAT-eGFP<sup>+</sup> ILC2s had an  
109 increased mean fluorescence intensity (MFI) for the IL-33 receptor subunit ST2 compared with ChAT-  
110 eGFP<sup>-</sup> cells at D4 and D7 p.i. (**Figure 1G**), and for inducible T cell co-stimulator (ICOS) at D7 p.i.  
111 (**Figure 1H**), suggesting that ChAT expression is associated with ILC2 activation state.

112

113 A striking degree of heterogeneity exists amongst ILC2s, including subtypes such as tissue-resident  
114 ‘natural’ ILC2s (nILC2s) and tissue-infiltrating ‘inflammatory’ ILC2s (iILC2s), which have been  
115 described and delineated on the basis of differential levels of phenotypic marker expression in the lung

116 (18). The functions of these subtypes have physiological relevance with regard to anti-helminth immune  
117 responses, such as pulmonary mucus production (9). To further characterize pulmonary ChAT-eGFP<sup>+</sup>  
118 ILC2s and probe whether these cells belong to a defined subtype of ILC2s, we infected ChAT-eGFP<sup>BAC</sup>  
119 mice with *N. brasiliensis* and analyzed pulmonary ILC2s at D7 p.i, utilizing an extended panel of  
120 phenotypic markers (**Figure 2A**). To assess whether ChAT-eGFP<sup>+</sup> ILC2s represented previously  
121 recognized nILC2 and iILC2 subsets, we used t-distributed stochastic neighbor embedding (t-SNE)  
122 analysis (omitting ChAT-eGFP expression as a component for clustering), to first identify populations  
123 of ILC2s (CD45<sup>+</sup>Lineage<sup>-</sup>CD127<sup>+</sup>ICOS<sup>+</sup> CD90<sup>+</sup> cells expressing either or both ST2 and IL-17RB) that  
124 most resembled conventional nILC2 (IL-17RB<sup>-</sup>ST2<sup>+</sup>CD90<sup>+</sup>Klrg1<sup>lo/-</sup>) and iILC2 (IL-17RB<sup>+</sup>ST2<sup>lo/-</sup>  
125 CD90<sup>lo</sup> Klrg<sup>+hi</sup>) subsets (**Figure 2A**). We identified additional clusters that we designated ‘nILCa’ and  
126 ‘iILC2a’ as these populations appeared to represent nILC2-like and iILC2-like cells in a higher state of  
127 cellular activation, given their differential expression of IL-17RB, dual expression of ST2 and Klrg1,  
128 and higher expression of CD90. Given our previous observation that ChAT-eGFP expression in ILC2s  
129 appeared to correlate with cellular activation, we reasoned that these groupings may be relevant for  
130 comparative analysis. The majority of ILC2s expressing high levels of ChAT-eGFP were located among  
131 these activated nILC2a and iILC2a populations (**Figure 2B**). Analysis was again carried out utilizing t-  
132 SNE (with ChAT-eGFP expression incorporated into clustering), and ChAT-eGFP<sup>+</sup> clusters could be  
133 segregated into 3 distinct populations, designated C1-C3 (**Figure 2C**). Based on a combination of  
134 marker expression and comparative assessment of the ChAT-eGFP<sup>+</sup> clusters against the 4 pre-defined  
135 reference subtypes, population C1 appeared most similar to conventional nILC2 cells, whereas C2  
136 shared the phenotypic profile of nILC2a, and C3 was most similar to iILC2a (**Figure 2D**). The greatest  
137 proportion of ChAT-eGFP<sup>+</sup> ILC2s were represented by population C2, followed by C3, then C1 (**Figure**  
138 **2E**). A similar analysis of marker expression of the very few ChAT-eGFP<sup>+</sup> cells in naïve ChAT-eGFP<sup>BAC</sup>  
139 lungs revealed that these cells clearly showed a nILC2-like profile, with no obvious differences in  
140 marker expression to that of total ChAT<sup>neg</sup> ILC2s, including ICOS and ST2 (**Figure 2F, 2G, 2H**). A  
141 different scenario was observed in infected mice however, with a disparate profile for total ChAT-eGFP<sup>+</sup>  
142 ILC2s relative to total ChAT<sup>neg</sup> ILC2s (**Figure 2F, 2G, 2H**), corroborating the findings of previous

143 analyses (**Figure 1G, 1H**). ChAT-eGFP<sup>+</sup> ILC2s do not therefore appear to represent a singular ILC2  
144 subtype during *N. brasiliensis* infection.

145

146 We examined ILC2s from mesenteric lymph nodes (MLNs) to determine if ChAT-eGFP expression by  
147 ILC2s was a unique feature of pulmonary tissues. ChAT-eGFP<sup>+</sup> ILC2 were found in MLNs of both  
148 naïve and *N. brasiliensis*-infected mice (**Figure 3A**). However, as in pulmonary populations, the  
149 proportion and total number of ILC2s expressing ChAT-eGFP increased during infection (**Figure 3B,**  
150 **3C**). ChAT-eGFP<sup>+</sup> ILC2s in the MLNs of infected mice displayed a different phenotypic profile to  
151 ChAT-eGFP<sup>neg</sup> ILC2s, particularly based on Klrg1 expression, which was restricted to ChAT-eGFP<sup>+</sup>  
152 cells, accompanied by higher expression of ST2, ICOS, and IL-17RB (**Figure 3D, 3E, 3F**). This  
153 difference in marker expression was also apparent between ChAT-eGFP<sup>+</sup> and ChAT-eGFP<sup>neg</sup> ILC2s of  
154 naïve MLNs, although expression levels were much greater following infection (**Figure 3E, 3F**). Given  
155 the difficulty in isolating viable leukocytes from the small intestinal lamina propria (siLP) of *N.*  
156 *brasiliensis* infected mice, we were not able to analyze ChAT-eGFP expression by flow cytometry from  
157 siLP ILC2 during infection. siLP ILC2s from naïve mice did not show any notable expression of ChAT-  
158 eGFP (**Figure 3G**), indicating that siLP ILC2s do not constitutively display a cholinergic phenotype in  
159 the absence of infection, akin to our observations in naïve lungs. Overall, these data indicate that ChAT-  
160 eGFP expression by ILC2s is not limited to pulmonary populations and support association of the  
161 cholinergic phenotype with cellular activation.

162

163 We evaluated if induction of the cholinergic phenotype in ILC2s was specific to parasite infection or a  
164 general feature of type 2 immunity. Mice exposed to extracts of *Alternaria alternata*, a fungal plant  
165 pathogen linked to exacerbation of asthma, develop rapid onset type 2-driven eosinophilic airway  
166 inflammation(19). ChAT-eGFP<sup>BAC</sup> and WT mice were dosed intranasally with *Alternaria* extract or  
167 phosphate buffered saline (PBS), culled 24 hours later, and lung cells were analyzed for ChAT-eGFP  
168 expression. Successful induction of a type 2 response was confirmed by pulmonary eosinophilia (**Figure**  
169 **S1B**). Challenge with *Alternaria* induced a small increase in ChAT-eGFP expression in some  
170 lymphocyte populations, including CD4<sup>+</sup> T cells and NKT cells, although expression in granulocytes

171 was unaffected (**Figure S1C**). As observed during nematode infection, the greatest proportional increase  
172 in ChAT-eGFP expression was observed in ILC2s (**Figures S1D, S1E**).

173

#### 174 **IL-25 and IL-33 induce the cholinergic phenotype of pulmonary ILC2s**

175 Our data suggested that ChAT expression was associated with cellular activation, leading us to  
176 investigate whether known activators of ILC2s could induce this phenotype. Ex vivo stimulation of  
177 CD45<sup>+</sup> cells isolated from naïve ChAT<sup>BAC</sup>-eGFP reporter mice with IL-33, but not IL-7, enhanced ILC2  
178 ChAT-eGFP expression, suggesting that activation through alarmin signaling pathways specifically  
179 drives the ILC2 cholinergic phenotype (**Figure 4A**). To explore this further, we dosed reporter mice  
180 intranasally with IL-33, IL-25 and thymic stromal lymphopoietin (TSLP), and analyzed ChAT-eGFP  
181 expression on pulmonary ILC2s 24 h later. IL-25 and IL-33 both induced ChAT-eGFP expression on  
182 ILC2s, although no effect was observed with TSLP (**Figure 4B-C**). Analysis of other leukocyte  
183 populations in the lung showed that activation of ChAT-eGFP expression by alarmins was only observed  
184 in ILC2s at the time point investigated (**Figure 4D**).

185

186

187 Lung ILC2s predominantly express the IL-33 receptor in naïve animals at immunological baseline,  
188 whereas iILC2s expressing the IL-25 receptor are thought to migrate to the lung from sites such as the  
189 gut following tissue damage such as that caused by helminth infection (18). It is possible that  
190 administration of recombinant IL-25 mobilized ILC2s from outside the lungs to migrate to the  
191 pulmonary tract and that these cells contributed to the increase in ChAT-eGFP<sup>+</sup> ILC2s, although this is  
192 unlikely as it would have to happen within 24 h. Although the proportion of IL-17RB-expressing ILC2s  
193 in the lungs increased following *N. brasiliensis* infection (**Figure 4E, 4F**), approximately 20% of ILC2s  
194 from the lungs of naïve ChAT-eGFP<sup>BAC</sup> mice expressed IL-17RB as well as ST2 (**Figure 4E, 4F**), and  
195 thus have the capacity to respond to administration of exogenous IL-25.

196

197 To assess the capacity of lung-resident ILC2s to upregulate ChAT-eGFP, we isolated CD45<sup>+</sup> cells from  
198 the lungs of naïve ChAT<sup>BAC</sup>-eGFP reporter mice and stimulated them *in vitro* with different



199 combinations of recombinant IL-33, IL-25, and IL-2, which are known to function as alarmins or  
200 promote proliferation and cytokine production (20, 21). We assayed ChAT-eGFP expression by ILC2s  
201 after 24 h, and stimulation with IL-25 and IL-33 enhanced ChAT-eGFP expression. IL-2 also induced  
202 ChAT-eGFP expression, and an additive effect of stimulation with IL-2 and either IL-33 or IL-25 was  
203 observed (**Figure 4G-H**).

204

## 205 **RoRa-driven disruption of ChAT expression impairs pulmonary type 2 immunity to *N.*** 206 ***brasiliensis***

207 To determine whether synthesis of ACh by ILC2s played a role in immunity to helminth infection, we  
208 generated *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice in which a portion of the coding domain of the *Chat* gene is floxed (22)  
209 and excised by Cre-recombinase expressed under the control of *Rora* regulatory elements (23) (**Figure**  
210 **S2A-B**). The use of *Rora*<sup>Cre+</sup> mice to selectively carry out gene deletion in ILC2s has been described  
211 previously (24). *Chat* deletion in *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> ILC2s was confirmed by PCR analysis and  
212 sequencing (**Figure S2C, S2D, S2E**). Infection of *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> and *Chat*<sup>LoxP</sup> littermate controls with  
213 *N. brasiliensis* revealed that the number of larvae recovered from the lungs were not significantly  
214 different between genotypes at 2 dpi, but higher intestinal worm burdens were observed at day 6 p.i. in  
215 *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice, indicating delayed parasite clearance in the absence of ILC2 ChAT expression  
216 (**Figure 5A**). *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice had reduced pulmonary eosinophilia compared with controls  
217 following *N. brasiliensis* infection, indicative of a suppressed type 2 immune response (**Figure 5B**).  
218 Reduced expression of *Il5* and *Il13* in total lung tissue of infected *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> samples, relative to  
219 controls supported this observation (**Figure 5C**). During the anti-helminth immune response, IL-13  
220 drives goblet cell hyperplasia and mucin production at epithelial barrier sites including the lung, where  
221 the predominant gel-forming mucins secreted by goblet cells are *Muc5b* and *Muc5ac* (25). Infected  
222 *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> at D6 p.i. demonstrated reduced expression of *Muc5b* and *Muc5ac* in total lung tissue  
223 (**Figure 5D**). PAS staining also revealed significantly reduced airway mucins in *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> lungs  
224 compared with the robust response observed in *Chat*<sup>LoxP</sup> airways (**Figure 5E-F**).

225

226 **Impaired immunity to *N. brasiliensis* in *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice is associated with defective intestinal**  
227 **barrier responses**

228 We assessed whether a defective response to *N. brasiliensis* infection following RoR $\alpha$ -mediated ChAT  
229 disruption was confined to pulmonary ILC2s and associated responses in the lung by evaluating  
230 responses in the small intestine. Intestinal epithelial effector responses characteristic of type 2 immunity  
231 include goblet and tuft cell hyperplasia. In the small intestine, we observed a decrease in periodic acid-  
232 Schiff (PAS)-positive goblet cells comparing *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> samples to *Chat*<sup>LoxP</sup> control samples  
233 (**Figure 6A, 6B**). We quantified tuft cells as analyzed through immunofluorescent staining of  
234 doublecortin-like kinase 1 (Dclk1) (**Figure 6C**) and observed that *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice did not have a  
235 statistically reduced number of tuft cells overall (**Figure 6D**), but the ratio of cells present in villus  
236 versus crypt regions was lower in *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice (**Figure 6E**). These data reflect a delayed and  
237 limited onset of type 2 immunity, which is in line with delayed worm expulsion (**Figure 5A**).

238

239 **ILC2-derived ACh promotes autocrine population expansion of ILC2s to facilitate optimal anti-**  
240 **helminth type 2 immunity.**

241 ILC2s are the major innate source of IL-13 during helminth infection, and ILC2-derived IL-13 is critical  
242 for expulsion of *N. brasiliensis* and induction of mucin expression in response to helminth infections (8,  
243 9, 26). We observed that RoR $\alpha$ -mediated disruption of *Chat* expression negatively impacted the type 2  
244 immune response to *N. brasiliensis* infection, and we next analyzed whether ILC2s themselves were  
245 affected by removing their capacity to synthesize ACh. The total number of ILC2s in the lung increased  
246 following *N. brasiliensis* infection regardless of genotype, but fewer ILC2s were found in the lungs of  
247 *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice (**Figure 7A**). Slightly fewer ILC2s were also observed in *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> lungs  
248 compared with *Chat*<sup>LoxP</sup> lungs at baseline (**Figure 7A**), and the fold change for infection-induced  
249 increases in ILC2 numbers at this timepoint was not significantly different between genotypes (**Figure**  
250 **S3A**). A similar finding was made in the MLNs of infected *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice, where fewer ILC2s  
251 were observed in comparison to infected controls (**Figure S3B**). Although ILC2s from *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup>  
252 mice could still express IL-5 and IL-13 (**Figure S3C-S3G**), the overall number of IL-13<sup>+</sup> and IL-5<sup>+</sup>  
253 ILC2s was significantly reduced in infected *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> lungs at day 6 p.i. (**Figure 7B**).

254

255 Ki67 staining of ILC2s revealed that *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> ILC2s proliferated less than *Chat*<sup>LoxP</sup> ILC2s  
256 following infection (**Figure 7C, 7D**), resulting in a smaller pool of proliferative ILC2s overall (**Figure**  
257 **7E**). Expression of ICOS was reduced on *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> ILC2s following infection, indicative of a  
258 decreased ILC2 activation state (**Figure 7F, 7G**). Similar observations were made for the activation  
259 markers ST2 and ICOS on ILC2s in the MLNs of infected *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> (**Figure S3H, S3I**),  
260 indicating that the effects caused by prevention of ACh synthesis were not confined to pulmonary ILC2s.  
261 We also analyzed the number of CD4<sup>+</sup> lung T cells (**Figure S4A**), their proliferative capacity (**Figure**  
262 **S4B, S4C**), and expression of IL-13 (**Figure S4D**), but found that these parameters were unaffected by  
263 genotype, indicative of an ILC2-specific effect of *Chat* deletion in *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice.

264

265 Lymphocytes are known to express acetylcholine receptors, although the full complement of muscarinic  
266 (mAChR) and nicotinic (nAChR) receptors expressed by ILC2s has not been defined to our knowledge  
267 (7, 27). Using cDNA prepared from FACS-purified ChAT-eGFP<sup>+</sup> and ChAT-eGFP<sup>neg</sup> lung ILC2s from  
268 *N. brasiliensis*-infected ChAT-eGFP<sup>BAC</sup> mice, we observed expression of transcripts for multiple  
269 mAChRs in addition to the  $\alpha 7$ nAChR (27). Interestingly, there appeared to be a degree of differential  
270 expression between ChAT-eGFP<sup>+</sup> and ChAT-eGFP<sup>neg</sup> with regards to AChR subtypes (**Figure 7H, 7I**).  
271 In order to determine whether ACh might act as an autocrine factor to influence proliferation and  
272 activation of the cells, we isolated WT ILC2s from the lungs of *N. brasiliensis*-infected C57BL/6J mice  
273 and cultured them in vitro with IL-7 and IL-2 alone or in the presence of the mAChR antagonist 1,1-  
274 dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP) or the nAChR antagonist mecamylamine.  
275 Addition of 4-DAMP restricted the proliferative capacity of the cells, whereas mecamylamine had no  
276 effect when compared with vehicle-treated control cultures (**Figure 7J, 7K, 7L**). These data suggest  
277 that activation-induced ACh synthesis by ILC2s plays a role in mAChR-mediated autocrine promotion  
278 of ILC2 proliferation and population expansion.

279

280 **Discussion**

281 ILC2s play a pivotal role in translating epithelial cell cytokine production into robust type 2 immune  
282 responses. Here we show that in addition to the cytokines IL-13 and IL-5, production of ACh by ILC2s  
283 is a requirement for optimal type 2-driven immunity to *N. brasiliensis*. The alarmin cytokines IL-25 and  
284 IL-33 upregulated ChAT-eGFP expression by ILC2s both in vivo and in vitro. As expression of ChAT  
285 by B cells is induced by MyD88-dependent Toll-like receptor signaling (5), we hypothesised that IL-33  
286 might regulate the ILC2 cholinergic phenotype, as members of the IL-1 family such as IL-33 also signal  
287 through this adapter protein. IL-25 was also a major regulator of ChAT expression, demonstrating that  
288 MyD88-dependent signaling is not essential for this in ILC2s. A factor common to both signaling  
289 pathways, such as the signal transducer TRAF6, may be required for inducible ChAT expression in  
290 ILC2s. TSLP, which does not signal through either MyD88 or TRAF6-dependent pathways, did not  
291 induce ChAT expression in ILC2s when administered in vivo at the same dose. TSLP has been reported  
292 to influence cutaneous ILC2 activation (28), but most studies identify IL-25 and IL-33 as the major  
293 inducers of ILC2 responses in the lung and gut (26, 29).

294

295 IL-2 was also shown to induce ChAT expression by itself or in combination with IL-25 and IL-33. IL-  
296 2 is a critical regulator of ILC2s, driving cell survival and proliferation and augmenting type 2 cytokine  
297 production (30). The cholinergic phenotype of ILC2s can be induced by a number of stimuli that activate  
298 these cells. Recently, neuropeptides such as NMU, vasoactive intestinal peptide (VIP) and CGRP and  
299 the small molecule neurotransmitter serotonin have also been shown to regulate ILC2 activation and  
300 effector activity (10–12, 16, 17, 31). If these molecules can also induce or modulate ChAT expression  
301 in ILC2s, this raises the possibility of bidirectional neuroimmune communication involving ILC2s and  
302 ACh-responsive neurons within NICUs.

303

304 Impairment of ACh synthesis by ILC2s resulted in a lower number of cells, a deficit in ILC2-derived  
305 effector cytokine production, and striking restriction of anti-helminth type 2 responses in the lungs and  
306 small intestine, tissue sites physiologically relevant to the parasite's life cycle. Lower numbers of ILC2s  
307 in the lungs and MLN of uninfected *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice is suggestive of a homeostatic requirement  
308 for ACh, and one interpretation of this may be that ILC2-derived ACh acts as an autocrine signal to aid

309 population expansion. ACh may promote expression of autocrine survival factors such as IL-9 (32), the  
310 expression of which has been shown to be promoted by neuropeptide signaling in ILC2s (33). The labile  
311 nature of ACh, due to high levels of circulating butyrylcholinesterase in tissue fluids (34) makes it likely  
312 that ILC2-derived ACh will function over relatively short distances, as would be the case during  
313 autocrine signaling. In support of this, we demonstrated that ILC2s express a range of nicotinic and  
314 muscarinic ACh receptors, and expression of the  $\alpha 7$  nAChR by ILC2s has also been previously  
315 described (27). In vitro culture of ILC2s with 4-DAMP led to a reduction in ILC2 proliferative capacity.  
316 Thus it is plausible that autocrine cholinergic signaling in ILC2s operates through mAChRs in order to  
317 promote their own expansion. Although 4-DAMP is frequently quoted as M3/M1-selective, studies on  
318 rat and human receptors show that it has potent effects on M1, M3, M4 and M5 receptors ( $K_i$  less than  
319 1 nM), and also good activity against M2 ( $K_i$  4-7 nM). (35, 36). Thus, at the concentration used in our  
320 experiments, 4-DAMP would be expected to antagonise all mAChR subtypes.

321  
322 Anticholinergics in the form of mAChR antagonists such as tiotropium are widely used to treat asthma  
323 and chronic obstructive pulmonary disease (37). These antagonists alleviate bronchoconstriction and  
324 mucus production, and are well documented to ameliorate allergen-induced airway inflammation and  
325 remodeling (38, 39), which may be accompanied by reduced type 2 cytokine production (40). In  
326 contrast, a recent report indicated that an  $\alpha 7$ -selective nAChR agonist reduced ILC2 effector function  
327 and airway hyperreactivity in an *Alternaria* allergic inflammation model (27). It is likely that signaling  
328 through different AChRs on ILC2s can result in disparate outcomes, and this could be affected by altered  
329 receptor expression under differing physiological conditions.

330  
331 A surprising observation was that ChAT expression by ILC2s was maintained several weeks after  
332 helminth eradication from the host (D21 p.i.). In addition to critical type 2 effector functions, ILC2s  
333 play important direct and indirect roles in promotion of wound healing and tissue repair (41, 42). It will  
334 be interesting to determine whether ACh production by ILC2s plays a role during later, pro-repair  
335 activities in addition to the acute inflammatory phase of infection.

336

337 A limitation of our study is that, despite the critical role of RoR $\alpha$  in ILC2 development (43, 44),  
338 expression of RoR $\alpha$  is not confined to ILC2s (45). Therefore, despite the fact that the *Rora*<sup>Cre+</sup> mouse  
339 has previously been used to successfully facilitate gene deletion in ILC2s in vivo (24), it is feasible that  
340 deletion in other cell types may also have occurred, and that this may have contributed to our  
341 observations. However, proliferation and Th<sub>2</sub> cytokine production by CD4<sup>+</sup> T cells, which are also  
342 known to express this transcription factor, were not affected during *N. brasiliensis* infection of  
343 *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice.

344

345 Helminths have evolved sophisticated strategies to promote survival in their hosts, targeted at key  
346 drivers of anti-parasite immunity (46). Secretion of acetylcholinesterases (AChEs) by parasitic  
347 nematodes has been postulated to promote parasite persistence via inhibition of cholinergic signaling in  
348 relation to the 'weep and sweep' response, characterized by intestinal smooth muscle contraction and  
349 fluid secretion from epithelial cells (47). Our current study demonstrates that production of ACh by  
350 ILC2s is a key factor in population expansion, driving maximal type 2 immunity and mucin expression,  
351 suggesting that hydrolysis of ACh by secreted AChEs may also act to suppress this to a level that allows  
352 for parasite establishment within the host.

353

354

## 355 **Materials and Methods**

356

357 **Study design.** The aim of this study was to determine the role of cholinergic signalling in the immune  
358 response to infection with a helminth parasite. We utilised ChAT-eGFP<sup>BAC</sup> reporter mice and flow  
359 cytometry to determine which cells synthesized acetylcholine (ACh), and conducted a kinetic analysis  
360 on their cholinergic phenotype throughout infection with the nematode parasite *Nippostrongylus*  
361 *brasiliensis*. Real-time qPCR was used to verify alterations in expression of *Chat*, and mass  
362 spectrometry used to confirm cellular secretion of ACh. Induction of a cholinergic phenotype in another  
363 setting characteristic of type 2 immunity was examined by intranasal administration of *Alternaria*  
364 *alternata* extracts and alarmin cytokines. The influence of ACh synthesis by ILC2s was investigated by  
365 generation of *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> transgenic mice, and the effect on immunity to parasite infection

366 determined in comparison to *Chat*<sup>LoxP</sup> littermate controls. Flow cytometry, cytokine ELISA, qPCR and  
367 histochemistry were used to characterise lung and gut barrier responses to parasite infection. Age and  
368 sex-matched mice were used for in vivo experiments, with group sizes between 3-6, conducted in  
369 replicates as indicated in figure legends. For flow cytometry experiments, negative controls were  
370 included to establish reliable gates for each marker. Parasite recoveries and histological scores were  
371 conducted in blinded conditions. No outliers were removed.

372  
373 **Animals and parasite infection.** This study was approved by the Animal Welfare Ethical Review Board  
374 at Imperial College London and was licensed by and performed under the UK Home Office Animals  
375 (Scientific Procedures) Act Personal Project Licence number 70/8193: 'Immunomodulation by helminth  
376 parasites'. C57BL/6J mice, aged 6-8 weeks old were purchased from Charles River. ChAT-eGFP<sup>BAC</sup> (3)  
377 were purchased from Jackson Laboratories and subsequently bred in-house. *Chat*<sup>LoxP</sup> mice were  
378 generated as previously described (22) and were backcrossed to F6-F10 generations on a B6 background  
379 with *Rora*<sup>Cre+</sup> (23) a kind gift from Andreas Zembrzycki (Salk Institute, La Jolla, CA) to generate the  
380 *Rora*<sup>Cre+</sup>*Chat*<sup>Loxp</sup> mice used in this study. Mice were infected with *N. brasiliensis* by sub-cutaneous (s.c.)  
381 inoculation with 500 infective larvae and parasites maintained by established methods (48).  
382

383  
384 **Murine model of allergic airway inflammation.** Extracts of *Alternaria alternata* were obtained as a  
385 gift from Henry McSorley (University of Edinburgh) or purchased as lyophilized protein extract from  
386 Greer Laboratories (USA). Mice were lightly dosed with isoflurane before intranasal administration  
387 with 50 µg *A. alternata* extract in a final volume of 50 µl PBS. Mice were exposed to a single dose of  
388 *A. alternata* for 24 hours. Control animals were dosed with 50 µl PBS following the same schedule.

389  
390 **Cytokines.** Recombinant murine cytokines were purchased from R&D (IL-25, TSLP) or Peprotech (IL-  
391 33, IL-2, IL-7) and used at 50 ng ml<sup>-1</sup> *in vitro* or administered in 50 µl doses at 10 µg ml<sup>-1</sup> *in vivo* as  
392 indicated.

393

394 **Tissue preparation.** For isolation of bronchoalveolar cells, lungs were lavaged twice in a total of 2 ml  
395 PBS with 0.2% BSA and 2 mM EDTA. Erythrocytes were lysed, leukocytes resuspended and counted.  
396 For lung single cell suspensions, lungs were perfused via cardiac puncture with 10 ml PBS then infused  
397 with 1.5 ml PBS containing 5 mg ml<sup>-1</sup> dispase II neutral protease (Sigma) via the trachea. The thymus  
398 and lung-draining lymph nodes were removed, lungs ligatured, removed into 1.5 ml digest solution,  
399 incubated at room temperature for 25 min, then for a further 30 min at 37°C. Lungs were mechanically  
400 dissociated in Dulbecco's Minimal Essential Medium (DMEM) with 25 mM HEPES and 100 U ml<sup>-1</sup>  
401 DNase I (Sigma), and incubated at room temperature for 10 min. Samples were passed through 100 µm  
402 cell strainers and erythrocytes lysed.

403 Single cell preparations of mesenteric lymph nodes were generated by mechanical dissociation through  
404 a 40 µm cell strainer followed by standard erythrocyte lysis. Small intestinal lamina propria (siLP)  
405 leukocytes were isolated with EDTA-based stripping of the intestinal epithelial layer followed by tissue  
406 digestion using collagenase-D (0.5 mg ml<sup>-1</sup>), Dispase-II (1.5 mg ml<sup>-1</sup>) and 10 µg ml<sup>-1</sup> DNase I in HBSS  
407 without Mg<sup>2+</sup> or Ca<sup>2+</sup> + 2% FCS. A 40-80% Percoll gradient separation was used to isolate a leukocyte  
408 enriched siLP sample.

409

410

411 **Flow cytometry and cell sorting.** Single cell suspensions were stained with fixable viability dyes  
412 (Invitrogen), then treated with rat anti-mouse CD32/CD16 (FcBlock, BD Biosciences), washed, then  
413 stained for extracellular markers using fluorophore conjugated monoclonal antibodies (eBioscience,  
414 Miltenyi Biotec or Biolegend). For intracellular staining, cells were fixed for 30 min at room  
415 temperature, then permeabilized using the FoxP3/transcription factor staining buffer kit (eBioscience)  
416 and stained with fluorochrome-conjugated antibodies. Unstained samples and fluorescence minus one  
417 controls were used as appropriate. When analyzing eGFP fluorescence from ChAT-eGFP<sup>BAC</sup> reporter  
418 mouse cells, WT (C57BL/6J) cells were used as negative controls to set eGFP gates. Samples were  
419 analyzed on a BD LSR Fortessa™ analyzer. For FACS sorting of ILC2s, lung tissue was processed to a  
420 single cell suspension as described, the lineage negative population enriched by magnetic activated cell



421 sorting, depleting other cells via a PE-conjugated lineage cocktail (Miltenyi Biotec), then ILC2s sorted  
422 on a BD FACS ARIA III cell sorter.

423

424 **Immunophenotyping of leukocyte populations.** Unless otherwise stated, leukocyte populations were  
425 identified by flow cytometry by gating live cells, followed by single cell and CD45<sup>+</sup> gating, and then  
426 using the following markers: ILC2; lineage<sup>-</sup>CD90<sup>+</sup>ICOS<sup>+</sup>ST2<sup>+</sup>CD127<sup>+</sup>. T cells; CD19<sup>-</sup>CD3ε<sup>+</sup>. CD4<sup>+</sup> T  
427 cells; CD19<sup>-</sup>CD3ε<sup>+</sup>DX5<sup>-</sup>CD4<sup>+</sup>CD8α<sup>-</sup>. CD8<sup>+</sup> T cells; CD19<sup>-</sup>CD3ε<sup>+</sup>DX5<sup>-</sup>CD4<sup>-</sup>CD8α<sup>+</sup>. B cells;  
428 CD19<sup>+</sup>CD3ε<sup>-</sup>B220<sup>+</sup>. γδ T cells; CD19<sup>-</sup>CD3ε<sup>+</sup>DX5<sup>-</sup>CD4<sup>-</sup>CD8α<sup>-</sup>GL3<sup>+</sup>. NK-T cells (NKT); CD19<sup>-</sup>  
429 CD3ε<sup>+</sup>DX5<sup>+</sup>. Natural Killer cells (NK); CD19<sup>-</sup>CD3ε<sup>-</sup>DX5<sup>+</sup>FcεR1<sup>-</sup>. Neutrophils; CD11b<sup>+</sup>SIGLEC-F<sup>-</sup>GR-  
430 1<sup>hi</sup>CD11c<sup>lo</sup> Eosinophils; CD11b<sup>+</sup>SIGLEC-F<sup>+</sup>GR-1<sup>lo</sup> CD11c<sup>lo</sup>. Basophils; CD19<sup>-</sup>CD3ε<sup>-</sup>DX5<sup>+</sup>FcεR1<sup>+</sup>.  
431 The lineage panel consisted of antibodies to CD3, CD4, CD8, B220, CD19, TER119, CD49b, FcεRI  
432 and CD11b. Additional markers analyzed for phenotyping and functional analysis were assessed using  
433 antibodies against Klrp1, IL-17RB, IL-5 and IL-13.

434

435 **Ki67 and intracellular cytokine staining.** To assess proliferative capacity of cells directly ex vivo,  
436 samples were processed to single cell suspension as described and rested in cDMEM (DMEM + 10%  
437 FCS, + 2mM L-glutamine + 100U ml<sup>-1</sup> Penicillin + 100ug ml<sup>-1</sup> streptomycin) for 1 hour at  
438 37°C/5%CO<sub>2</sub>, before extracellular staining, fixing and permeabilization as detailed. Intracellular Fc  
439 receptor blocking followed by anti-Ki67 staining was then carried out with fluorophore conjugated  
440 mAbs in permeabilization buffer. To assess cytokine production, single cell suspensions were diluted to  
441 5x 10<sup>6</sup> cells ml<sup>-1</sup> in cDMEM and either stimulated for 4 h at 37°C/5% CO<sub>2</sub> with 1 ug ml<sup>-1</sup> PMA/100 ng  
442 ml<sup>-1</sup> ionomycin with 1x Brefeldin-A (GolgiPlug, BD Biosciences) + 1μM Monensin (Sigma) or left  
443 unstimulated (golgi -inhibitors alone). Samples were stained, fixed and permeabilized as described and  
444 intracellular staining for Fc receptor blocking followed by fluorophore conjugated mAbs against IL-5  
445 and IL-13 was carried out in permeabilization buffer.

446

447 **ILC2 in vitro culture and proliferation analysis with acetylcholine receptor antagonists.** Female  
448 C57BL/6J mice aged 6-8 weeks were infected with *N. brasiliensis* and lungs were processed and ILC2

449 FACS sorted at between D5 to D9 p.i. as described. For each experimental run, lungs from 5 mice were  
450 pooled and sorted as a single sample and isolated ILC2 were then split equally between experimental  
451 treatment conditions. Cells were cultured for 72 hours at 37°C / 5% CO<sub>2</sub> in U bottom 96 well plates  
452 (Greiner, Cellstar) in 200 µl of cRPMI (RPMI + 10% FCS + 2 mM L-glutamine + 100 U ml<sup>-1</sup> Penicillin  
453 + 100 ug ml<sup>-1</sup> streptomycin) containing 50 ng ml<sup>-1</sup> recombinant human IL-2 (Biolegend) and 50 ng ml<sup>-1</sup>  
454 recombinant murine IL-7 (Biolegend) and either 10 µM 1,1-dimethyl-4-diphenylacetoxypiperidinium  
455 iodide (4-DAMP) (Abcam), 10 µM mecamlamine hydrochloride (mecamlamine) (Sigma), or an equal  
456 volume of drug vehicle (dH2O and DMSO) (vehicle control). Finally, cells were stained as described,  
457 including re-staining using the extracellular ILC2 marker panel used to initially sort the cells (to ensure  
458 purity of the cultured population), followed by intranuclear Ki67 staining. Samples were analyzed on a  
459 BD LSR Fortessa™ analyzer.

460

461 **Endpoint PCR.** Total RNA was extracted by TRIzol/chloroform phase-separation and DNase-1  
462 treatment. Prior to RNA extraction, mouse brain tissue (used as a for positive control for all acetylcholine  
463 receptor subunit expressions) was homogenized using a TissueLyser II (Qiagen). Reverse transcription  
464 (RT) of RNA was carried out in a 20 µl reaction volume with 50 ng RNA using the Superscript III  
465 reverse transcriptase protocol (Invitrogen) according to the manufacturer's instructions. Polymerase  
466 chain reaction (PCR) was carried out in a 20 µl reaction volume containing: 0.25 pmol forward and  
467 reverse primers, 1.25 mM dNTPs, 0.5 U Taq polymerase (New England Biolabs), 1x Thermopol  
468 reaction buffer (New England Biolabs), 2 µl cDNA and dH2O. PCR products were visualized by agarose  
469 gel electrophoresis on a gel of appropriate agarose percentage (1-3%) made with a standard Tris-acetate-  
470 EDTA buffer (pH 8.0), utilising GelRed ® nucleic acid gel stain (Biotium) and a GelDOC-IT TS  
471 imaging system (UVP). Primers used for endpoint PCR are as follows:

472 *Chrm1*: 5'- GGACAACAACACCAGAGGAGA-3'/5'-CGAGGTCACCTTAGGGTAGGG-3'

473 *Chrm2*: 5'- TGAAAACACGGTTTCCACTTC-3'/5'- GATGGAGGAGGCTTCTTTTTG-3'

474 *Chrm3*: 5'- TTTACATGCCTGTCACCATCA-3'/5'- ACAGCCACCATACTTCCTCCT-3'

475 *Chrm4*: 5'- TGCCTCTGTCATGAACCTTCT-3'/5'- TGGTTATCAGGCACTGTCCTC-3'

476 *Chrm5*: 5'- CTCTGCTGGCAGTACTTGGTC-3'/5'-GTGAGCCGGTTTTCTCTTCTT-3'

477 *Chrna1*: 5'- GACCATGAAGTCAGACCAGGA-3'/5'- TTAGCTCAGCCTCTGCTCATC-3'  
478 *Chrna2*: 5'- TGAGGTCTGAGGATGCTGACT-3'/5'- AGAGATGGCTCCAGTCACAGA-3'  
479 *Chrna3*: 5'- GTTGTCCCTGTCTGCTCTGTC-3'/5'- CCATCAAGGGTTGCAGAAATA-3'  
480 *Chrna4*: 5'- AGATGATGACGACCAACGTGT-3'/5'- ATAGAACAGGTGGGCTTTGGT-3'  
481 *Chrna5*: 5'- TGGGCCTTGCAATATCTCAGT-3'/5'- TGACAGTGCCATTGTACCTGA-3'  
482 *Chrna7*: 5'- TCAGCAGCTATATCCCCAATG-3'/5'- CAGCAAGAATACCAGCAAAGC-3'  
483 *Chrb1*: 5'- CTCACTGTGTTCTTGCTGCTG-3'/5'- GAGTTGGTCTCTCTCGGGTTT-3'  
484 *Chrb2*: 5'- GGACCATATGCGAAGTGAAGA-3'/5'- ATTTCCAGGGAAAAAGAAGCA-3'  
485 *Chrb4*: 5'- TGGCTGCCTGACATAGTTCTC-3'/5'- AGTCCAGGATCCGAACTTCAT-3'  
486

487 **RT-qPCR.** Total lung tissue was homogenized using a TissueLyser II (Qiagen). FACS purified cells  
488 were lysed directly in TRIzol (Sigma). Total RNA was extracted by TRIzol/chloroform phase-  
489 separation, DNase-1 treated, then reverse transcribed using the iScript cDNA synthesis kit (Biorad).  
490 RT-qPCR reactions were carried out using either the PowerUp SYBR Green Mix (ThermoFisher) (*Il13*,  
491 *Il5*, *Muc5ac*, *Muc5b*, *Gapdh*, *Actb*, *Hprt*) or the Quantitect SYBR Green PCR kit (Qiagen) (*Chat*, *18s*)  
492 in a ABI 7500 Fast Real-time PCR thermocycler (Applied Biosystems). RT-qPCR reactions were run  
493 in triplicate, with no template and no RT controls. Relative expression of each gene was calculated by  
494 the comparative cycle threshold (Ct) method ( $2^{-\Delta\Delta CT}$ ) using *Actb*, *Hprt* and *Gapdh* (*Il13*, *Il5*, *Muc5ac*,  
495 *Muc5b*) and *18s* (*Chat*) as reference genes. Primer sequences used for RT-qPCR were as follows:

496 *Chat*; 5'-GGCCATTGTGAAGCGGTTTG-3'/5'-GCCAGGCGGTTGTTTAGATACA-3',  
497 *18s*; 5'-TAACGAACGAGACTCTGGCAT-3'/CGGACATCTAAGGGCATCACAG-3'.  
498 *Il13*; 5'-TCACTGTAGCCTCCAGGTCTC-3'/5'-TTTCATGGCTGAGGGCTGGTT-3'.  
499 *Il5*; 5'-AGCTGGATTTTGGAAAAGAAAAGGG-3'/5'-GCTTTCTGTTGGCATGGGGT-3'.  
500 *Muc5ac*; 5'-GACACAAGCCATGCAGAGTCC-3'/5'-CTGGAAAGGCCCAAGCATGT-3'.  
501 *Muc5b*; 5'-AGCATCAAAGAGGGTGGTGGG-3'/5'-CTTGCTGTGGGGAGCCTTAAC-3'.  
502 *Gapdh*; 5'-GTCATCCCAGAGCTGAACGG-3'/5'-TACTTGGCAGGTTTCTCCAGG-3'.  
503 *Actb*; 5'-TTCCTTCTTGGGTATGGAATCCT-3'/5'-TTTACGGATGTCAACGTCACAC-3'.  
504 *Hprt*; 5'-ACAGGCCAGACTTTGTTGGA-3'/5'-ACTTGCGCTCATCTTAGGCT-3'

505

506 **Genotyping.** Genomic DNA (gDNA) was isolated from FACS-purified lung ILC2 from  
507 *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> and WT mice at day 6 p.i. with *N. brasiliensis* using a DNeasy Blood and Tissue kit  
508 (Qiagen). PCR was performed with gDNA template using Q5 DNA polymerase (NEB) and the primers  
509 5'-TGAGGGATGATGGATGAATGAG-3'/5'-CTAGGGTTGTTTCCAGAAGGC-3', situated within  
510 intronic regions flanking coding exon 5 of murine *Chat*. Amplified products were separated by agarose  
511 gel electrophoresis, and bands corresponding to the WT allele (2076 bp) and the deleted allele (546 bp)  
512 were excised, purified by standard procedures, and sequenced by Eurofins Genomics.

513

514 **Detection of acetylcholine release.** FACS-purified ILC2s were incubated at 37°C for 30 min in 96 well  
515 round-bottomed plates (10<sup>5</sup> cells in 150 µl), centrifuged, supernatants removed, the AChE inhibitor  
516 BW284C51 (Sigma) added at 10 µM and samples stored at -80°C until analysis by HPLC-mass  
517 spectrometry. Control samples were spiked with 50 nM internal standard (acetylcholine -1,1,2,2,-D4  
518 chloride, QMX laboratories).

519

520 **Histology and PAS staining.** Lung or small intestinal tissues were harvested, fixed in 10% buffered  
521 formalin, paraffin embedded and sectioned using standard techniques. Sections were stained with  
522 periodic acid-Schiff's and Haematoxylin and Eosin reagents, photographed at 40x or 100x magnification  
523 using a Zeiss Primo Star microscope and analyzed using Image J to determine the Histological Mucus  
524 Index (HMI) by established methods (49). Lung sections were analyzed using ImageJ. Briefly, lung  
525 images were overlaid with a standard grid (2000 Units) and the number of grid units containing PAS  
526 positive epithelial cells were divided by all units containing epithelial cells to establish the Histological  
527 Mucus index (HMI). Intestinal images were analyzed with ImageJ and overlaid with a standard grid  
528 (1500 Units); 15 crypt-villus segments were selected and the percentage of PAS-positive units per crypt-  
529 villus segment were divided by total units in each crypt-villus to establish HMI.

530

531 **Immunofluorescence.** Jejunal sections were paraffin embedded and sectioned according to standard  
532 techniques. For immunofluorescent staining, tissue slides were incubated at 60°C for 30 min. Next,  
533 paraffin was removed in Neo-clear (Sigma-Aldrich) by washing twice for 5 min. Then, tissue was  
534 rehydrated in decreasing ethanol concentrations (2x 100%, 1x 95%, 1x 80 %, 1x 70 %, (all 3 min) and  
535 then in distilled water. Subsequently, tissue slides were heated and boiled in pH 6 citrate buffer for 15  
536 min using a microwave. The citrate buffer was cooled to room temperature for 20 min, and slides were  
537 washed with distilled water. Next, tissues sections were marked using a hydrophobic pen (PAP pen,  
538 ab2601, Abcam) to prevent leakage. Tissue sections were incubated with blocking buffer (1% BSA, 2%  
539 normal goat serum, 0.2% Triton X-100 in PBS) for 1 hour at room temperature in a humified chamber.  
540 Next, slides were incubated with primary antibody for anti-DCLK1 (Abcam, ab31704) in antibody  
541 dilution buffer at 1:250 (0.5% bovine serum albumin, 1% normal goat serum, 0.05% Tween 20 in PBS)  
542 at 4°C overnight in a humified chamber. The next day, slides were washed 3 times in 0.2% Triton X-  
543 100 prepared in PBS for 10 min each. Slides were then incubated with the secondary antibody (1:500,  
544 Goat anti-Rabbit IgG Alexa Fluor 488, Invitrogen, A-11034,) and DAPI (1:1000) for 1 hour at room  
545 temperature in a dark humified chamber. After incubation, slides were washed 3 times with 0.2% Triton  
546 X-100 prepared in PBS for 10 min each. Finally, slides were washed with distilled water and were  
547 mounted using Fluoromount G medium (ThermoFisher Scientific) using cover slips. All the images  
548 were acquired with 20x and 40x objectives using a ZEISS confocal microscope LSM 880 and DCLK1  
549 (green) cells were quantified for  $\geq 30$  crypt-villus pairs per mouse.

550

551 **Statistical analysis.** Flow cytometry data was analyzed and t-SNE analysis was conducted using FlowJo  
552 software (Treestar). Graphs and statistical tests were carried out using GraphPad Prism software  
553 (GraphPad). Normality of data distribution was analyzed by Shapiro-Wilk test. Parametric data were  
554 analyzed by Welch's t-test, non-parametric data were analyzed by Mann-Whitney-U test. Data represent  
555 mean  $\pm$  SEM unless otherwise stated. Statistical significance between groups is indicated as \* $p < 0.05$ ,  
556 \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , n.s. = non-significant difference ( $p > 0.05$ ).

557

558 **H2: Supplementary Materials**

559 **Figure S1.** Pulmonary ILC2s acquire a cholinergic phenotype following exposure to *Alternaria*  
560 *alternata*

561 **Figure S2.** Generation of *Rora*<sup>Cre+</sup>*Chat*<sup>loxP</sup> and validation of *Chat* deletion in lung ILC2s.

562 **Figure S3.** Pulmonary ILC2 cytokine measurements and assessment of numbers and activation  
563 markers of mesenteric lymph node ILC2s in *Chat*<sup>LoxP</sup> and *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice.

564 **Figure S4.** Pulmonary CD4<sup>+</sup> T cell numbers and capacity for Th<sub>2</sub> cytokine expression remains  
565 intact in *Rora*<sup>Cre+</sup>*Chat*<sup>loxP</sup> mice

566 **Figure S5.** Material Transfer Agreement with INEM/CNRS.

567

568

## 569 **References and Notes**

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761 **Figure Legends**

762

763 **Figure 1. Pulmonary ILC2s acquire a cholinergic phenotype associated with an enhanced**

764 **activation state during infection with *Nippostrongylus brasiliensis*. A)** Proportion and total number

765 of CD45<sup>+</sup> leukocytes expressing ChAT-eGFP in the lungs of naïve ChAT<sup>BAC</sup>-eGFP mice or animals

766 infected with *N. brasiliensis* (*Nb*) at day (D) 2,4,7, and 21 post infection (p.i.). **B)** Proportion of parental

767 leukocyte populations expressing ChAT-eGFP in the lungs of naïve ChAT<sup>BAC</sup>-eGFP mice or animals

768 infected with *Nb* at D4 p.i. **C)** Representative flow cytometry plots of ChAT-eGFP expression by ILC2s

769 (CD45<sup>+</sup>CD90<sup>+</sup>Lineage<sup>-</sup>CD127<sup>+</sup>ICOS<sup>+</sup>ST2<sup>+</sup>) in wild type C57BL/6J mice infected with *Nb* (eGFP gating

770 control), naïve ChAT<sup>BAC</sup>-eGFP animals or infected ChAT<sup>BAC</sup>-eGFP animals at D7 p.i. **D)** Dynamics of

771 ChAT expression by ILC2s in the lungs and BAL throughout infection with *Nb*. **E)** Expression of *Chat*

772 transcripts assayed by RT-qPCR in FACS-sorted eGFP<sup>+</sup> and eGFP<sup>-</sup> pulmonary ILC2s from *Nb*-infected

773 ChAT<sup>BAC</sup>-eGFP animals, normalised to 18s rRNA expression and relative to expression from ILC2s

774 from naïve controls. **F)** Quantification of basal acetylcholine (ACh) release from FACS-sorted

775 pulmonary ILC2s from C57BL6/J naïve and *Nb*-infected animals. **G)** Mean fluorescence intensity (MFI)

776 of ST2 expressed by ChAT-eGFP negative and ChAT-eGFP<sup>+</sup> ILC2s in the lungs throughout infection

777 with *Nb*. **H)** Geometric MFI (gMFI) of ICOS expressed by ChAT-eGFP<sup>-</sup> and ChAT-eGFP<sup>+</sup> ILC2s in

778 the lungs throughout infection with *Nb*. Joined data points in (H-G) represent ChAT<sup>+</sup> and ChAT<sup>-</sup> ILC2

779 from individual mice. n = 3 to 5 mice/group. Data are representative of N = 3 and graphs present mean

780 ± SEM. \*p<0.05, \*\*p<0.01, \*\*\*p< 0.001, \*\*\*\*p<0.0001, n.s. = non-significant (p>0.05).

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782 **Figure 2. ChAT-eGFP<sup>+</sup> ILC2s display a range of phenotypes which support positive association**

783 **of ChAT expression by ILC2s with cellular activation state. A)** Identification of population clusters

784 (left) representing natural ILC2 (nILC2: IL17RB<sup>-</sup>ST2<sup>+</sup>Klrg1<sup>-</sup>CD90<sup>hi</sup>), inflammatory ILC2 (iILC2:

785 IL17RB<sup>+</sup>ST2<sup>lo</sup>Klrg1<sup>+</sup>CD90<sup>lo</sup>) and nILC2/iILC2 with an activated-like phenotypic profile (nILC2a:

786 IL17RB<sup>-</sup>ST2<sup>+</sup>Klrg1<sup>+</sup>CD90<sup>+</sup>, iILC2a: IL17RB<sup>+</sup>ST2<sup>+</sup>Klrg1<sup>+</sup>CD90<sup>+</sup>) within the lungs of *N. brasiliensis*

787 (*Nb*)-infected ChAT-eGFP<sup>BAC</sup> mice at day 7 post infection (D7 p.i.). Clustering analysis was conducted

788 using t-distributed stochastic neighbour embedding (t-SNE), with the parameter of ChAT-eGFP

789 expression omitted, carried out on total lung ILC2s (identified as live, single cells,

790 CD45<sup>+</sup>Lineage<sup>neg</sup>CD127<sup>+</sup>ICOS<sup>+</sup>CD90<sup>+</sup> and expressing either or both ST2 and/or IL-17RB) with  
791 indicated populations identified on the basis of various phenotypic markers and marker expression levels  
792 as indicated by the heatmap plots shown (right). **B)** Heatmap plot showing locations of greatest ChAT-  
793 eGFP expression amongst ILC2 clusters as defined through t-SNE in (A). **C)** Clustering analysis carried  
794 out by t-distributed stochastic neighbour embedding (t-SNE) as in (A) but with the parameter of ChAT-  
795 eGFP expression included (left) and heatmap plot of ChAT-eGFP expression to identify ChAT<sup>+</sup> cell  
796 clusters, designated C1, C2 and C3 (right). **D)** Representative histogram overlays of indicated marker  
797 expression for the indicated populations identified within a given biological samples. **E)** Contributions  
798 of populations C1, C2, C3 to total ChAT-eGFP<sup>+</sup> ILC2 present in the lungs of ChAT-eGFP<sup>BAC</sup> mice at  
799 D7 p.i, expressed as the contributing proportion of each population to the total. **F)** Representative  
800 histogram overlays for the indicated marker expressions of total ChAT-eGFP<sup>+</sup> and ChAT-eGFP<sup>neg</sup>  
801 ILC2s (identified as live, single cells, CD45<sup>+</sup>Lineage<sup>neg</sup>CD127<sup>+</sup>ICOS<sup>+</sup>CD90<sup>+</sup> and expressing either or  
802 both ST2 and/or IL-17RB) present in the lungs of naïve and infected ChAT-eGFP<sup>BAC</sup> mice at D7 p.i..  
803 **G)** Mean fluorescence intensity (MFI) of ST2 expressed by total ILC2 and ChAT-eGFP negative and  
804 ChAT-eGFP<sup>+</sup> ILC2s in the lungs of naïve and infected mice as in (F). **H)** As for (G) but for ICOS  
805 geometric MFI (gMFI). Data are representative of observations and population comparisons made in n  
806 = 3 male ChAT-eGFP<sup>BAC</sup> mice in both naïve and infected treatment groups. Data shown as mean ± SEM  
807 and analyzed by Shapiro-Wilk normality testing followed by Welch's t-test, \*p<0.05, \*\*p<0.01. n.s. =  
808 non-significant (p>0.05).

809

810 **Figure 3. Mesenteric lymph node ILC2s upregulate expression of ChAT following infection with**  
811 ***Nippostrongylus brasiliensis*.** **A)** Representative flow cytometry gating indicating identification of total  
812 ILC2s and ChAT-eGFP<sup>+</sup> and ChAT-eGFP<sup>neg</sup> ILC2s from the mesenteric lymph nodes (MLNs) of naïve  
813 and infected ChAT-eGFP<sup>BAC</sup> mice at D7 p.i. *N. brasiliensis* -infected C57BL/6J control sampled  
814 included to indicate ChAT-eGFP<sup>+</sup> gating control. **B)** Quantification of ChAT-eGFP<sup>+</sup> ILC2s from naïve  
815 and D7 p.i. ChAT-eGFP<sup>BAC</sup> mice expressed as a proportion of total ILC2s as shown in (A). **C)**  
816 Quantification of the number of total, ChAT-eGFP<sup>+</sup> and ChAT-eGFP<sup>neg</sup> ILC2s from MLNs of naïve and  
817 D7 p.i. ChAT-eGFP<sup>BAC</sup> mice. **D)** Representative histogram overlays for the indicated marker

818 expressions of total ChAT-eGFP<sup>+</sup> and ChAT-eGFP<sup>neg</sup> MLN ILC2s of naïve and infected ChAT-  
819 eGFP<sup>BAC</sup> mice at D7 p.i.. **E**) Mean fluorescence intensity (MFI) of ST2 expressed by total ILC2 and  
820 ChAT-eGFP negative and ChAT-eGFP<sup>+</sup> ILC2s in the MLNs of naïve and infected mice as in (D). **F**) As  
821 for (E) but for ICOS geometric MFI (gMFI). **G**) Representative flow cytometry plot of ChAT-eGFP  
822 expression by small intestinal lamina propria (siLP) ILC2s in naïve ChAT<sup>BAC</sup>-eGFP animals. Gate  
823 number represents proportion of ILC2 parental gate. Data are representative of observations and  
824 population comparisons made in n = 3 male ChAT-eGFP<sup>BAC</sup> mice in both naïve and infected treatment  
825 groups, other than data shown in (G) which are representative of results in n = 2 mice. Data shown as  
826 mean  $\pm$  SEM and analyzed by Shapiro-Wilk normality testing followed by Welch's t-test, \*p<0.05,  
827 \*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

828

829 **Figure 4. The cholinergic phenotype of pulmonary ILC2s is induced by IL-25 and IL-33 and is**  
830 **augmented by IL-2.** **A**) Proportion of pulmonary ILC2s expressing ChAT-eGFP following sorting by  
831 FACS and 24 hr culture with medium only (control), IL-33 or IL-7. **B**) Proportion of ILC2s in the lungs  
832 of ChAT<sup>BAC</sup>-eGFP mice, 24 h following intranasal dosing with PBS, IL-33, IL-25, or thymic stromal  
833 lymphopietin (TSLP). **C**) As for (B) but for mean fluorescence intensity (MFI) of eGFP expressed by  
834 ILC2s. **D**) As for (A) but for the parental leukocyte populations indicated. **E**) Representative flow  
835 cytometry plot of IL-17RB expression by lung ILC2s in naïve ChAT<sup>BAC</sup>-eGFP animals. Gate number  
836 represents proportion of ILC2 (CD45<sup>+</sup>CD90<sup>+</sup>Lineage<sup>-</sup>CD127<sup>+</sup>ICOS<sup>+</sup> then ST2<sup>+</sup> and/or IL-17RB<sup>+</sup> live  
837 single cells). **F**) Quantification of IL-17RB<sup>+</sup> lung ILC2s as in (E). **G**) Proportion of ChAT-eGFP<sup>+</sup> ILC2s  
838 following *in vitro* culture of FACS purified total lung CD45<sup>+</sup> cells for 24 h with the indicated  
839 combinations of IL-33, IL-25 and IL-2. **H**) As for (G) but for eGFP MFI of ILC2s. n = 3-5 animals per  
840 group. Data (A-D) and (G-H) are representative of N = 2. Data (E-F) are representative of n = 3 mice  
841 per treatment group. Graphs present mean  $\pm$  SEM. Analysis carried out by Shapiro-Wilk test followed  
842 by Welch's t-test. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, n.s. = non-significant (p>0.05).

843

844 **Figure 5. RoR $\alpha$ -driven disruption of ChAT expression impairs pulmonary type 2 immunity and**  
845 **expulsion of *Nippostrongylus brasiliensis* from the gut.** **A**) Recovery of worms from the lungs and

846 intestines of *Chat*<sup>LoxP</sup> and *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice at Day 2 (D2) and D6 post infection (p.i.) respectively,  
847 with *N. brasiliensis* (*Nb*) (N = 2-3). **B**) Total number of eosinophils in the lungs of *Nb*-infected *Chat*<sup>LoxP</sup>  
848 and *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice at D6 p.i (N = 1). **C**) RT-qPCR analysis of *Il13* (left) and *Il5* (right) transcript  
849 expression in lung tissue of *Nb*-infected *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice, represented as fold change against  
850 expression in infected *Chat*<sup>LoxP</sup> lung samples. Each data point represents results from 1 animal. **D**) as for  
851 (C) but for analysis of *Muc5ac* and *Muc5b* expression. **E**) Representative lung sections from D6 *Nb*-  
852 infected *Chat*<sup>LoxP</sup> and *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice (2 per genotype) showing Periodic Acid-Schiff<sup>®</sup> (PAS) and  
853 Hematoxylin and Eosin (H&E) staining (scale bar: 100  $\mu$ m). **F**) Quantification of PAS stained lung  
854 sections (n = 5 mice per group, data representative of N = 2). Data show mean  $\pm$  SEM and analyzed by  
855 Mann-Whitney U test, \* $p < 0.05$ , \*\* $p < 0.01$ , n.s. = non-significant ( $p > 0.05$ ).

856

857 **Figure 6. Impaired immunity to *Nippostrongylus brasiliensis* in *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice is associated**  
858 **with defective intestinal epithelial effector responses.** **A**) Representative intestinal sections from *N.*  
859 *brasiliensis* (*Nb*)-infected *Chat*<sup>LoxP</sup> and *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice at D7 p.i. (2 per genotype) showing  
860 Periodic Acid-Schiff<sup>®</sup> (PAS) staining (scale bar: 50  $\mu$ m) at 40x and 100x magnification. **B**)  
861 Quantification of PAS stained sections from (A). **C**) Representative immunofluorescence staining for  
862 doublecortin-like kinase 1 (*Dclk1*) expressing Tuft cells (green) with DAPI counterstain (blue) in jejunal  
863 sections of *Nb*-infected *Chat*<sup>LoxP</sup> and *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice at D7 p.i. at 20x magnification (scale bar:  
864 50  $\mu$ m). **D**) Quantification of *Dclk1*<sup>+</sup> Tuft cells in crypt (left), villus (center) and crypt+villus (right)  
865 regions of sections as in (C). **E**) Quantification of *Dclk1*<sup>+</sup> Tuft cells as in (H), expressed as the ratio of  
866 cells in villus/ crypt regions per biological replicate sample. Data represent n= 5-6 mice per experimental  
867 group, N = 2. Data shown as mean  $\pm$  SEM and analyzed by Mann-Whitney U test, \* $p < 0.05$ , \*\* $p < 0.01$ ,  
868 \*\*\* $p < 0.001$ , n.s. = non-significant ( $p > 0.05$ ).

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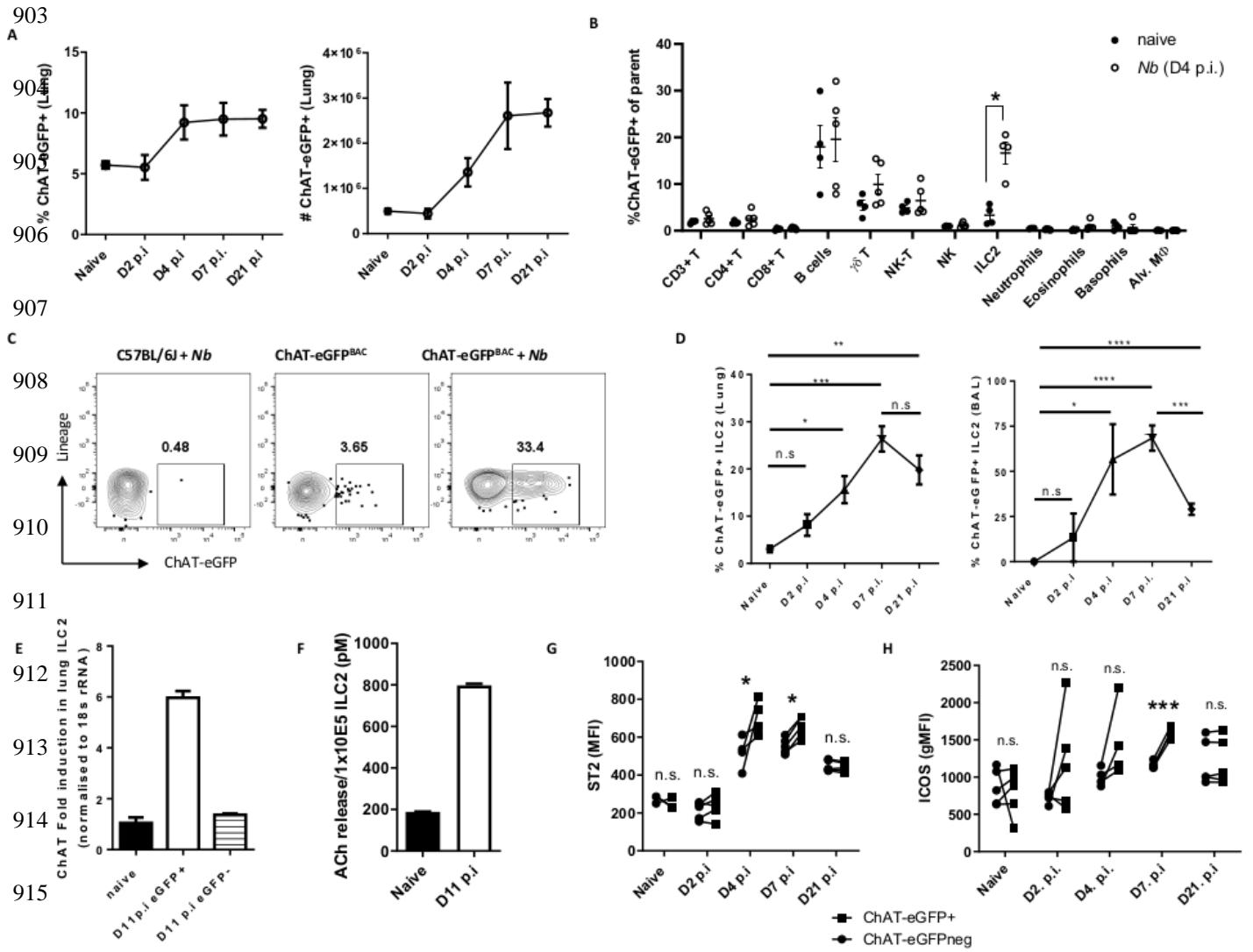
870 **Figure 7. RoRa-driven disruption of ChAT expression in ILC2 limits ILC2 proliferation.** **A**)  
871 Number of ILC2s in the lungs of naïve and *N. brasiliensis* (*Nb*)-infected *Chat*<sup>LoxP</sup> and *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup>  
872 mice at D6 p.i. **B**) Total number of IL-13<sup>+</sup> (left), IL-5<sup>+</sup> (center) and IL-13<sup>+</sup>IL-5<sup>+</sup> ILC2s (right) in the  
873 lungs of naïve and *Nb*-infected (D6 p.i.) *Chat*<sup>LoxP</sup> and *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice. **C**) Representative flow



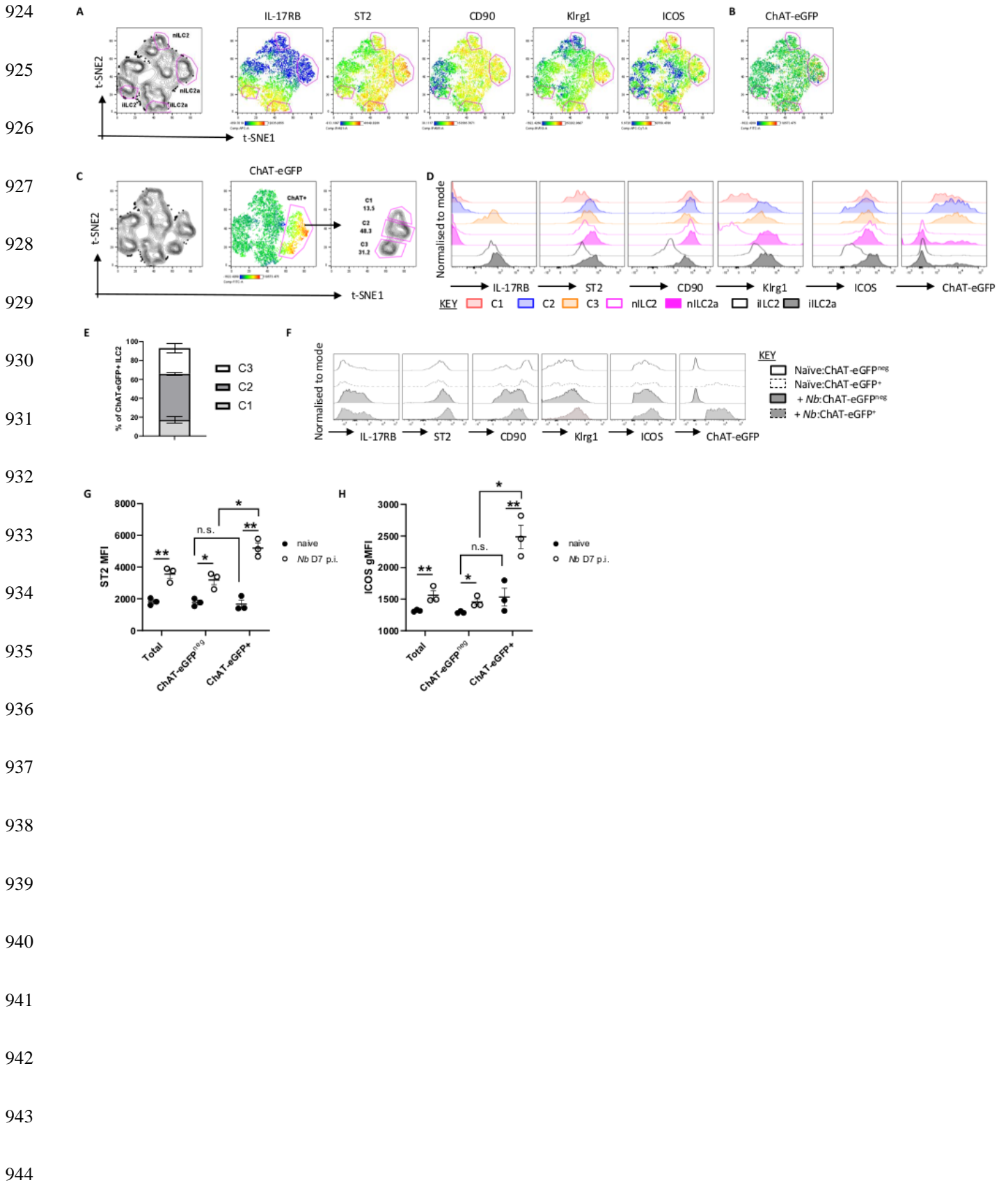
874 cytometry plots of Ki67 staining in lung ILC2s from naïve and *Nb*-infected *Chat*<sup>LoxP</sup> and  
875 *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice at D6 p.i. Gate numbers represent proportion of ILC2 parental gate. Positive gate  
876 set with fluorescence-minus-one control for Ki67. **D)** Summary data for proportion of Ki67<sup>+</sup> lung ILC2s  
877 from naïve and infected *Chat*<sup>LoxP</sup> and *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice. **E)** as for (D) but for total number of Ki67<sup>+</sup>  
878 ILC2. **F)** Representative histogram overlays for ICOS expression by lung ILC2s from naïve and *Nb*-  
879 infected *Chat*<sup>LoxP</sup> and *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice at D6.p.i. As all ILC2s are ICOS<sup>+</sup>, ICOS<sup>neg</sup> ILCs  
880 (CD45<sup>+</sup>CD90<sup>+</sup>Lineage<sup>-</sup>CD127<sup>+</sup>ICOS<sup>neg</sup>) from *Nb*-infected *Chat*<sup>LoxP</sup> mice are also shown as a biological  
881 negative control for ICOS expression. **G)** Summary data for ICOS mean fluorescence intensity (MFI)  
882 of lung ILC2s from naïve and infected *Chat*<sup>LoxP</sup> and *Rora*<sup>Cre+</sup>*Chat*<sup>LoxP</sup> mice. **H)** Muscarinic acetylcholine  
883 receptor (mAChR) subtype expression analysis (*Chrm1-5*) conducted on cDNA from FACS sorted  
884 ChAT-eGFP<sup>+</sup> and ChAT-eGFP<sup>neg</sup> lung ILC2s of *Nb*-infected ChAT-eGFP<sup>BAC</sup> mice, by endpoint PCR  
885 analysis, visualized by agarose gel electrophoresis. C57BL/6J brain tissue cDNA from was prepared  
886 and used as a positive control for AChR gene expressions. **I)** As for (H) but for analysis of nicotinic  
887 acetylcholine receptor (nAChR) alpha subunits (*Chrna1-5,7*) and beta subunits (*Chrb1-4*) expressions.  
888 **J)** Representative flow cytometry plots and summary data (**K)** for Ki67 expression (represented as %  
889 of ILC2 parent) by FACS sorted C57BL/6J female lung ILC2s from *N. brasiliensis* -infected mice,  
890 cultured for 72 hours *in vitro* with recombinant IL-7 and IL-2 only (50 ng ml<sup>-1</sup>) or in the presence of 10  
891 µM of the muscarinic receptor antagonist 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-  
892 DAMP) or the nicotinic receptor antagonist mecamylamine hydrochloride (mecamylamine), or with an  
893 equivalent volume of reagent vehicles (dH2O, DMSO) added to the culture medium (vehicle). **L)** Data  
894 from (K) expressed as vehicle control normalized values for each independent experimental run. Data  
895 A-G n = 5 mice/ group, N = 2. Data H-I are representative of cells pooled from n = 6 *Nb*-infected ChAT-  
896 eGFP<sup>BAC</sup> mice, sorted as ChAT-eGFP<sup>+</sup> and ChAT-eGFP<sup>neg</sup> lung ILC2s at D11 p.i. Data J-L are  
897 representative of N = 4 with similar results (raw data shown in K, using cells pooled from n = 5 mice  
898 per experiment). Data show mean ± SEM and analyzed by Mann-Whitney U test, \*p<0.05, \*\*p<0.01,  
899 \*\*\*p< 0.001, \*\*\*\*p<0.0001, n.s. = non-significant (p>0.05).

901 **Figures**

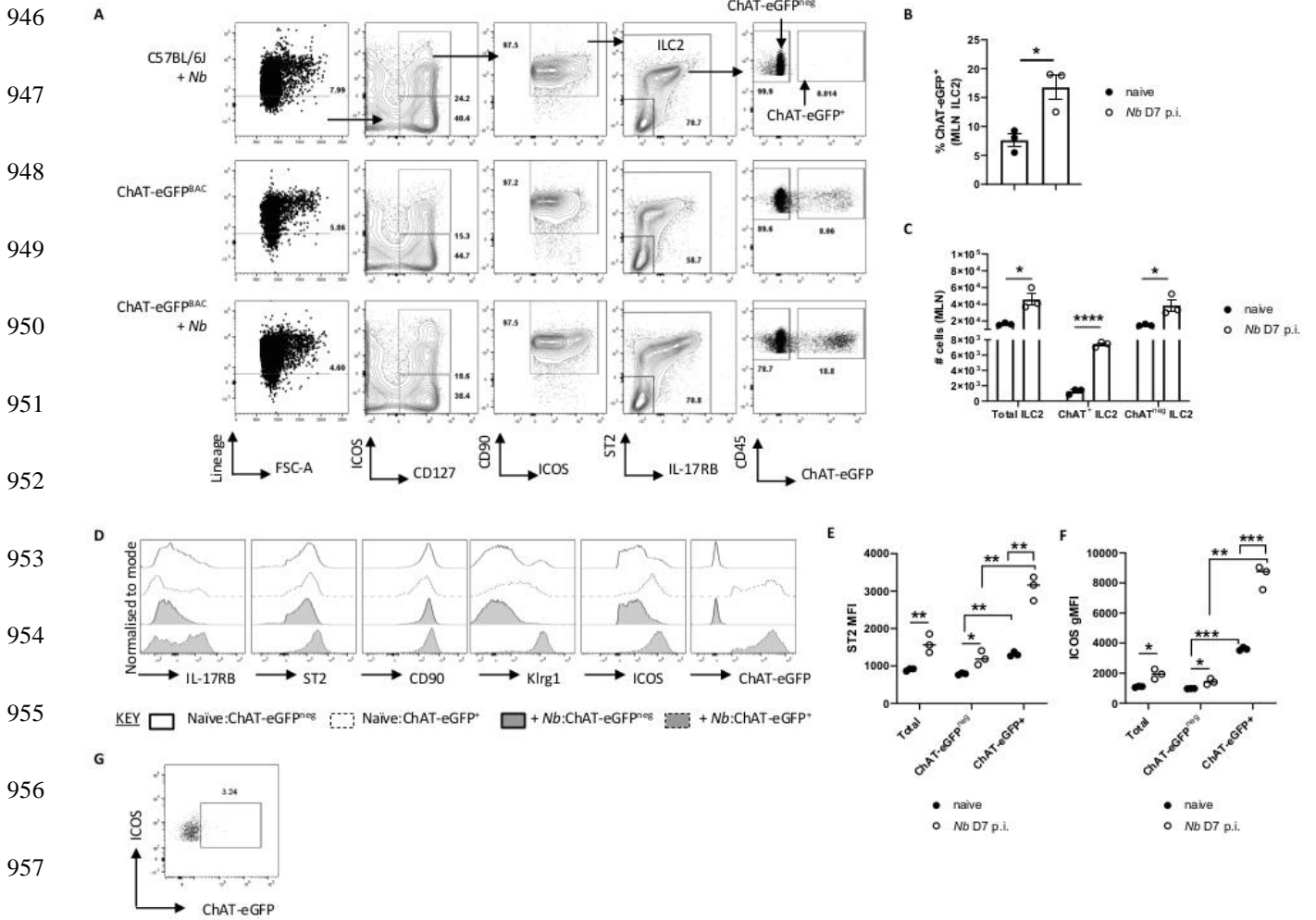
902 **Figure 1**



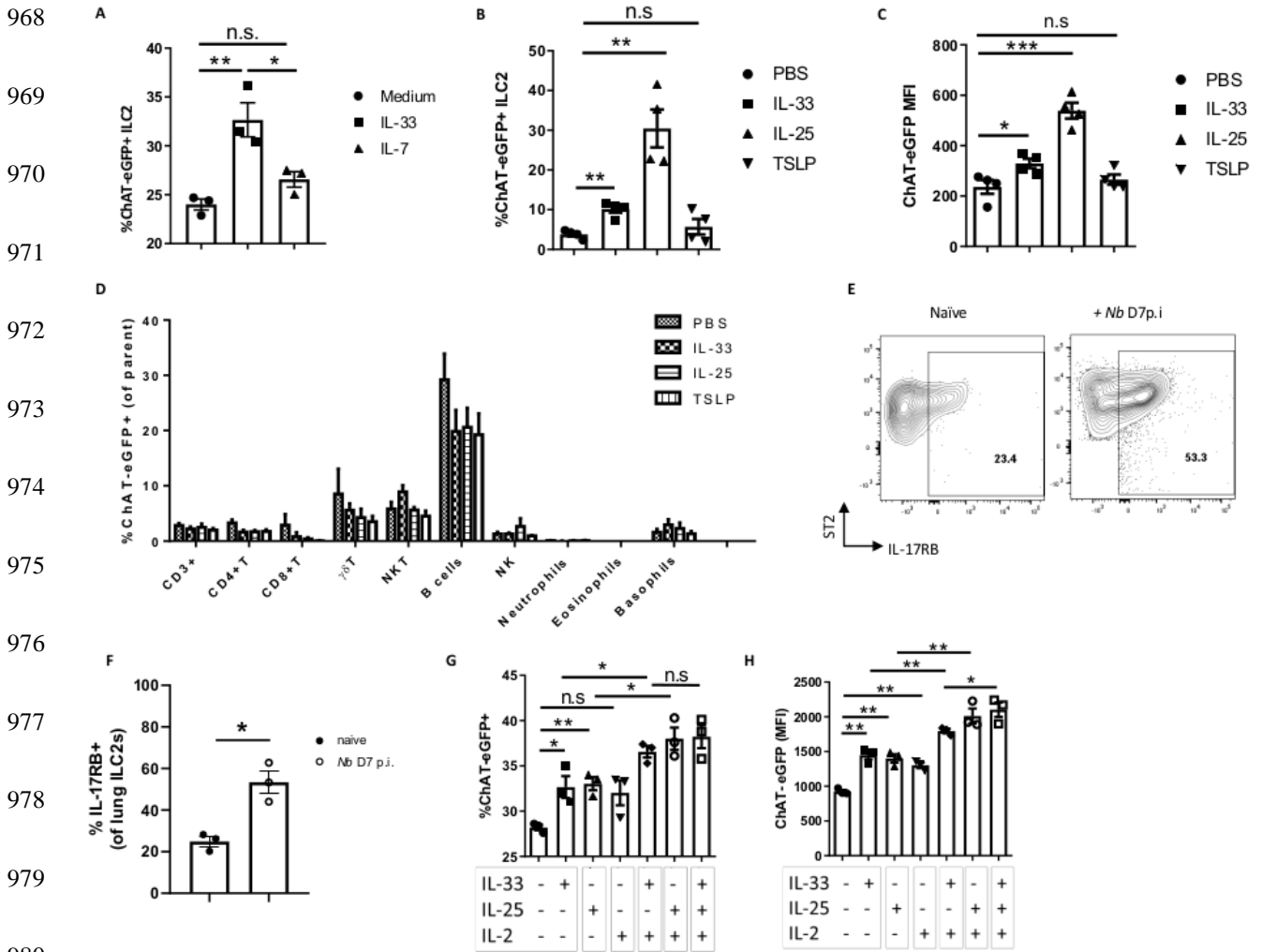
923 **Figure 2**



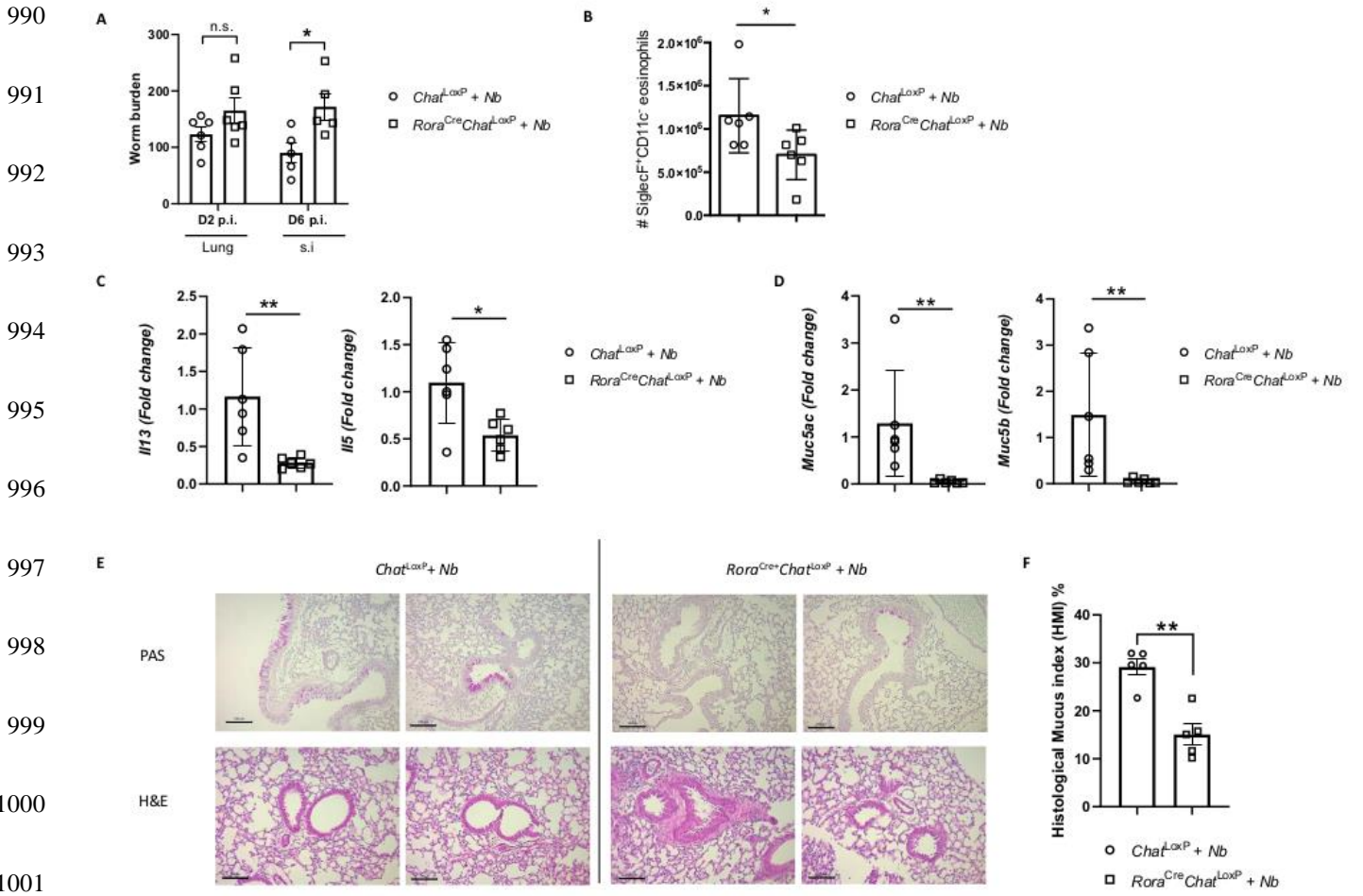
945 **Figure 3**



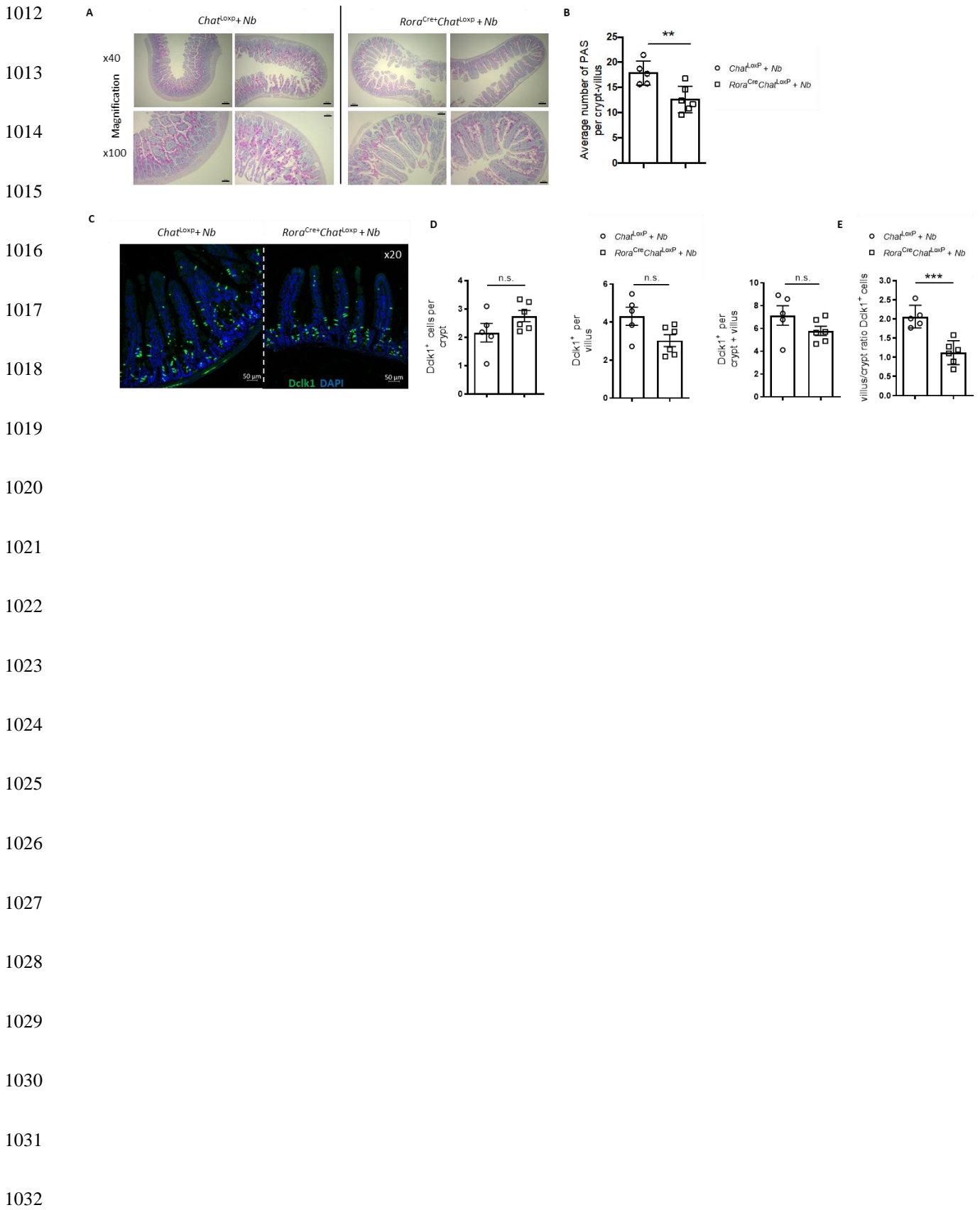
967 **Figure 4**



989 **Figure 5**



1011 **Figure 6**



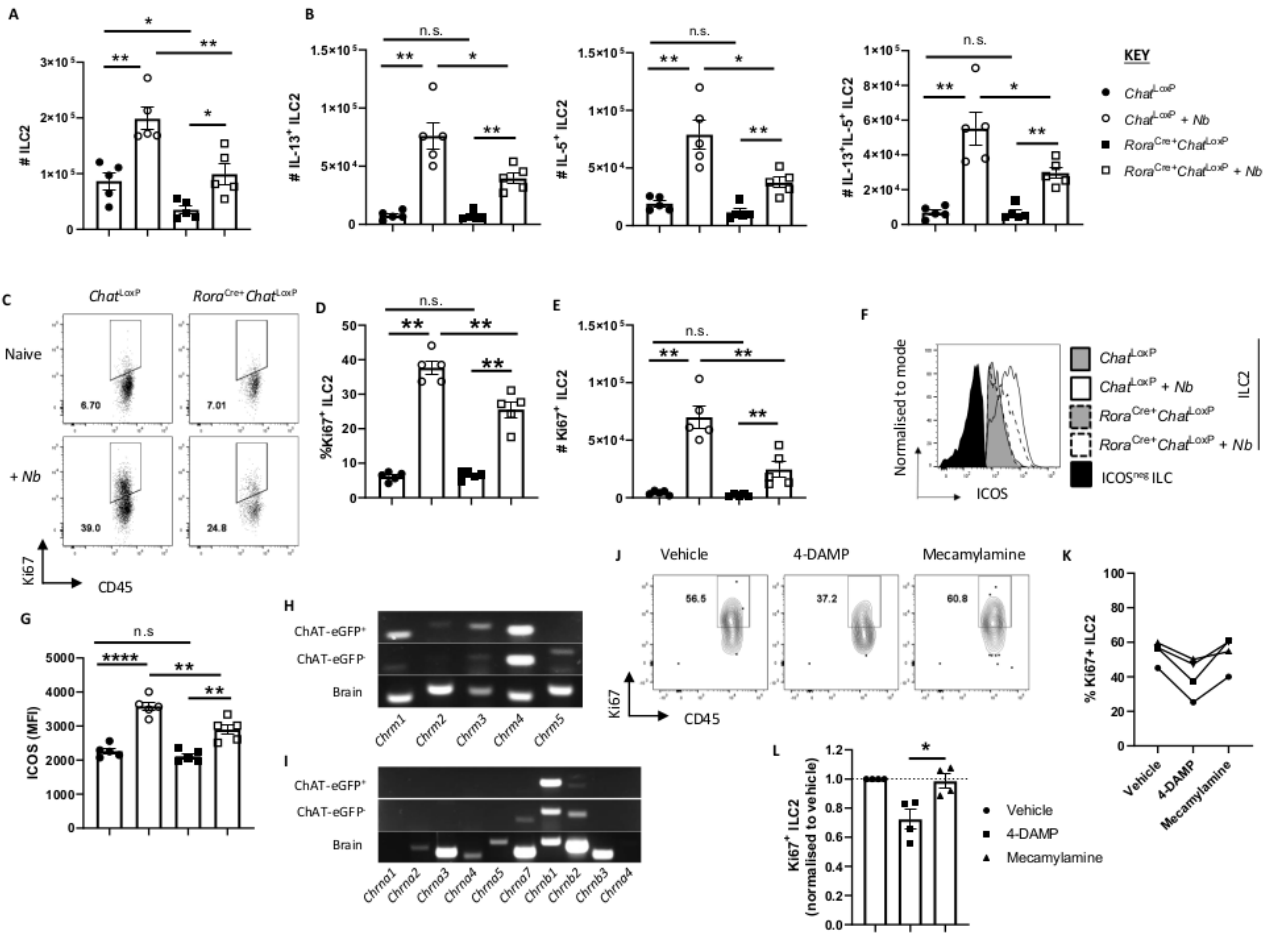
1033 **Figure 7**

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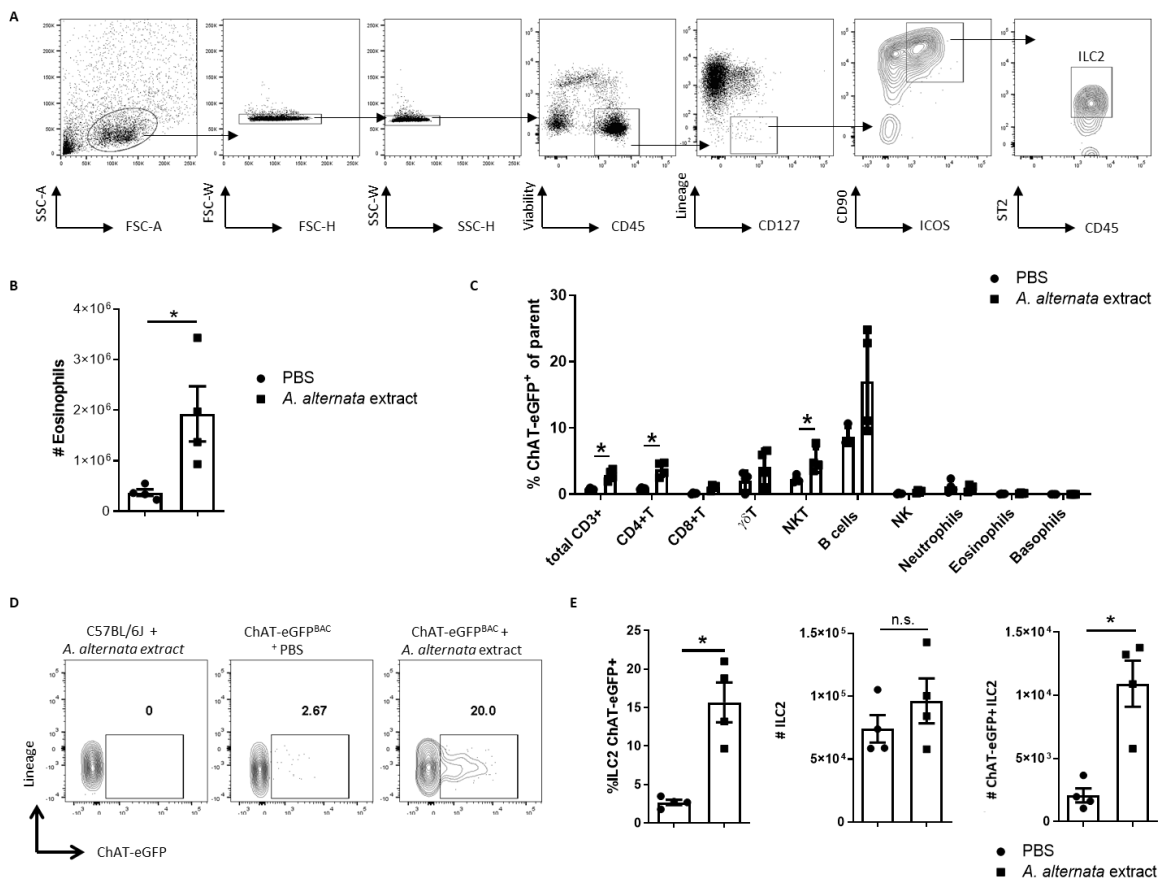
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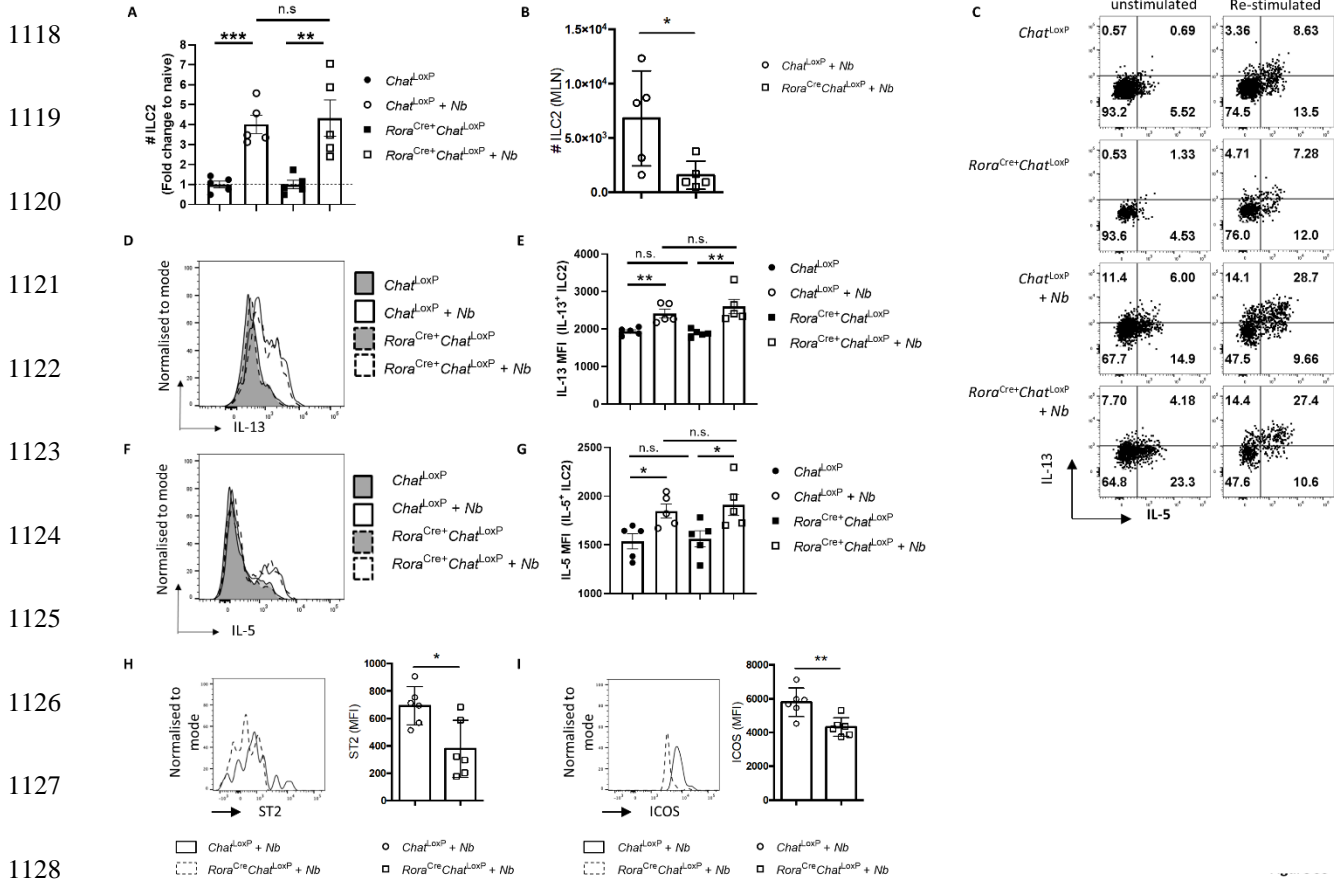
1055 **Supplementary Materials**

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1082 **Figure S1. Pulmonary ILC2s acquire a cholinergic phenotype following exposure to *Alternaria***  
 1083 ***alternata*.** **A)** Representative gating strategy for identification of ILC2s by flow cytometry, as used in  
 1084 this study, unless otherwise stated. Tissue shown here is mouse lung. **B)** Intranasal administration of  
 1085 *Alternaria alternata* extract stimulates pulmonary eosinophilia. **C)** Proportion of parental cell  
 1086 populations expressing ChAT-eGFP in the lungs of ChAT<sup>BAC</sup>-eGFP mice 24 hrs following intranasal  
 1087 dosing with PBS (vehicle control) or *A. alternata* extract. **D)** Representative flow cytometry plots of  
 1088 ChAT-eGFP expression by ILC2s in lungs of wild type C57BL/6J mice exposed to *A. alternata* extract  
 1089 (eGFP gating control), or ChAT<sup>BAC</sup>-eGFP mice dosed with PBS or *A. alternata* allergenic extract. **E)**  
 1090 ILC2 responses in the lungs of ChAT<sup>BAC</sup>-eGFP mice 24 hrs following intranasal dosing with PBS or *A.*  
 1091 *alternata* extract including (from left to right): proportion (%) of ChAT-eGFP<sup>+</sup> ILC2s, total number (#)  
 1092 of ILC2s in the lung, and total number of ChAT-eGFP<sup>+</sup> ILC2s. n = 4 mice/group. Data are representative  
 1093 of N=2 and represent mean  $\pm$  SEM. \*p<0.05, n.s. = non-significant (p>0.05).





1129 **Figure S3. Pulmonary ILC2 cytokine measurements and assessment of numbers and activation**  
 1130 **markers of mesenteric lymph node ILC2s in  $Chat^{LoxP}$  and  $Rora^{Cre+} Chat^{LoxP}$  mice.** **A)** Number of  
 1131 ILC2s in naïve and *N. brasiliensis* (*Nb*)-infected  $Rora^{Cre+} Chat^{LoxP}$  and  $Chat^{LoxP}$  mice expressed as the  
 1132 fold change from the average number of ILC2s in naïve mice of each genotype (normalized to a value  
 1133 of 1). **B)** Number of ILC2s from mesenteric lymph nodes (MLN) of  $Rora^{Cre+} Chat^{LoxP}$  and  $Chat^{LoxP}$  mice  
 1134 infected with *Nb*. **C)** Representative flow cytometry plots for intracellular staining of lung ILC2 for IL-  
 1135 13 and IL-5 in unstimulated (Monensin only) and re-stimulated (PMA/Ionomycin+ Monensin) culture  
 1136 conditions. Quadrants were set using fluorescence minus one controls for IL-13 and IL-5. Quadrant  
 1137 numbers represent the proportion of the ILC2 parental population. **D)** Representative histogram overlays  
 1138 of IL-13 intracellular staining of lung ILC2 from re-stimulated lung samples and **(E)** summary of IL-13  
 1139 mean fluorescence intensity (MFI) analysis of those samples. **F)** and **(G)** as for **(D)** and **(E)** respectively,  
 1140 but for intracellular staining of IL-5. **H)** Representative histogram overlays for ST2 expression by MLN  
 1141 ILC2s from infected  $Chat^{LoxP}$  and  $Rora^{Cre+} Chat^{LoxP}$  mice (left) and summary data for ST2 mean  
 1142 fluorescence intensity (MFI) of MLN ILC2s (right). **I)** As for **(H)** but for ICOS expression by MLN

1143 ILC2s. Timepoints of infection were D6 p.i. in (A), (C-G) ( n = 5 mice per group, N = 2) and D7 p.i. in  
1144 (B), (H-I) (n = 6 mice per group, N=1). Timepoints Data represent mean  $\pm$  SEM and analysed by Mann  
1145 Whitney U test. \* $p < 0.05$ , n.s. = non-significant ( $p > 0.05$ ).

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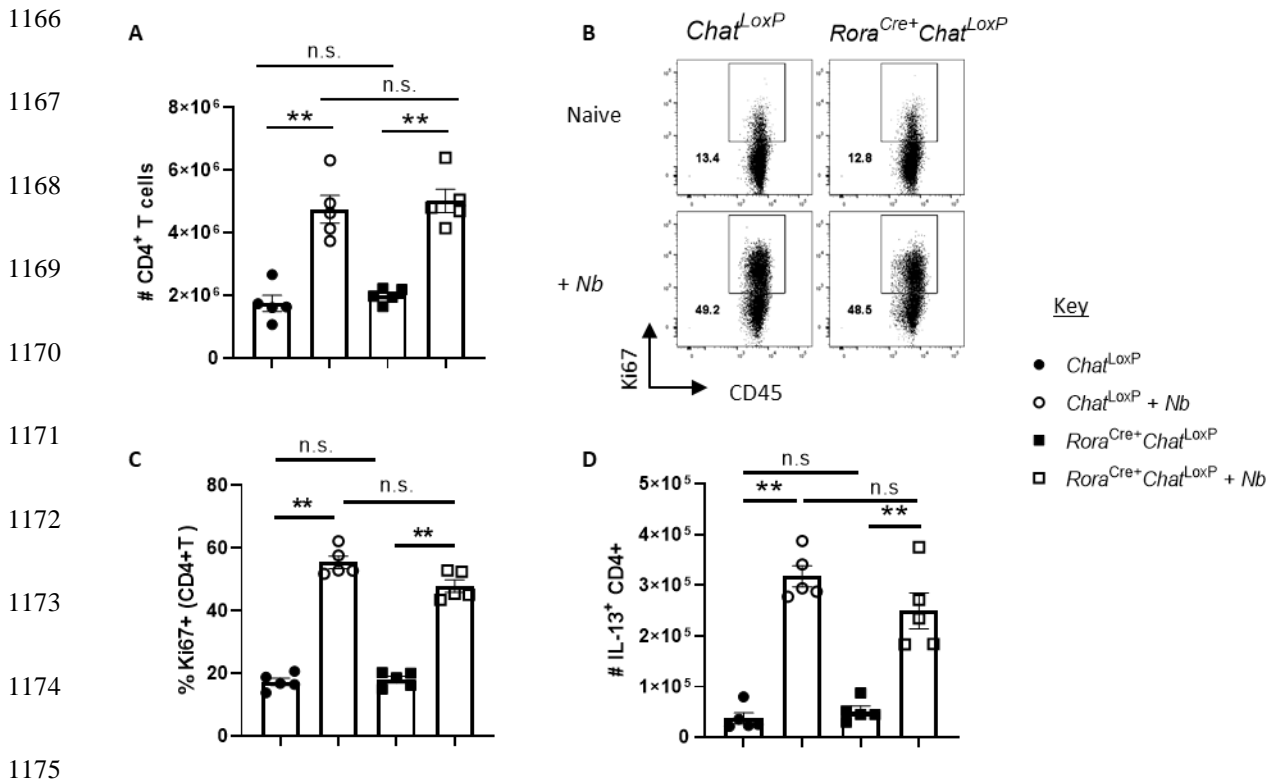
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1176 **Figure S4. Pulmonary CD4<sup>+</sup> T cell numbers and capacity for Th<sub>2</sub> cytokine expression remains**  
 1177 **intact in *Rora<sup>Cre+</sup>Chat<sup>loxP</sup>* mice. A)** Total number of CD4<sup>+</sup> T cells in the lungs of naïve and infected  
 1178 *Chat<sup>LoxP</sup>* and *Rora<sup>Cre+</sup>Chat<sup>LoxP</sup>* mice. **B)** Representative flow cytometry plots of Ki67 staining in lung  
 1179 CD4<sup>+</sup> T cells from naïve and infected *Chat<sup>LoxP</sup>* and *Rora<sup>Cre+</sup>Chat<sup>LoxP</sup>* mice. Gate numbers represent  
 1180 proportion of ILC2 parental gate. Positive gate set with fluorescence minus one control for Ki67. **C)**  
 1181 Proportion of lung CD4<sup>+</sup> T cells from naïve and *N. brasiliensis* (*Nb*)-infected *Chat<sup>LoxP</sup>* and  
 1182 *Rora<sup>Cre+</sup>Chat<sup>LoxP</sup>* mice expressing Ki67. **D)** Number of IL-13<sup>+</sup> CD4<sup>+</sup> T cells in the lungs of naïve and  
 1183 infected *Chat<sup>LoxP</sup>* and *Rora<sup>Cre+</sup>Chat<sup>LoxP</sup>* mice. n = 5 mice per group, N = 2. Timepoints of infection were  
 1184 D6 p.i. Data represent mean ± SEM and analysed by Mann Whitney U test. \*p<0.05, \*\*p<0.01, n.s. =  
 1185 non-significant (p>0.05).

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Dated

2015

- (1) Partner XY
- (2) INEM - UMR7355, MOLECULAR IMMUNOLOGY, UNIVERSITY AND CNRS

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## Materials Transfer Agreement

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## **MATERIALS TRANSFER AGREEMENT**

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1195 **THIS AGREEMENT** is made on

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### **BETWEEN**

1197

1. The University XY

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2. INEM - UMR7355, Molecular Immunology, University and CNRS, 3b rue de la

1199

Ferollerie F-45071 Orleans - Cedex 2, France ("the Recipient Organisation")

1200

### **BACKGROUND**

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1202

The parties have agreed that the University will provide the Recipient Organisation with

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the biological materials as described below and the related Confidential Information as

1204 defined below, which is the confidential and proprietary property of the University, upon  
1205 the terms and conditions set out in this Agreement.

1206 **OPERATIVE PROVISIONS**

1207 **1. INTERPRETATION**

1208 1.1 In this Agreement the following expressions have the following meanings unless  
1209 inconsistent with the context:



|                                   |   |
|-----------------------------------|---|
| <b>“Confidential Information”</b> | any and all knowledge, know-how, information, and/or techniques of a confidential or proprietary nature disclosed by the University to the Recipient Organisation relating to the Materials including, without limitation, all products, inventions, biological material, systems of production, research, data, specifications, software programs and samples, designs, photographs, drawings, plans, prototypes, models, documents, recordings, instructions, formulae, methodologies, processes, manuals, papers or other materials of any nature whatsoever, whether written or otherwise, relating to same |
| <b>“Intellectual Property”</b>    | all intellectual and industrial property rights, including without limitation, patents, rights in know-how, trade marks, registered designs, models, unregistered design rights, unregistered trade marks and copyright (whether in drawings, plans, specifications, designs and computer software or otherwise), database rights, topography rights, any rights in any invention, discovery or process, and applications for and rights to apply for any of the foregoing, in each case in the United Kingdom and all countries in the world   |
| <b>“Materials”</b>                | <i>Rora<sup>Cre</sup>Chat<sup>LoxP</sup> mice</i> , supplied by the University to the Recipient Organisation and all unmodified progeny generated from the materials supplied and that part of all derivatives and the derivatives progeny which contains any of the materials supplied or its progeny  |
| <b>“Modifications”</b>            | substances created by the Recipient Organisation which contain or incorporate the Materials   |
| <b>“Purpose”</b>                  | has the meaning set out in <b>clause 4.1.1</b>  |
| <b>“Recipient Scientist”</b>      | the principal scientist employed by the Recipient Organisation whose name is specified in the <b>Appendix</b>   |

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1.2 All references to the Materials shall be taken to include any and all information and Intellectual Property to which the Recipient Organisation may be given access to by the University relating to or in connection with the Materials, including without limitation, data, formulae, processes, designs, photographs, drawings, specifications, software programs and samples and any other such material, in each case, however disclosed.

**2. OWNERSHIP**

2.1 *All Intellectual Property in the Materials and the physical embodiment of the Materials themselves will remain the property of the University.*

2.2 *All rights (including all Intellectual Property) relating to the Materials and all Modifications shall remain the property of or rest in the University.*

1228 **3. DELIVERY OF MATERIALS**

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1230 3.1 The University shall send the Materials to the Recipient Organisation.

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1232 3.2 The University shall provide the Recipient Organisation with a copy of any  
1233 protocols which the University may have concerning the handling, storage and  
1234 safety of the Materials.

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1236 3.3 Should any government permit or licence be required for the transfer of the  
1237 Materials to the Recipient Organisation, the Recipient Organisation shall obtain  
1238 such permit or licence at its entire cost and expense and shall supply the same to  
1239 the University prior to the despatch of the Materials.

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1241 **4. USE OF THE MATERIALS**

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1243 4.1 The Recipient Organisation agrees that:

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1245 *4.1.1 the Materials are provided to the Recipient Organisation on a non-exclusive*  
1246 *basis only for the purposes of research use only in laboratory animals or in*  
1247 *vitro experiments ("**the Purpose**") and not for administration to human*  
1248 *subjects, for clinical or diagnostic purposes involving human subjects, or*  
1249 *for commercial purposes;*

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1251 *4.1.2 the Materials are to be used only at the Recipient Organisation's premises*  
1252 *and only in the Recipient Scientist's laboratory at those premises;*

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1254 *4.1.3 the Materials will be handled and stored in accordance with any reasonable*  
1255 *protocols provided to the Recipient Organisation in accordance with **clause***  
1256 ***3.2;***

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1258 4.1.4 the Materials will be used only by individuals working within the Recipient  
1259 Organisation, and will not be transferred, distributed, or released to any  
1260 other person, firm or institution; *and*

1261 *4.1.5 the Materials are not made available to anyone other than employees of*  
1262 *the Recipient Organisation engaged in carrying out the Purpose and shall*  
1263 *not be further distributed to others without the University's prior written*  
1264 *consent. The Recipient Organisation shall refer any request for the*  
1265 *Materials to the University.*

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1267 4.2 The Recipient Organisation agrees to use the Materials in compliance with all  
1268 applicable statutes and regulations and under suitable containment conditions.

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1271 **5. CONFIDENTIALITY**

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1273 5.1 In consideration of the University disclosing the Materials to the Recipient  
1274 Organisation, the Recipient Organisation agrees to keep all Confidential  
1275 Information associated with the Materials which is disclosed by the University to  
1276 the Recipient Organisation secret and confidential and not to disclose or transfer  
1277 the same or permit the same to be disclosed or transferred to any third party for  
1278 any reason whatsoever.

1279

1280 5.2 The Recipient Organisation will keep any confidential materials passed to the  
1281 Recipient Organisation by the University at the premises of the Recipient  
1282 Organisation in a secure environment, protected against theft, damage, loss,  
1283 misuse or unauthorised access.

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1285 **6. RESULTS & COMMERCIALISATION**

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1287 6.1 The Recipient Organisation will inform the University in confidence of research  
1288 results relating to or created using the Materials by written communication or by  
1289 providing the University with a manuscript describing the results of such research  
1290 at the time the manuscript is submitted for publication. If publication results from  
1291 research using the Materials, acknowledgement of and/or credit will be given to  
1292 the University as the source of the Materials.

1293

1294 6.2 If the Recipient Organisation or any of its employees, including the Recipient  
1295 Scientist, wishes to include in a publication any information which has been  
1296 provided by the University and which is "confidential" the Recipient Organisation  
1297 shall not publish without written permission from the University and shall provide  
1298 the University with a copy of the proposed text before publication takes place.

1299

1300 6.3 The Recipient Organisation shall have no right to use or permit the use of any  
1301 products or processes containing, using or directly derived from the Materials for  
1302 profit making or commercial purposes ("**Commercial Use**"). If the Recipient  
1303 Organisation wishes to make Commercial Use of the Materials or any product  
1304 directly derived from the Materials it shall request a licence from the University.  
1305 The University shall have no obligation to grant any such licence to the Recipient  
1306 Organisation.

1307

1308 6.4 Nothing in this Agreement, including any Intellectual Property protection being  
1309 sought by the Recipient Organisation on any new use made of the Materials, shall  
1310 prevent the University from being able to distribute the Materials to other  
1311 commercial or non-commercial entities.

1312

1313

1314 **7. CONSIDERATION**

1315

1316 7.1 The Materials are provided at no cost.

1317

1318 **8. TERM AND TERMINATION**

1319

1320 8.1 Unless terminated in accordance with **clause 8.2**, this Agreement shall take effect  
1321 from the date set out at the beginning of this Agreement and shall remain in full  
1322 force and effect for a period of 10 years.

1323

1324 8.2 The University may terminate this Agreement if the Recipient Organisation is in  
1325 material breach of any of its terms and, where the breach is capable of remedy,  
1326 the Recipient Organisation has failed to remedy the same within one month of  
1327 service of a written notice from the University specifying the breach and requiring  
1328 it to be remedied.

1329

1330 8.3 Notwithstanding any early termination of this Agreement, the obligations on the  
1331 Recipient Organisation created in this Agreement shall survive and continue to be  
1332 binding upon the Recipient Organisation, its successors and assigns for 3 years  
1333 from the date of termination or expiry of this Agreement.

1334

1335 8.4 Upon the termination or expiry of this Agreement, the Recipient Organisation shall  
1336 cease using the Confidential Information and the Materials in any manner  
1337 whatsoever and, upon written request by the University, the Recipient  
1338 Organisation shall deliver up to the University or destroy all of the Confidential  
1339 Information and Materials in or under the Recipient Organisation's possession or  
1340 control.

1341

1342 **9. LIABILITY**

1343

1344 9.1 All characteristics of the Materials are not fully understood and their use may  
1345 involve risks or dangers that are not known or fully appreciated. The Materials are  
1346 being provided on an "as is" basis, without warranty of any sort, express or implied  
1347 and the University will not be liable for any use made of the Materials or any claim  
1348 that the Materials infringe the intellectual property rights of third parties.

1349

1350 9.2 So far as is permitted by law, the Recipient Organisation assumes all liability for  
1351 damages which may arise from its receipt, use, storage or disposal of the Materials  
1352 and it will hold the University and its employees harmless from any loss, claim,  
1353 damage or liability, of any kind which may arise from or in connection with this  
1354 Agreement or the use, handling or storage of the Materials. In no event shall the  
1355 University be liable for any use by the Recipient Organisation of the Materials or

1356 any loss, claim, damage or liability, of any kind which may arise from or in  
1357 connection with this Agreement or the use, handling or storage of the Materials.

1358

1359 **10. GENERAL**

1360 10.1 The Recipient Organisation shall not assign, transfer, charge or otherwise dispose  
1361 of any or all of the rights, duties or obligations granted to it under this Agreement  
1362 without the prior written consent of the University.

1363

1364 10.2 This Agreement may be executed in one or more counterparts each of which shall  
1365 for all purposes be deemed to be an original and all of which shall constitute one  
1366 and the same instrument. Each party agrees that executed counterparts may be  
1367 exchanged by email as scanned pdf copies.

1368

1369 10.3 This Agreement and any non-contractual obligations arising out of or in connection  
1370 with it shall be governed by and construed in all respects in accordance with the  
1371 laws of England and the parties hereby submit to the non-exclusive jurisdiction of  
1372 the English Courts.

1373

1374 **On behalf of The University XY**

1375

1376 Name:

1377

1378 Signature:

1379

1380 Position:

1381

1382 Date:

1383

1384 **On behalf of INEM - UMR7355, Molecular Immunology, University and CNRS**

1385

1386 Name:

1387

1388 Signature:

1389

1390 Position:

1391

1392 Date:

1393

## APPENDIX 1

|                     | <b>Recipient Organisation's Scientist</b>  |
|---------------------|--|
| <b>Name</b>         | <b>Bernhard Ryffel</b>   |
| <b>Title</b>        | <b>MD, PhD</b>   |
| <b>Full address</b> | <b>INEM - UMR7355, Molecular Immunology, University and CNRS,<br/>3b rue de la Ferrollerie F-45071 Orleans - Cedex 2, France</b> |