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.

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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-13$^+$ T cells and IL-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-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-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-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 *Cd4-cre Il-4Il13*$^{fl/fl}$ mice to inhaled HDM or *Alternaria*. T cells from *Cd4-cre Il-4Il13*$^{fl/fl}$ 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 ***Cd4-cre Il-4Il13***$^{fl/fl}$ 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 ***Cd4-cre Il-4Il13***$^{fl/fl}$ 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

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

Discussion

An essential clinical feature of asthma and wheezing in childhood, including infancy and the pre-school years, is the presence of AHR (*37*). In addition, cohort studies have shown the single most important factor that determines development of asthma in children is AHR in early life, which may be apparent even before the onset of the manifest symptom of wheezing (*38*). In order to identify mechanisms mediating allergen induced disease inception, and targets for intervention to allow asthma prevention, achieving a reduction in AHR is essential. However, the underlying molecular mechanisms remained unclear. AHR was the focus of this study in order to optimally reflect paediatric symptoms. We determined that effector IL-13$^+$ CD4$^+$ cells are critical for the development of AHR following exposure to either the ILC2 promoting cytokine rIL-33 or clinically relevant allergens HDM and *Alternaria* in the first weeks of life. IL-13$^+$ ILCs were insufficient to compensate for an absence of IL-13$^+$CD4$^+$ T cells in early life, demonstrated by the lack of AHR in neonatal SCID mice or in mice specifically lacking IL-13 in CD4$^+$ T cells following exposure to inhaled allergen. Mice lacking functional T and B cells or IL-13$^+$CD4$^+$ T cells did not develop increased levels of IL-13 following allergen exposure from day 3 of life despite comparable numbers of IL-13$^+$ ILCs. Moreover, administration of inhaled farm dust bacteria during exposure to HDM to neonatal mice resulted in complete protection from AHR with a significant reduction in pulmonary IL-13$^+$CD4$^+$ T cells and levels of IL-13, but sustained elevation of IL-33 and IL-13$^+$ ILCs. Collectively these data show that in neonatal mice, T cells are an essential early source of IL-13 to drive AHR. Our results indicate that although ILCs have the potential to generate IL-13 when stimulated with PMA 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. ***Cd4-cre Il-4Il13$^{fl/fl}$* 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*).

Mice were anesthetized with pentobarbital sodium (50mg/kg intra-peritoneal) and ketamine (100mg/kg intra-muscular), tracheostomised and connected to the flexivent ventilator via a blunt-ended 21-gauge needle (neonate) or 19-gauge needle (adult). The mice were ventilated with an average breathing frequency of 150 breaths/minute; tidal volume of 10ml/kg body weight; positive end-expiratory pressure approximately 2cm H_2O. Changes in resistance to increasing to increasing concentrations of nebulized methacholine (3 -3 100mg/ml were calculated from the snapshot perturbation measurements.Resultant data was fitted using multiple linear regression to the single compartment model in the form: pressure = resistance x flow + elastance x volume + fitting constant.

Inflammation and cell recovery

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

Flow cytometry

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

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

Quantification of cytokines

Lung tissue was homogenized at 50mg/ml in HBSS (Gibco) containing protease inhibitor tablets (Roche Diagnostics), centrifuged at 800 x *g* for 20minutes and the supernatant was 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 $^{***}p < 0.001$

Supplementary Materials

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.

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

3 day old wild type BALB/c (WT) and ***Il*33$^{-/-}$** mice were exposed to intermittent doses of house dust mite (HDM) or PBS. **(A)** Airway responsiveness to methacholine (Mch) in neonatal WT and ***Il*33$^{-/-}$** 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.