

1 **Title: Inception of early life allergen induced airway hyperresponsiveness is reliant on**

2 **IL-13⁺CD4⁺ T cells**

3

4 **One Sentence Summary:**

5 **The lymphoid cellular source of IL-13 driving AHR is age dependent.**

6

7

8

9 **Authors:**

10 S. Saglani^{1,2*}, L. G. Gregory¹, A. K. Manghera¹, W. J. Branchett¹, F. Uwadiae¹, L. J.
11 Entwistle¹, R. A. Oliver¹, J. E. Vasiliou¹, R. Sherburn¹, S. Lui¹, F. Puttur¹, Vöhringer D⁴, S.
12 A. Walker¹, J. Buckley¹, R. Grychtol¹, V. Fainardi¹, L. Denney¹, A. Byrne¹, E. von Mutius³,
13 A. Bush², C. M. Lloyd^{1*}

14

15 **Affiliations:**

16 1 Inflammation, Repair & Development, National Heart & Lung Institute, Imperial College
17 London, London, United Kingdom.

18 2 Respiratory Paediatrics, Royal Brompton Hospital & National Heart & Lung Institute,
19 Imperial College London, London, United Kingdom.

20 3 Asthma and Allergy Research Group, Munich University, Lindwurmstrasse 4, D 80337
21 Munich, Germany.

22 4 Universitätsklinikum Erlangen, Friedrich-Alexander Universität Erlangen-Nürnberg
23 Wasserturmstrasse 3-5, 91054 Erlangen, Germany

24

25 **Corresponding Authors:**

26 Professor Clare M Lloyd or Professor Sejal Saglani

27 Sir Alexander Fleming Building

28 Imperial College London

29 Exhibition Road

30 London SW7 2AZ

31 Tel: +44 2075943102, Fax: +44 2045943118

32 Email: c.lloyd@imperial.ac.uk, s.saglani@imperial.ac.uk

33

34

35

36

37

38

39

40

41

42

43

44

45

46

47

48

49

50

51

52 **Abstract**

53 Airway hyperresponsiveness (AHR) is a critical feature of wheezing and asthma in children,
54 but the initiating immune mechanisms remain unconfirmed. We demonstrate that both rIL-33
55 and allergen (house dust mite (HDM), or *Alternaria alternata*) exposure from day 3 of life
56 resulted in significantly increased pulmonary IL-13⁺CD4⁺ T cells which were indispensable for

57 the development of AHR. In contrast, adult mice had a predominance of pulmonary
58 Lin^{neg}CD45⁺CD90⁺IL-13⁺ innate lymphoid cells (ILC2s) following administration of rIL-33.
59 HDM exposure of neonatal IL-33KO mice still resulted in AHR. However, neonatal CD4^{cre}IL-
60 13 KO mice (lacking IL-13⁺CD4⁺ T cells) exposed to allergen from day 3 of life were protected
61 from AHR despite persistent pulmonary eosinophilia, elevated IL-33 levels and IL-13⁺ ILCs.
62 Moreover, neonatal mice were protected from AHR when inhaled *Acinetobacter Iwoffii* (an
63 environmental bacterial isolate found in cattle farms which is known to protect from childhood
64 asthma) was administered concurrent with HDM. *A. Iwoffii* blocked the expansion of
65 pulmonary IL-13⁺CD4⁺ T cells while IL-13⁺ ILCs and IL-33 remained elevated. Administration
66 of *A. Iwoffii* mirrored the findings from the CD4^{cre}IL-13 KO mice, providing a translational
67 approach for disease protection in early life. These data demonstrate that IL-13⁺CD4⁺ T cells,
68 rather than IL-13⁺ ILCs or IL-33 are critical for inception of allergic airways
69 hyperresponsiveness in early life.

70

71

72

73

74 **Introduction**

75 The key pathophysiological abnormalities of allergic asthma include airway
76 hyperresponsiveness (AHR), eosinophilic inflammation and remodelling (1). Childhood onset
77 disease is common, affecting approximately 10% of children, and is characterised by the key
78 clinical symptom of recurrent wheeze (2). Approximately one-third of all children develop
79 wheezing in the first 5 years of life, but only one-third of those will develop asthma (3).
80 However, the mechanisms by which allergic immune responses are initiated and the factors
81 that mediate onset of pre-school wheezing and progression to asthma are currently unidentified
82 (4, 5). AHR is a central feature of recurrent wheezing in children who develop asthma, and
83 impaired lung function (6, 7) and AHR shortly after birth (8, 9), are known to be associated
84 with asthma in adolescence and adulthood (10).

85

86 The importance of innate immunity, specifically innate lymphoid cells (ILCs), in the inception
87 of allergic asthma is increasingly proposed (11). However, during pregnancy there is a change
88 in the uterine environment towards a Th2 cytokine profile and the thymic microenvironment is
89 Th2-skewed in the early postnatal period and undergoes age-related suppression in favor of
90 increasing Th1 maturation (12). Despite this, the current dogma is that pulmonary type 2 ILCs,
91 not CD4⁺ T cells, are the primary cellular source of type 2 cytokines (IL-5 and IL-13) in early
92 life (13). Even though allergic asthma begins in childhood (14), mechanistic studies of allergic
93 airways disease had predominantly used adult experimental models (15-17), thus disregarding
94 the specific developmental effects of postnatal immune maturation (18). A number of recent
95 studies have utilized age appropriate murine neonatal models and have demonstrated that in
96 C57BL/6 mice perinatal type 2 immunity depends on IL-33 which is immediately upregulated
97 from the first day of life and drives accumulation and activation of IL-13 secreting ILC2s and
98 pulmonary eosinophils following house dust mite exposure (13, 19, 20). However, the

99 predominant clinical manifestation in infants and pre-school children is recurrent wheezing
100 with associated AHR and reduced lung function (21), but neonatal studies to date have not
101 investigated the mechanisms driving AHR.

102

103 Age dependent maturation of the immune system occurs following birth once the neonate
104 encounters the antigen rich external environment (22). The composition of the airway bacterial
105 profile *in utero* and in early life is also important since exposure to a diverse bacterial mix,
106 such as that found on traditional cattle farms has been shown to protect from the development
107 of allergy and asthma (23, 24). The capacity of the adaptive immune system to induce memory
108 responses is limited and is thought to gradually develop following early-life environmental
109 exposure to microbes, pollutants and allergens (25). Interestingly, a population of fetally
110 derived CD4⁺ T cells with an effector memory phenotype are present in cord blood. These cells
111 develop during fetal life but have a variety of effector inflammatory functions associated with
112 CD4⁺ T helper cells at birth (26). However, little is known about the phenotype or function of
113 tissue specific (pulmonary) effector T cells in early life. Studies in infants have shown allergen
114 induced immune responses in whole blood mononuclear cells can be detected at birth with IL-
115 13 predominating following stimulation with the egg protein ovalbumin (27). A differential
116 developmental pattern of IL-13 vs IL-4, IL-5, and IFN- γ production was evident in infants in
117 the first 3 months of life (28). Although these data implicate IL-13 in the inception of early
118 life allergic immune responses in children, there is little direct mechanistic evidence,
119 particularly for identification of a cellular source for IL-13 during this crucial period.

120

121 We have previously demonstrated that exposure of neonatal mice from day 3 of life to inhaled
122 house dust mite (HDM) promotes robust eosinophilia, Th2 type immune responses and AHR
123 (29). We show here that the cellular source of type 2 mediators in neonatal mice is not restricted

124 to ILCs but that IL-13 secreting CD4⁺ T cells are crucial for the development of AHR in early
125 life. Additionally, we show that IL-33, which is elevated in school-age children with severe
126 asthma, and has been linked to airway remodelling, is not a requirement for the initiation of
127 allergic airways disease. Moreover, protection from AHR was achieved in neonatal mice using
128 inhaled farmyard bacteria administered concomitantly with HDM, with a selective reduction
129 in IL-13⁺CD4⁺ T cells and IL-13, despite elevated IL-33 and IL-13⁺ ILCs. Our data
130 demonstrate the cellular source of IL-13 is essential in determining the development of early
131 life AHR and underpins the concept of a window of immune development in early life that has
132 implications for development of AHR.

133

134 **Results**

135 *IL-13⁺CD4⁺T cells are induced by IL-33 in neonatal mice.*

136 IL-33 is sufficient to generate allergic airway responses in adult mice via the induction of type
137 2 ILCs, and without the development of an adaptive immune response (30). Whilst ILCs have
138 been shown to drive type 2 immunity in some models, their role in initiating AHR in early life
139 has not been investigated. In order to determine whether ILCs are also the predominant cellular
140 source of IL-13 in neonatal mice, we delivered intra-nasal rIL-33 for 2 weeks to adult mice or
141 neonatal mice from day 3 of life (Fig. 1A) and enumerated IL-13⁺CD4⁺ T cells and ILCs by
142 flow cytometry (fig. S1, A and B). There are numerous published strategies to define an ILC,
143 using combinations of extracellular markers and intracellular cytokine or transcription factor
144 expression (31). However, expression of cell surface markers by ILCs is variable and context-
145 dependent (32). Given the importance of IL-13 in driving the pathological features of early life
146 airway disease we focussed on cytokine secreting cells, initially gating on pulmonary
147 Lin^{neg}CD45⁺IL-13⁺ cells and then examined the expression of the extracellular ILC markers
148 CD127, CD90, CD25, ST2 and ICOS within this population (fig. S1C). We found that in

149 neonatal BALB/c mice only CD90 reliably marked the IL-13⁺ ILC population at steady state
150 and during IL-33-driven inflammation (fig. S1C), therefore we used Lin^{neg}CD45⁺CD90⁺IL-13⁺
151 as our definition for ILC2. ST2, CD25 and ICOS were variable expressed, being present on
152 only 30-40% of ILC2 at baseline (PBS treated) although this increased to 75% on rIL-33
153 induced ILC2 (fig. S1C & D). CD127 staining showed little separation from fluorescence
154 minus one controls in BALB/c ILC2s under all conditions tested (fig. S1E), precluding its
155 usefulness as a definitive marker of ILC2 populations in this strain of mice. Notably, more
156 substantial CD127 staining was observed on ILC2 in C57BL/6 mice, both at steady state and
157 during allergic airway inflammation (fig. S1E), suggesting that strain differences exist in
158 murine ILC2 surface phenotype. Importantly, Lin^{neg}CD45⁺CRTH2⁺ ILC2 in children with
159 STRA are also predominantly CD127^{neg} (33). Collectively, these findings rationalise the use of
160 Lin^{neg}CD45⁺CD90⁺ IL-13⁺ as a robust definition of functional IL-13 producing ILC2 in
161 neonatal BALB/c mice.

162 There was a significant difference in the balance of IL-13⁺ lymphoid cellular phenotypes
163 observed in the lungs of adult and neonatal mice exposed to the innate cytokine rIL-33. In adult
164 mice, as expected the effect was predominantly on induction of IL-13⁺ ILCs, compared to IL-
165 13⁺CD4⁺ T cells (CD3⁺CD4⁺) (Fig. 1B). In contrast, neonatal mice responded to rIL-33 with
166 comparable increases in levels of both IL-13⁺CD4⁺ T cells and IL-13⁺ ILCs (Fig. 1C). Both
167 adult and neonatal mice developed significant eosinophilia following challenge with rIL-33
168 and this response was greatest in adult mice (Fig 1D). In addition, rIL-33 increased airway
169 resistance at both ages, concomitant with increased IL-13 levels (Fig. 1 E-H). Levels of IL-5
170 were also increased in mice of both ages although the magnitude of the response was greatest
171 in adult mice (Fig. 1I). Although 2 weeks exposure to cytokine is too short to observe
172 phenotypic changes in airway remodelling, increases in the mucin genes Muc5ac and Muc5b,
173 the principle components of airway mucous, were observed in both neonatal and adult mice

174 (Fig. 1 J&K). Muc5ac has been demonstrated to be necessary for the development of AHR, is
175 increased in asthmatic patients including children and is regulated by levels of IL-13 (34).

176

177 *Both T cells and ILC comprise the neonatal response to allergen.*

178 To determine the cellular source of IL-13 following exposure to an antigenically complex,
179 clinically relevant allergen, we compared intra-nasal HDM exposure for 2 weeks in neonatal
180 mice from day 3 of life to adult mice (Fig. 2). Duration of allergen exposure was limited to 2
181 weeks in order to focus on mechanisms underlying disease inception, before established
182 adaptive allergic immunity with elevated IgE levels. Both neonatal and adult mice developed
183 AHR (Fig. 2, A and B) and eosinophilia (Fig. 2C) in response to allergen. In contrast to the
184 immune response to rIL-33, which in adult mice skewed the IL-13 secreting cells towards an
185 ILC2 dominated response, HDM resulted in significantly elevated numbers of IL-13⁺CD4⁺ T
186 cells (Fig. 2D). In contrast, neonatal mice exposed to HDM had a mixed response with
187 induction of both Th2 and ILC2s (Fig. 2E). However, significantly more IL-13⁺CD4⁺ T cells
188 were induced compared to IL-13⁺ ILCs (Fig 2E). The increase in IL-13⁺ cells, irrespective of
189 their source resulted in elevated levels of pulmonary IL-13 in adult and neonatal mice (Fig.
190 2F). To extend our observations to another clinically relevant allergen we enumerated ILCs
191 and T cells following exposure to the fungal allergen *Alternaria alternata* (fig. S2A), which is
192 associated with severe paediatric asthma (35) and with elevated pulmonary IL-33 (36). In both
193 adult and neonatal mice inhaled *Alternaria* induced a strong inflammatory response with
194 significant elevations of both IL-13⁺CD4⁺ T cells and IL-13⁺ ILCs (Fig. 2, G and H) with
195 associated increased levels of IL-13 (Fig. 2I). The elevated IL-13 concentration in the lung
196 correlated with increased AHR (fig. S2, B and C). Congruent with a type 2 inflammatory
197 response both adult and neonatal mice exhibited significant pulmonary eosinophilia (fig. S2D).

198

199 We next phenotyped the allergen induced T cells present in neonatal lungs and compared them
200 to adults. In the CD4⁺IL-13^{neg} population the proportion of cells with a memory like phenotype
201 (CD44⁺ CD62L^{low/-}) was equivalent in adults and neonates (Fig. 3, A and B), whereas the
202 percentage expressing CD103, an integrin associated with epithelial lymphocyte localisation,
203 and the activation markers CD69 and ICOS was greater in adults compared to neonates (Fig.
204 3, C - F). Examination of the CD4⁺IL-13⁺ T cells revealed similar patterns of extracellular
205 marker expression in cells recovered from both HDM and *Alternaria* treated mice although the
206 proportions of cells expressing specific markers was greatest in mice administered *Alternaria*.
207 In contrast to the IL-13^{neg} population, the proportion of IL-13⁺CD4⁺ T cells with a memory like
208 phenotype was significantly higher in neonates than adults (Fig. 3, A and B). Likewise, more
209 neonatal IL-13⁺CD4⁺ T cells expressed CD103 than adults after HDM (Fig. 3, C and D). A
210 very high proportion of cells expressed CD69 and ICOS in adults and neonatal mice exposed
211 to either HDM or *Alternaria* (Fig. 3, E and F). Thus, the effector T cells in neonatal lungs, far
212 from having an immature phenotype appear analogous to adult T cells.

213 We next characterised the intra-cellular cytokine profile of the T cells. At baseline (PBS control
214 mice) the majority of neonatal cytokine producing CD3⁺CD4⁺ cells had the capacity to secrete
215 IFN- γ and IL-17, whereas this was more limited in cells from adult mice (Fig. 3, G and H).
216 HDM elicited a mixed T cell response with an increase in the proportion of Th2 (IL-5⁺ or IL-
217 13⁺), Th1 (IFN- γ ⁺) and regulatory (IL-10⁺) T cells in both adults and neonates (Fig. 3, I and J).
218 One notable difference in the T cell response to HDM between adults and neonates is that in
219 neonates a high proportion of IL-13⁺CD4⁺ T cells co-express IL-5 but in adults these dual
220 positive cells are relatively rare. Exposure to *Alternaria* stimulated the greatest proliferation of
221 CD4⁺ T cells which predominantly generated IL-13 or IL-17 in neonates (Fig. 3K). In adult
222 mice *Alternaria* polarised T cells to a type 2 phenotype with very few Th17 cells compared to
223 neonates (Fig. 3, K and L).

224 CD4⁺ effector cells have recently been identified in human cord blood (3). We therefore
225 investigated the relative proportions of CD4⁺IL-13⁺ T cells and IL-13⁺ ILCs in cord blood. 50%
226 of CD45⁺ cells were CD4⁺, while only 0.02% were Lin⁻CD161⁺ ILCs (Fig. 3M). In keeping
227 with our observations regarding pulmonary IL-13⁺ cells in neonatal mice, ILC2s in cord blood
228 (from healthy term infants) were not the predominant source of IL-13. Instead CD4⁺ T cells
229 contributed to the potential pool of neonatal IL-13 (Fig. 3N). Neonatal T cells were positive
230 for the T-cell receptor and negative for the CD1d dimer expressed on natural killer T cells (Fig.
231 3O).

232

233

234 *Allergen exposure in neonatal SCID mice protects from AHR, while IL33^{-/-} mice are unaffected.*

235 To determine the functional significance of the pulmonary IL-13⁺CD4⁺ T cells in early life, we
236 exposed neonatal SCID mice, which lack T, B and NK cells, to HDM for 2 weeks (fig. S3A).

237 While the WT mice showed the expected increase in AHR (fig. S3B), the neonatal SCID mice
238 did not exhibit any AHR (fig. S3C). The observed AHR in WT mice correlated with increased

239 levels of IL-13 and IL-33 which were not apparent in SCID mice (fig. S3, D and E). IL-13 is
240 known to be critical for the generation of AHR. However, the dependence of AHR on the innate

241 cytokine IL-33 in neonates has not been investigated. Therefore, we exposed neonatal WT and
242 IL33^{-/-} mice to HDM from day 3 of life. Neonatal IL33^{-/-} mice developed equivalent AHR and

243 an inflammatory response of similar composition and magnitude to WT mice (Fig. 4, A-D).

244 IL-33 was absent in the IL33^{-/-} mice as expected but the type 2 cytokines IL-5 and IL-13 were
245 induced to similar levels in WT and KO mice (Fig. 4, E-G), demonstrating that in neonatal

246 mice AHR is independent of IL-33 but totally reliant on IL-13. These data underscore the
247 functional importance of perinatal IL-13⁺CD4⁺ T cells in inducing AHR in early life and

248 suggest pulmonary T cells are a critical source of IL-13 in the immediate postnatal period.

249

250 *Mice lacking IL-13 in CD4⁺ T cells do not develop AHR in response to allergen.*

251 **In order to confirm the importance of T cell derived IL-13 on the generation of allergen**

252 **induced AHR we exposed either neonatal WT or *Cd4-cre Il-4Il13^{fl/fl}* mice to inhaled HDM**

253 **or *Alternaria*. T cells from *Cd4-cre Il-4Il13^{fl/fl}* mice are unable to generate IL-13 or IL-4**

254 **so permit the specific role of T cell derived IL-4/IL-13 to be investigated.** Initial experiments

255 confirmed the previously observed allergen induced increase in IL-13⁺CD4⁺ T cells in WT

256 mice and established that these cells were absent in the *Cd4-cre Il-4Il13^{fl/fl}* mice (Fig. 5A).

257 Confirming our hypothesis, mice lacking these cells did not develop AHR following exposure

258 to either HDM or *Alternaria* (Fig. 5, B and C). We next determined the composition of the

259 pulmonary cellular infiltrate in these mice. The number of eosinophils (Fig. 5, D and E) and

260 IL-13⁺ ILC (Fig. 5, F and G) were not significantly different between WT and *Cd4-cre Il-*

261 *4Il13^{fl/fl}* mice. Likewise, T cells expressing IL-5 were not affected by the specific loss of IL-

262 13⁺CD4⁺ T cells (Fig. 5, H and I). Despite the presence of increased IL-13⁺ ILC2 with the

263 capacity to secrete IL-13 (Fig. 5, F and G), there was only a minimal increase in pulmonary

264 IL-13 levels following HDM or *Alternaria* exposure (Fig. 5, J and K) in the absence of IL-

265 13⁺CD4⁺ T cells. Expression of Muc5ac was increased in neonates exposed to both HDM and

266 *Alternaria* (Fig. 5, L and M). In mice lacking IL-13⁺CD4⁺ T cells the HDM induced increase

267 in Muc5ac was ablated (Fig. 5L) indicating that expression is dependent on IL-13 levels.

268 However, in mice administered *Alternaria*, allergen induced Muc5ac gene expression was

269 maintained (Fig. 5M). This suggests IL-13⁺ ILC2 alone are not sufficient to generate levels of

270 IL-13 necessary to drive AHR in the absence of IL-13⁺CD4⁺ T cells in early life. Thus, mice

271 lacking IL-13 specifically in CD4⁺ T cells were completely protected from developing AHR

272 following exposure to different allergens, underscoring the importance of these cells in the

273 inception of early life AHR.

274

275 *Therapeutic manipulation of IL-13⁺CD4⁺ T cells protects neonatal mice from developing AHR.*

276 Whilst studies using knockout mice show proof of principle, in order to determine whether it
277 is possible to translate these findings and therapeutically manipulate neonatal IL-13⁺CD4⁺ T
278 cells which drive allergen induced changes to lung function, we investigated the mechanism of
279 action of an immunomodulatory agent, the lyophilised bacteria *Acinetobacter iwoffii*, which is
280 associated with protection from asthma in children (17) and AHR in adult mouse models (20).
281 We administered *A. iwoffii* (a farmyard isolate with strong allergy protective properties) intra-
282 nasally concomitant with HDM to neonatal BALB/c mice from day 3 of life for 3 weeks (Fig.
283 6A). Neonatal mice that received both the inhaled bacteria and HDM were completely
284 protected from the development of AHR (Fig. 6B). Moreover, eosinophils were significantly
285 reduced in the mice that received *A. iwoffii* (Fig. 6C). Pulmonary IL-33 levels remained
286 elevated in all mice that received HDM even those that received the bacterial isolate (Fig. 6D),
287 but in keeping with the AHR results, allergen induced IL-13 levels were almost completely
288 abrogated in the mice that received *A. iwoffii* (Fig. 6E). When the lymphoid cellular source of
289 IL-13 was assessed, numbers of IL-13⁺ ILCs were increased in the mice that received bacteria
290 irrespective of allergen exposure (Fig. 6F), but IL-13⁺CD4⁺ T cells were significantly reduced
291 (Fig. 6G). Levels of allergen induced IL-5 were unaffected by the *A. iwoffii* (Fig. 6H). To
292 further investigate how the *A. iwoffii* prevented the recruitment of IL-13⁺CD4⁺ T cells we
293 enumerated pulmonary dendritic cells. In agreement with data in the literature, exposure to
294 HDM alone resulted in a significant increase in the number of CD11b⁺ cDCs (Fig. 6I). pDC
295 and MoDC numbers were also elevated. However, concomitant exposure to *A. iwoffii*
296 completely blocked the expansion of CD11b⁺ cDCs and MoDCs (Fig.6I) correlating with the
297 reduction in IL-13⁺CD4⁺ T cells and improvement in AHR in these mice. These data confirm
298 the critical role of IL-13 from IL-13⁺CD4⁺ T cells for the generation of neonatal AHR and

299 show that IL-13⁺ ILCs cannot compensate for the absence of IL-13 from T cells in inducing
300 neonatal AHR.

301

302 **Discussion**

303 An essential clinical feature of asthma and wheezing in childhood, including infancy and the
304 pre-school years, is the presence of AHR (37). In addition, cohort studies have shown the
305 single most important factor that determines development of asthma in children is AHR in early
306 life, which may be apparent even before the onset of the manifest symptom of wheezing (38).

307 In order to identify mechanisms mediating allergen induced disease inception, and targets for
308 intervention to allow asthma prevention, achieving a reduction in AHR is essential. However,
309 the underlying molecular mechanisms remained unclear. AHR was the focus of this study in
310 order to optimally reflect paediatric symptoms. We determined that effector IL-13⁺ CD4⁺ cells
311 are critical for the development of AHR following exposure to either the ILC2 promoting
312 cytokine rIL-33 or clinically relevant allergens HDM and *Alternaria* in the first weeks of life.
313 IL-13⁺ ILCs were insufficient to compensate for an absence of IL-13⁺CD4⁺ T cells in early life,
314 demonstrated by the lack of AHR in neonatal SCID mice or in mice specifically lacking IL-13
315 in CD4⁺ T cells following exposure to inhaled allergen. Mice lacking functional T and B cells
316 or IL-13⁺CD4⁺ T cells did not develop increased levels of IL-13 following allergen exposure
317 from day 3 of life despite comparable numbers of IL-13⁺ ILCs. Moreover, administration of
318 inhaled farm dust bacteria during exposure to HDM to neonatal mice resulted in complete
319 protection from AHR with a significant reduction in pulmonary IL-13⁺CD4⁺ T cells and levels
320 of IL-13, but sustained elevation of IL-33 and IL-13⁺ ILCs. Collectively these data show that
321 in neonatal mice, T cells are an essential early source of IL-13 to drive AHR. Our results
322 indicate that although ILCs have the potential to generate IL-13 when stimulated with PMA
323 and ionomycin *in vitro*, in the absence of IL-13⁺CD4⁺ T cells *in vivo* functional levels of IL-13

324 are not generated. Thus, despite the assumption that ILC2s initiate pulmonary allergic immune
325 responses, this is in fact dependent on age, and in neonatal mice IL-13⁺CD4⁺ T cells are critical
326 for disease inception in early life. This has significant implications for therapies to prevent
327 wheeze and asthma inception in childhood, since molecular targets that prevent the induction
328 of type 2 ILCs are unlikely to be effective in preventing early onset disease.

329

330 Key distinctions between adult and neonatal immune responses following HDM exposure were
331 also shown in a neonatal model that used a single dose of allergen to achieve sensitisation at
332 day 3, 14 or adult life, followed by allergen challenge a week later (13) . Similarly, previous
333 neonatal murine studies have shown the importance of the first 2 weeks of life in causing
334 exaggerated immune responses (39), but did not examine associated AHR or interrogate the
335 cellular source of the mediators that potentially generate AHR. We used a model of continuous,
336 low dose allergen exposure, rather than sensitisation followed by allergen challenge several
337 days later, as this reflects the type of exposure that young children likely experience with
338 perennial allergens such as HDM and *Alternaria*. Although ILC2 have been shown to be
339 important in generating Th2 immunity in experiments with papain or helminths in adult mice
340 (40, 41) our data indicate that in neonatal mice IL-13⁺CD4⁺ T cells drive early life allergen
341 induced AHR. **We cannot rule out the role of T cell derived IL-4 in neonatal allergen-**
342 **driven responses, and given the potnetial importance of IL-4, future studies are needed**
343 **to clarify the relative importance of IL-4 and IL-13 production in this process.** In adult
344 mice, an elegant series of experiments using ILC deficient mice reconstituted with naïve CD4⁺
345 T cells has also shown that activation of primed Th2 cells is independent of ILC2s (42).
346 However, in contrast to our findings in neonatal mice, the adult Th2 cell activation was
347 dependent on pulmonary IL-33. Similarly, in response to HDM or papain, adult Th2 cells
348 secreting IL-13 but not IL-4 have been shown to mediate TCR independent, IL-33 dependent

349 innate-like immune responses (43). Thus, interactions between Th2 cells and ILC2 are vital in
350 developing pathophysiology, but are likely to be contextual depending on environmental or
351 temporal factors.

352

353 The *in utero* environment is biased towards Th2 immunity in order to support a successful
354 pregnancy. CD4⁺ effector cells with a memory phenotype have previously been identified in
355 human cord blood (26) and we have also shown the presence of IL-13⁺ CD4⁺ cells, but very
356 few IL-13⁺ILCs in cord blood from healthy newborns. A specific subpopulation of IL-4⁺CD4⁺
357 T cells that are present in cord blood from naïve human neonates, but are lost during ageing,
358 has also been described (44). This distinct subpopulation of IL4⁺CD4⁺ cells was only found in
359 neonates, but not in adults, and supports the hypothesis of an endogenously poised type 2
360 cytokine profile of T cells in neonates and a link between cytokine production and
361 developmental stage (44). Using neonatal BALB/c mice we have shown that CD4⁺ T cells make
362 a vital contribution to the pool of IL-13 in the lung which drives AHR. Caution should therefore
363 be exercised when interpreting data from C57BL/6 mice where allergen appears to induce
364 ILC2s as the major source of IL-13 secreting cells in an IL-33 dependent manner (13). An
365 important factor that determined our use of BALB/c mice is the direct reflection of the disease
366 phenotype of our patients with severe wheezing and asthma, incorporating a marked airway
367 eosinophilia, AHR and remodelling (45, 46), in this strain and protocol of allergen exposure.
368 Previous murine studies, also in BALB/c mice, have shown that polyclonal stimulation of lung
369 T cells results in a bias towards IL-4 and IL-5, and increased ratio of GATA3⁺ T cells compared
370 to T-bet⁺ T cells (47). Interestingly, this would appear to be an intrinsic feature of neonatal T
371 cells since BCG primed lung dendritic cells from either neonates or adults prime adult naïve T
372 cells towards Th1 whereas a Th2 cytokine response is observed from naïve neonatal T cells.
373 Cord blood cells, which are reflective of fetal blood, showed a substantial IL-13 response to

374 allergen stimulation *in vitro*, and the newborns had a Th2 cytokine bias that was restricted to
375 IL-13 (28). Although these results were in peripheral blood rather than the lung, they do
376 indicate that early life is indeed associated with a skewed IL-13 response. We now show that
377 T cell derived IL-13 is critical for the inception of allergen induced lung function changes.

378

379 Our data highlight the critical role of T cell derived IL-13 in the neonatal period. Neonatal mice
380 have a population of IL-13⁺CD4⁺ T cells which have the capacity to rapidly promote AHR
381 when exposed to allergen. Modulating the allergen induced increase in dendritic cells in the
382 lung, and consequently the IL-13⁺CD4⁺ T cells, via a farm dust bacterial isolate specifically
383 abrogates AHR, even while IL-33 and IL-13⁺ ILCs are maintained. The concept of the neonatal
384 ‘window of opportunity’ is gathering momentum with regard to the mucosal microbiota (39)
385 and we now know that life-long immune homeostasis and susceptibility to immune mediated
386 diseases (asthma, allergies, bronchiectasis) can be shaped during the postnatal period (22, 48,
387 49). The specialized neonatal adaptive immune response after birth also has a predisposition to
388 higher expression of GATA-3⁺, type 2 cytokine producing pulmonary T cells (47) as a result
389 of both normal development and in response to environmental exposures. In the current study
390 we have shown that interventional approaches to prevent AHR and asthma in early life need to
391 focus on reducing IL-13⁺CD4⁺ T cells, rather than IL-13⁺ ILCs, highlighting the need for age-
392 specific therapeutic approaches in infants and young children compared to adults.

393

394

395

396

397

398

399

400 **Materials and Methods**

401 *Study Design*

402 This study aimed to determine the immune mechanisms that drive the inception of airway
403 hyperresponsiveness in neonatal mice.

404 *Research samples.*

405 Immune cells were collected from the lung tissue of adult and neonatal mice at the times
406 indicated depending on the experimental setup. Cord blood was collected at delivery of full
407 term pregnancies.

408

409 *Experimental design.*

410 *Randomization*

411 In all experiments, mice from the control and experimental groups came from the same cohorts,
412 were reared under the same environmental conditions, and were age-matched. Adult female
413 mice were randomly placed in either the control group or the experimental group. Neonatal
414 mice were of either sex and litters were randomly assigned to control or experimental groups.

415

416 *Sample size.*

417 The number of mice analyzed for each different experimental approach is indicated on each
418 figure. All experiments were repeated at least once with similar sample sizes and a minimum
419 number of 4 mice per group.

420

421 *Animals and reagents*

422 Female BALB/c wild-type and Beige SCID mice were initially obtained from Charles River
423 (Saffron Walden, UK) and maintained by in-house breeding. *Cd4-cre Il-4^{fl/fl}* mice(50) on

424 a BALB/c background were a kind gift from David Voehringer. *IL33^{-/-}* mice were a kind gift
425 from MedImmune Inc. Each mother with its litter was housed separately. Mice were
426 maintained in specific pathogen-free conditions and given food and water *ad libitum*. In
427 individual experiments all mice were matched exactly for age and background strain. All
428 procedures were conducted in accordance with the Animals (Scientific procedures) Act 1986.
429 Recombinant mouse IL-33 (50µg/kg) for intra-nasal administration was purchased from R&D
430 Systems (UK).

431

432 ***Allergen challenge***

433 In experiments to assess the effect of allergen challenge on allergic airways disease 3 day old
434 neonatal mice and adults (6-8 weeks) received intra-nasal administration of either HDM or
435 *Alternaria alternata* (Greer, Lenior, NC, USA). From birth to 2 weeks of age mice were
436 administered 20µg house dust mite (HDM) extract, 5µg *Alternaria* or 10µl phosphate buffered
437 saline (PBS), three times a week. Adult mice received 25µg HDM or 10µg *Alternaria*. All
438 outputs were assessed at 24 hours after allergen challenge (51).

439

440 ***Bacteria and allergen co-exposure***

441 BALB/c mice were exposed to intermittent intra-nasal *Acinetobacter iwoffii* F78 (*A. iwoffii*
442 F78) (1.3x10⁸cfu first 2 weeks, then 2x10⁸cfu (52) (a kind gift from Johann Bauer) or PBS
443 followed by HDM (10µg first 2 weeks, then 15µg) or PBS for 3 weeks starting on day 3 of life
444 for 3 weeks. All outputs were assessed 24 hours post final challenge as described below.

445

446 ***Measurement of airway hyperresponsiveness***

447 Airways resistance was calculated using the flexivent small animal ventilator (Scireq) using
448 our established protocols (29).

449 Mice were anesthetized with pentobarbital sodium (50mg/kg intra-peritoneal) and ketamine
450 (100mg/kg intra-muscular), tracheostomised and connected to the flexivent ventilator via a
451 blunt-ended 21-gauge needle (neonate) or 19-gauge needle (adult). The mice were ventilated
452 with an average breathing frequency of 150 breaths/minute; tidal volume of 10ml/kg body
453 weight; positive end-expiratory pressure approximately 2cm H₂O. Changes in resistance to
454 increasing to increasing concentrations of nebulized methacholine (3 -3 100mg/ml were
455 calculated from the snapshot perturbation measurements. Resultant data was fitted using
456 multiple linear regression to the single compartment model in the form: pressure = resistance x
457 flow + elastance x volume + fitting constant.

458

459 ***Inflammation and cell recovery***

460 Bronchoalveolar lavage (BAL) was performed with PBS via a tracheal cannula. The volume of
461 BAL fluid instilled was 3 x 200µl aliquots for neonatal mice, 3 x 300 µl for 3 week old mice
462 and 3 x 400µl in adults (29). After lavage, the large left lobe of the lung was mechanically
463 chopped and incubated at 37°C for 1 hour in complete media (RPMI + 10% fetal calf serum,
464 2mM L-glutamine, 100U/ml penicillin/streptomycin) containing 0.15mg/mL collagenase
465 (Type D, Roche Diagnostics) and 25mg/mL DNase (Type 1, Roche Diagnostics). Cells were
466 recovered by filtration through a 70-µm nylon sieve (Falcon, BD Biosciences, MA), washed
467 twice, resuspended in 1ml complete media, and counted in a haemocytometer (Immune
468 Systems). The total cell yield was quantified by haemocytometer. All cell counts were
469 performed blind by the same observer.

470

471 ***Flow cytometry***

472 To reduce non-specific binding, cells were incubated with rabbit serum (Sigma) for 15 minutes
473 before staining. Where staining for intracellular cytokines, single cell suspensions were

474 incubated at 37 °C in complete RPMI for 4 hours, in the presence of 20 ng/ml phorbol 12-
475 myristate 13-acetate (PMA, Sigma-Aldrich), 1.5µg/ml ionomycin free acid from Streptomyces
476 conglobatus (Merck) and 5µg/ml Brefeldin A (Sigma-Aldrich). Cells were subsequently
477 washed in PBS and stained with LIVE/DEAD™ Fixable Blue Dead Cell Stain (Thermo
478 Fisher/Life Technologies), as per manufacturer's directions, before washing twice in PBS. Cell
479 suspensions were then stained with fluorochrome-conjugated monoclonal antibodies to surface
480 markers (see table/supplementary methods) in staining buffer (PBS containing 1% BSA and
481 0.01% sodium azide) for 20 minutes at 4 °C. Cells were then washed twice in staining buffer
482 and fixed in IC Fixation Buffer (Thermo Fisher/eBioscience) for 15 minutes at room
483 temperature. Where necessary, fixed cells were permeabilized using Permeabilization Buffer
484 (Thermo Fisher/eBioscience) and stained with fluorochrome-conjugated antibodies to
485 intracellular cytokines (see table/supplementary methods) in Permeabilization buffer for 20
486 minutes at 4 °C. 'Fluorescence minus one' (FMO) controls for extracellular and intracellular
487 antigens were used on matched tissue samples for quality control purposes and to assist with
488 gating. Data were acquired on an LSR Fortessa using FACSDIVA™ software (both BD) and
489 analysed using FlowJo software (v10, Tree Star). For ILC identification in mouse samples,
490 lineage exclusion gates consisting of the surface markers TCRβ, TCRγδ, CD3e, CD5, CD19,
491 CD11b, CD11c, FCεR1, GR-1, F4/80, NKp46 and TER-119 were used (Supplementary table
492 1). For ILC identification in human samples, a lineage exclusion gate consisting of CD14,
493 CD16, CD19, CD20, CD3 and CD56 was employed.

494

495 ***Quantification of cytokines***

496 Lung tissue was homogenized at 50mg/ml in HBSS (Gibco) containing protease inhibitor
497 tablets (Roche Diagnostics), centrifuged at 800 x g for 20minutes and the supernatant was
498 collected. Cytokines were analyzed in lung homogenate supernatants. Paired antibodies for

499 mouse interleukin IL-33 (R&D Systems) and IL-5 (BD Biosciences) were used in
500 standardized sandwich ELISA's according to the manufacturer's protocols. IL-13 was
501 measured using a Quantikine kit (R&D Systems) as per the manufacturer's protocol.

502

503 *qPCR*

504 RNA was extracted from the lung using the Qiagen miRNeasy Plus Mini Kit, following the
505 manufacturer's instructions. Reverse transcription was performed with 1-2 µg RNA using the
506 High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), following the
507 manufacturer's instructions. Generated cDNA was used for quantitative real-time PCR analysis
508 using TaqMan® Fast Advanced Master Mix (Applied Biosystems) and quantified on the ViiA
509 7 (Applied Biosystems). Relative gene expression was determined via normalisation to the
510 housekeeping gene Gapdh. All TaqMan® primers were purchased from ThermoFisher
511 Scientific. Primers: Gapdh (Mm99999915_g1), Dye: FAM-MGB. Il5 (Mm00439646_m1),
512 Dye: FAM-MGB. Il13 (Mm00434204_m1), Dye: FAM-MGB. Muc5ac (Mm01276726_g1),
513 Dye: FAM-MGB. Muc5b (Mm00466391_m1), Dye: FAM-MGB.

514

515 *Statistical analysis*

516 All results were expressed as median and interquartile range and data were analyzed using
517 GraphPad Prism 7 software (GraphPad Software). Non-parametric tests (Mann Whitney U)
518 were used to detect differences between groups and statistical significance accepted when $p <$
519 0.05 . * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$

520

521 **Supplementary Materials**

522 **Fig. S1. Defining IL-13⁺ CD4⁺ T cells and ILCs in neonatal mice.**

523 **Fig. S2. *Alternaria* induces AHR in neonatal mice.**

524 **Fig. S3. Allergen exposure in neonatal SCID mice does not result in AHR.**

525 **Table S1. Antibodies used for flow cytometry.**

526

527

528

529 **References and notes:**

530

531 1. A. Papi, C. Brightling, S. E. Pedersen, H. K. Reddel, Asthma. *Lancet* **391**, 783-800
532 (2018).

533 2. L. J. Griffiths, R. A. Lyons, A. Bandyopadhyay, K. S. Tingay, S. Walton, M. Cortina-
534 Borja, A. Akbari, H. Bedford, C. Dezateux, Childhood asthma prevalence: cross-
535 sectional record linkage study comparing parent-reported wheeze with general
536 practitioner-recorded asthma diagnoses from primary care electronic health
537 records in Wales. *BMJ Open Respir Res* **5**, e000260 (2018).

538 3. R. Granell, A. J. Henderson, J. A. Sterne, Associations of wheezing phenotypes with
539 late asthma outcomes in the Avon Longitudinal Study of Parents and Children: A
540 population-based birth cohort. *J Allergy Clin Immunol* **138**, 1060-1070 e1011
541 (2016).

542 4. C. E. Berry, D. Billheimer, I. C. Jenkins, Z. J. Lu, D. A. Stern, L. B. Gerald, T. F. Carr, S.
543 Guerra, W. J. Morgan, A. L. Wright, F. D. Martinez, A Distinct Low Lung Function
544 Trajectory from Childhood to the Fourth Decade of Life. *Am J Respir Crit Care Med*
545 **194**, 607-612 (2016).

546 5. I. D. Pavord, R. Beasley, A. Agusti, G. P. Anderson, E. Bel, G. Brusselle, P. Cullinan, A.
547 Custovic, F. M. Ducharme, J. V. Fahy, U. Frey, P. Gibson, L. G. Heaney, P. G. Holt, M.
548 Humbert, C. M. Lloyd, G. Marks, F. D. Martinez, P. D. Sly, E. von Mutius, S. Wenzel,
549 H. J. Zar, A. Bush, After asthma: redefining airways diseases. *Lancet* **391**, 350-400
550 (2018).

551 6. F. D. Martinez, W. J. Morgan, A. L. Wright, C. J. Holberg, L. M. Taussig, Diminished
552 lung function as a predisposing factor for wheezing respiratory illness in infants.
553 *N Engl J Med* **319**, 1112-1117 (1988).

554 7. S. W. Turner, L. J. Palmer, P. J. Rye, N. A. Gibson, P. K. Judge, M. Cox, S. Young, J.
555 Goldblatt, L. I. Landau, P. N. Le Souef, The relationship between infant airway
556 function, childhood airway responsiveness, and asthma. *Am J Respir Crit Care Med*
557 **169**, 921-927 (2004).

558 8. L. J. Palmer, P. J. Rye, N. A. Gibson, P. R. Burton, L. I. Landau, P. N. Lesouef, Airway
559 responsiveness in early infancy predicts asthma, lung function, and respiratory
560 symptoms by school age. *Am J Respir Crit Care Med* **163**, 37-42 (2001).

561 9. H. Bisgaard, S. M. Jensen, K. Bonnelykke, Interaction between asthma and lung
562 function growth in early life. *Am J Respir Crit Care Med* **185**, 1183-1189 (2012).

563 10. V. Hovland, A. Riiser, P. Mowinckel, K. H. Carlsen, K. C. Lodrup Carlsen, The
564 significance of early recurrent wheeze for asthma outcomes in late childhood. *Eur*
565 *Respir J* **41**, 838-845 (2013).

566 11. S. T. Holgate, Innate and adaptive immune responses in asthma. *Nat Med* **18**, 673-
567 683 (2012).

568 12. M. K. Tulic, D. Andrews, M. L. Crook, A. Charles, M. R. Tourigny, R. Moqbel, S. L.
569 Prescott, Changes in thymic regulatory T-cell maturation from birth to puberty:
570 differences in atopic children. *J Allergy Clin Immunol* **129**, 199-206 e191-194
571 (2012).

572 13. I. M. de Kleer, M. Kool, M. J. de Bruijn, M. Willart, J. van Moorlehem, M. J. Schuijs,
573 M. Plantinga, R. Beyaert, E. Hams, P. G. Fallon, H. Hammad, R. W. Hendriks, B. N.
574 Lambrecht, Perinatal Activation of the Interleukin-33 Pathway Promotes Type 2
575 Immunity in the Developing Lung. *Immunity* **45**, 1285-1298 (2016).

576 14. S. Guerra, F. D. Martinez, Epidemiology of the origins of airflow limitation in
577 asthma. *Proc Am Thorac Soc* **6**, 707-711 (2009).

- 578 15. J. L. Barlow, A. Bellosi, C. S. Hardman, L. F. Drynan, S. H. Wong, J. P. Cruickshank, A.
579 N. McKenzie, Innate IL-13-producing nuocytes arise during allergic lung
580 inflammation and contribute to airways hyperreactivity. *J Allergy Clin Immunol*
581 **129**, 191-198 e191-194 (2012).
- 582 16. R. G. Klein Wolterink, A. Kleinjan, M. van Nimwegen, I. Bergen, M. de Bruijn, Y.
583 Levani, R. W. Hendriks, Pulmonary innate lymphoid cells are major producers of
584 IL-5 and IL-13 in murine models of allergic asthma. *Eur J Immunol* **42**, 1106-1116
585 (2012).
- 586 17. Y. J. Chang, H. Y. Kim, L. A. Albacker, N. Baumgarth, A. N. McKenzie, D. E. Smith, R.
587 H. Dekruyff, D. T. Umetsu, Innate lymphoid cells mediate influenza-induced airway
588 hyper-reactivity independently of adaptive immunity. *Nat Immunol* **12**, 631-638
589 (2011).
- 590 18. P. D. Sly, P. G. Holt, Role of innate immunity in the development of allergy and
591 asthma. *Curr Opin Allergy Clin Immunol* **11**, 127-131 (2011).
- 592 19. S. Saluzzo, A. D. Gorki, B. M. J. Rana, R. Martins, S. Scanlon, P. Starkl, K. Lakovits, A.
593 Hladik, A. Korosec, O. Sharif, J. M. Warszawska, H. Jolin, I. Mesteri, A. N. J. McKenzie,
594 S. Knapp, First-Breath-Induced Type 2 Pathways Shape the Lung Immune
595 Environment. *Cell Rep* **18**, 1893-1905 (2017).
- 596 20. C. A. Steer, I. Martinez-Gonzalez, M. Ghaedi, P. Allinger, L. Matha, F. Takei, Group 2
597 innate lymphoid cell activation in the neonatal lung drives type 2 immunity and
598 allergen sensitization. *J Allergy Clin Immunol* **140**, 593-595 e593 (2017).
- 599 21. D. C. M. Belgrave, R. Granell, S. W. Turner, J. A. Curtin, I. E. Buchan, P. N. Le Souef,
600 A. Simpson, A. J. Henderson, A. Custovic, Lung function trajectories from pre-
601 school age to adulthood and their associations with early life factors: a
602 retrospective analysis of three population-based birth cohort studies. *Lancet*
603 *Respir Med*, (2018).
- 604 22. N. Torow, B. J. Marsland, M. W. Hornef, E. S. Gollwitzer, Neonatal mucosal
605 immunology. *Mucosal Immunol* **10**, 5-17 (2017).
- 606 23. M. M. Stein, C. L. Hrusch, J. Gozdz, C. Igartua, V. Pivniouk, S. E. Murray, J. G. Ledford,
607 M. Marques Dos Santos, R. L. Anderson, N. Metwali, J. W. Neilson, R. M. Maier, J. A.
608 Gilbert, M. Holbreich, P. S. Thorne, F. D. Martinez, E. von Mutius, D. Vercelli, C. Ober,
609 A. I. Sperling, Innate Immunity and Asthma Risk in Amish and Hutterite Farm
610 Children. *N Engl J Med* **375**, 411-421 (2016).
- 611 24. J. Genuneit, E. von Mutius, Do farm-grown lungs breathe better? *Thorax* **72**, 202-
612 203 (2017).
- 613 25. J. G. Lisciandro, A. H. van den Biggelaar, Neonatal immune function and
614 inflammatory illnesses in later life: lessons to be learnt from the developing
615 world? *Clin Exp Allergy* **40**, 1719-1731 (2010).
- 616 26. X. Zhang, B. Mozeleski, S. Lemoine, E. Deriaud, A. Lim, D. Zhivaki, E. Azria, C. Le Ray,
617 G. Roguet, O. Launay, A. Vanet, C. Leclerc, R. Lo-Man, CD4 T cells with effector
618 memory phenotype and function develop in the sterile environment of the fetus.
619 *Sci Transl Med* **6**, 238ra272 (2014).
- 620 27. D. J. Martino, A. Bosco, K. L. McKenna, E. Hollams, D. Mok, P. G. Holt, S. L. Prescott,
621 T-cell activation genes differentially expressed at birth in CD4+ T-cells from
622 children who develop IgE food allergy. *Allergy* **67**, 191-200 (2012).
- 623 28. J. Rothers, M. Halonen, D. A. Stern, I. C. Lohman, S. Mobley, A. Spangenberg, D.
624 Anderson, A. L. Wright, Adaptive cytokine production in early life differentially
625 predicts total IgE levels and asthma through age 5 years. *J Allergy Clin Immunol*
626 **128**, 397-402 e392 (2011).

- 627 29. S. Saglani, S. A. Mathie, L. G. Gregory, M. J. Bell, A. Bush, C. M. Lloyd,
628 Pathophysiological features of asthma develop in parallel in house dust mite-
629 exposed neonatal mice. *Am J Respir Cell Mol Biol* **41**, 281-289 (2009).
- 630 30. J. L. Barlow, S. Peel, J. Fox, V. Panova, C. S. Hardman, A. Camelo, C. Bucks, X. Wu, C.
631 M. Kane, D. R. Neill, R. J. Flynn, I. Sayers, I. P. Hall, A. N. McKenzie, IL-33 is more
632 potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid
633 cells) and airway contraction. *J Allergy Clin Immunol* **132**, 933-941 (2013).
- 634 31. M. Ebbo, A. Crinier, F. Vely, E. Vivier, Innate lymphoid cells: major players in
635 inflammatory diseases. *Nat Rev Immunol* **17**, 665-678 (2017).
- 636 32. B. W. S. Li, R. Stadhouders, M. J. W. de Bruijn, M. Lukkes, D. Beerens, M. D. Brem, A.
637 KleinJan, I. Bergen, H. Vroman, M. Kool, I. W. F. J. van, T. N. Rao, H. J. Fehling, R. W.
638 Hendriks, Group 2 Innate Lymphoid Cells Exhibit a Dynamic Phenotype in Allergic
639 Airway Inflammation. *Front Immunol* **8**, 1684 (2017).
- 640 33. P. Nagakumar, L. Denney, L. Fleming, A. Bush, C. M. Lloyd, S. Saglani, Type 2 innate
641 lymphoid cells in induced sputum from children with severe asthma. *J Allergy Clin*
642 *Immunol* **137**, 624-626 e626 (2016).
- 643 34. L. R. Bonser, D. J. Erle, Airway Mucus and Asthma: The Role of MUC5AC and
644 MUC5B. *J Clin Med* **6**, (2017).
- 645 35. S. Castanhinha, R. Sherburn, S. Walker, A. Gupta, C. J. Bossley, J. Buckley, N.
646 Ullmann, R. Grychtol, G. Campbell, M. Maglione, S. Koo, L. Fleming, L. Gregory, R. J.
647 Snelgrove, A. Bush, C. M. Lloyd, S. Saglani, Pediatric severe asthma with fungal
648 sensitization is mediated by steroid-resistant IL-33. *J Allergy Clin Immunol* **136**,
649 312-322 e317 (2015).
- 650 36. R. J. Snelgrove, L. G. Gregory, T. Peiro, S. Akthar, G. A. Campbell, S. A. Walker, C. M.
651 Lloyd, Alternaria-derived serine protease activity drives IL-33-mediated asthma
652 exacerbations. *J Allergy Clin Immunol* **134**, 583-592 e586 (2014).
- 653 37. F. D. Martinez, D. Vercelli, Asthma. *Lancet* **382**, 1360-1372 (2013).
- 654 38. N. Tagiyeva, G. Devereux, S. Fielding, S. Turner, G. Douglas, Outcomes of Childhood
655 Asthma and Wheezy Bronchitis. A 50-Year Cohort Study. *Am J Respir Crit Care Med*
656 **193**, 23-30 (2016).
- 657 39. E. S. Gollwitzer, S. Saglani, A. Trompette, K. Yadava, R. Sherburn, K. D. McCoy, L. P.
658 Nicod, C. M. Lloyd, B. J. Marsland, Lung microbiota promotes tolerance to allergens
659 in neonates via PD-L1. *Nat Med* **20**, 642-647 (2014).
- 660 40. T. Y. Halim, C. A. Steer, L. Matha, M. J. Gold, I. Martinez-Gonzalez, K. M. McNagny, A.
661 N. McKenzie, F. Takei, Group 2 innate lymphoid cells are critical for the initiation
662 of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity* **40**, 425-
663 435 (2014).
- 664 41. C. J. Oliphant, Y. Y. Hwang, J. A. Walker, M. Salimi, S. H. Wong, J. M. Brewer, A.
665 Englezakis, J. L. Barlow, E. Hams, S. T. Scanlon, G. S. Ogg, P. G. Fallon, A. N. McKenzie,
666 MHCII-mediated dialog between group 2 innate lymphoid cells and CD4(+) T cells
667 potentiates type 2 immunity and promotes parasitic helminth expulsion.
668 *Immunity* **41**, 283-295 (2014).
- 669 42. S. J. Van Dyken, J. C. Nussbaum, J. Lee, A. B. Molofsky, H. E. Liang, J. L. Pollack, R. E.
670 Gate, G. E. Haliburton, C. J. Ye, A. Marson, D. J. Erle, R. M. Locksley, A tissue
671 checkpoint regulates type 2 immunity. *Nat Immunol* **17**, 1381-1387 (2016).
- 672 43. L. Guo, Y. Huang, X. Chen, J. Hu-Li, J. F. Urban, Jr., W. E. Paul, Innate immunological
673 function of TH2 cells in vivo. *Nat Immunol* **16**, 1051-1059 (2015).
- 674 44. K. Hebel, S. Weinert, B. Kuropka, J. Knolle, B. Kosak, G. Jorch, C. Arens, E. Krause, R.
675 C. Braun-Dullaes, M. C. Brunner-Weinzierl, CD4+ T cells from human neonates

- 676 and infants are poised spontaneously to run a nonclassical IL-4 program. *J*
677 *Immunol* **192**, 5160-5170 (2014).
- 678 45. C. J. Bossley, L. Fleming, A. Gupta, N. Regamey, J. Frith, T. Oates, L. Tsartsali, C. M.
679 Lloyd, A. Bush, S. Saglani, Pediatric severe asthma is characterized by eosinophilia
680 and remodeling without T(H)2 cytokines. *J Allergy Clin Immunol* **129**, 974-982
681 e913 (2012).
- 682 46. S. Saglani, D. N. Payne, J. Zhu, Z. Wang, A. G. Nicholson, A. Bush, P. K. Jeffery, Early
683 detection of airway wall remodeling and eosinophilic inflammation in preschool
684 wheezers. *Am J Respir Crit Care Med* **176**, 858-864 (2007).
- 685 47. X. Roux, A. Remot, A. Petit-Camurdan, M. A. Nahori, H. Kiefer-Biasizzo, G. Marchal,
686 M. Lagranderie, S. Riffault, Neonatal lung immune responses show a shift of
687 cytokines and transcription factors toward Th2 and a deficit in conventional and
688 plasmacytoid dendritic cells. *Eur J Immunol* **41**, 2852-2861 (2011).
- 689 48. N. Torow, M. W. Hornef, The Neonatal Window of Opportunity: Setting the Stage
690 for Life-Long Host-Microbial Interaction and Immune Homeostasis. *J Immunol*
691 **198**, 557-563 (2017).
- 692 49. S. H. Chotirmall, S. L. Gellatly, K. F. Budden, M. Mac Aogain, S. D. Shukla, D. L. Wood,
693 P. Hugenholtz, K. Pethe, P. M. Hansbro, Microbiomes in respiratory health and
694 disease: An Asia-Pacific perspective. *Respirology* **22**, 240-250 (2017).
- 695 50. K. Oeser, J. Maxeiner, C. Symowski, M. Stassen, D. Voehringer, T cells are the critical
696 source of IL-4/IL-13 in a mouse model of allergic asthma. *Allergy* **70**, 1440-1449
697 (2015).
- 698 51. L. G. Gregory, B. Causton, J. R. Murdoch, S. A. Mathie, V. O'Donnell, C. P. Thomas, F.
699 M. Priest, D. J. Quint, C. M. Lloyd, Inhaled house dust mite induces pulmonary T
700 helper 2 cytokine production. *Clin Exp Allergy* **39**, 1597-1610 (2009).
- 701 52. M. Kauth, H. Heine, Allergy protection by cowshed bacteria - recent findings and
702 future prospects. *Pediatr Allergy Immunol* **27**, 340-347 (2016).
- 703
704

705 **Acknowledgements:**

706 The authors thank Jessica Rowley and Jane Srivastava of the Imperial College Core Flow
707 Cytometry facility for assistance. We also thank Johann Bauer (**Lehrstuhl für Tierhygiene,**
708 **Technische Universität München,**) for his kind gift of *A. iwoffii*. We are grateful to Prof Mark
709 Johnson (**Chelsea and Westminster Hospital**) for the cord blood samples.

710

711 **Funding:** This work was supported by the Wellcome Trust (grant 083586/Z/07/Z,
712 087618/Z/08/Z and 107059/Z/15/Z). CML is a Wellcome Senior Fellow in Basic Biomedical
713 Sciences, AB is an NIHR Senior Investigator, SS is an NIHR Career Development Fellow.

714

715

716 **Author Contributions:**

717 S.S. wrote the manuscript draft, conceived and designed the experiments. E.v.M supplied the
718 *A. iwoffii* and provided intellectual input on the farmyard dust. J.E.V., A.K.M., R. G., R.S.,
719 A.B., S.L. S.A.W., J.B., V.F., L.D., F.P., F.U., L.J.E., W.J.B., R.A.O. and L.G.G. performed
720 the experiments. **S.A.W., R.A.O. and L.G.G. carried out the statistical analyses.** A.K.M., A.B.
721 and L.G.G. revised the manuscript. C.M.L. conceived the study, designed the experiments and
722 edited the manuscript.

723

724 **Competing Interests:**

725 The authors **declare that they** have no competing interests.

726

727 **Data and materials availability:**

728 All data needed to evaluate the conclusions in the paper are present in the paper and/or the
729 Supplementary Materials.

730

731 **Figures captions:**

732 **Fig. 1. T cells and ILCs are equally important sources of IL-13 in neonatal mice exposed**
733 **to rIL-33.**

734 (A) BALB/c mice ages 3 days and 6-8 weeks were exposed to intermittent intra-nasal
735 recombinant IL-33 (rIL-33) (50µg/kg) or PBS for 2 weeks (↓). Harvest was carried out 24
736 hours post final dose. Numbers of CD3⁺CD4⁺IL-13⁺ T cells compared to innate lymphoid cells
737 (Lin^{neg}CD45⁺CD90⁺IL-13⁺) in lungs of (B) adult and (C) neonatal BALB/c mice. (D)
738 Pulmonary eosinophils (SiglecF⁺CD11c^{low/neg}) enumerated by flow cytometry. Airway
739 resistance to methacholine (Mch) in (E) neonatal and (F) adult mice exposed to intra-nasal rIL-
740 33. (G) IL-13 gene expression in the lung. (H) Levels of IL-13 in the lungs. (I) IL-5 gene
741 expression in the lung. Expression of (J) Muc5ac and (K) Muc5b. N=6-8 rIL-33, N=4-5 PBS.
742 Data representative of at least 2 experiments. *p<0.05, *p<0.01, ***p<0.001.

743

744 **Fig. 2. In response to allergen T cells and ILCs are equally important sources of IL-13.**

745 Airway responsiveness to methacholine (Mch) in (A) neonatal and (B) adult mice. (C)
746 Pulmonary eosinophils (SiglecF⁺CD11c^{low/neg}). Numbers of Lin^{neg}CD45⁺CD90⁺IL-13⁺ILCs
747 and CD3⁺CD4⁺IL-13⁺ T cells in lungs of (D) adult and (E) neonatal mice. (F) Levels of IL-13
748 in the lung of mice exposed to HDM. . IL-13⁺ILCs and T cells in lungs of (G) adult and (H)
749 neonatal mice exposed to *ALT*. (I) Levels of IL-13 in the lung of mice exposed to *ALT*. N=6-
750 8 allergen exposed, N=4-6 PBS. Data representative of 2 experiments. *p<0.05, **<0.01,
751 ***p<0.001.

752

753 **Fig. 3. IL-13 is a feature of allergen activated neonatal T cells.**

754 CD4⁺ T cells from house dust mite HDM and *Alternaria alternata* (ALT) treated neonatal and
755 adult mice were classified as either IL-13⁺ or IL-13^{neg} and analysed for surface markers. Cells

756 were defined as either (A & B) memory-like (CD44⁺CD62L^{low}), (C & D) epithelial associated
757 (CD103⁺) or (E & F) activated (CD69⁺/ICOS⁺). The proportion of neonatal and adult CD4⁺ T
758 cells from (G & H) PBS control, (I & J) HDM treated or (K & L) ALT exposed mice
759 expressing cytokine were also enumerated. N=7-9 allergen exposed, N=5 PBS. Data
760 representative of 2 experiments. Human cord blood was analysed for (M) total CD4⁺ T cells
761 and total ILC2 (Lin⁻CD161⁺CD127⁺CRTH2⁺CD56⁻) and (N) IL-13⁺ CD4⁺ T cells and IL-13⁺
762 ILC2. (O) Neonatal T cells expressed TCR-β but lacked expression of CD1d dimer. Values are
763 expressed as a percentage of live CD45⁺ lymphoid cells. N=20. *p<0.05, **<0.01, ***p<0.001.
764

765 **Fig. 4. IL-33 is not critical for initiation of allergic airways disease phenotype.**

766 **3 day old** wild type BALB/c (WT) and **IL33**^{-/-} mice were exposed to intermittent doses of house
767 dust mite (HDM) or PBS. (A) Airway responsiveness to methacholine (Mch) in neonatal WT
768 and **IL33**^{-/-} mice. (B) Pulmonary eosinophils (SiglecF⁺CD11c^{low/neg}). Numbers of pulmonary
769 (C) Lin^{neg}CD45⁺ IL-13⁺ILCs and (D) CD3⁺CD4⁺IL-13⁺ T cells. Levels of (E) IL-33, (F) IL-5
770 and (G) IL-13 in the lung. N=7-12 HDM, N=6-8 PBS. Data representative of 2 experiments.
771 *p<0.05, **p<0.01, ***p<0.001.

772

773 **Fig. 5. T cell derived IL-13 is essential for the inception of AHR.**

774 **3 day old** wild type BALB/c (WT) and **Cd4-cre IL-4/IL13^{fl/fl}** (CD4Cre) mice were exposed to
775 intermittent doses of house dust mite (HDM), *Alternaria alternata* (ALT) or PBS for 2 weeks.
776 (A) Representative flow cytometry plots of cytokine expressing (IL-5 and/or IL-13) CD3⁺CD4⁺
777 T cells. Airway responsiveness to methacholine (Mch) in neonatal mice exposed to (B) HDM
778 or (C) ALT. Lung eosinophils enumerated by flow cytometry in mice exposed to (D) HDM or
779 (E) ALT. Lin^{neg}CD45⁺CD90⁺IL-13⁺ILCs in mice exposed to (F) HDM or (G) ALT. IL-5⁺T
780 cells to in mice exposed to (H) HDM or (I) ALT. IL-13 levels in mice exposed (J) HDM or

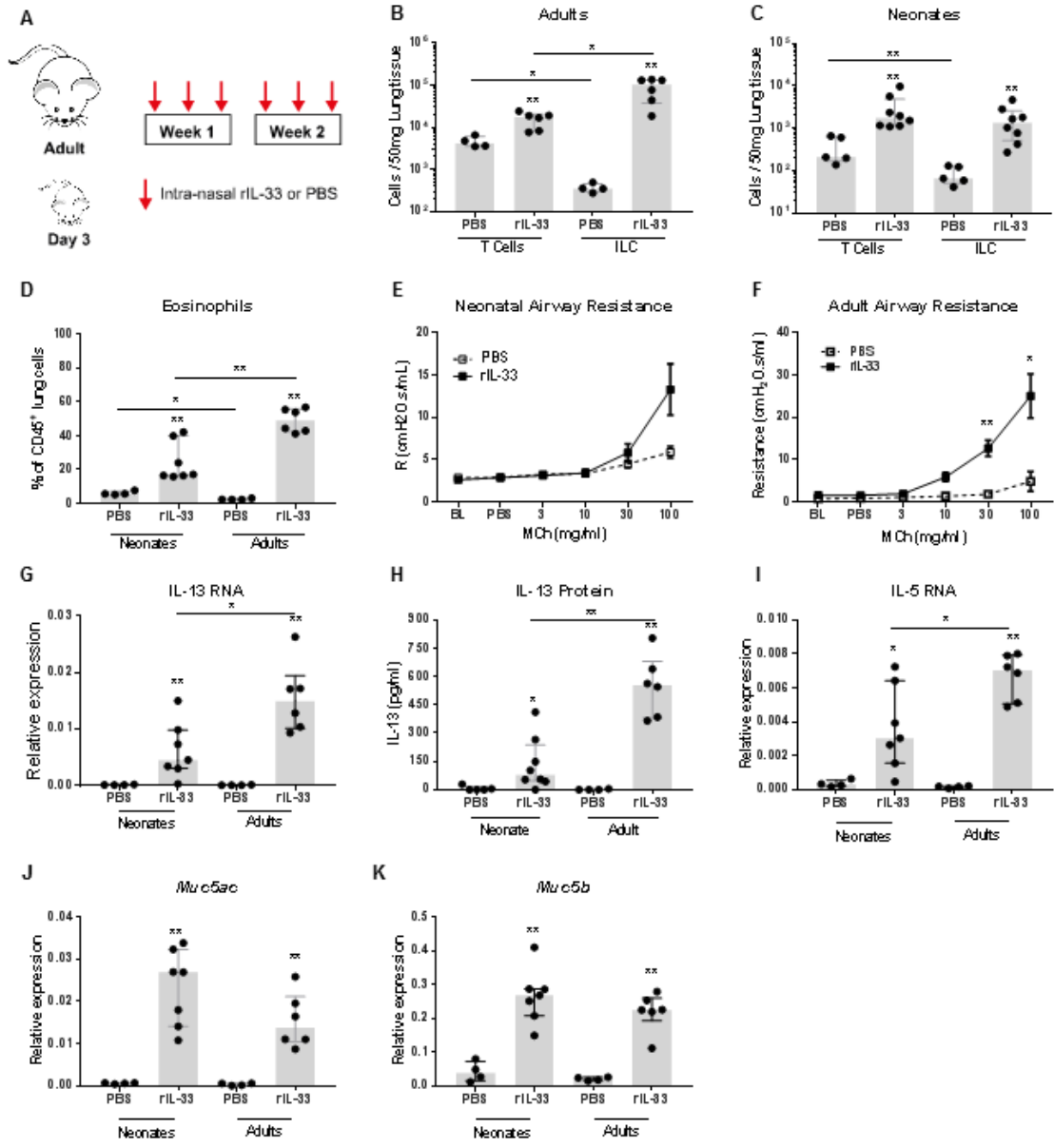
781 **(K)** ALT. Muc5ac gene expression in mice exposed to **(L)** HDM or **(M)** ALT. N=6-8 allergen
782 exposed, N=4-6 PBS. Data representative of at least 2 experiments. *p<0.05, **<0.01,
783 ***p<0.001.

784

785 **Fig. 6. Protection from AHR in neonatal mice can be achieved by reduction of IL-13⁺ T**
786 **cells.**

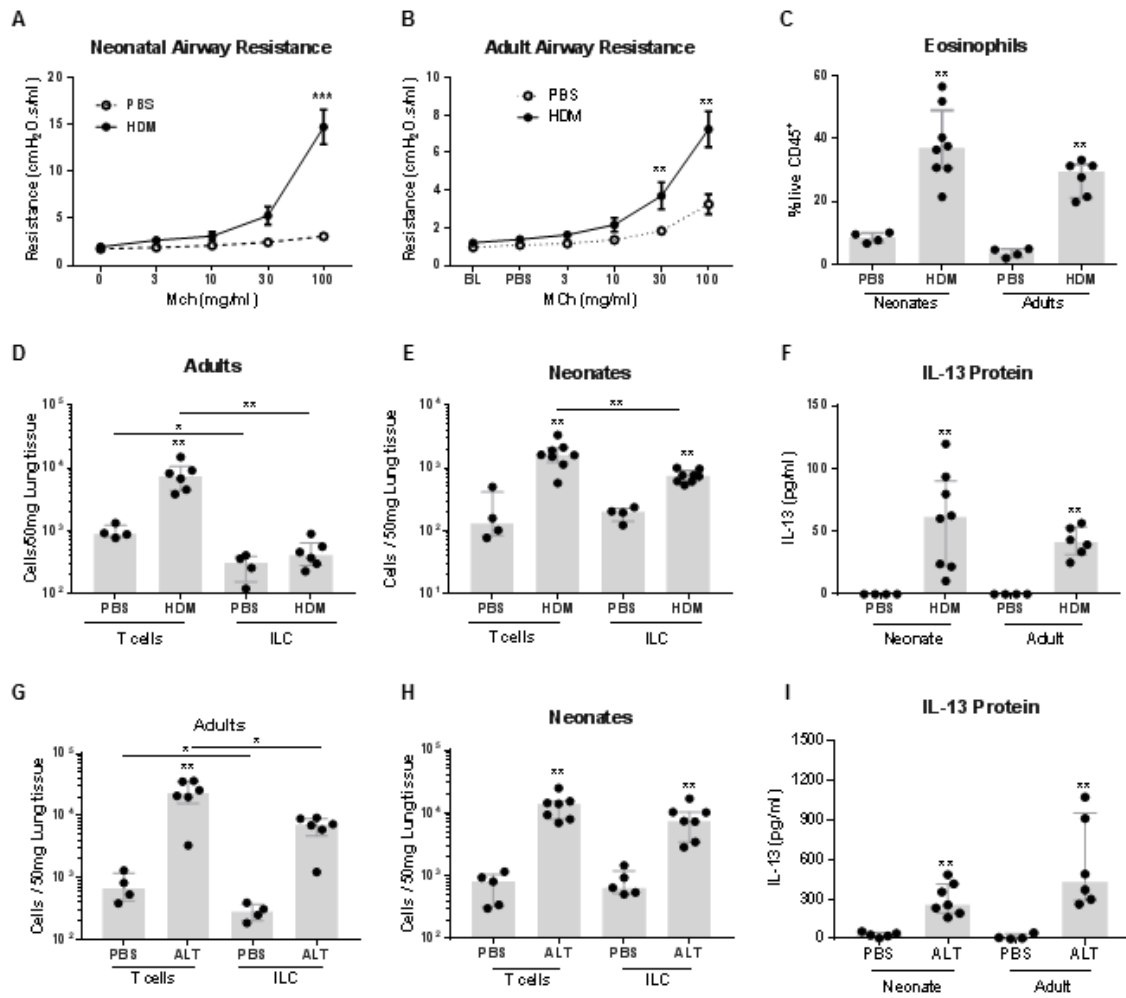
787 **(A)** BALB/c mice aged 3 days were exposed to intermittent intra-nasal *Acinetobacter iwoffii*
788 F78 (*A. iwoffii* F78) (1.3x10⁸cfu first 2 weeks, then 2x10⁸cfu) or PBS (↑) followed by house
789 dust mite (HDM) (10μg first 2 weeks, then 15μg) or PBS for 3 weeks (↓). Analysis was carried
790 out 24 hours post final dose. **(B)** Airway hyperresponsiveness (AHR) to methacholine (Mch)
791 after 3 weeks of *A. iwoffii* F78 and HDM co-exposure. **(C)** Numbers of eosinophils
792 (SiglecF⁺CD11c^{low/neg}) in lung. **(D)** IL-33 and **(E)** IL-13 levels from lung homogenate. **(F)**
793 Numbers of innate lymphoid cells (Lin⁻CD45⁺IL-13⁺) and **(G)** CD3⁺CD4⁺IL-13⁺ T cells. **(H)**
794 IL-5 levels in the lung tissue. **(I)** Dendritic cell (DC) populations in the lung enumerated by
795 flow cytometry. cDC (CD11b⁺CD11c^{high}), pDC (CD11c^{int}Ly6c⁺CD64⁻), MoDC
796 (CD11c⁺Ly6c⁺CD64⁺). N=8 for HDM and bacteria exposed groups, N=5 for PBS. *p<0.05,
797 **p<0.01, ***p<0.001. Data representative of 3 experiments.

798



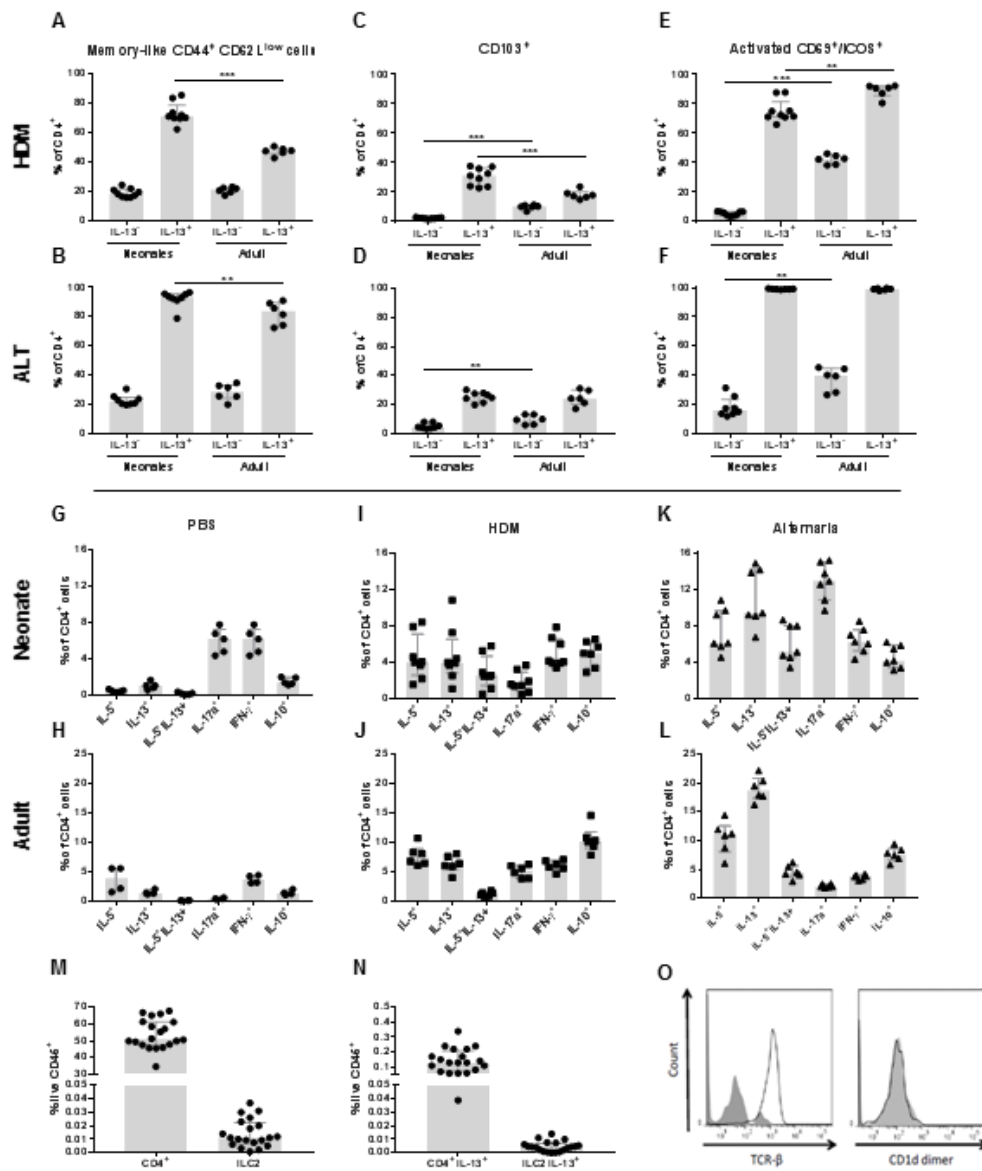
799

800



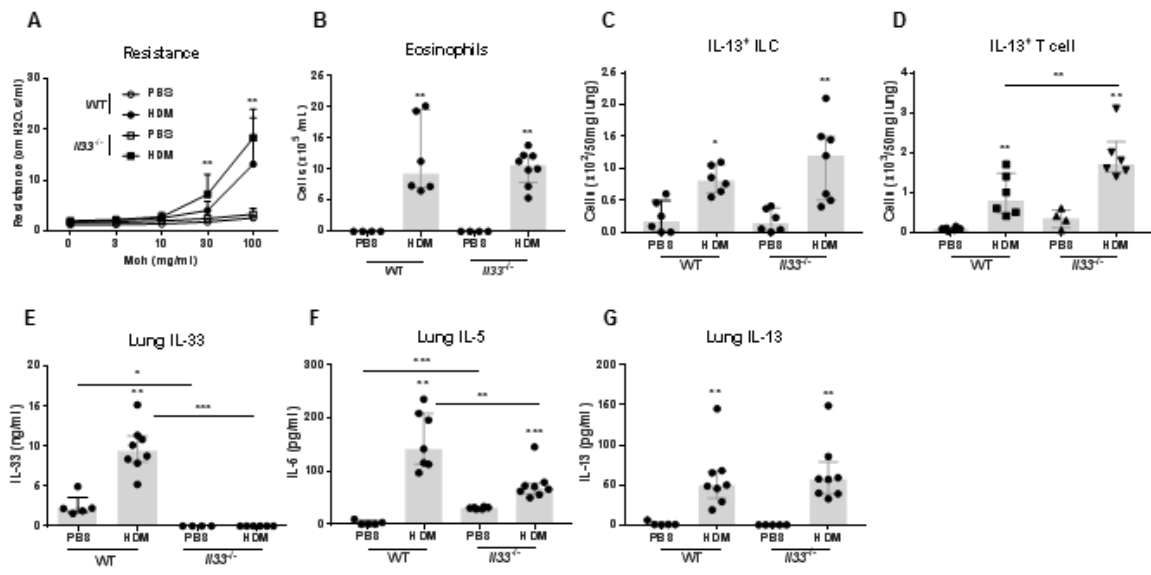
801

802



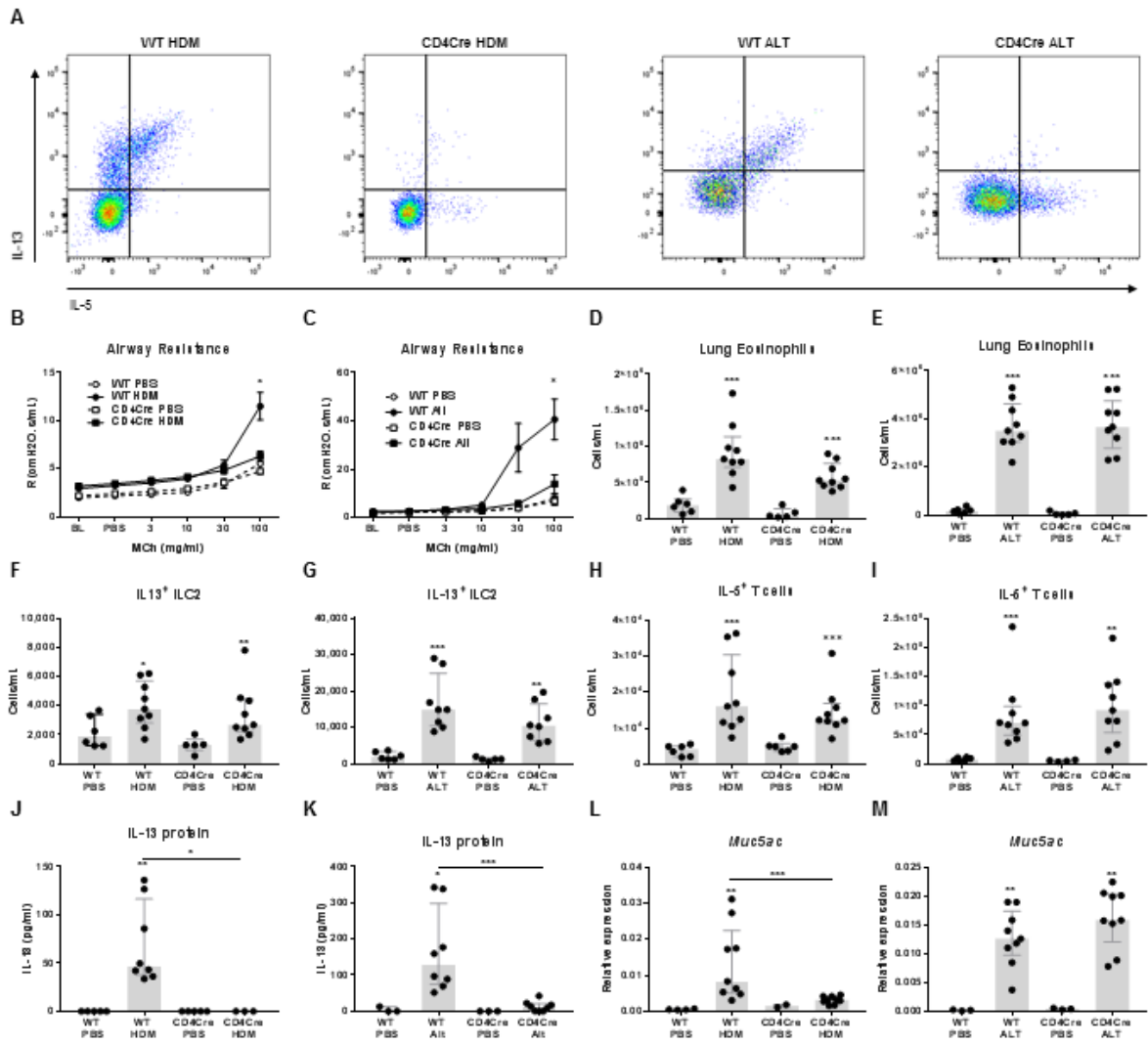
803

804



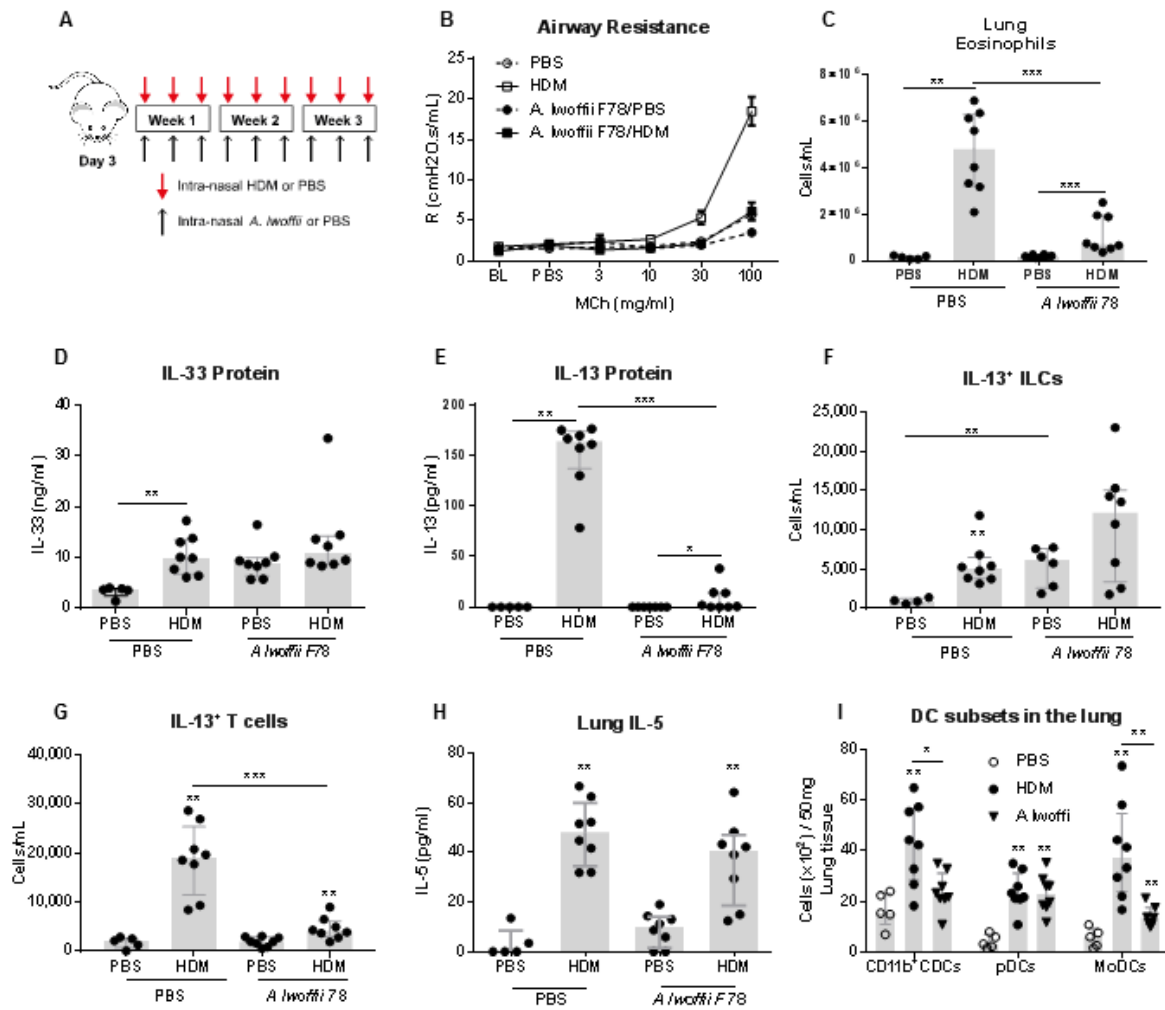
805

806



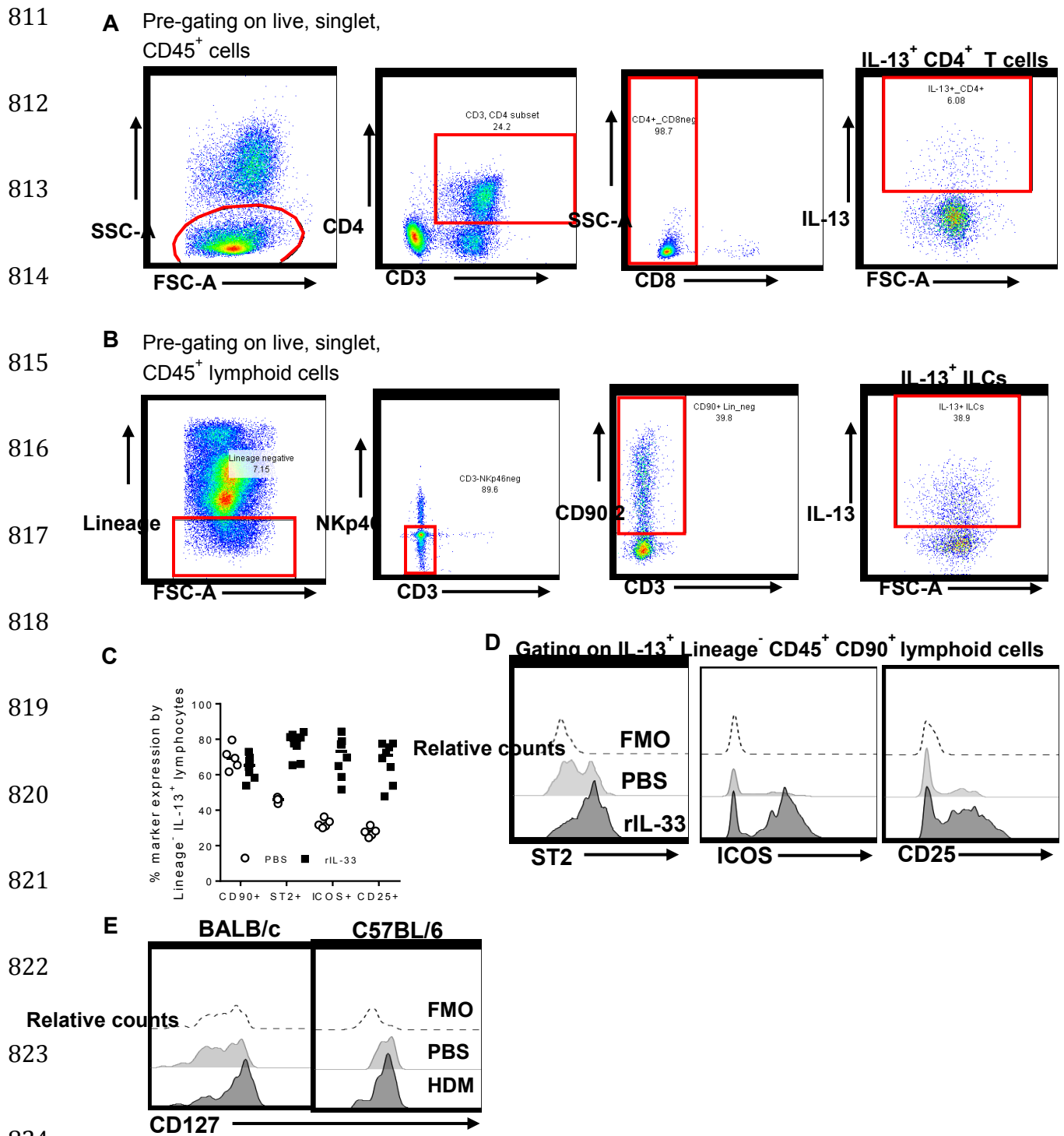
807

808



809

810

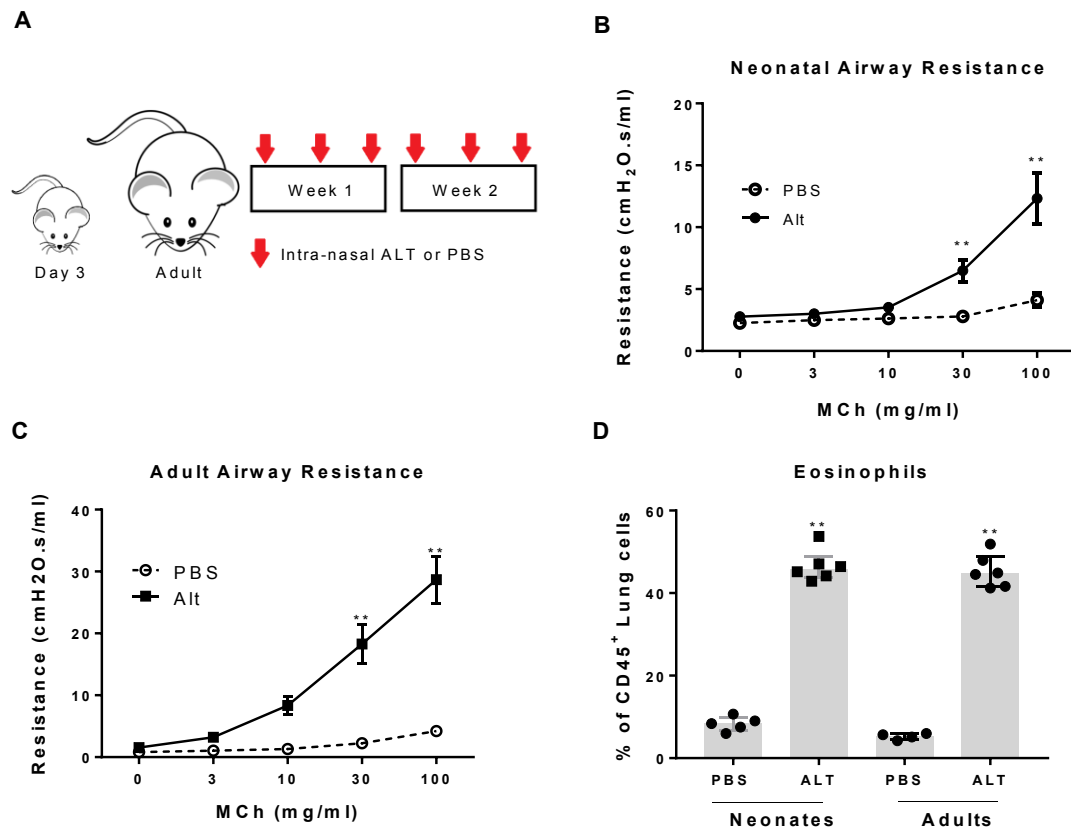


825 **Supplementary Fig. 1. Defining IL-13⁺ CD4⁺ T cells and ILCs in neonatal mice**

826 **(A)** Hierarchical (left to right) gating strategy for IL-13⁺ CD4⁺ T cells, defined as live, singlet,
 827 CD45⁺, FSC/SSC low (lymphoid), CD4⁺, CD3⁺, CD8⁻ cells with positive intracellular IL-13
 828 staining were then selected. **(B)** Hierarchical (left to right) gating strategy for IL-13⁺ ILCs,

829 defined as live, singlet, CD45⁺ lymphoid, lineage- (TCR β , TCR $\gamma\delta$, CD3e, CD5, CD19, CD11b,
830 CD11c, FC ϵ R1, GR-1, F4/80, NKp46 and TER-119) –negative, CD90.2⁺ cells with positive
831 intracellular IL-13 staining. Plots in **(A)** and **(B)** are from representative lung samples from IL-
832 33-treated neonatal mice. **(C)** Percentages of IL-13⁺ Lineage⁻ CD45⁺ lymphoid cells in lung
833 tissue expressing typical ILC surface markers. **(D)** Representative histograms showing surface
834 expression of ST2, ICOS and CD25 on pulmonary IL-13⁺ CD90.2⁺ CD45⁺ Lineage⁻ ILCs,
835 relative to FMO controls. **(E)** Representative histograms showing CD127 surface staining on
836 IL-13⁺ CD90.2⁺ Lineage⁻ ILC2 from lungs of neonatal BALB/c (left) and C57BL/6 mice
837 treated with HDM or PBS for 2 weeks, relative to FMO controls. Data are representative of 4-
838 8 replicate animals per group.

839



840

841

842

843 **Supplementary Fig. 2. *Alternaria* induces AHR in neonatal mice**

844 (A) Day 3 old and adult BALB/c mice were exposed to intermittent doses of *Alternaria*

845 *alternata* (ALT) or PBS for 2 weeks (↓). Analysis was carried out 24 hours post final dose.

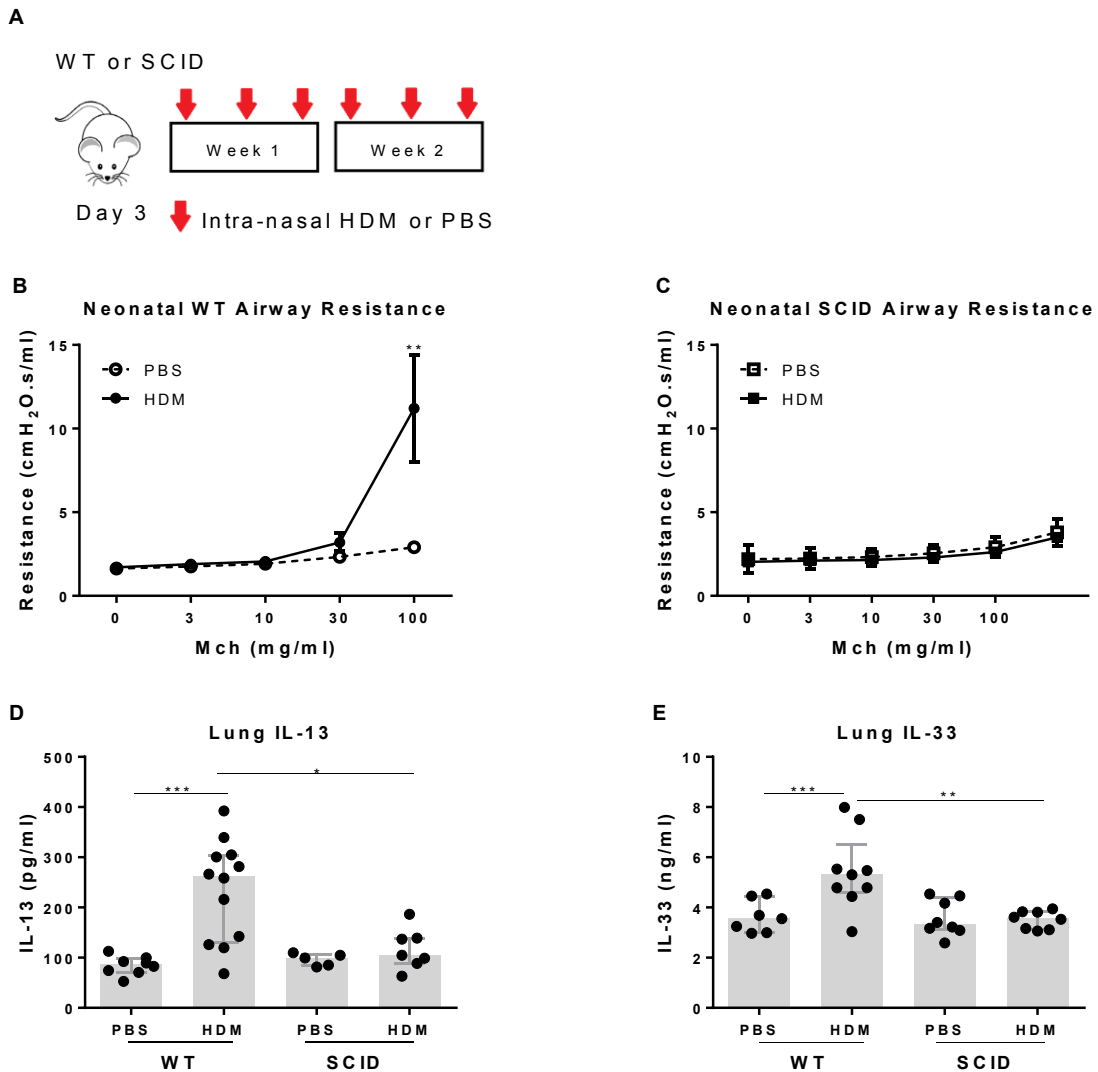
846 Airway resistance to methacholine (Mch) in (B) neonatal and (C) adult mice. (D) Numbers of

847 eosinophils (SiglecF⁺CD11c^{low/neg}) in lung enumerated by flow cytometry. N=6-8 ALT, n=4-6

848 PBS. Data representative of 2 experiments. *p<0.05, **<0.01, ***p<0.001.

849

850



851

852 **Supplementary Fig. 3. Allergen exposure in neonatal SCID mice does not result in AHR.**

853 **(A)** Day 3 old WT BALB/c and beige SCID mice were exposed to intermittent doses of house

854 dust mite (HDM) or PBS for 2 weeks (↓). Analysis was carried out 24 hours post final dose.

855 Airway resistance to methacholine (Mch) in **(B)** WT mice and **(C)** SCID mice. Levels of **(D)**

856 IL-13 and **(E)** IL-33 from lung homogenates of WT mice compared to SCID mice. N=7-12

857 HDM, n=6-8 PBS. Data representative of 2 experiments. *p<0.05, **p<0.01, ***p<0.001.

858

Antigen	Clone	Fluorochrome	Target species	Manufacturer
CD103	2E7	FITC	Mouse	Thermo Fisher/ eBiosciences
CD11b	M1/70	APC	Mouse	Biolegend
CD11c	N418	APC or APC Cy7	Mouse	Biolegend
CD127	A7R34	BV711	Mouse	Biolegend
CD19	ebio 1D3	APC	Mouse	Biolegend
CD25	PC61	BV510	Mouse	Biolegend
CD3e	145-2C11	APC cy7 or PE Cy7	Mouse	Biolegend
CD4	RM4-5	BV421 or APC Cy7	Mouse	Biolegend
CD44	IM7	PERCP Cy5.5	Mouse	Biolegend
CD45	30-F11	PerCP Cy5.5 or BV711 or PE/Dazzle™ 594	Mouse	Biolegend
CD5	53-7.3	APC	Mouse	Biolegend
CD62L	MEL-14	BV605	Mouse	Biolegend
CD64	X54-5/7.1	BV421	Mouse	Biolegend
CD8a	53-6.7	BV605	Mouse	Biolegend
CD90.2	53-2.1	BV605	Mouse	Biolegend
F4/80	BM8	APC	Mouse	Biolegend
FCεR1	MAR1	APC	Mouse	Biolegend
GR-1	RB6-8C5	APC	Mouse	Biolegend
IA/IE	M5/114.15.2	PECRP cy5.5	Mouse	Biolegend
ICOS	7E.17G9	PE cy7	Mouse	Biolegend

IFN γ	XMG1.2	FITC	Mouse	Biolegend
IL-10	JES5-16E3	APC	Mouse	Biolegend
IL-13	ebio 13A	PE	Mouse	Thermo Fisher/ eBiosciences
IL-17A	TC11-18H10	Alexa Fluor 700 or PERCP Cy5.5	Mouse	Biolegend
IL-5	TRFK5	BV421	Mouse	Biolegend
Ly6G	1A8	BV510	Mouse	Biolegend
Nkp46	29A1.4	PE/Dazzle™ 594 or BV605	Mouse	Biolegend
Siglec F	E50-2440	PE	Mouse	BD
ST2	DJ8	FITC	Mouse	MD Biosciences
TCR β	H57-597	APC	Mouse	Biolegend
TCR $\gamma\delta$	ebio GL3	APC	Mouse	Biolegend
TER-119	TER-119	APC	Mouse	Biolegend
CD11c	3.9	FITC	Human	Biolegend
CD123	6H6	FITC	Human	Biolegend
CD127	A019D5	PERCP Cy5.5	Human	Biolegend
CD14	63D3	FITC	Human	Biolegend
CD16	B73.1	FITC	Human	Biolegend
CD161	HP-3G10	PE Cy7	Human	Biolegend
CD19	HIB19	FITC	Human	Biolegend
CD1a	HI149	FITC	Human	Biolegend
CD20	2H7	FITC	Human	Biolegend

CD3	OKT3	BV510	Human	Biolegend
CD34	581	FITC	Human	Biolegend
CD4	OKT4	Alexa Fluor 700	Human	Biolegend
CD45	HI30	BV605	Human	Biolegend
CD5	L17F12	FITC	Human	Biolegend
CD56	HCD56	PE Texas Red	Human	Biolegend
CD8	SK1	FITC	Human	Biolegend
CRTH2	BM16	APC Cy7	Human	Biolegend
FCεR1	AER-37	FITC	Human	Biolegend
IL-13	JES10-5A2	PE	Human	Biolegend

859

860 Table S1. Antibodies used for flow cytometry

861