Title: Inception of early life allergen induced airway hyperresponsiveness is reliant on IL-13^{+}CD4^{+} T cells

One Sentence Summary:

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

Authors:

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Abstract

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

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

Introduction

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

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

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

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

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

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

Results

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

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

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

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

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

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

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

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

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

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

*Allergen exposure in neonatal SCID mice protects from AHR, while **Il33**$^{-/-}$ mice are unaffected.*

To determine the functional significance of the pulmonary $IL\text{-}13^+CD4^+$ T cells in early life, we exposed neonatal SCID mice, which lack T, B and NK cells, to HDM for 2 weeks (fig. S3A). While the WT mice showed the expected increase in AHR (fig. S3B), the neonatal SCID mice did not exhibit any AHR (fig. S3C). The observed AHR in WT mice correlated with increased levels of IL-13 and IL-33 which were not apparent in SCID mice (fig. S3, D and E). IL-13 is known to be critical for the generation of AHR. However, the dependence of AHR on the innate cytokine IL-33 in neonates has not been investigated. Therefore, we exposed neonatal WT and ***Il33***$^{-/-}$ mice to HDM from day 3 of life. Neonatal ***Il33***$^{-/-}$ mice developed equivalent AHR and an inflammatory response of similar composition and magnitude to WT mice (Fig. 4, A-D). IL-33 was absent in the ***Il33***$^{-/-}$ mice as expected but the type 2 cytokines IL-5 and IL-13 were induced to similar levels in WT and KO mice (Fig. 4, E-G), demonstrating that in neonatal mice AHR is independent of IL-33 but totally reliant on IL-13. These data underscore the functional importance of perinatal $IL\text{-}13^+CD4^+$ T cells in inducing AHR in early life and suggest pulmonary T cells are a critical source of IL-13 in the immediate postnatal period.

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

In order to confirm the importance of T cell derived IL-13 on the generation of allergen induced AHR we exposed either neonatal WT or *Cd*4-*cre Il*-4*Il*13$^{fl/fl}$ mice to inhaled HDM or *Alternaria*. T cells from *Cd*4-*cre Il*-4*Il*13$^{fl/fl}$ mice are unable to generate IL-13 or IL-4 so permit the specifc role of T cell derived IL-4/IL-13 to be investigated. Initial experiments confirmed the previously observed allergen induced increase in IL-13$^+$CD4$^+$ T cells in WT mice and established that these cells were absent in the ***Cd*4-*cre Il*-4*Il*13$^{fl/fl}$** mice (Fig. 5A). Confirming our hypothesis, mice lacking these cells did not develop AHR following exposure to either HDM or *Alternaria* (Fig. 5, B and C). We next determined the composition of the pulmonary cellular infiltrate in these mice. The number of eosinophils (Fig. 5, D and E) and IL-13$^+$ ILC (Fig. 5, F and G) were not significantly different between WT and ***Cd*4-*cre Il*-4*Il*13$^{fl/fl}$** mice. Likewise, T cells expressing IL-5 were not affected by the specific loss of IL-13$^+$CD4$^+$ T cells (Fig. 5, H and I). Despite the presence of increased IL-13$^+$ ILC2 with the capacity to secrete IL-13 (Fig. 5, F and G), there was only a minimal increase in pulmonary IL-13 levels following HDM or *Alternaria* exposure (Fig. 5, J and K) in the absence of IL-13$^+$CD4$^+$ T cells. Expression of Muc5ac was increased in neonates exposed to both HDM and *Alternaria* (Fig. 5, L and M). In mice lacking IL-13$^+$CD4$^+$ T cells the HDM induced increase in Muc5ac was ablated (Fig. 5L) indicating that expression is dependent on IL-13 levels. However, in mice administered *Alternaria,* allergen induced Muc5ac gene expression was maintained (Fig. 5M). This suggests IL-13$^+$ ILC2 alone are not sufficient to generate levels of IL-13 necessary to drive AHR in the absence of IL-13$^+$CD4$^+$ T cells in early life. Thus, mice lacking IL-13 specifically in CD4$^+$ T cells were completely protected from developing AHR following exposure to different allergens, underscoring the importance of these cells in the inception of early life AHR.

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

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

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

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

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

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

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

Materials and Methods

Study Design

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

Research samples.

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

Experimental design.

Randomization

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

Sample size.

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

Animals and reagents

Female BALB/c wild-type and Beige SCID mice were initially obtained from Charles River (Saffron Walden, UK) and maintained by in-house breeding. ***Cd*4-*cre Il*-4*Il*13$^{fl/fl}$ mice**(*50*) on

a BALB/c background were a kind gift from David Voehringer. ***Il33***$^{-/-}$ mice were a kind gift from MedImmune Inc. Each mother with its litter was housed separately. Mice were maintained in specific pathogen–free conditions and given food and water *ad libitum*. In individual experiments all mice were matched exactly for age and background strain. All procedures were conducted in accordance with the Animals (Scientific procedures) Act 1986. Recombinant mouse IL-33 (50µg/kg) for intra-nasal administration was purchased from R&D Systems (UK).

Allergen challenge

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

Bacteria and allergen co-exposure

BALB/c mice were exposed to intermittent intra-nasal *Acinetobacter iwoffii* F78 (*A. iwoffii* F78) ($1.3x10^8$cfu first 2 weeks, then $2x10^8$cfu (*52*) (a kind gift from Johann Bauer) or PBS followed by HDM (10µg first 2 weeks, then 15µg) or PBS for 3 weeks starting on day 3 of life for 3 weeks. All outputs were assessed 24 hours post final challenge as described below.

Measurement of airway hyperresponsiveness

Airways resistance was calculated using the flexivent small animal ventilator (Scireq) using 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 H_2O. 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 ºC 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 ºC. 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 fluorochome-conjugated antibodies to
485 intracellular cytokines (see table/supplementary methods) in Permeablization buffer for 20
486 minutes at 4 ºC. '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

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

qPCR

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

Statistical analysis

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

<u>Supplementary Materials</u>

Fig. S1. Defining IL-13^{+} CD4^{+} T cells and ILCs in neonatal mice.

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

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

Table S1. Antibodies used for flow cytometry.

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Author Contributions:

S.S. wrote the manuscript draft, conceived and designed the experiments. E.v.M supplied the *A. iwoffii* and provided intellectual input on the farmyard dust. J.E.V., A.K.M., R. G., R.S., 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 the experiments. ***S.A.W., R.A.O. and L.G.G. carried out the statistical analyses***. A.K.M., A.B. and L.G.G. revised the manuscript. C.M.L. conceived the study, designed the experiments and edited the manuscript.

Competing Interests:

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

Data and materials availability:

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

Figures captions:

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

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

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

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

Fig. 3. <u>IL-13 is a feature of allergen activated neonatal T cells</u>.

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

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

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

3 day old wild type BALB/c (WT) and ***Il33*$^{-/-}$** mice were exposed to intermittent doses of house dust mite (HDM) or PBS. **(A)** Airway responsiveness to methacholine (Mch) in neonatal WT and ***Il33*$^{-/-}$** mice. **(B)** Pulmonary eosinophils ($SiglecF^+CD11c^{low/neg}$). Numbers of pulmonary **(C)** $Lin^{neg}CD45^+$ IL-13^+ILCs and **(D)** $CD3^+CD4^+$IL-13^+ T cells. Levels of **(E)** IL-33, **(F)** IL-5 and **(G)** IL-13 in the lung. N=7-12 HDM, N=6-8 PBS. Data representative of 2 experiments. *p<0.05, **p<0.01, ***p<0.001.

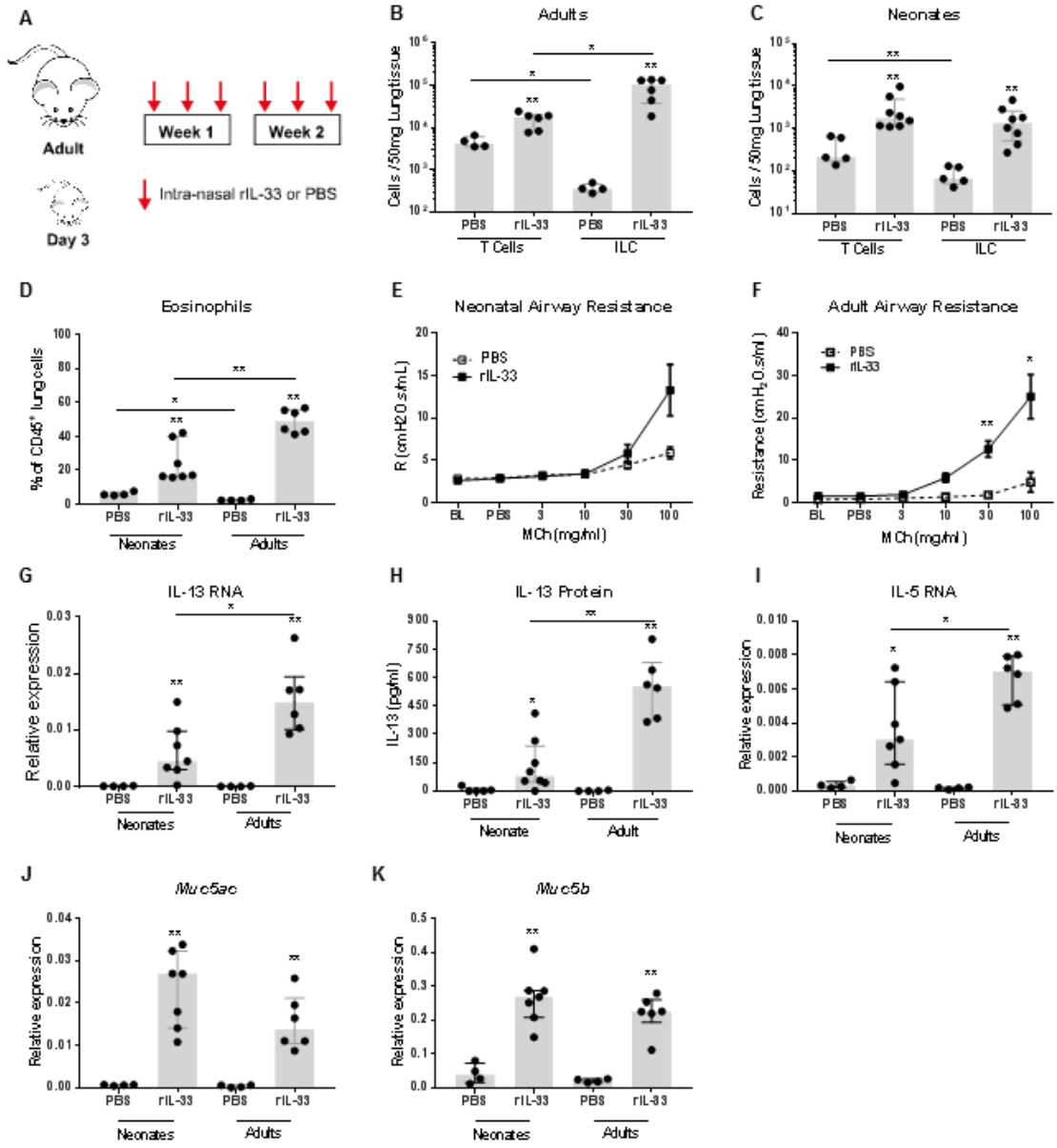
Fig. 5. T cell derived IL-13 is essential for the inception of AHR.

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

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

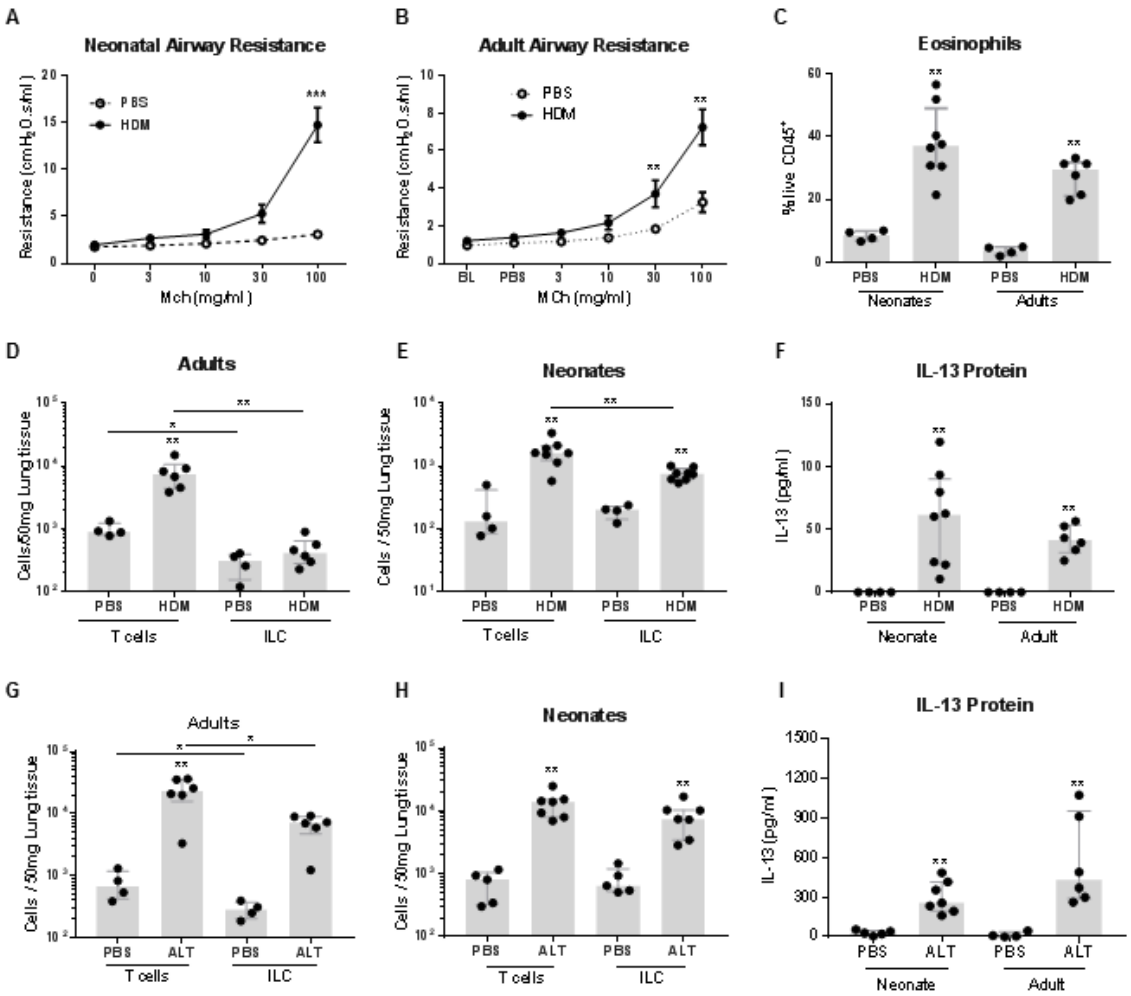
Fig. 6. Protection from AHR in neonatal mice can be achieved by reduction of IL-13^{+} T cells.

(A) BALB/c mice aged 3 days were exposed to intermittent intra-nasal *Acinetobacter iwoffii* F78 (*A. iwoffii* F78) (1.3x10^{8}cfu first 2 weeks, then 2x10^{8}cfu) or PBS (⇧) followed by house dust mite (HDM) (10µg first 2 weeks, then 15µg) or PBS for 3 weeks (⬇). Analysis was carried out 24 hours post final dose. **(B)** Airway hyperresponsivness (AHR) to methacholine (Mch) after 3 weeks of *A. iwoffii* F78 and HDM co-exposure. **(C)** Numbers of eosinophils (SiglecF^{+}CD11c$^{low/neg}$) in lung. **(D)** IL-33 and **(E)** IL-13 levels from lung homogenate. **(F)** Numbers of innate lymphoid cells (Lin^{-}CD45^{+}IL-13^{+}) and **(G)** CD3^{+}CD4^{+}IL-13^{+} T cells. **(H)** IL-5 levels in the lung tissue. **(I)** Dendritic cell (DC) populations in the lung enumerated by flow cytometry. cDC (CD11b^{+}CD11c^{high}), pDC (CD11c^{int}Ly6c^{+}CD64^{-}), MoDC (CD11c^{+}Ly6c^{+}CD64^{+}). N=8 for HDM and bacteria exposed groups, N=5 for PBS. *p<0.05, **p<0.01, ***p<0.001. Data representative of 3 experiments.


A
Adult
Day 3
Week 1
Week 2
Intra-nasal rIL-33 or PBS
B
Adults
Cells /50mg Lung tissue
PBS
rIL-33
T Cells
ILC
C
Neonates
D
Eosinophils
%of CD45+ lung cells
Neonates
Adults
E
Neonatal Airway Resistance
R (cmH2O.s/mL)
MCh (mg/ml)
F
Adult Airway Resistance
Resistance (cmH2O.s/ml)
G
IL-13 RNA
Relative expression
H
IL-13 Protein
IL-13 (pg/ml)
Neonate
Adult
I
IL-5 RNA
J
Muc5ac
K
Muc5b

799

800


A
Neonatal Airway Resistance
Resistance (cmH2O.s/ml)
PBS
HDM
***
Mch (mg/ml)
B
Adult Airway Resistance
Resistance (cmH2O.s/ml)
PBS
HDM
**
BL
Mch (mg/ml)
C
Eosinophils
%live CD45+
PBS
HDM
Neonates
Adults
D
Adults
Cells/50mg Lung tissue
T cells
ILC
E
Neonates
Cells / 50mg Lung tissue
T cells
ILC
F
IL-13 Protein
IL-13 (pg/ml)
Neonate
Adult
G
Adults
Cells / 50mg Lung tissue
ALT
T cells
ILC
H
Neonates
Cells / 50mg Lung tissue
ALT
T cells
ILC
I
IL-13 Protein
IL-13 (pg/ml)
ALT
Neonate
Adult

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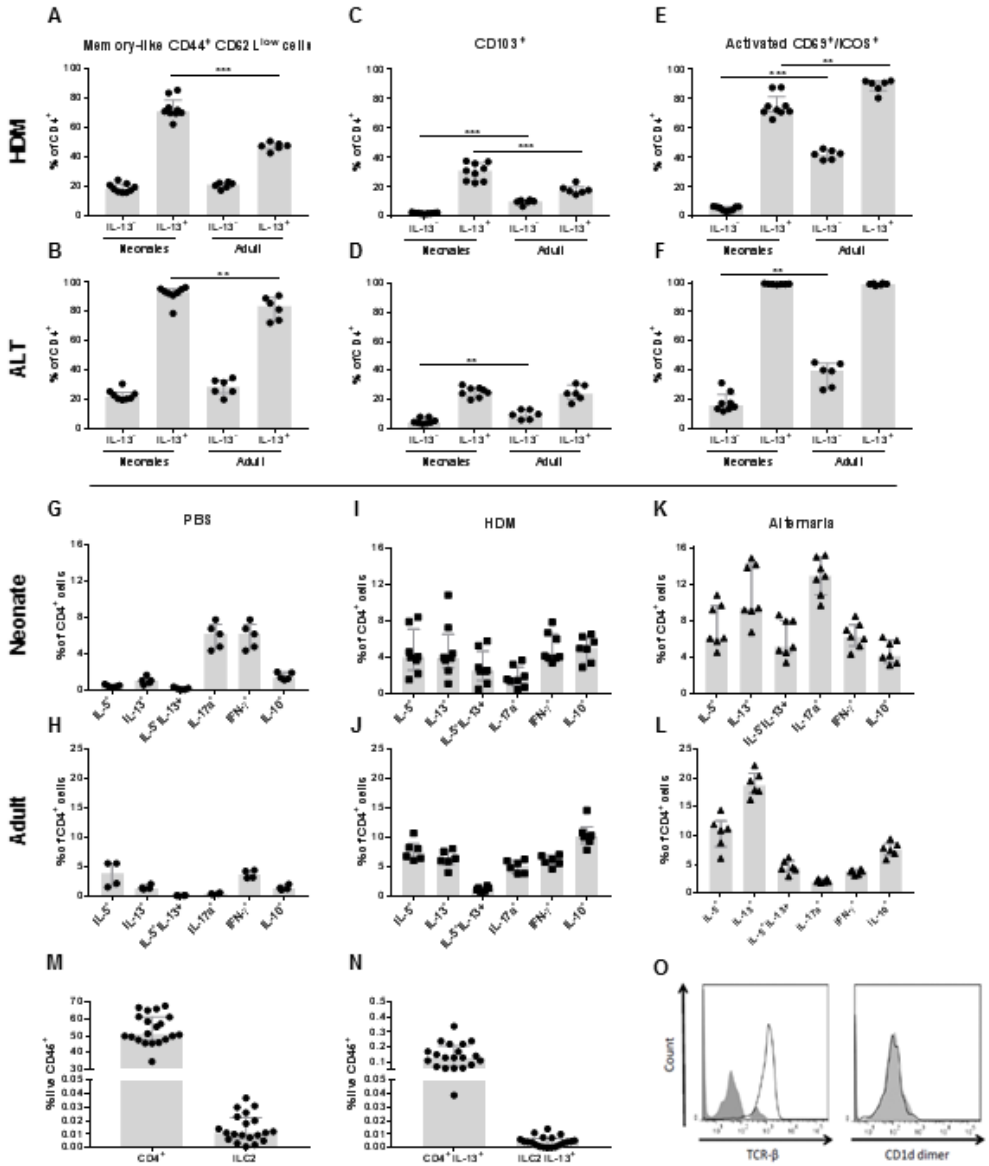
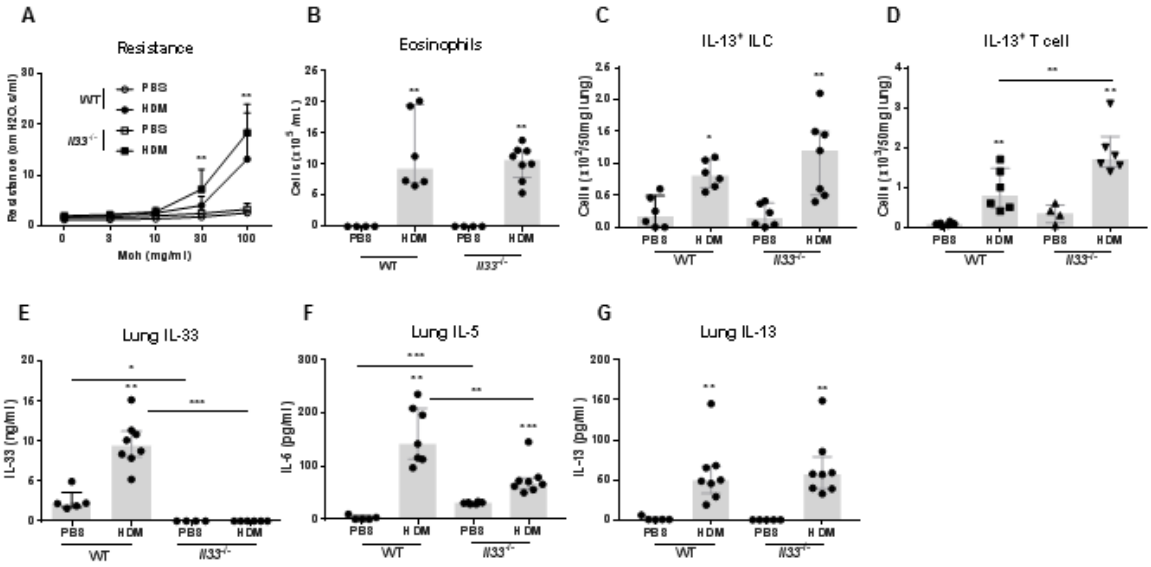

A
Memory-like CD44+ CD62 Llow cells
C
CD103+
E
Activated CD69+/ICOS+
HDM
B
D
F
ALT
Neonates
Adult
IL-13-
IL-13+
G
PBS
I
HDM
K
Alternaria
Neonate
H
J
L
Adult
IL-5+
IL-13+
IL-5+IL-13+
IL-17a+
IFN-γ+
IL-10+
M
N
O
%of CD4+ cells
%of fCD4+ cells
%live CD45+
CD4+
ILC2
CD4+IL-13+
ILC2 IL-13+
Count
TCR-β
CD1d dimer

803

804


A
Resistance
WT
PBS
HDM
Il33-/-
PBS
HDM
Resistance (cm H2O.s/ml)
Mch (mg/ml)
B
Eosinophils
Cells (x10^3 /ml)
C
IL-13+ ILC
Cells (x10^3/50mg Lung)
D
IL-13+ T cell
Cells (x10^3/50mg Lung)
E
Lung IL-33
IL-33 (ng/ml)
F
Lung IL-5
IL-5 (pg/ml)
G
Lung IL-13
IL-13 (pg/ml)

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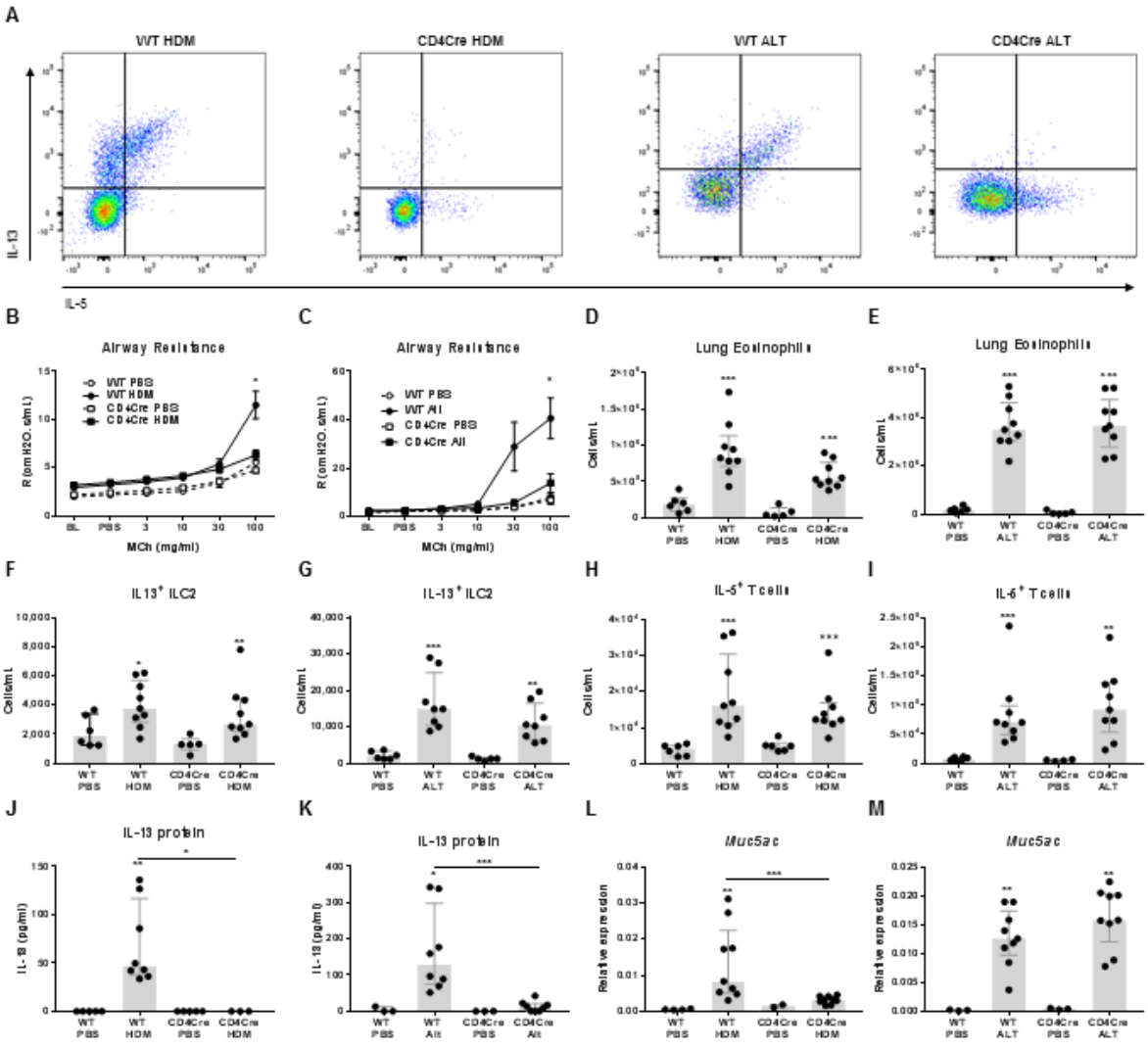
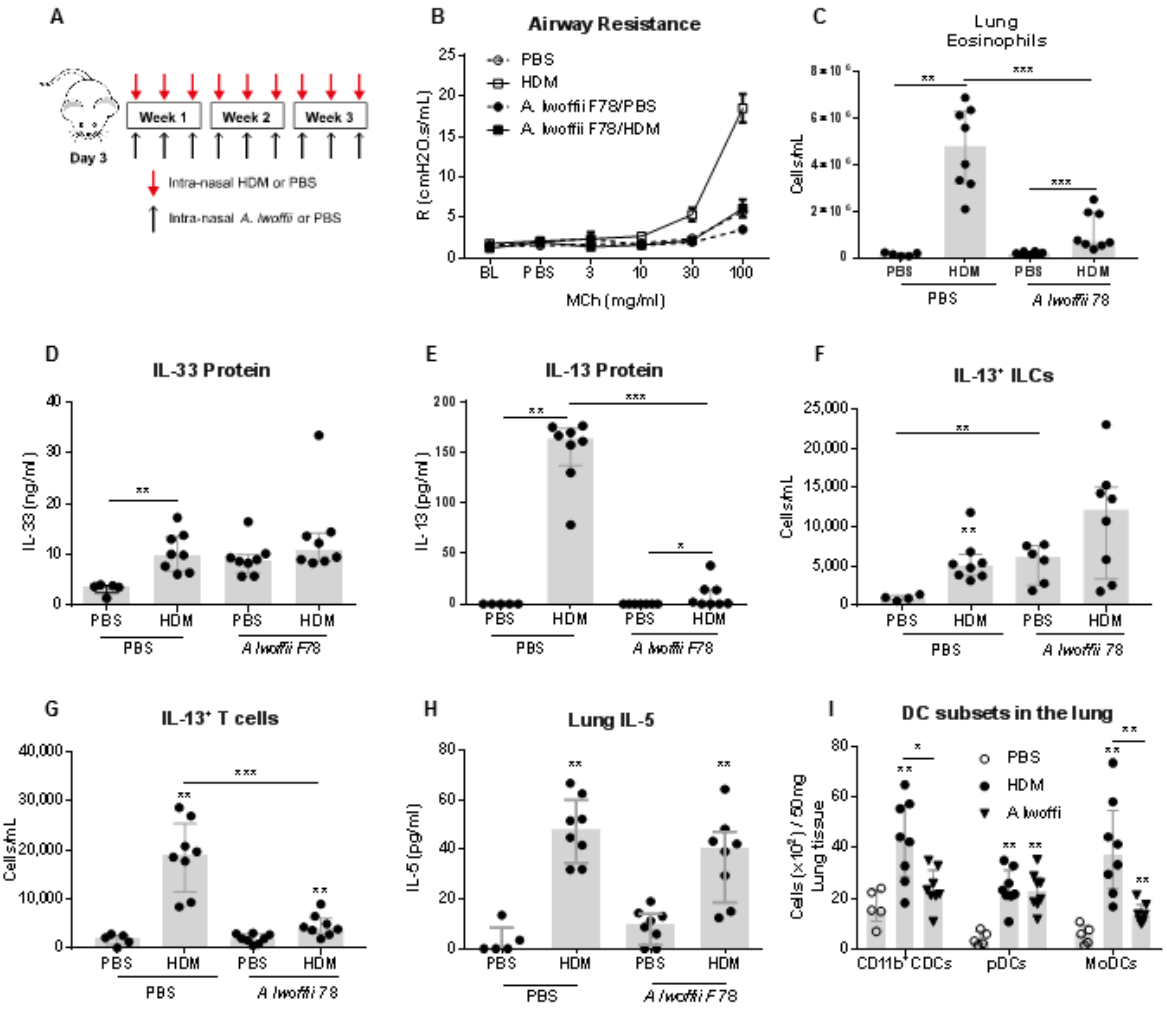
806


A
WT HDM
CD4Cre HDM
WT ALT
CD4Cre ALT
IL-13
IL-5
B
Airway Resistance
WT PBS
WT HDM
CD4Cre PBS
CD4Cre HDM
R (cm H2O. s/mL)
BL PBS 3 10 30 100
MCh (mg/ml)
C
Airway Resistance
WT PBS
WT Alt
CD4Cre PBS
CD4Cre Alt
MCh (mg/ml)
D
Lung Eosinophils
Cells/mL
E
Lung Eosinophils
F
IL13+ ILC2
G
IL-13+ ILC2
H
IL-5+ T cells
I
IL-6+ T cells
J
IL-13 protein
IL-13 (pg/ml)
K
IL-13 protein
L
Muc5ac
Relative expression
M
Muc5ac
WT PBS
WT HDM
CD4Cre PBS
CD4Cre HDM
WT ALT
CD4Cre ALT

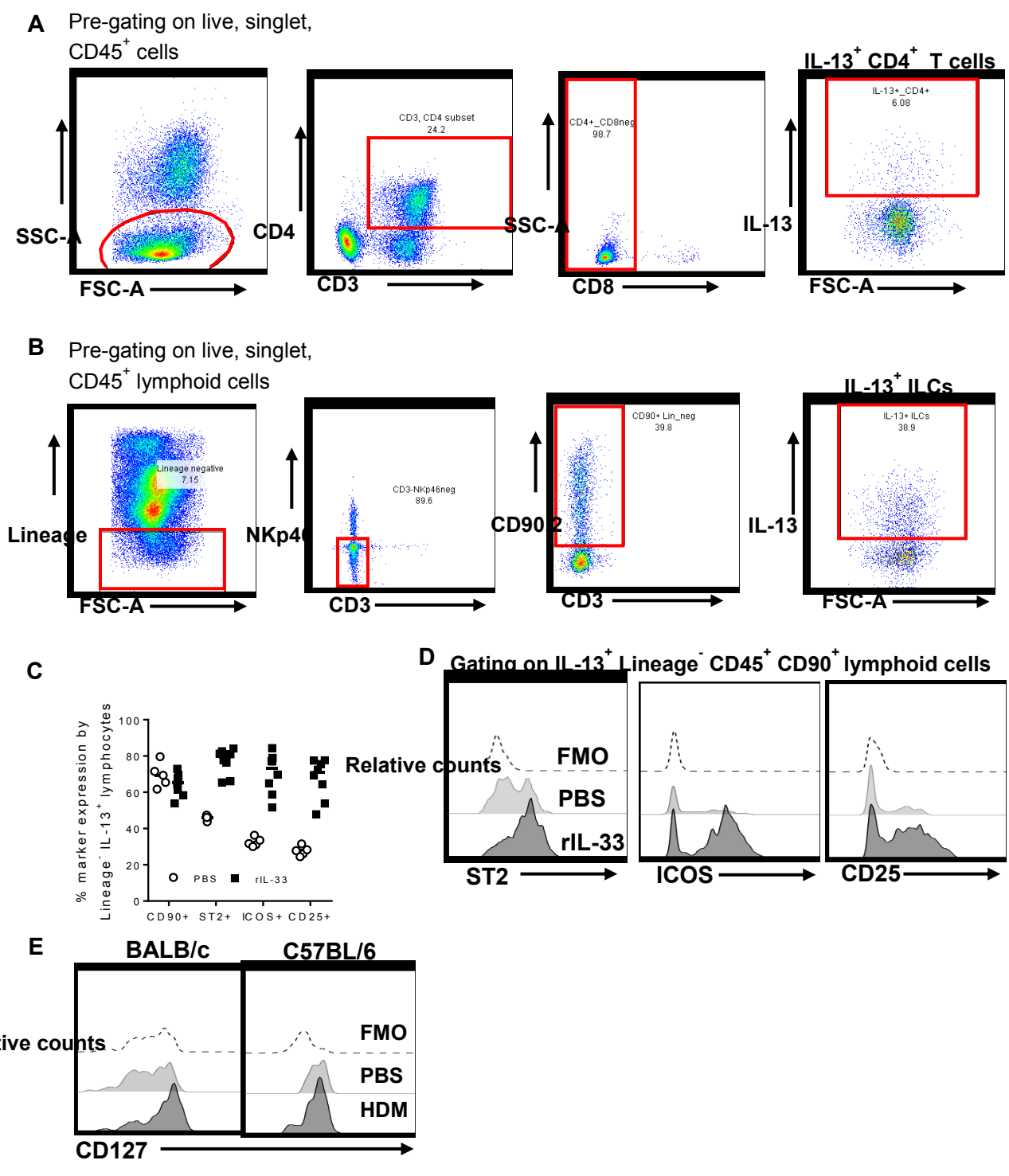
807

808


A
Week 1
Week 2
Week 3
Day 3
Intra-nasal HDM or PBS
Intra-nasal A. lwoffii or PBS
B
Airway Resistance
PBS
HDM
A. lwoffii F78/PBS
A. lwoffii F78/HDM
R (cmH2O.s/mL)
BL
PBS
3
10
30
100
MCh (mg/ml)
C
Lung Eosinophils
Cells/mL
PBS
HDM
A lwoffii 78
D
IL-33 Protein
IL-33 (ng/ml)
E
IL-13 Protein
IL-13 (pg/ml)
F
IL-13+ ILCs
Cells/mL
G
IL-13+ T cells
Cells/mL
H
Lung IL-5
IL-5 (pg/ml)
I
DC subsets in the lung
Cells (x10^3) / 50mg Lung tissue
CD11b+ CDCs
pDCs
MoDCs

809

810

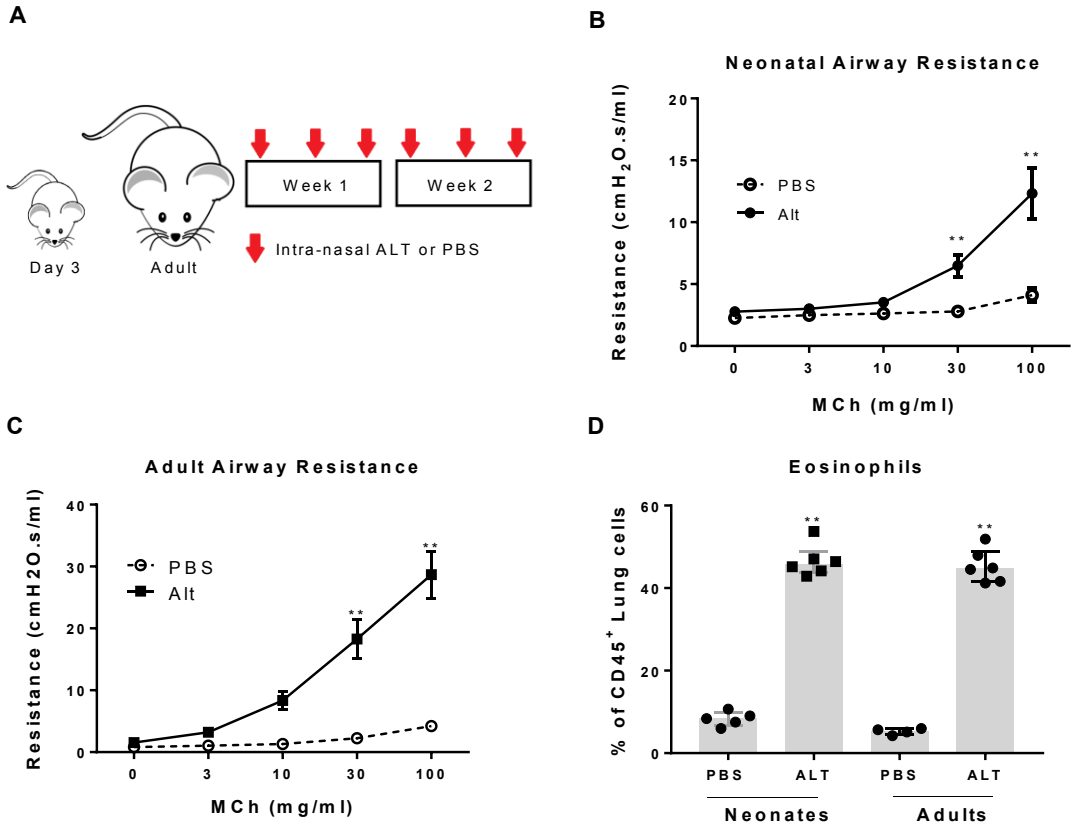


Supplementary Fig. 1. Defining IL-13$^+$ CD4$^+$ T cells and ILCs in neonatal mice

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

829 defined as live, singlet, $CD45^+$ lymphoid, lineage- (TCRβ, TCRγδ, CD3e, CD5, CD19, CD11b,
830 CD11c, FCεR1, 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 $IL\text{-}13^+$ $Lineage^-$ $CD45^+$ 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 $IL\text{-}13^+$ $CD90.2^+$ $CD45^+$ Lineage- ILCs,
835 relative to FMO controls. **(E)** Representative histograms showing CD127 surface staining on
836 $IL\text{-}13^+$ $CD90.2^+$ $Lineage^-$ 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



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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.

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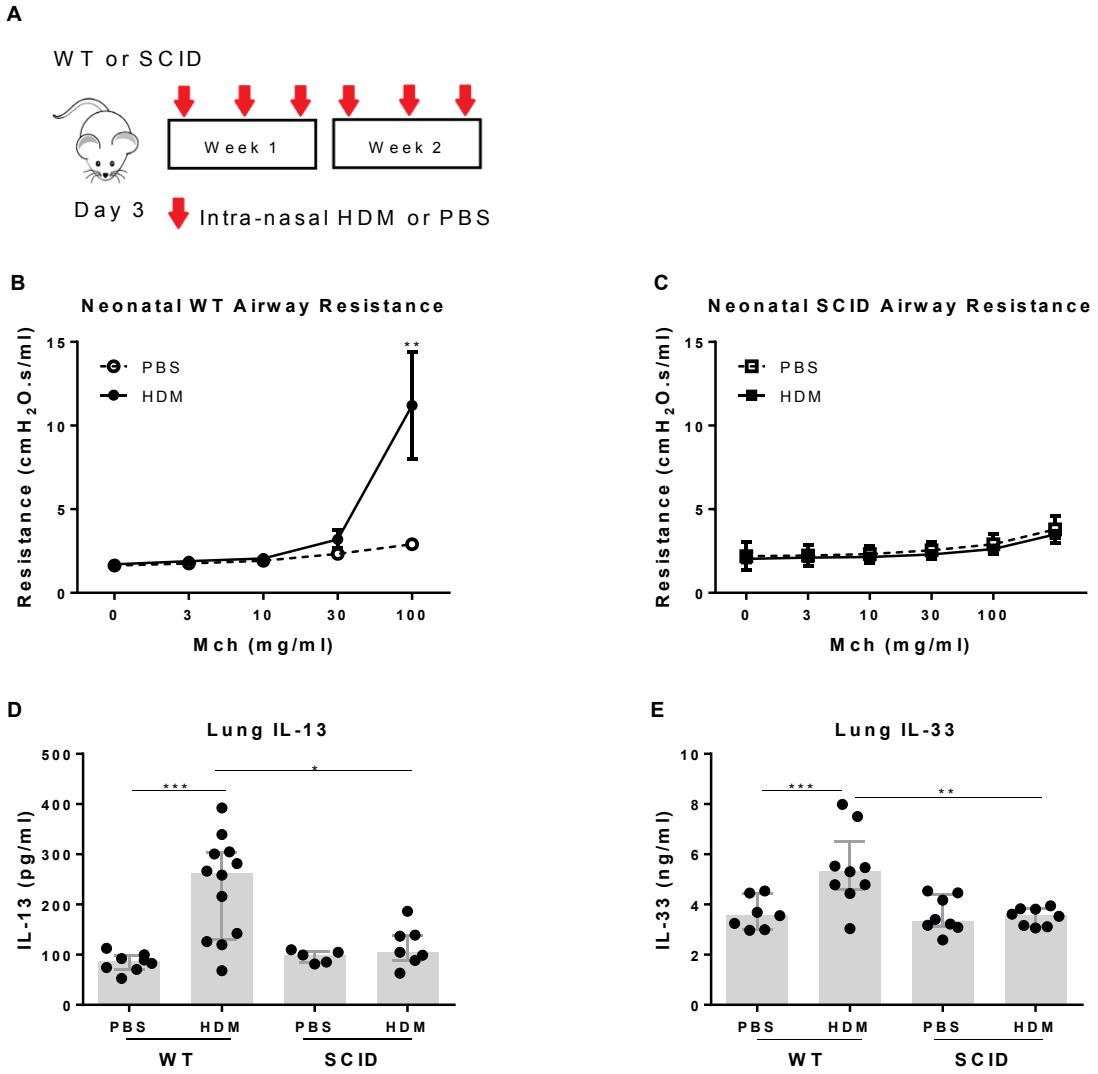

851

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

IFNγ	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
TCRβ	H57-597	APC	Mouse	Biolegend
TCRγδ	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

Table S1. Antibodies used for flow cytometry