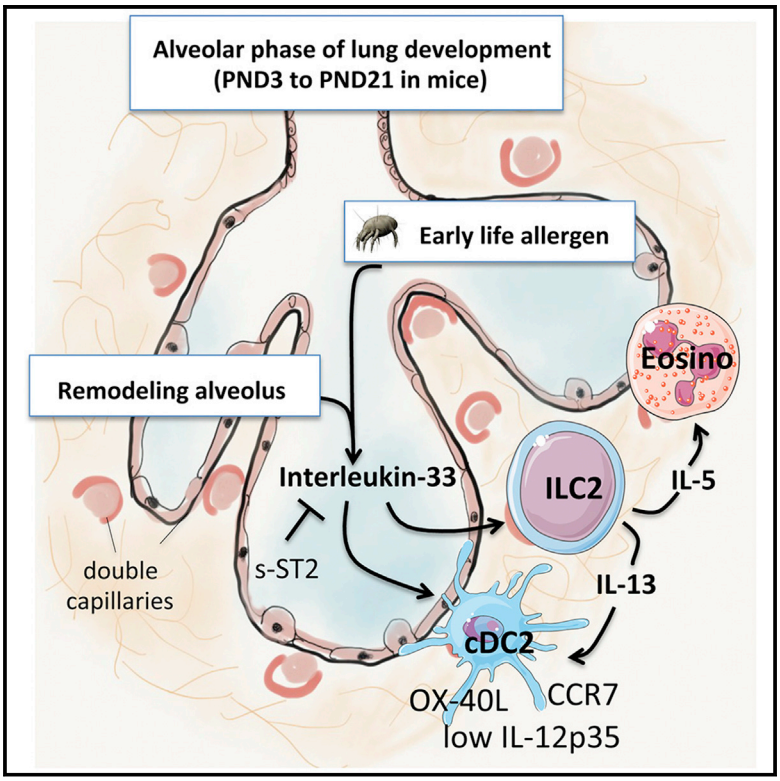
Article

Immunity

Perinatal Activation of the Interleukin-33 Pathway Promotes Type 2 Immunity in the Developing Lung

Graphical Abstract



Highlights

- There is spontaneous accumulation of type 2 immune cells in the developing lung
- Perinatal type 2 immunity depends on IL-33 and not on T or B cells
- Postnatal lung DCs are very scarce yet very efficient at inducing Th2 cell adaptive immunity
- The IL-33 axis is more important for asthma development at young age

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In Brief

It is not known why allergy often begins in early life. de Kleer et al. show that shortly after birth, type 2 innate immune cells accumulate in an IL-33-dependent manner in the developing lung. Interleukin-33 furthermore boosts the function of neonatal dendritic cells to promote long-lasting Th2-cell-mediated immunity.

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Perinatal Activation of the Interleukin-33 Pathway Promotes Type 2 Immunity in the Developing Lung

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SUMMARY

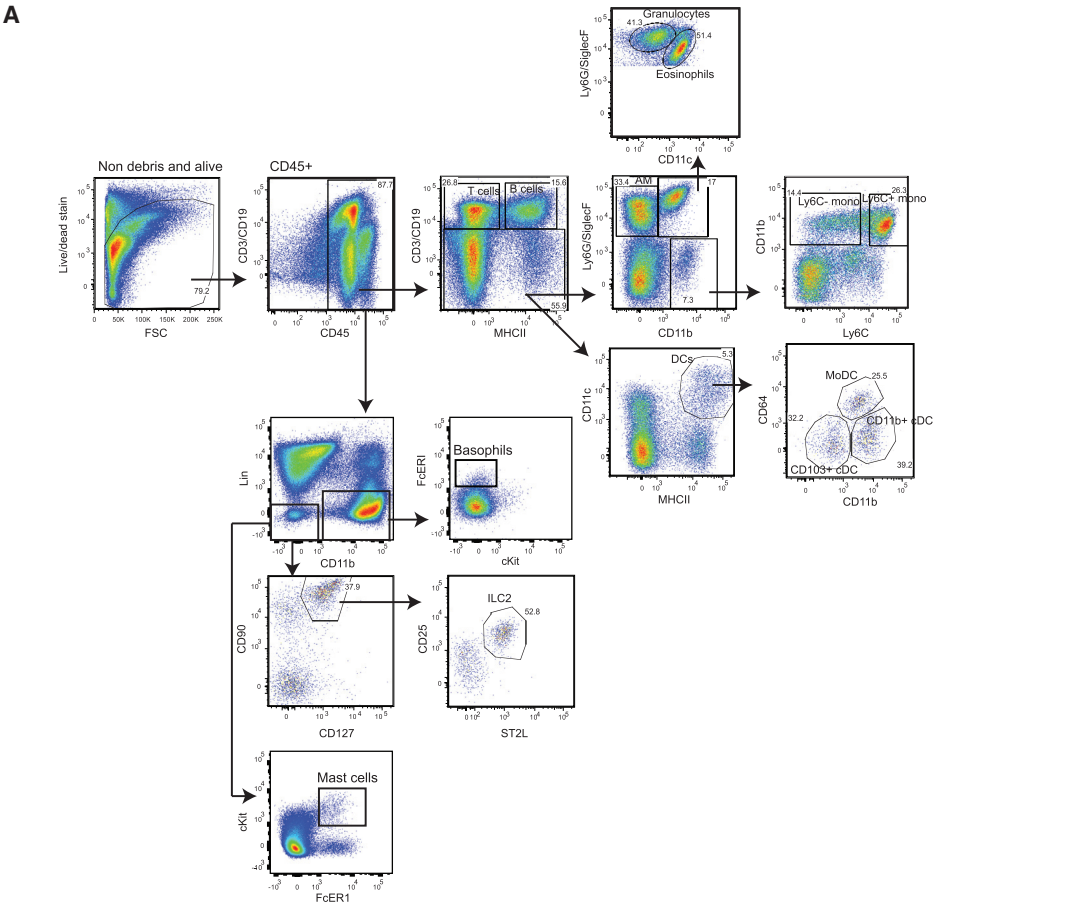
Allergic disease originates in early life and polymorphisms in interleukin-33 gene (*IL33*) and *IL1RL1*, coding for IL-33R and decoy receptor sST2, confer allergy risk. Early life T helper 2 (Th2) cell skewing and allergy susceptibility are often seen as remnants of feto-maternal symbiosis. Here we report that shortly after birth, innate lymphoid type 2 cells (ILC2s), eosinophils, basophils, and mast cells spontaneously accumulated in developing lungs in an IL-33-dependent manner. During the phase of postnatal lung alveolarization, house dust mite exposure further increased IL-33, which boosted cytokine production in ILC2s and activated CD11b$^+$ dendritic cells (DCs). IL-33 suppressed IL-12p35 and induced OX40L in neonatal DCs, thus promoting Th2 cell skewing. Decoy sST2 had a strong preventive effect on asthma in the neonatal period, less so in adulthood. Thus, enhanced neonatal Th2 cell skewing to inhaled allergens results from postnatal hyperactivity of the IL-33 axis during a period of maximal lung remodeling.

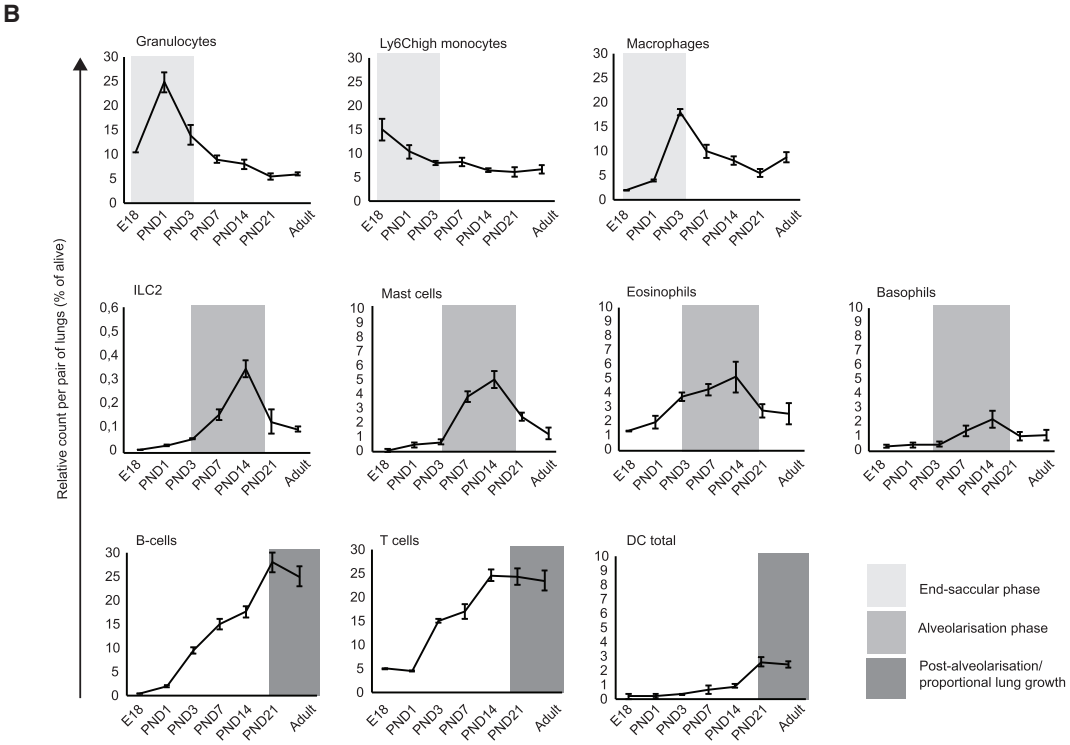
INTRODUCTION

Allergic asthma often starts in early childhood, affecting around 5%–10% of school-aged children. Most cases of pediatric asthma are driven by a type 2 immune response, rich in eosinophils, mast cells, and basophils and controlled by allergen-specific CD4$^+$ T helper 2 (Th2) lymphocytes. The type 2 response induces goblet cell metaplasia (GCM), airway hyperresponsiveness (AHR), and airway remodeling (Lambrecht and Hammad, 2015; Saglani et al., 2013). House dust mite (HDM) allergen represents the most common trigger of allergic asthma (Celedón et al., 2007) and Th2 cell sensitization to HDM has been modeled in the mouse. In adult mice, lung CD11b$^+$ conventional dendritic cells (DCs) and bronchial epithelial cells control sensitization to HDM (Hammad and Lambrecht, 2015; Schuijs et al., 2015). DCs take up inhaled HDM allergen and transport it to the draining mediastinal lymph nodes (MLNs), where they polarize the Th cell immune response, helped by basophils, innate lymphoid cells type 2 (ILC2s), and B cells (Coquet et al., 2015; Hammad et al., 2010; Oliphant et al., 2014; Plantinga et al., 2013). Activation of DCs occurs through the release of epithelial cytokines like thymic stromal lymphopoietin (TSLP), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin (IL)-25, and the IL-1 family members IL-1α and IL-33 (Hammad et al., 2009; Kool et al., 2011; Phipps et al., 2009; Willart et al., 2012). Some of these lung-tissue-derived cytokines like IL-33 also induce cytokine production by ILC2s and tissue-resident memory Th2 cells (Coquet et al., 2015; Endo et al., 2015; Guo et al., 2015; Klein Wolterink et al., 2012).

Early postnatal T cell reactivity to HDM allergens is a major risk factor for the development of asthma in children, suggesting that persistent Th2 cell sensitization occurs early after birth (Celedón et al., 2007; Lodge et al., 2011). Why young children are at increased risk to become sensitized is largely unknown. The immune system is undergoing important shifts in its composition and function during the postnatal period and early childhood. Newborns demonstrate enhanced Th2 cell function, which is believed to be the result of a complex feto-maternal symbiosis. Th2 cell immunity suppresses alloresponses during pregnancy and promotes growth of the placenta (Fock et al., 2013; Forsthuber et al., 1996). The enhanced Th2 cell bias of neonatal T cells is in part T cell intrinsic and transient (Li et al., 2004; Sornasse et al., 1996; White et al., 2002), but cord blood DCs have also been shown to lack production of the Th1-cell-polarizing cytokine IL-12p70 (Goriely et al., 2001; Upham et al., 2002).

When allergens are first encountered, the lungs of young children are still developing and undergo a postnatal phase of alveolarization, characterized by epithelial cell differentiation, angiogenesis, and extracellular matrix remodeling. This alveolar phase of lung growth lasts until 3 years of age and is controlled by many growth factors and cytokines that could also influence





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the immune system. Here we found that IL-33 was spontaneously produced during the alveolar phase of lung development, causing accumulation of type 2 innate immune cells. In this period, HDM exposure caused a vicious circle of IL-33-mediated activation of innate type 2 cells and DCs. IL-33 suppressed IL-12 production and induced OX40-ligand in neonatal DCs, explaining the Th2 cell bias in this period. Consequently, the soluble IL-33 decoy receptor (sST2) had a much stronger preventive effect on allergy development in neonatal than in adult mice. As polymorphisms in the IL-33 or IL-1RL1 gene have been consistently found to confer allergy and asthma risk, we propose that the IL-33 pathway is a crucial regulator of asthma development in early life that alters communication between epithelial cells, DCs, and innate type 2 immunity.

RESULTS

Developing Lungs Accumulate Type 2 Immune Cells

Lung development proceeds via a pseudoglandular stage (embryonic [E] day 9.5–16.5), a canalicular stage (E16.5–17.5), a saccular stage (E18.5 to post natal day 5 [PND5]), an alveolar stage (PND4–21), and a stage of microvascular maturation (PND14–21) (Schittny et al., 2008). Fetal lung is first populated around E12 by CD45$^+$ yolk sac-derived macrophages (De Kleer et al., 2014; Guilliams et al., 2013; Schulz et al., 2012; van de Laar et al., 2016). We recently have shown that this first wave of yolk sac-derived macrophages is followed by a consecutive wave of fetal liver-derived monocytes that enter the lung around E18, the start of the saccular phase in mice, and give rise to alveolar macrophages (AM) under the influence of GM-CSF (Guilliams et al., 2013; van de Laar et al., 2016). The prenatal influx of fetal liver-derived monocytes was accompanied by a rapid influx of granulocytes that peaked in number at PND1 (Figures 1A, 1B, and S1). At PND3 coinciding with the start of the alveolar phase of lung development, ILC2s, mast cells, eosinophils, and basophils (hereinafter collectively referred to as "type 2 innate cells") entered the lung and rapidly increased in numbers at PND14, outnumbering the number of adult type 2 cells by a factor of 4 to 10. Type 2 cells declined again after weaning. Numbers of DCs and T and B cells were very low directly after birth and gradually increased over time. DCs reached adult numbers around PND21, around the time of weaning. Thus, the postnatal phase of lung alveolarization is characterized by a spontaneous influx of high numbers of type 2 innate cells.

Type 2 Immunity to Allergens Is Promoted during the Alveolarization Period

DCs are crucial for mounting adaptive Th2-cell-mediated immunity to HDM in adult mice (Plantinga et al., 2013). As adaptive immune cells and DCs were severely impaired in the neonatal respiratory tract (Figure 1B), we hypothesized that sensitization to inhaled HDM would be defective during this period. We compared the effect of a single HDM sensitization in the early postnatal period (PND3, protocol A), alveolarization period (PND14, protocol B), or adult age (6–8 weeks, protocol C) (Figure 2A). All mice were challenged 7–11 days and analyzed 14 days after the sensitization. Despite severely reduced DC numbers at PND3 and PND14, HDM sensitization at these early time points followed by challenge resulted in significantly increased eosinophilia and lymphocytosis in the bronchoalveolar lavage (BAL) fluid (Figure 2B), increased Th2-cell-associated cytokines in MLN (Figure 2D), and increased total IgE and HDM-specific IgG1 in serum (Figure 2E), compared with PBS-sensitized mice. The degree of eosinophilia and lymphocytosis of mice sensitized at PND14 (protocol B) was even more marked than in mice sensitized at adult age (protocol C, Figure 2B). Mice in protocol B also demonstrated more inflammatory, eosinophil-rich lesions around the bronchi and pulmonary blood vessels (Figure 2C), higher IL-5 and IL-13 cytokines in the mediastinal LNs (Figure 2D), and higher concentration of serum HDM-specific IgG1 (Figure 2E) than mice in protocol C at adult age. Lungs of mice in protocol B contained much higher numbers of IL-5- and IL-13-producing CD4$^+$ T cells, as measured by intracellular staining, indicative of strongest adaptive immune response induction to HDM around PND14 (Figure 2F).

Mice in protocol A that were sham-sensitized at PND3 with PBS and challenged between PND10 and 14 with HDM (PBS-HDM group) showed significant eosinophilia in the BAL (Figure 2B) and goblet cell hyperplasia on lung sections (Figure 2C), an effect not seen in the PBS-HDM mice of protocols B and C. These signs of airway inflammation were accompanied by a strong increase in IL-5- and IL-13-producing ILC2s (Figure 2G) and were also observed in the lungs of PBS-HDM and HDM-HDM *Ccr7*$^{-/-}$ age-matched controls undergoing protocol A (Figures S2 and S3). *Ccr7*$^{-/-}$ mice lack migration of DCs to the MLNs and therefore cannot become actively sensitized to mount airway eosinophilia or an adaptive Th2 cell response in the MLN (Figures S3 and S4). We conclude therefore that the observed eosinophilia, goblet cell hyperplasia, and increase in IL-5- and IL-13-producing ILC2s in the PBS-HDM group in protocol A represents an innate immune response to HDM challenge. Indeed, administration of three consecutive doses of HDM (10 μg) in naive mice was sufficient to induce the strong accumulation and activation of IL-5- and IL-13-producing ILC2s around PND14, but much less so around PND3 or adulthood (Figure 2H). These data indicate that (1) the alveolarization period favors Th2 cell sensitization and (2) an exaggerated innate immune response to allergens in this period also causes immediate CCR7-independent type 2 airway inflammation.

Conventional CD11b$^+$ DCs Present Allergen in the Alveolarization Period

We next studied in greater detail how DCs developed postnatally. On tracheal whole mounts of MHCII-GFP mice, the network of highly dendritic MHCII$^+$ DCs that is found in large conducting airways of adult mice was virtually absent in neonatal mice

Figure 1. Three Waves of Perinatal Pulmonary Immune Cell Development

(A) Gating strategy by 12-color flow cytometry on dispersed lung cells.

(B) Relative cell counts in naive lungs of fetal (E20), neonatal, infant, and adult mice.

Figure S1 shows these data in absolute cell counts.

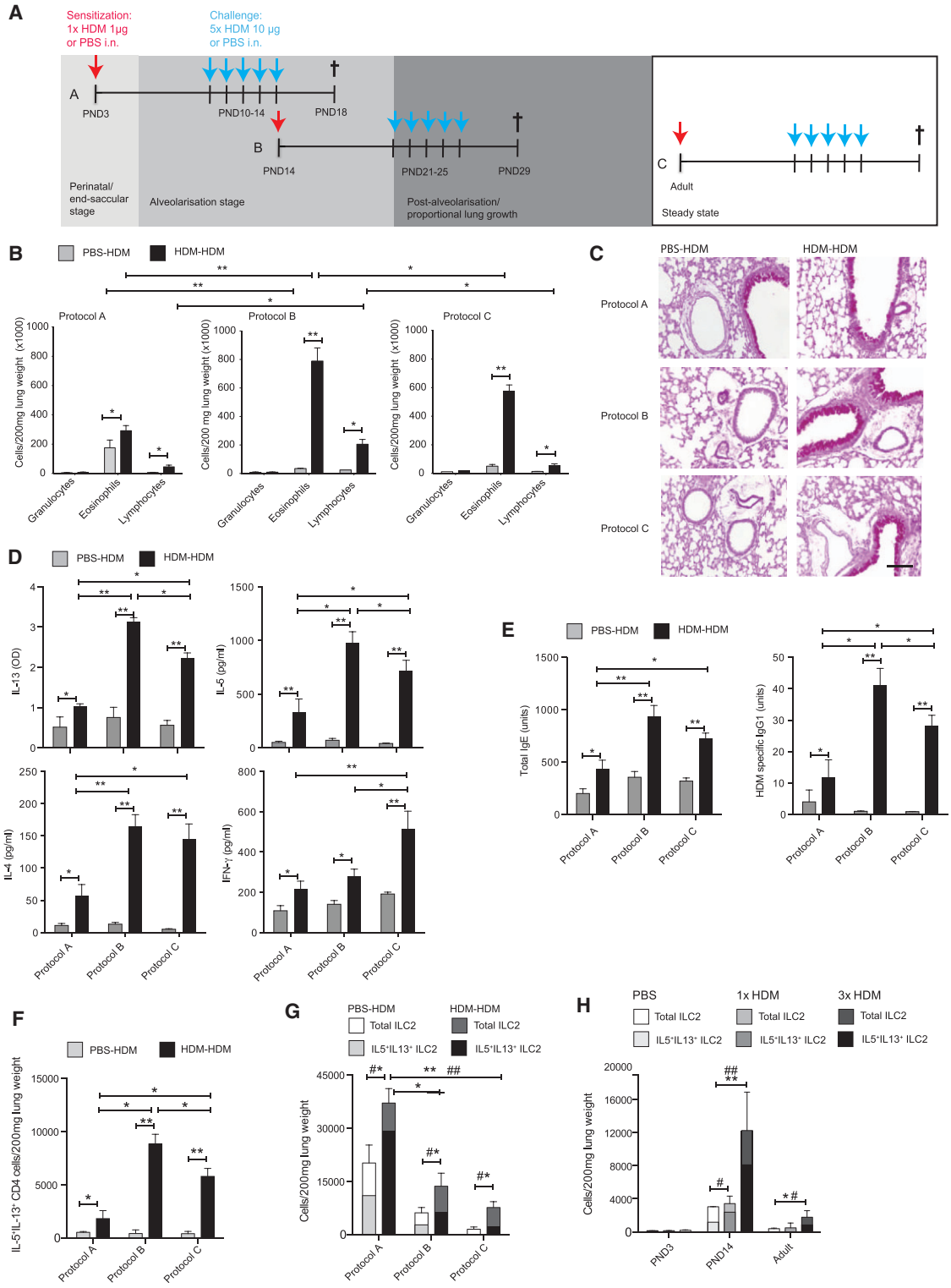

Figure 2. HDM Sensitization and Challenge in Various Age Groups

(A) Protocol of allergic airway induction. PND3, PND14, and adult (PND42–49) mice were sensitized on PND3 (protocol A), PND14 (protocol B), or PND42–49 (protocol C) with HDM or PBS and were challenged with HDM on 5 consecutive days 1 week after sensitization. 2 weeks after the start of the protocol, the mice were sacrificed.

(B) Differential cell counts in the BAL were determined by flow cytometry 72 hr after the last HDM challenge (day 14).

(C) PAS staining of lung sections. Scale bar represents 100 µM.

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(Figure 3A). A small number of studies have described increased numbers of CD11b$^+$ DCs in neonatal lungs (Gollwitzer et al., 2014; Roux et al., 2011), but a careful distinction between CD11b$^+$ cDCs and moDCs has never been made. Using a panel that discriminates cDCs from monocyte-derived DCs (Guilliams et al., 2013), we found that CD11b$^+$CD64$^+$ moDCs represented the largest DC subset in the lung from E20 until PND2 (Figure 3B). Among the two conventional pulmonary DC subsets, CD11b$^+$ cDCs developed more slowly, leading to a predominance of CD103$^+$ cDCs between PND1 and PND12. Naive MLNs at PND3, PND14, and adult age showed differences in total cellularity but equal ratios of T cells, B cells, and total DCs were noticed (Figure 3C). Also in the MLN, there was a clear predominance of CD103$^+$ cDCs at PND3. Monocyte-derived DCs were hardly present in steady-state MLNs, consistent with the idea that these cells are poorly migratory (Plantinga et al., 2013).

Fluorescently labeled HDM-AF647 was injected intranasally (i.n.) to measure the antigen uptake and migratory capacity of DCs in various age groups. In the lung of both adult and neonatal mice, most of the fluorescent HDM was taken up by CD11c$^+$ cells including alveolar macrophages (AMs) and DCs (Figure 3D). The capacity to take up antigen was not age dependent. Overall, moDCs took up HDM most efficiently, whereas the uptake of HDM allergen by lung CD103$^+$ and CD11b$^+$ cDCs was lower. There was very little uptake of allergen by CD45$^-$ lung cells, which include airway epithelial cells, across all ages tested (Figure 3D).

After HDM antigen uptake in the lung, DCs migrate to the MLN in adult mice (Plantinga et al., 2013). The i.n. administration of 10 μg HDM-AF647 resulted in a 2-fold, 9-fold, and 3.5-fold increase in CD11c$^+$MHC$^+$ DC numbers in PND3, PND14, and adult MLN, respectively (Figure 3E). Within the MLN, MHCII$^+$CD11c$^+$ cells highly expressed the chemokine receptor CCR7, and this was even more the case in HDM-AF647-carrying DCs (Figure 3F). Finally, a study of *Ccr7*$^{-/-}$ mice indicated that also at PND3 and PND14, migration of HDM$^+$ DCs to the MLN after 10 μg of HDM was CCR7 dependent, like in adult mice (Figure 3G). Despite the high predominance of CD103$^+$ DCs in neonatal lungs, the migratory CCR7$^+$ HDM$^+$ DC population in the MLN mainly consisted of CD11b$^+$ cDCs (Figure 3H). By increasing the dose of HDM antigen 10-fold, the proportion of moDCs in the MLN increased and the total number of migratory DCs increased, but still CD11b$^+$ cDCs predominated the response across all age groups. These data indicate that in the neonatal period, the low numbers of CD11b$^+$ cDCs are highly efficient in taking up HDM allergen and migrating to the MLN in a CCR7-dependent manner.

IL-33 Blockade Inhibits Neonatal Type 2 Immunity and Enhanced DC Function

We next sought to understand why migration of neonatal DCs is enhanced. In steady-state conditions, high numbers of activated IL-5- and IL-13-producing ILC2s cells, as well as type 2 cytokine-producing mast cells, basophils, and eosinophils, were found in the lungs on PND14, but not on PND3 or in adulthood (Figures 4A, 4B, and 1B). We found no IL-5- and IL-13-expressing CD4$^+$ T cells in steady-state mice. The innate type 2 cells all express ST2L (IL-33R) and proliferate in response to IL-33 (Saglani et al., 2013; Schmitz et al., 2005; Willart et al., 2012). We therefore investigated the concentration of IL-33 protein in lysed lung cells in steady state (PBS) or after administration of HDM to unsensitized mice. In steady state, IL-33 protein concentration peaked around PND14 (Figure 4C; van de Laar et al., 2016). At 16 hr after the administration of HDM, the expression of IL-33 was significantly higher in lung cell homogenates of PND14 lungs compared to PND3 or adult lungs (Figure 4C). Other cytokines like KC, MCP1, GM-CSF, IL-1β, and TSLP did not show similar peak responses at PND14 (data not shown). IL-33 mRNA was detected mainly in sorted CD45$^-$CD31$^-$Epcam$^+$ epithelial cells (ECs), and mRNA was further boosted by stimulation with HDM (Figure 4D). IL-33 mRNA was not detected in AM, DCs, or monocytes. Because IL-33 released from ECs is an important driver of type 2 immunity (Hammad and Lambrecht, 2015), we checked whether spontaneous type 2 immunity at PND14 was dependent on secreted IL-33. Whereas WT mice developed spontaneous tissue eosinophilia and accumulation of ILC2s in the lung, *Il1rl1*$^{-/-}$ mice (lacking the IL-33R, also known as T1/ST2) had severely reduced spontaneous type 2 immunity of the lung at PND14 (Figure 4E). This tissue eosinophilia was largely independent of cells of the adaptive immune response, as *Rag2*$^{-/-}$ mice had equal eosinophilia at PND14 compared with wild-type controls (Figure 4F), and ILC2 numbers were increased compared with wild-type controls, as described previously(Roediger et al., 2015).

We next questioned whether HDM also led to ILC2 activation in neonatal mice in an IL-33-dependent manner. After an innate immune response to a single administration of HDM at PND14, ILC2 numbers were not increased, but ILC2s were clearly activated as indicated by a rise in intracellular IL-5 and IL-13 protein compared with PBS-exposed mice (Figures 2H and 4G). The increase in intracellular IL-5 and IL-13 production was blocked by in vivo administration of recombinant sST2 (r-sST2), a recombinant decoy receptor for IL-33, made to exactly mimic the splice variant of ST2 and only neutralizing extracellular IL-33. Therefore, released IL-33 was responsible for mediating ILC2 activation (Figure 4G).

IL-13 produced by activated ILC2 has been shown to enhance the migratory capacity of CD11b$^+$ cDCs in adult mice (Halim et al., 2016). PND14 CD11b$^+$ cDCs and moDCs expressed more IL-13R and ST2L compared with adult or PND3 DCs (Figure 4H). Similarly, PND14 ILC2 expressed more ST2L than adult or PND3 ILC2 (Figure 4I). Treatment with blocking anti-IL-13 or r-sST2 resulted in a strong reduction in DC migration to the

(D) Cytokines in MLN cells restimulated for 3 days with 15 μg/mL HDM.

(E) Total IgE- and HDM-specific IgG1 in serum.

(F and G) Number of IL-5$^+$IL-13$^+$CD4$^+$ T cells (F) and total ILC2 and IL-5$^+$IL-13$^+$ ILC2s (G) in the lungs.

(H) Number of ILC2s after 1 or 3 daily administrations of PBS or HDM (10 μg). Results show one representative experiment out of three. Six (adult), eight (PND14), and ten (PND3) mice/group were used. Results are shown as mean ± SEM.

Asterisks in (G) and (H) indicate statistically significant differences between total ILC2 numbers. Hashtags indicate statistically significant differences between number of IL-5$^+$IL-13$^+$ ILC2s. $^*p < 0.05$, $^{**}p < 0.01$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$. Figures S3 and S4 shows these analysis in *Ccr7*$^{-/-}$ mice.

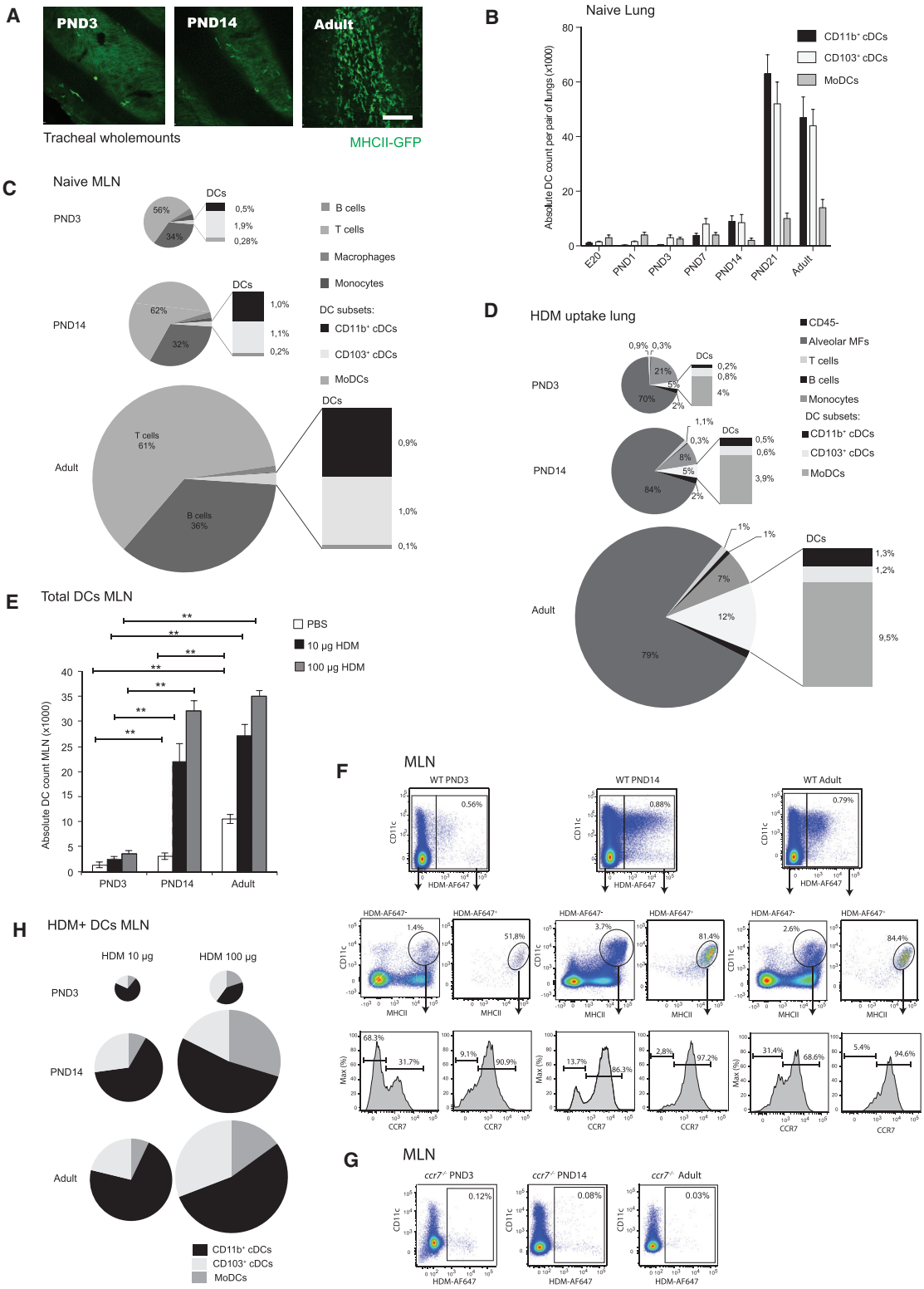

Figure 3. DC Numbers, Antigen Uptake, and Migration in Neonatal, Infant, and Adult Mice

(A) Tracheal whole mounts of naive MHCII-GFP mice at PND3, 14, and adult age. Scale bar represents 20 µM.

(B) Total number of moDCs, CD11b$^+$ cDCs, and CD103$^+$ cDCs in naive fetal, neonatal, infant, and adult lungs.

(C) Pie charts depicting the relative proportions of lymphocytes and myeloid cells within total cells present in naive MLN at PND3, PND14, and adult age.

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MLN (Figure 4J). One of the molecules induced by IL-33 on DCs is OX40L, a TNFR family molecule involved in polarizing naive Th cells into Th2 cells (Salek-Ardakani et al., 2003). We found no constitutive OX40L expression on DCs in naive MLN and lungs but upon HDM exposure, strong induction was observed on migratory CD11b$^+$ cDCs. Expression of OX40L was again most prominent at PND14 (Figure 4K). The CD11b$^+$ cDCs that still migrated to the MLN despite r-sST2 blockade at PND14 had lower OX40L expression (Figure 4L) consistent with previous in vivo findings that OX40L expression of adult pulmonary DCs is controlled by IL-33 (Besnard et al., 2011). Combining the two blocking antibodies did not have an additional effect on migration compared to r-sST2 alone (data not shown). These data indicate that during the innate response to HDM at PND14, IL-33 promotes CD11b$^+$ DC migration via a direct effect on DCs and via the induction of IL-13 production in ILC2s.

Neonatal DCs Preferentially Induce Th2 Cell Polarization

It is well known that the immune system of neonates shows a preferential polarization toward Th2 cell development, caused by delayed production of IL-12p70 by neonatal (cord blood) DCs or defective IFN-γ production by neonatal T cells (Goriely et al., 2001; Upham et al., 2002; Vekemans et al., 2002). Due to practical limitations, studies on the function of neonatal mucosal DCs are missing. We therefore set up an experiment to compare the intrinsic Th2 cell skewing capacity of the three main pulmonary DC subsets at different ages after activation and migration to the MLN, avoiding the use of HDM that has the potential to activate DCs. We administered 100 μg of the harmless antigen ovalbumin (OVA) i.n. to PND3, PND14, and adult WT mice. Three days later we flow-sorted DC subsets from the MLN to stimulate adult OVA-specific T cell receptor (TCR) Tg CD4$^+$ T cells (OT-II cells) in vitro. Each of the neonatal and adult DC subset was proficient in inducing all prototypic Th1 (IFN-γ), Th2 (IL-4, IL-5, IL-13), Th17 (IL-17), and T regulatory (IL-10) cytokines (Figures 5A and S5). However, CD11b$^+$ cDCs and moDCs derived from neonatal mice induced a stronger tendency for Th2 cell-associated cytokine production compared to their adult counterparts (Figures 5A and S5). Also, IL-17 and IL-10 production were slightly increased. As adult T cells were used in the readout, only slightly lower IFN-γ levels were measured. These data show that neonatal DCs have a pro-Th2 cell bias.

IL-33 Causes IL-12p35 Deficiency in Neonatal DCs

We next investigated the reason for the Th2 cell bias of neonatal DCs. IL-33 could promote Th2 cell-mediated immunity via the induction of OX40L on DCs (Figure 4L) and a reduction in IL-12p70 production (Besnard et al., 2011; Kurowska-Stolarska et al., 2008; Rank et al., 2009). Sorted neonatal mediastinal moDCs and CD11b$^+$ cDCs and to a lesser extent CD103$^+$ cDCs expressed less mRNA IL-12p35, when compared to their adult counterparts (Figure 5B). Expression of IL-6 mRNA was identical across age groups.

To study the Th2 cell skewing effect of IL-33, we isolated DC subsets from naive PND14 and adult lung and co-cultured the DCs with OT-II cells in the presence of OVA and increasing concentrations of IL-33 (Figure 5C). Correlating with the highest ST2L expression on PND14 DCs, IL-33 induced strongest IL-4 induction in co-cultures containing PND14 CD11b$^+$ cDCs or moDCs, indicative of Th2 cell polarization.

ST2L expression on PND3 DCs was very low and not upregulated upon HDM exposure, suggesting that ST2L signaling has no role in the well-known IL-12 deficiency of the early postnatal period. To test to what extent IL-33 contributed to the IL-12p35 deficiency in neonatal DCs, we sorted migratory DCs from the MLN of PND3, PND14, and adult mice after treatment with HDM-AF647 with or without r-sST2. Of interest, r-sST2 treatment increased IL-12p35 mRNA levels in adult and PND14 migratory DCs but not in PND3 DCs (Figure 5D). These data indicate that IL-12p35 deficiency in neonatal pulmonary DCs in the early postnatal phase is IL-33 independent. During the phase of alveolarization, however, IL-12p35 is actively suppressed by IL-33 made by epithelial cells.

sST2 Prevents Neonatal Type 2 Immunity to Allergens

Having observed that IL-33 plays a prominent role in ILC2 activation, DC migration, and Th2 cell skewing in early life, we next questioned whether IL-33 blockade through r-sST2 treatment would also prevent asthma in an age-dependent manner. We blocked IL-33 signaling by injecting neonatal and adult mice with r-sST2 during HDM sensitization or challenge, in protocol A (sensitization at PND3), B (sensitization at PND14), and C (sensitization in adulthood). In protocol B, r-sST2 treatment at sensitization led to complete abrogation of the number of eosinophils and lymphocytes in the BAL (Figures 6A and 6B), goblet cell metaplasia in lung epithelial cells (Figure 6C), cytokine production in the MLN (Figures 6D and 6E), and total IgE and HDM-specific IgG1 in the serum (Figure 6F). In protocol A and C, treatment with r-sST2 at time of sensitization resulted in a significant but clearly less-effective reduction in these parameters. In contrast, treatment with r-sST2 during the challenge phase of the protocol had effect on eosinophilia only in protocol A, and not in protocol B or C (Figure S6A), most likely as r-sST2 treatment during challenge in protocol A coincided with the peak of innate immunity to HDM in this period (see PBS-HDM group in protocol A). Th2-cell-associated cytokine production in the MLN (Figure S6B) and serum immunoglobulins (Figure S6C) were not affected.

(D) Pie charts depicting the uptake of Alexa Fluor 647 (AF647)-labeled HDM by CD45$^+$ and CD45$^-$ cells in the lung of neonatal, infant, and adult mice administered with the allergen 72 hr earlier.

(E) Total number of CD11c$^+$MHCII$^+$ in neonatal, infant, and adult MLN 72 hr after allergen challenge.

(F) CCR7 expression on total CD11c$^+$MHCII$^+$ DCs and on HDM-AF647$^+$ CD11$^+$MHCII^{hi} DCs in neonatal, infant, and adult MLN 72 hr after the administration of allergen.

(G) Presence of HDM-AF647-labeled cells in MLN 72 hr after the i.n. administration of allergen (10 μg) to neonatal, infant, and adult *Ccr7*$^{-/-}$ mice.

(H) Pie charts depicting the relative proportion of HDM-AF647$^+$ DC subsets in the MLN of mice 72 hr after 10 μg or 100 μg HDM allergen administration.

Data represent two independent experiments with at least six mice per timepoint. Results are shown as mean ± SEM.

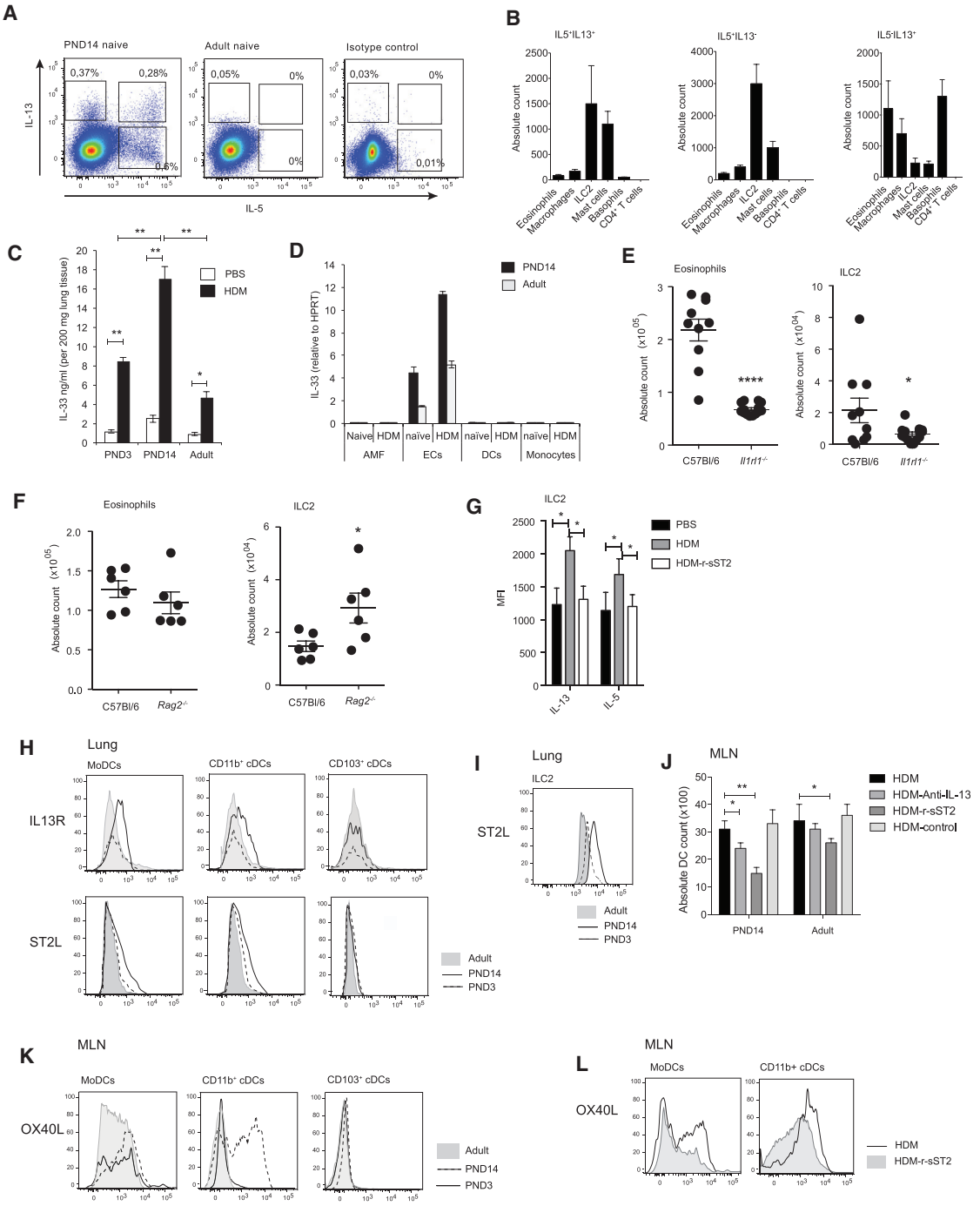

Figure 4. Hyperactive IL-33-ST2L Axis in PND14 Mice

(A) Intracellular IL-5 and IL-13 staining on naive adult and PND14 lungs.

(B) Absolute cell number of IL-5 and IL-13 single- and double-positive cells in the lungs of naive PND14 mice.

(C) IL-33 in lung homogenates 16 hr after PBS or 100 μg HDM i.n. IL-33 concentration was corrected for dry lung weight.

(D) IL-33 mRNA expression in lung cells flow cytometry sorted from naive mice or 6 hr after the administration of HDM (100 μg).

(E) Absolute numbers of eosinophils and ILC2s in the lungs of PND14 C57BL/6 and *Il1rl1*$^{-/-}$ mice.

(F) Absolute numbers of eosinophils and ILC2s in the lungs of PND14 C57BL/6 and *Rag2*$^{-/-}$ mice.

(G) Mean fluorescence intensity (MFI) of IL-13^{+} and IL-5^{+} ILC2s 24 hr after PBS, HDM i.n. (100 μg), or HDM and r-sST2 i.p. (200 μg).

(H) Expression of IL-13R and ST2L on naive DC subsets in the lungs of adult mice (gray solid graph), PND14 mice (black line), and PND3 mice (dashed line).

(I) ST2L expression on lung ILC2s 24 hr after the administration of HDM (100 μg).

(J) Absolute numbers of DCs in the lung draining lymph nodes of PND14 and adult mice 72 hr after i.n. HDM exposure (100 μg). Anti-IL13, r-sST2, and the isotype control were given i.p.

(legend continued on next page)

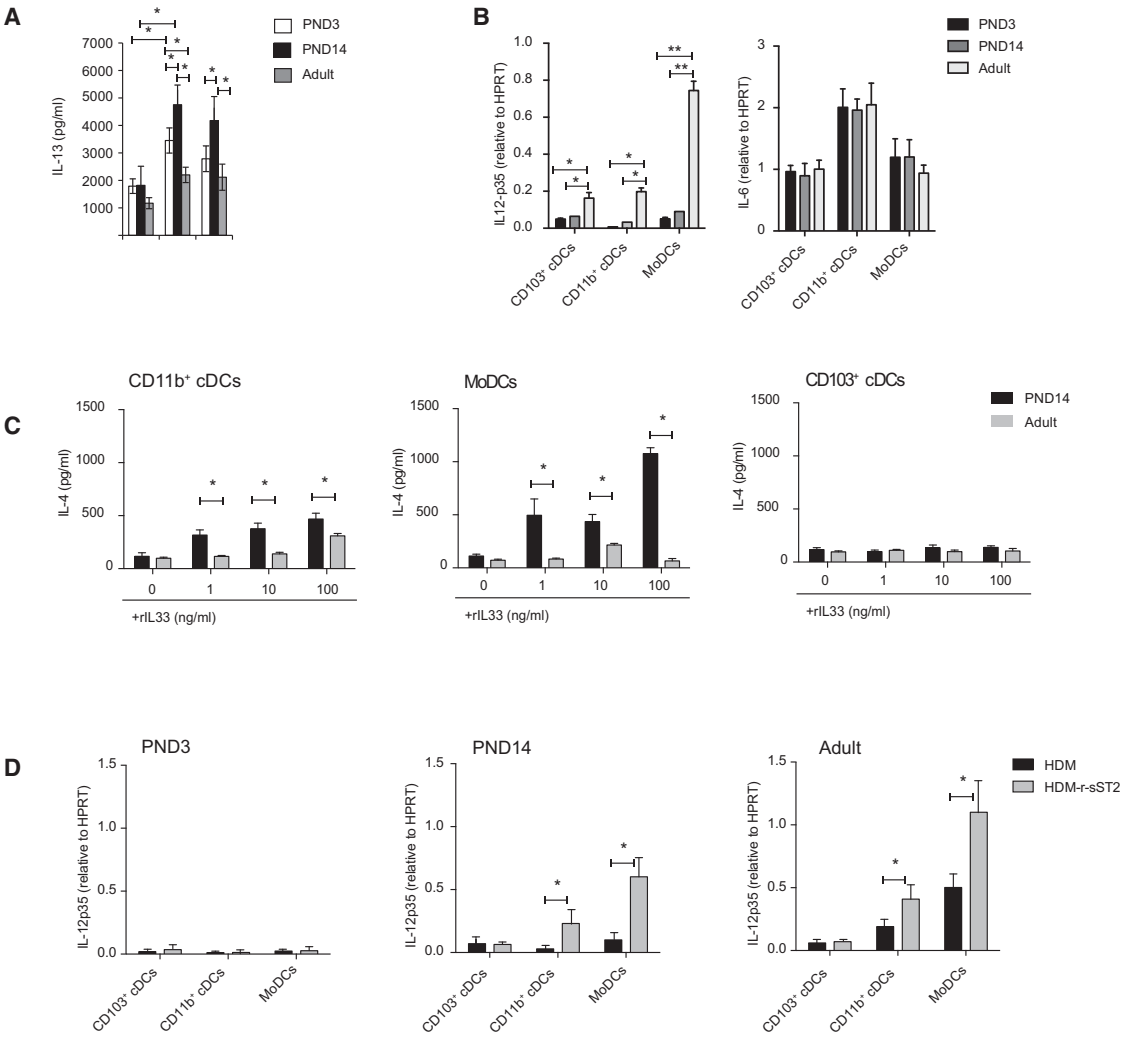

Figure 5. Th2 Cell Skewing Capacity of Neonatal versus Adult DCs

(A) Migratory DCs were sorted from MLNs 3 days after i.n. OVA (100 μg) exposure and co-cultured with naive adult OT-II CD4$^+$ T cells in vitro. After 4 days, IL-13 was measured by ELISA in supernatants. Data represent two independent experiments with at least 6 (adult), 12 (PND14), or 14 (PND3) mice per group. See Figure S5 for data on other cytokines produced.

(B) mRNA IL-12p35 and IL-6 expression determined by RT-PCR in migratory DCs sorted from MLN 3 days after HDM exposure. Data represent three independent experiments with six mice per group.

(C) Cytokine production in supernatants of co-cultures of naive lung-derived PND14 and adult DCs with naive CD4$^+$ T cells from adult OT-II mice, cultured in the presence of OVA protein and different concentrations of IL-33. Data represent two independent experiments with six adult and ten PND14 mice per time point.

(D) mRNA IL-12p35 expression determined by RT-PCR in migratory DCs sorted from MLN 3 days after i.n. HDM exposure with or without r-sST2 i.p.

Results are shown as mean ± SEM. *$p < 0.05$.

Overall, these data indicate that a hyperactive IL-33 axis that coincides with maximal lung remodeling during lung development drives activation of innate and adaptive immunity to allergens in the neonatal period, by driving activation of ILC2s and conventional cDCs.

DISCUSSION

Children under the age of 3 are at risk of developing allergic disease to inhaled allergens, at a time when their lungs are still developing. Based on studies of immune cells obtained from

(K) OX40L expression on migratory DC subsets in the MLN of adult mice (gray solid graph), PND14 mice (thin black line), and PND3 mice (thick black line) 3 days after HDM (100 μg) exposure.

(L) OX40L expression in DCs in lung draining lymph nodes 24 hr after HDM i.n. (100 μg) or HDM i.n. and r-sST2 i.p. (200 μg).

Data represent two independent experiments with at least six mice per time point. Results are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$.

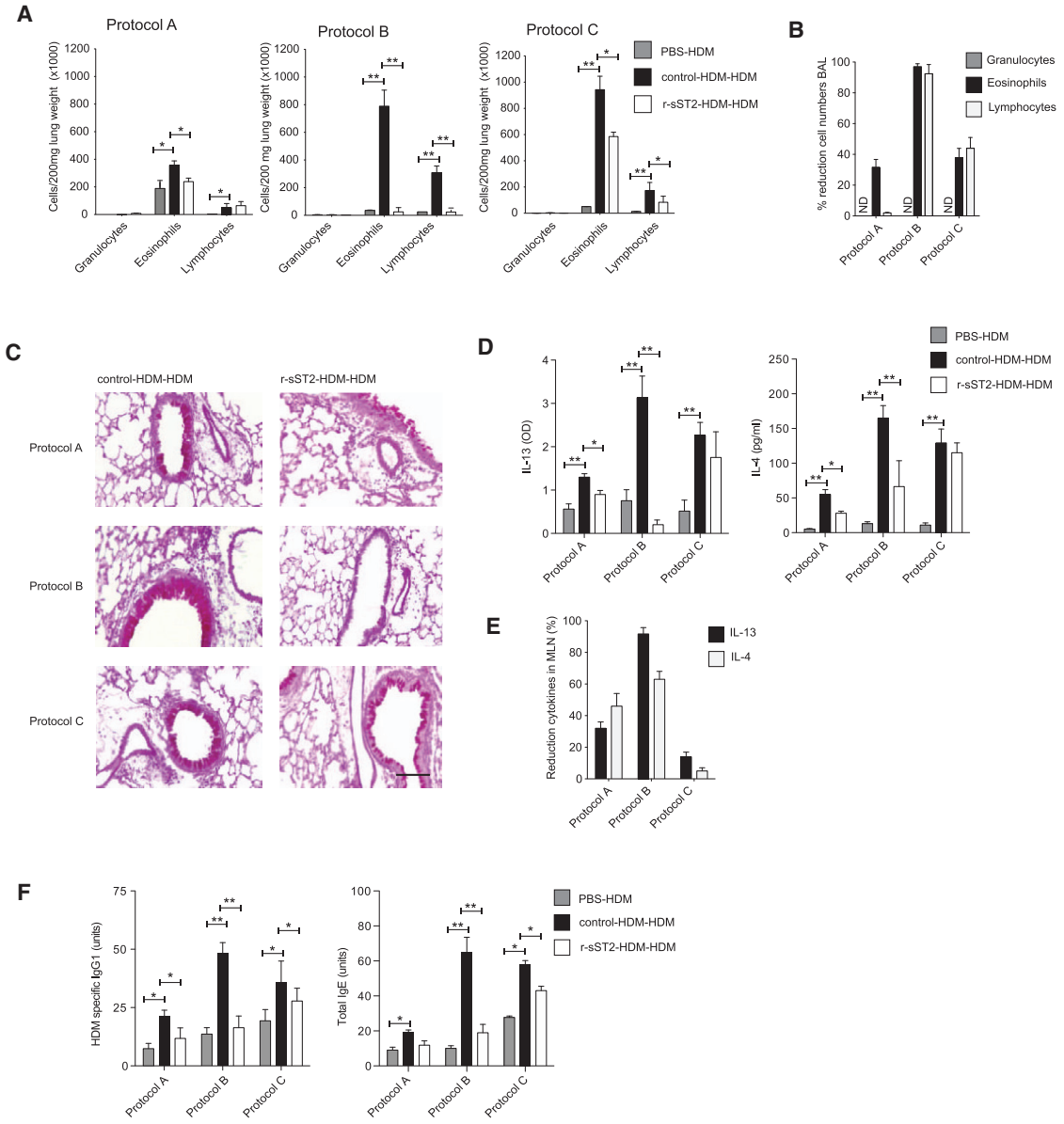

Figure 6. Blockade of IL-33 during Sensitization Is Most Effective in PND14 Mice

PND3 (protocol A) and PND14 (protocol B) and adult WT (protocol C) mice were sensitized on day 0 with HDM or PBS i.n. and in the presence of blocking r-sST2 or control i.p. Subsequently, the mice were challenged with HDM on 5 consecutive days starting 1 week later and sacrificed on day 14 after starting the protocol. See also Figure 2A.

(A) Absolute differential cell counts in BAL fluid determined by FACS 72 hr later.

(B) Reduction in cell numbers in BAL fluid due to r-sST2 treatment at time of sensitization. ND indicates not detectable.

(C) PAS staining of lung sections. Scale bar represents 100 μM.

(D) Cytokines in supernatants of MLN cell cultures, restimulated for 3 days with 15 μg/mL HDM.

(E) Reduction in IL-13 and IL-4 production in supernatants of MLN cell cultures due to r-sST2 treatment at time of sensitization.

(F) Serum Igs. Results show one representative experiment out of two. Six to eight mice/group were used.

Results are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$. See Figure S6 for the effects of r-sST2 administration during challenge.

cord blood, it was proposed that delayed postnatal maturation of Th cell function and developmental defects within the innate immune system that result from feto-maternal adaptation predisposes young children to Th2 cell sensitization (Bot et al., 1998; De Wit et al., 2003; Li et al., 2004; Sarzotti et al., 1996; Sornasse et al., 1996; Tonon et al., 2002). However, a thorough understanding of the allergic sensitization process in the context of postnatal immune maturation and lung development is still lacking. The structural features of postnatal lung development (mainly consisting of formation and remodeling of alveoli) are very similar in humans and mice although the time span is obviously different. The most active phase of alveolar septation

occurs through the second postnatal week in mice and between 2 and 3 years in humans, a time frame where many atopic children also become allergic (Burri, 2006; Wahn and von Mutius, 2001). We here found highly increased production of the epithelial cytokine IL-33 and accumulation of innate type 2 cells during the alveolar period when the lung is maximally remodeling. The spontaneous activation of type 2 cells around this period required extracellular IL-33 and ST2. In this period, innate and adaptive immune responses to HDM were strongly increased through activation of CD11b$^+$ cDCs that migrated vigorously to the MLNs and polarized Th2 cells. HDM-induced IL-33 had a direct effect on CD11b$^+$ cDCs but also stimulated CD11b$^+$ cDCs indirectly via activating innate type 2 cells that produced IL-13. Our finding that IL-13 promotes neonatal DC migration to lung draining lymph nodes is compatible with recent studies employing the model allergen papain in adult mice (Halim et al., 2014, 2016). Although memory Th2 cells have also been found to produce IL-13 after stimulation with epithelial cytokines IL-33 and IL-25 (Endo et al., 2015; Guo et al., 2015), we did not find evidence of this pathway at this very young age, most likely because adaptive T cell immunity becomes fully competent only by the time of weaning. In support, the spontaneous wave of type 2 immunity around PND14 was intact in Rag2-deficient mice, and ILC2 accumulations were even enhanced in these mice, most likely due to increased availability of IL-2 in *Rag2*$^{-/-}$ mice.

Studies in neonatal rodents and humans reported scarcity as well as immaturity of pulmonary DCs, hyporesponsiveness to PAMPs, and low lymphocyte numbers and therefore suggested incompetence of the early postnatal pulmonary immune system to mount adaptive immune responses, an effect that was even exacerbated in germ-free mice (Belderbos et al., 2009; Gollwitzer et al., 2014; Nelson and Holt, 1995; Nelson et al., 1994; Roux et al., 2011; Tschernig et al., 2006). In accordance with these studies, we found that DCs were indeed severely impaired in neonatal lungs, and the few CD11b$^+$ DCs in neonatal lungs during the first post-natal week were sessile CD11b$^+$CD64$^+$ moDCs that closely resemble macrophages (Guilliams et al., 2013; Plantinga et al., 2013). Of the two conventional DC subsets, CD11b$^+$ cDCs developed with slower kinetics compared with CD103$^+$ cDCs and numbers of CD11b$^+$ cDCs remained severely impaired until weaning. The efficiency of PND14 CD11b$^+$ cDCs to drive Th2 cell sensitization in CD4$^+$ T cells was determined by a strong capacity to migrate to the mediastinal nodes in a CCR7-dependent manner. Thus, even during lung development when epithelial barrier function might be incomplete and there might be passive leakage of antigen into the systemic circulation, induction of adaptive immunity occurs by regulated migration of DCs into lung draining nodes, driven by the same chemokine network as in adult mice. Compared to their adult counterparts and compared to CD103$^+$ DCs, neonatal CD11b$^+$ DC subsets also had a strong intrinsic capacity to induce Th2-cell-mediated responses. This Th2 cell skewing capacity was present directly after birth but increased further during the second post-natal week. This specific Th2 cell polarization bias could be explained by two independent but superimposed mechanisms. First, there was increased expression of OX40L, a molecule involved in polarization of Th2 cells, on DCs in the alveolarization phase. Although TSLP is mainly known as the prototypical cytokine-driving expression of OX40L on human and murine DCs (Hammad and Lambrecht, 2015; Ito et al., 2005), we found this increased expression to be IL-33 dependent in the alveolarization phase of lung development. Second, we found that neonatal lung DCs were severely impaired in IL-12p35 expression. IL-12p35 is the rate limiting subunit of IL-12p70, the predominant cytokine driving Th1 cell development. These data are in line with previous work showing that human cord blood DCs (De Wit et al., 2003; Goriely et al., 2001; Tonon et al., 2002) and mouse splenic DCs (Lee et al., 2008) are deficient in IL-12 production. In neonatal lung DCs, IL-12p35 deficiency was strongest in CD11b$^+$ cDCs and CD64$^+$ moDCs, the two main HDM-presenting DC subsets. We could not account the Th2 cell bias to the delayed development of CD103$^+$ cDCs, the mucosal equivalent of splenic CD8α^+CD4$^-$ cDCs that seem to be defective in neonatal spleens (Lee et al., 2008). In neonatal lungs, CD103$^+$ cDCs were most abundant in neonatal lungs and least deficient for IL-12p35, consistent with the idea that CD103$^+$ cDCs have an intrinsic bias to produce more IL-12 (Everts et al., 2016).

Reduced IL-12p70 synthesis in cord blood moDCs has been associated with impaired chromatin remodeling in the IL-12p35 promotor region, suggesting epigenetic regulation of gene transcription (Goriely et al., 2004). Here we identified an additional extrinsic cause for IL-12p35 deficiency in neonatal DCs. In the second postnatal week, IL-12p35 deficiency in lung DCs was caused by a direct and dose-dependent suppressive effect of epithelial IL-33 on IL-12 production (Besnard et al., 2011; Plantinga et al., 2013; Rank et al., 2009).

Altogether, it appears that IL-33 has a high contribution to the development of allergic airway inflammation at young age. This was also shown in a model of sensitization to fungal allergens, in which IL-33 was shown to be high in mice exposed to *Alternaria* allergen (Castanhinha et al., 2015). In our hands, treatment with r-sST2 to block IL-33 at the time of sensitization completely abolished eosinophilic airway inflammation, Th2-cell-associated cytokine production, and GCM particularly when administered in the period of maximal lung alveolarization. Consistently, polymorphisms in *IL1RL1*, coding for ST2 (IL-33R), as well as the *IL33* gene itself have been found associated with asthma and blood eosinophil counts, particularly in childhood asthma (Bønnelykke et al., 2014; Castanhinha et al., 2015; Gordon et al., 2016; Moffatt et al., 2010; Saglani et al., 2013; Savenije et al., 2011; Torgerson et al., 2011; Traister et al., 2015). In asthmatics, polymorphisms in *IL1RL1* also control the relative abundance of the cell-bound IL-33R (ST2L) versus the soluble IL-33 receptor (sST2), that acts as a decoy receptor and antagonist of the IL-33-IL-33R axis (Grotenboer et al., 2013; Traister et al., 2015). It would be interesting to now analyze whether carriers of the *IL33* and *IL1RL1* risk alleles are at increased risk to specifically develop allergic asthma at young age, and whether SNPs associated with high expression of sST2 are associated with protection.

Why would there be spontaneous Th2 cell immunity in the developing lung? We speculate that type 2 immunity controls lung development and/or remodeling. IL-33 boosts the function of ILC2s that can promote tissue remodeling, repair, and homeostasis. Activated ILC2s, mast cells, and basophils produce growth factors such as amphiregulin and epidermal growth factor (EGF) that promote epithelial homeostasis (Groschwitz et al.,

2009; Monticelli et al., 2012). IL-33 is also a known activator of alternatively activated M2 macrophages that control tissue remodeling and postnatal branching morphogenesis of the lung (Jones et al., 2013; Kurowska-Stolarska et al., 2009). We and others did observe increased staining of lung mononuclear cells with the M2 marker Ym1 in the neonatal period (data not shown and Hung et al., 2002). Despite this circumstantial evidence, we were unable to measure gross abnormalities in lung alveolarization in IL-33-deficient animals (I.K., unpublished observations).

In conclusion, our data indicate that neonatal Th2 cell skewing is reminiscent of not just maternal-fetal immune adaptation. High IL-33 production in the postnatal alveolar phase of lung development promotes a wave of type 2 immunity and lowers the threshold for innate immune responses to allergens. It increases the risk for Th2 cell sensitization by boosting the function of DCs and suppressing IL-12 production. Our findings help explain how particular polymorphisms at the *IL1RL1* locus promote or suppress asthma development in childhood. As other genetic polymorphisms seem to control epithelial integrity and genes involved in lung growth, and as respiratory syncytial virus infection at a very young age promotes sensitization while affecting lung growth, it will also be interesting to study whether the IL-33 axis remains hyperactive in some forms of asthma that are accompanied by excessive and altered lung regeneration and remodelling.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 WT mice were obtained from Harlan. MHCII-EGFP mice were provided by H. Ploegh (Harvard Medical School) (Boes et al., 2002). *Ccr7*$^{-/-}$ and *Rag2*$^{-/-}$ mice were derived from Jackson Laboratories. *Il1rl1*$^{-/-}$ mice were housed at Trinity Biomedical Sciences Institute. Mice were housed under specific-pathogen-free conditions. All experiments were approved by the animal ethics committee of Ghent University or Erasmus Medical Center, Rotterdam.

Reagents

HDM extracts were obtained from Greer Laboratories. Recombinant sST2 (r-sST2) was produced and provided by H. Braun (Flanders Institute for Biotechnology) (Willart et al., 2012). Blocking antibody against IL-13 (clone eBio1316H) was obtained by eBioscience. We obtained ELISA Duoset for mIL-13, mKC, mCCL20, and mIL-33 from R&D Systems. APC Cy7-labeled antibody to MHCII, PE-Cy5-labeled, and PE-labeled antibodies to CD3, CD19, and CD8a, PE-Cy7-labeled antibody against CD11c, APC-labeled antibodies against FcεRIα and CD64, PE-Cy7-labeled antibody against CD127, AF647-labeled antibody against EPCAM, biotin-labeled antibody against F4/80, and ELISA sets for mMCP1, mTSLP, and mIL-17A were acquired from eBioscience. FITC-labeled antibody to Ly6C, PE-labeled antibody against Siglec-F, Ly6G, B220, NK1.1, and Ter-119, Horizon V450-labeled antibody against CD11b, PE-TxRed-labeled antibody against CD45, APC Cy7-labeled antibody against cKit, as well as mIL-10, mIL-1β, mIL-4, mIL-5, and mIFN-γ ELISA sets and antibody pairs to mouse IgE, IgG1, and IgG2a in serum were obtained from BD. FITC-labeled antibody to IL-13RA1 was obtained from Antibodies-online. Aqua, CFSE, streptavadin PE-Texas red, OVA Alexa Fluor 488, and OVA AF647 were purchased from Molecular Probes/Invitrogen. HDM (*Dermatophagoidespteronyssinus* extracts, Greer Laboratories) was labeled with an AF647 labeling kit (Invitrogen).

OVA Experiments

DC migration was investigated by injecting 100 μg OVA Alexa Fluor 488 i.n. and dissecting MLNs after 24 hr. Cells were stained for flow cytometry, and DCs positive for Alexa Fluor 488 and highly expressing MHCII and CD11c were considered to be migratory DCs. For DC-OT-II, coculture DCs were sorted from the lungs of naive WT mice and cocultured in a 1:10 ratio with MACS-sorted lymphocytes from LNs and spleen of OT-II TCR Tg mice. 3 days later, supernatants were collected and analyzed on IL-4, IL-5, IL-13, and IFN-γ by ELISA.

Model of HDM-Induced Asthma

HDM extracts were from Greer Laboratories. PND3, PND14, and adult mice were anesthetized with isoflurane, sensitized i.n. with 1 μg HDM in resp. 5, 10, or 40 μL PBS i.n. After 7 days, mice were challenged with 10 μg HDM on 5 consecutive days under anesthesia. 3 days after the last challenge, mice were sacrificed and organs were dissected for analysis. BAL was performed using 3 × 0.7−1 mL EDTA-containing PBS and analyzed, and lungs were inflated with a 1:1 mixture of PBS and OCT solution and snap frozen in liquid nitrogen. Single-cell suspensions of MLNs were prepared by pressing tissue through a 100-μm cell sieve and restimulated in vitro with 15 μg/mL HDM for 3 days. Supernatant was collected from these cultures, and cytokine profiles were assayed by ELISA. In neutralization experiments, IL-33 was blocked at the time of sensitization to HDM by use of 200 μg r-sST2 in 50 (PND3), 100 (PND14), or 150 (adult) μL PBS injected i.p. In other experiments 100 μg r-sST2 was injected i.p. on the first, third, and fifth day of the challenge phase. Recombinant sST2 (r-sST2) was produced and provided by H. Braun (Willart et al., 2012). IL-13 antibody (clone eBio1316H) was from eBioscience.

Early Innate Immune Response to HDM

Mice were i.n. instilled with PBS or 100 μg HDM and sacrificed 16 hr after the injection. Lungs were snap frozen and then homogenized with a tissue homogenizer in 500 μL of cold lysis buffer (20 mM Tris-HCl [pH 8.0], 0.14 M NaCl, 10% glycerol, and 20 mg/mL leupeptin) using a tissue homogenizer (IKA) with the addition of 1% Igepal after homogenization. Samples where then kept on ice for 30 min with agitation each 10 min, followed by a centrifugation to pellet debris. Cleared lysate was quantified for protein concentration with NanoOrange reagent (Invitrogen) according to the manufacturer's protocol.

Cell suspensions were made of the right lung and used for FACS analysis. Innate cytokines were measured by ELISA on lung homogenates, and concentrations were corrected for the protein content.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2016.10.031.

AUTHOR CONTRIBUTIONS

I.K. designed and performed experiments and wrote the manuscript; M.K., M.J.W.B., M.W., J.V.M., M.J.S., M.P., and E.H. performed experiments; R.B. generated key tools; H.H., P.G.F., and R.W.H. designed and supervised part of the experiments; and B.N.L. designed and supervised the study and wrote the manuscript.

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Supplemental Information

Perinatal Activation of the Interleukin-33 Pathway Promotes Type 2 Immunity in the Developing Lung

Ismé M. de Kleer, Mirjam Kool, Marjolein J.W. de Bruijn, Monique Willart, Justine van Moorleghem, Martijn J. Schuijs, Maud Plantinga, Rudi Beyaert, Emily Hams, Padraic G. Fallon, Hamida Hammad, Rudi W. Hendriks, and Bart N. Lambrecht

Supplemental material

Supplementary figure legends

Supplementary Figure 1, related to Figure 1. Absolute cell counts in naïve lungs of fetal (E20), neonatal, infant and adult mice.

Supplementary Figure 2, related to Figure 2. Protocol A, B and C refer to the protocols described in Fig. 2A. Total ILC2 and $IL5^{+}IL13^{+}$ ILC2 in the lungs of WT and *Ccr7*$^{-/-}$ mice.

Supplementary Figure 3, related to figure 2B. Protocol A, B and C refer to the protocols described in Fig. 2A. Differential cell counts in the BAL of *Ccr7*$^{-/-}$ mice after induction of allergic airway inflammation (for protocol see Fig. 2A). For better comparison we added the WT data of age-matched mice to the graphs (identical data points as in Fig. 2B). Note that the WT and *Ccr7*$^{-/-}$ experiments were done on separate days.

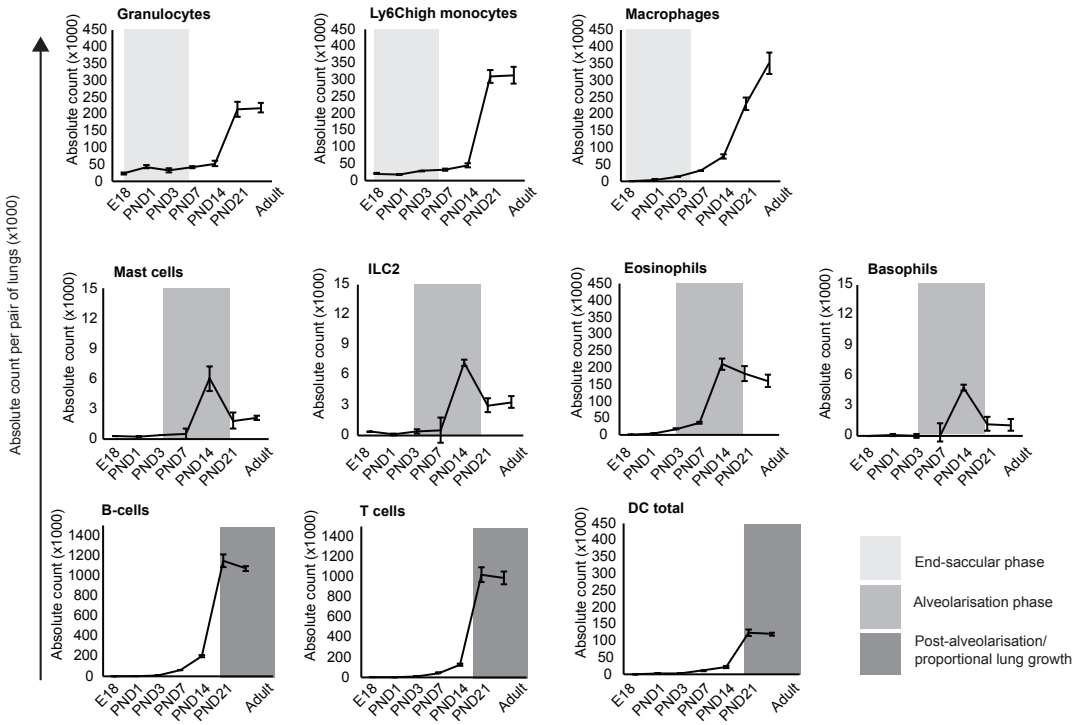
Supplementary Figure 4, related to Figure 2D. Protocol A, B and C refer to the protocols described in Fig. 2A. Cytokine concentration in MLN cells of *Ccr7-/-* mice restimulated for 3 d with 15 µg/ml HDM. For better comparison we added WT data to the graphs (see Fig. 2B). Note that the WT and *Ccr7*$^{-/-}$ experiments were done on separate days.

Supplementary Figure 5, related to Figure 5A. Migratory DC were sorted from MLN 3 days after i.n. OVA (100 µg) exposure and co-cultured with naïve adult OTII $CD4^{+}$ T cells *in vitro*. After 4 days cytokines were measured by ELISA in supernatants. Data represent two independent experiments with at least 6 (adult), 12 (PND14) or 14 (PND3) mice per group.

Supplementary Figure 6, related to Figure 6. Effect of blocking r-sST2 during the challenge phase. Protocol A, B and C refer to the protocols described in Fig. 2A. WT adult, PND14 and PND3 mice were sensitized on day 0 with HDM or PBS i.n. The mice were challenged with HDM on days 7–11. Blocking r-sST2 or isotype control was co-administered on days 7, 9 and 11 i.p. (A) Differential cell counts were determined in the BAL by flow cytometry 72 h later. (B) Cytokines in MLN cells restimulated for 3 d with 15 µg/ml HDM. (C) Total Igs in the serum.

Supplementary figures

S1



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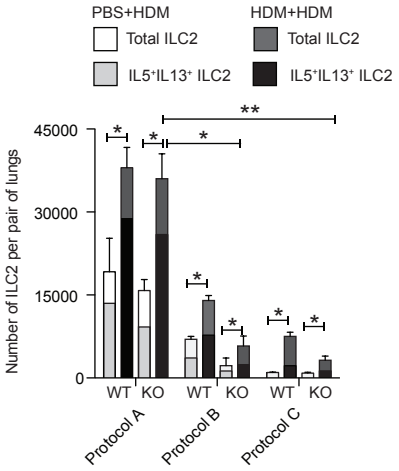

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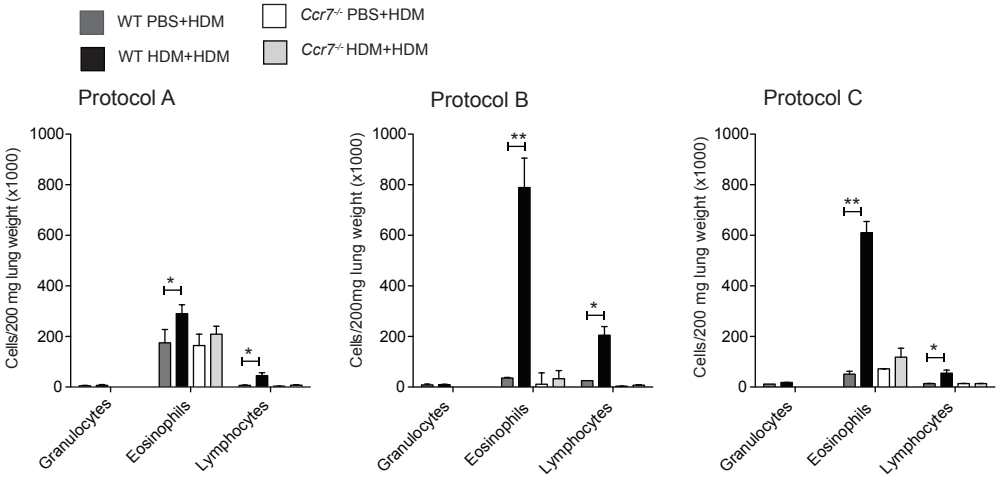

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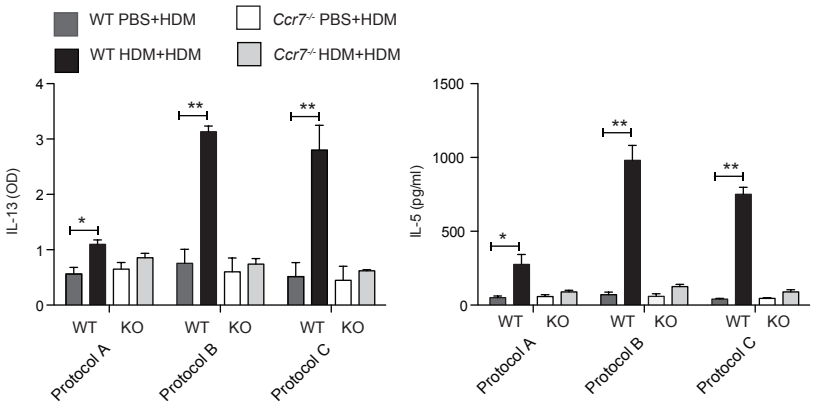

Supplementary figures

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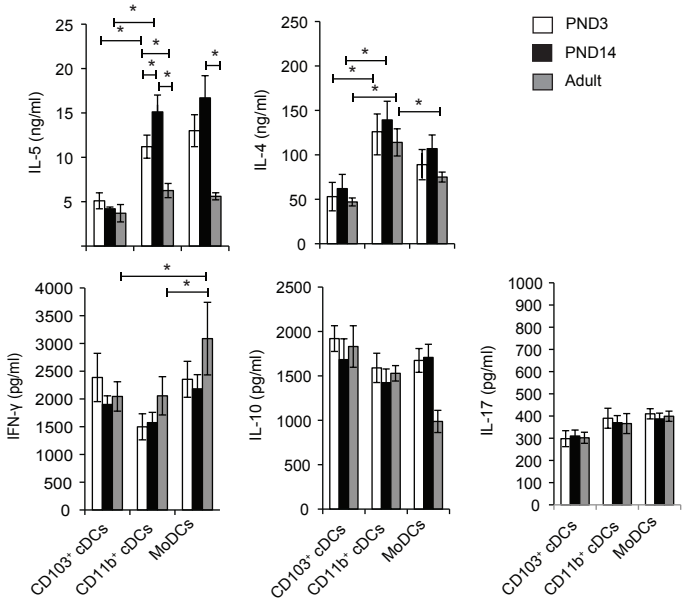

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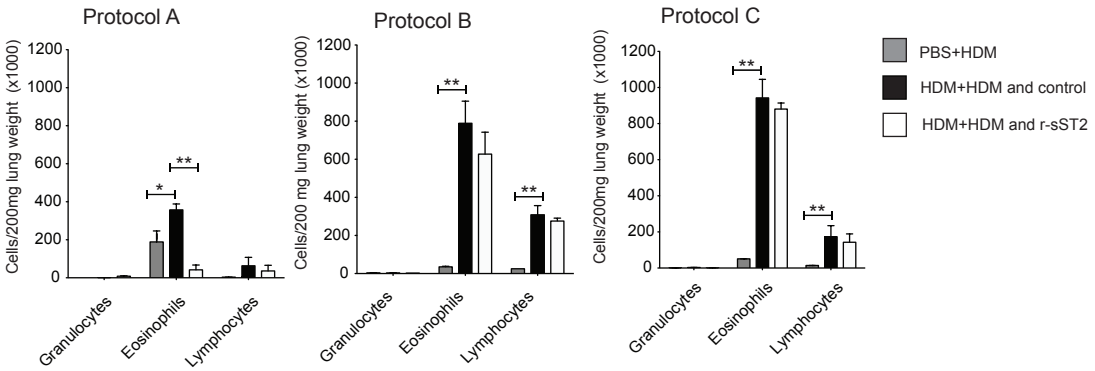

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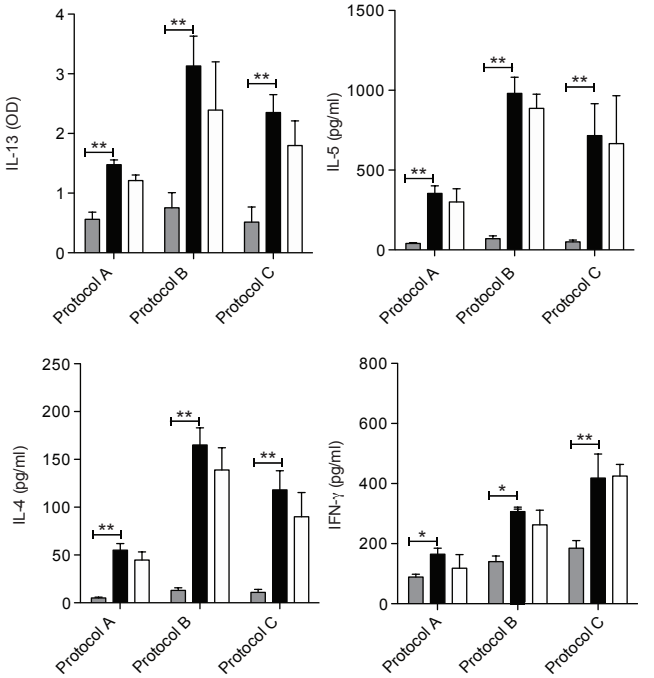

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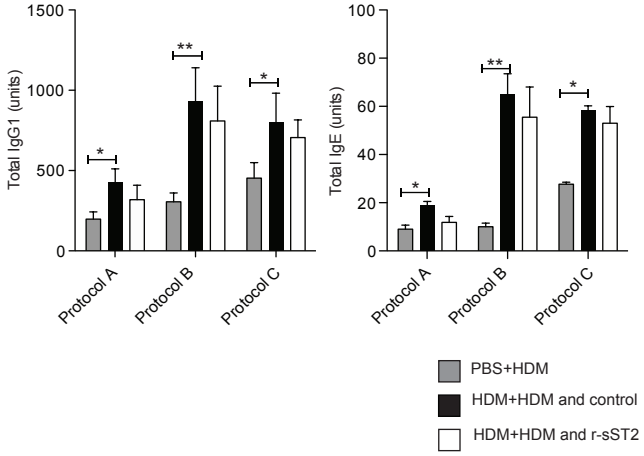
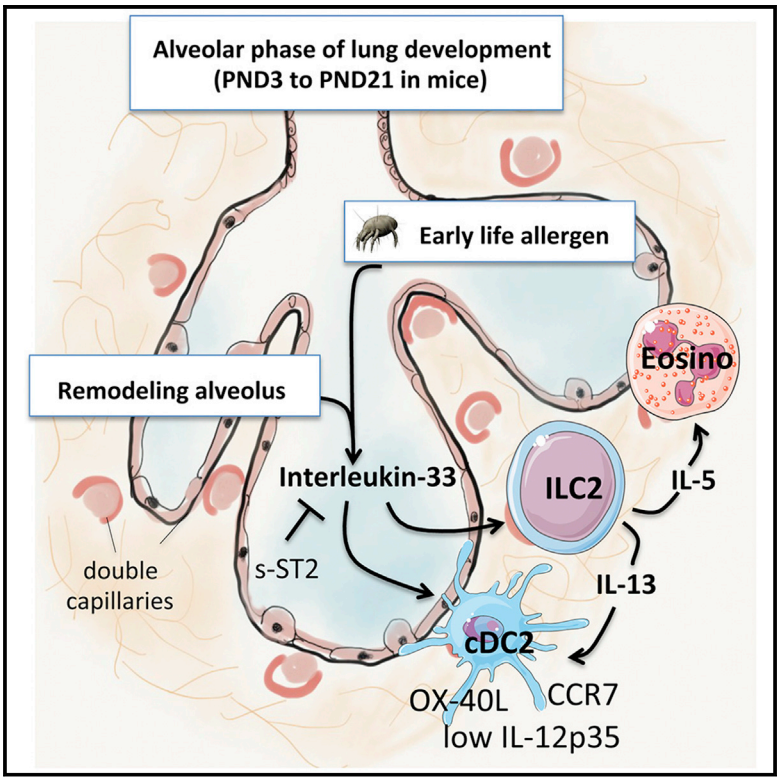


Article

Immunity

Perinatal Activation of the Interleukin-33 Pathway Promotes Type 2 Immunity in the Developing Lung

Graphical Abstract



Highlights

- There is spontaneous accumulation of type 2 immune cells in the developing lung
- Perinatal type 2 immunity depends on IL-33 and not on T or B cells
- Postnatal lung DCs are very scarce yet very efficient at inducing Th2 cell adaptive immunity
- The IL-33 axis is more important for asthma development at young age

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In Brief

It is not known why allergy often begins in early life. de Kleer et al. show that shortly after birth, type 2 innate immune cells accumulate in an IL-33-dependent manner in the developing lung. Interleukin-33 furthermore boosts the function of neonatal dendritic cells to promote long-lasting Th2-cell-mediated immunity.

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Perinatal Activation of the Interleukin-33 Pathway Promotes Type 2 Immunity in the Developing Lung

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SUMMARY

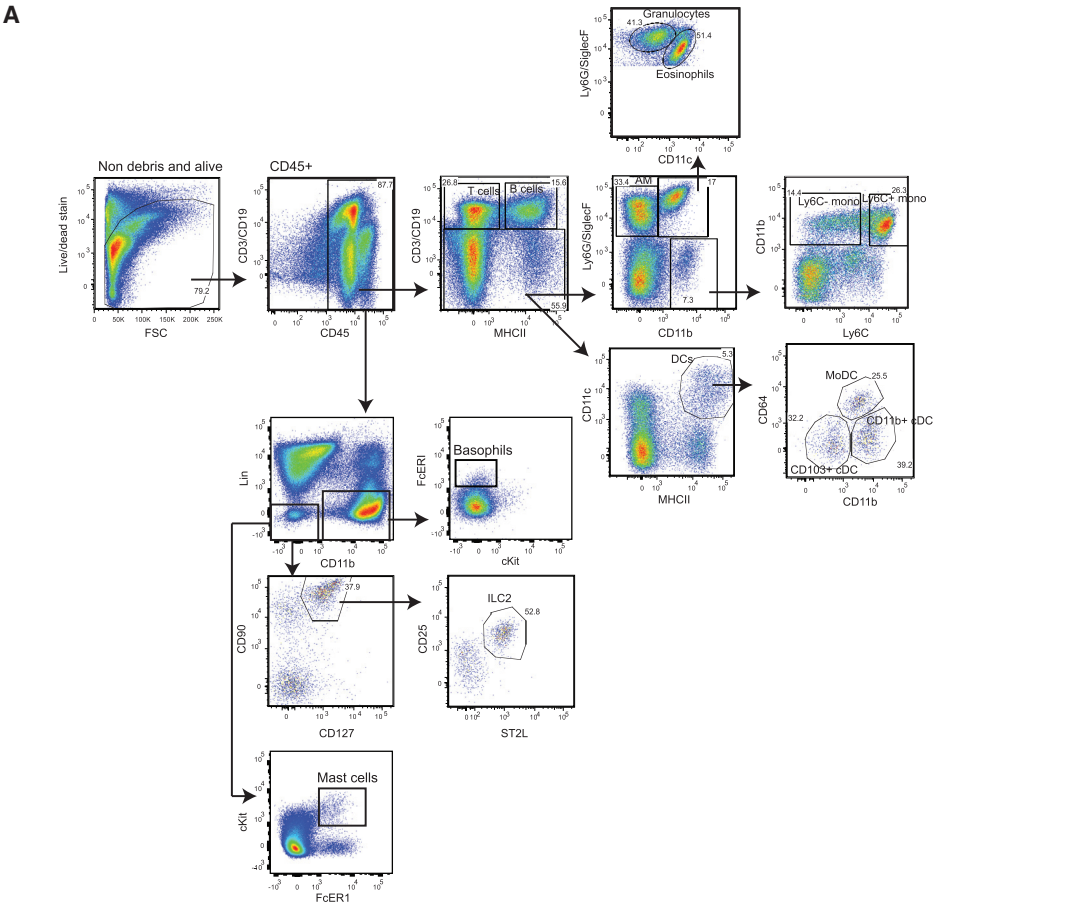
Allergic disease originates in early life and polymorphisms in interleukin-33 gene (*IL33*) and *IL1RL1*, coding for IL-33R and decoy receptor sST2, confer allergy risk. Early life T helper 2 (Th2) cell skewing and allergy susceptibility are often seen as remnants of feto-maternal symbiosis. Here we report that shortly after birth, innate lymphoid type 2 cells (ILC2s), eosinophils, basophils, and mast cells spontaneously accumulated in developing lungs in an IL-33-dependent manner. During the phase of postnatal lung alveolarization, house dust mite exposure further increased IL-33, which boosted cytokine production in ILC2s and activated CD11b$^+$ dendritic cells (DCs). IL-33 suppressed IL-12p35 and induced OX40L in neonatal DCs, thus promoting Th2 cell skewing. Decoy sST2 had a strong preventive effect on asthma in the neonatal period, less so in adulthood. Thus, enhanced neonatal Th2 cell skewing to inhaled allergens results from postnatal hyperactivity of the IL-33 axis during a period of maximal lung remodeling.

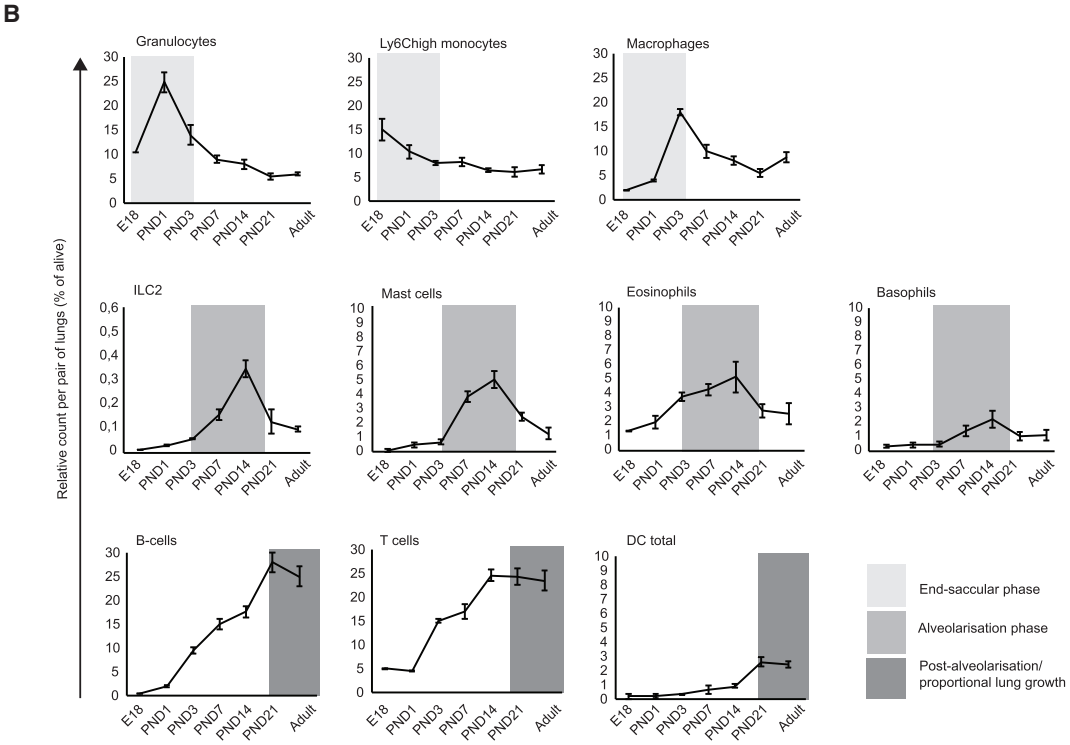
INTRODUCTION

Allergic asthma often starts in early childhood, affecting around 5%–10% of school-aged children. Most cases of pediatric asthma are driven by a type 2 immune response, rich in eosinophils, mast cells, and basophils and controlled by allergen-specific CD4$^+$ T helper 2 (Th2) lymphocytes. The type 2 response induces goblet cell metaplasia (GCM), airway hyperresponsiveness (AHR), and airway remodeling (Lambrecht and Hammad, 2015; Saglani et al., 2013). House dust mite (HDM) allergen represents the most common trigger of allergic asthma (Celedón et al., 2007) and Th2 cell sensitization to HDM has been modeled in the mouse. In adult mice, lung CD11b$^+$ conventional dendritic cells (DCs) and bronchial epithelial cells control sensitization to HDM (Hammad and Lambrecht, 2015; Schuijs et al., 2015). DCs take up inhaled HDM allergen and transport it to the draining mediastinal lymph nodes (MLNs), where they polarize the Th cell immune response, helped by basophils, innate lymphoid cells type 2 (ILC2s), and B cells (Coquet et al., 2015; Hammad et al., 2010; Oliphant et al., 2014; Plantinga et al., 2013). Activation of DCs occurs through the release of epithelial cytokines like thymic stromal lymphopoietin (TSLP), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin (IL)-25, and the IL-1 family members IL-1α and IL-33 (Hammad et al., 2009; Kool et al., 2011; Phipps et al., 2009; Willart et al., 2012). Some of these lung-tissue-derived cytokines like IL-33 also induce cytokine production by ILC2s and tissue-resident memory Th2 cells (Coquet et al., 2015; Endo et al., 2015; Guo et al., 2015; Klein Wolterink et al., 2012).

Early postnatal T cell reactivity to HDM allergens is a major risk factor for the development of asthma in children, suggesting that persistent Th2 cell sensitization occurs early after birth (Celedón et al., 2007; Lodge et al., 2011). Why young children are at increased risk to become sensitized is largely unknown. The immune system is undergoing important shifts in its composition and function during the postnatal period and early childhood. Newborns demonstrate enhanced Th2 cell function, which is believed to be the result of a complex feto-maternal symbiosis. Th2 cell immunity suppresses alloresponses during pregnancy and promotes growth of the placenta (Fock et al., 2013; Forsthuber et al., 1996). The enhanced Th2 cell bias of neonatal T cells is in part T cell intrinsic and transient (Li et al., 2004; Sornasse et al., 1996; White et al., 2002), but cord blood DCs have also been shown to lack production of the Th1-cell-polarizing cytokine IL-12p70 (Goriely et al., 2001; Upham et al., 2002).

When allergens are first encountered, the lungs of young children are still developing and undergo a postnatal phase of alveolarization, characterized by epithelial cell differentiation, angiogenesis, and extracellular matrix remodeling. This alveolar phase of lung growth lasts until 3 years of age and is controlled by many growth factors and cytokines that could also influence





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the immune system. Here we found that IL-33 was spontaneously produced during the alveolar phase of lung development, causing accumulation of type 2 innate immune cells. In this period, HDM exposure caused a vicious circle of IL-33-mediated activation of innate type 2 cells and DCs. IL-33 suppressed IL-12 production and induced OX40-ligand in neonatal DCs, explaining the Th2 cell bias in this period. Consequently, the soluble IL-33 decoy receptor (sST2) had a much stronger preventive effect on allergy development in neonatal than in adult mice. As polymorphisms in the IL-33 or IL-1RL1 gene have been consistently found to confer allergy and asthma risk, we propose that the IL-33 pathway is a crucial regulator of asthma development in early life that alters communication between epithelial cells, DCs, and innate type 2 immunity.

RESULTS

Developing Lungs Accumulate Type 2 Immune Cells

Lung development proceeds via a pseudoglandular stage (embryonic [E] day 9.5–16.5), a canalicular stage (E16.5–17.5), a saccular stage (E18.5 to post natal day 5 [PND5]), an alveolar stage (PND4–21), and a stage of microvascular maturation (PND14–21) (Schittny et al., 2008). Fetal lung is first populated around E12 by CD45$^+$ yolk sac-derived macrophages (De Kleer et al., 2014; Guilliams et al., 2013; Schulz et al., 2012; van de Laar et al., 2016). We recently have shown that this first wave of yolk sac-derived macrophages is followed by a consecutive wave of fetal liver-derived monocytes that enter the lung around E18, the start of the saccular phase in mice, and give rise to alveolar macrophages (AM) under the influence of GM-CSF (Guilliams et al., 2013; van de Laar et al., 2016). The prenatal influx of fetal liver-derived monocytes was accompanied by a rapid influx of granulocytes that peaked in number at PND1 (Figures 1A, 1B, and S1). At PND3 coinciding with the start of the alveolar phase of lung development, ILC2s, mast cells, eosinophils, and basophils (hereinafter collectively referred to as "type 2 innate cells") entered the lung and rapidly increased in numbers at PND14, outnumbering the number of adult type 2 cells by a factor of 4 to 10. Type 2 cells declined again after weaning. Numbers of DCs and T and B cells were very low directly after birth and gradually increased over time. DCs reached adult numbers around PND21, around the time of weaning. Thus, the postnatal phase of lung alveolarization is characterized by a spontaneous influx of high numbers of type 2 innate cells.

Type 2 Immunity to Allergens Is Promoted during the Alveolarization Period

DCs are crucial for mounting adaptive Th2-cell-mediated immunity to HDM in adult mice (Plantinga et al., 2013). As adaptive immune cells and DCs were severely impaired in the neonatal respiratory tract (Figure 1B), we hypothesized that sensitization to inhaled HDM would be defective during this period. We compared the effect of a single HDM sensitization in the early postnatal period (PND3, protocol A), alveolarization period (PND14, protocol B), or adult age (6–8 weeks, protocol C) (Figure 2A). All mice were challenged 7–11 days and analyzed 14 days after the sensitization. Despite severely reduced DC numbers at PND3 and PND14, HDM sensitization at these early time points followed by challenge resulted in significantly increased eosinophilia and lymphocytosis in the bronchoalveolar lavage (BAL) fluid (Figure 2B), increased Th2-cell-associated cytokines in MLN (Figure 2D), and increased total IgE and HDM-specific IgG1 in serum (Figure 2E), compared with PBS-sensitized mice. The degree of eosinophilia and lymphocytosis of mice sensitized at PND14 (protocol B) was even more marked than in mice sensitized at adult age (protocol C, Figure 2B). Mice in protocol B also demonstrated more inflammatory, eosinophil-rich lesions around the bronchi and pulmonary blood vessels (Figure 2C), higher IL-5 and IL-13 cytokines in the mediastinal LNs (Figure 2D), and higher concentration of serum HDM-specific IgG1 (Figure 2E) than mice in protocol C at adult age. Lungs of mice in protocol B contained much higher numbers of IL-5- and IL-13-producing CD4$^+$ T cells, as measured by intracellular staining, indicative of strongest adaptive immune response induction to HDM around PND14 (Figure 2F).

Mice in protocol A that were sham-sensitized at PND3 with PBS and challenged between PND10 and 14 with HDM (PBS-HDM group) showed significant eosinophilia in the BAL (Figure 2B) and goblet cell hyperplasia on lung sections (Figure 2C), an effect not seen in the PBS-HDM mice of protocols B and C. These signs of airway inflammation were accompanied by a strong increase in IL-5- and IL-13-producing ILC2s (Figure 2G) and were also observed in the lungs of PBS-HDM and HDM-HDM *Ccr7*$^{-/-}$ age-matched controls undergoing protocol A (Figures S2 and S3). *Ccr7*$^{-/-}$ mice lack migration of DCs to the MLNs and therefore cannot become actively sensitized to mount airway eosinophilia or an adaptive Th2 cell response in the MLN (Figures S3 and S4). We conclude therefore that the observed eosinophilia, goblet cell hyperplasia, and increase in IL-5- and IL-13-producing ILC2s in the PBS-HDM group in protocol A represents an innate immune response to HDM challenge. Indeed, administration of three consecutive doses of HDM (10 μg) in naive mice was sufficient to induce the strong accumulation and activation of IL-5- and IL-13-producing ILC2s around PND14, but much less so around PND3 or adulthood (Figure 2H). These data indicate that (1) the alveolarization period favors Th2 cell sensitization and (2) an exaggerated innate immune response to allergens in this period also causes immediate CCR7-independent type 2 airway inflammation.

Conventional CD11b$^+$ DCs Present Allergen in the Alveolarization Period

We next studied in greater detail how DCs developed postnatally. On tracheal whole mounts of MHCII-GFP mice, the network of highly dendritic MHCII$^+$ DCs that is found in large conducting airways of adult mice was virtually absent in neonatal mice

Figure 1. Three Waves of Perinatal Pulmonary Immune Cell Development

(A) Gating strategy by 12-color flow cytometry on dispersed lung cells.

(B) Relative cell counts in naive lungs of fetal (E20), neonatal, infant, and adult mice.

Figure S1 shows these data in absolute cell counts.

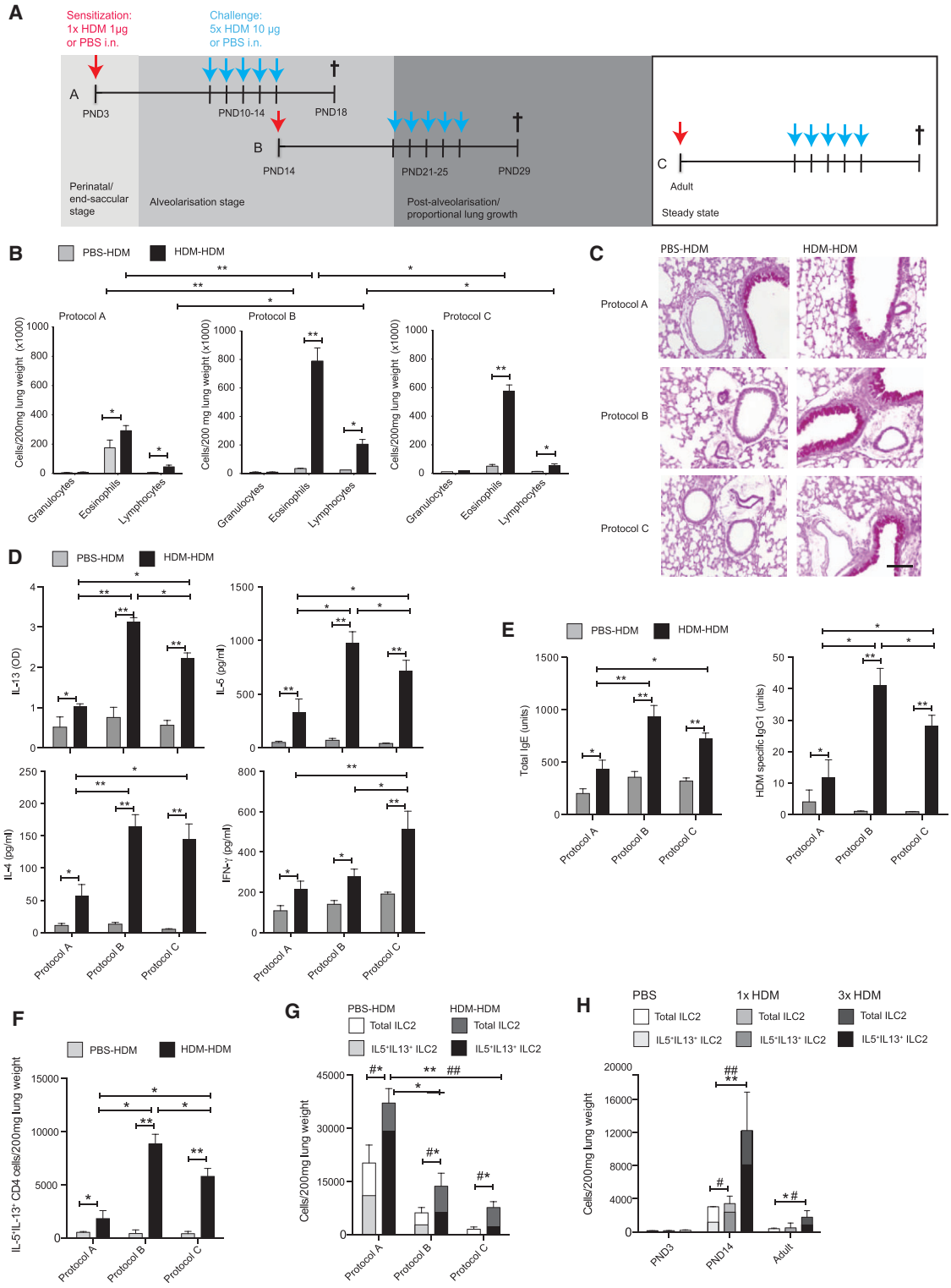

Figure 2. HDM Sensitization and Challenge in Various Age Groups

(A) Protocol of allergic airway induction. PND3, PND14, and adult (PND42–49) mice were sensitized on PND3 (protocol A), PND14 (protocol B), or PND42–49 (protocol C) with HDM or PBS and were challenged with HDM on 5 consecutive days 1 week after sensitization. 2 weeks after the start of the protocol, the mice were sacrificed.

(B) Differential cell counts in the BAL were determined by flow cytometry 72 hr after the last HDM challenge (day 14).

(C) PAS staining of lung sections. Scale bar represents 100 µM.

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(Figure 3A). A small number of studies have described increased numbers of CD11b$^+$ DCs in neonatal lungs (Gollwitzer et al., 2014; Roux et al., 2011), but a careful distinction between CD11b$^+$ cDCs and moDCs has never been made. Using a panel that discriminates cDCs from monocyte-derived DCs (Guilliams et al., 2013), we found that CD11b$^+$CD64$^+$ moDCs represented the largest DC subset in the lung from E20 until PND2 (Figure 3B). Among the two conventional pulmonary DC subsets, CD11b$^+$ cDCs developed more slowly, leading to a predominance of CD103$^+$ cDCs between PND1 and PND12. Naive MLNs at PND3, PND14, and adult age showed differences in total cellularity but equal ratios of T cells, B cells, and total DCs were noticed (Figure 3C). Also in the MLN, there was a clear predominance of CD103$^+$ cDCs at PND3. Monocyte-derived DCs were hardly present in steady-state MLNs, consistent with the idea that these cells are poorly migratory (Plantinga et al., 2013).

Fluorescently labeled HDM-AF647 was injected intranasally (i.n.) to measure the antigen uptake and migratory capacity of DCs in various age groups. In the lung of both adult and neonatal mice, most of the fluorescent HDM was taken up by CD11c$^+$ cells including alveolar macrophages (AMs) and DCs (Figure 3D). The capacity to take up antigen was not age dependent. Overall, moDCs took up HDM most efficiently, whereas the uptake of HDM allergen by lung CD103$^+$ and CD11b$^+$ cDCs was lower. There was very little uptake of allergen by CD45$^-$ lung cells, which include airway epithelial cells, across all ages tested (Figure 3D).

After HDM antigen uptake in the lung, DCs migrate to the MLN in adult mice (Plantinga et al., 2013). The i.n. administration of 10 μg HDM-AF647 resulted in a 2-fold, 9-fold, and 3.5-fold increase in CD11c$^+$MHC$^+$ DC numbers in PND3, PND14, and adult MLN, respectively (Figure 3E). Within the MLN, MHCII$^+$CD11c$^+$ cells highly expressed the chemokine receptor CCR7, and this was even more the case in HDM-AF647-carrying DCs (Figure 3F). Finally, a study of *Ccr7*$^{-/-}$ mice indicated that also at PND3 and PND14, migration of HDM$^+$ DCs to the MLN after 10 μg of HDM was CCR7 dependent, like in adult mice (Figure 3G). Despite the high predominance of CD103$^+$ DCs in neonatal lungs, the migratory CCR7$^+$ HDM$^+$ DC population in the MLN mainly consisted of CD11b$^+$ cDCs (Figure 3H). By increasing the dose of HDM antigen 10-fold, the proportion of moDCs in the MLN increased and the total number of migratory DCs increased, but still CD11b$^+$ cDCs predominated the response across all age groups. These data indicate that in the neonatal period, the low numbers of CD11b$^+$ cDCs are highly efficient in taking up HDM allergen and migrating to the MLN in a CCR7-dependent manner.

IL-33 Blockade Inhibits Neonatal Type 2 Immunity and Enhanced DC Function

We next sought to understand why migration of neonatal DCs is enhanced. In steady-state conditions, high numbers of activated IL-5- and IL-13-producing ILC2s cells, as well as type 2 cytokine-producing mast cells, basophils, and eosinophils, were found in the lungs on PND14, but not on PND3 or in adulthood (Figures 4A, 4B, and 1B). We found no IL-5- and IL-13-expressing CD4$^+$ T cells in steady-state mice. The innate type 2 cells all express ST2L (IL-33R) and proliferate in response to IL-33 (Saglani et al., 2013; Schmitz et al., 2005; Willart et al., 2012). We therefore investigated the concentration of IL-33 protein in lysed lung cells in steady state (PBS) or after administration of HDM to unsensitized mice. In steady state, IL-33 protein concentration peaked around PND14 (Figure 4C; van de Laar et al., 2016). At 16 hr after the administration of HDM, the expression of IL-33 was significantly higher in lung cell homogenates of PND14 lungs compared to PND3 or adult lungs (Figure 4C). Other cytokines like KC, MCP1, GM-CSF, IL-1β, and TSLP did not show similar peak responses at PND14 (data not shown). IL-33 mRNA was detected mainly in sorted CD45$^-$CD31$^-$Epcam$^+$ epithelial cells (ECs), and mRNA was further boosted by stimulation with HDM (Figure 4D). IL-33 mRNA was not detected in AM, DCs, or monocytes. Because IL-33 released from ECs is an important driver of type 2 immunity (Hammad and Lambrecht, 2015), we checked whether spontaneous type 2 immunity at PND14 was dependent on secreted IL-33. Whereas WT mice developed spontaneous tissue eosinophilia and accumulation of ILC2s in the lung, *Il1rl1*$^{-/-}$ mice (lacking the IL-33R, also known as T1/ST2) had severely reduced spontaneous type 2 immunity of the lung at PND14 (Figure 4E). This tissue eosinophilia was largely independent of cells of the adaptive immune response, as *Rag2*$^{-/-}$ mice had equal eosinophilia at PND14 compared with wild-type controls (Figure 4F), and ILC2 numbers were increased compared with wild-type controls, as described previously(Roediger et al., 2015).

We next questioned whether HDM also led to ILC2 activation in neonatal mice in an IL-33-dependent manner. After an innate immune response to a single administration of HDM at PND14, ILC2 numbers were not increased, but ILC2s were clearly activated as indicated by a rise in intracellular IL-5 and IL-13 protein compared with PBS-exposed mice (Figures 2H and 4G). The increase in intracellular IL-5 and IL-13 production was blocked by in vivo administration of recombinant sST2 (r-sST2), a recombinant decoy receptor for IL-33, made to exactly mimic the splice variant of ST2 and only neutralizing extracellular IL-33. Therefore, released IL-33 was responsible for mediating ILC2 activation (Figure 4G).

IL-13 produced by activated ILC2 has been shown to enhance the migratory capacity of CD11b$^+$ cDCs in adult mice (Halim et al., 2016). PND14 CD11b$^+$ cDCs and moDCs expressed more IL-13R and ST2L compared with adult or PND3 DCs (Figure 4H). Similarly, PND14 ILC2 expressed more ST2L than adult or PND3 ILC2 (Figure 4I). Treatment with blocking anti-IL-13 or r-sST2 resulted in a strong reduction in DC migration to the

(D) Cytokines in MLN cells restimulated for 3 days with 15 μg/mL HDM.

(E) Total IgE- and HDM-specific IgG1 in serum.

(F and G) Number of IL-5$^+$IL-13$^+$CD4$^+$ T cells (F) and total ILC2 and IL-5$^+$IL-13$^+$ ILC2s (G) in the lungs.

(H) Number of ILC2s after 1 or 3 daily administrations of PBS or HDM (10 μg). Results show one representative experiment out of three. Six (adult), eight (PND14), and ten (PND3) mice/group were used. Results are shown as mean ± SEM.

Asterisks in (G) and (H) indicate statistically significant differences between total ILC2 numbers. Hashtags indicate statistically significant differences between number of IL-5$^+$IL-13$^+$ ILC2s. $^*p < 0.05$, $^{**}p < 0.01$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$. Figures S3 and S4 shows these analysis in *Ccr7*$^{-/-}$ mice.

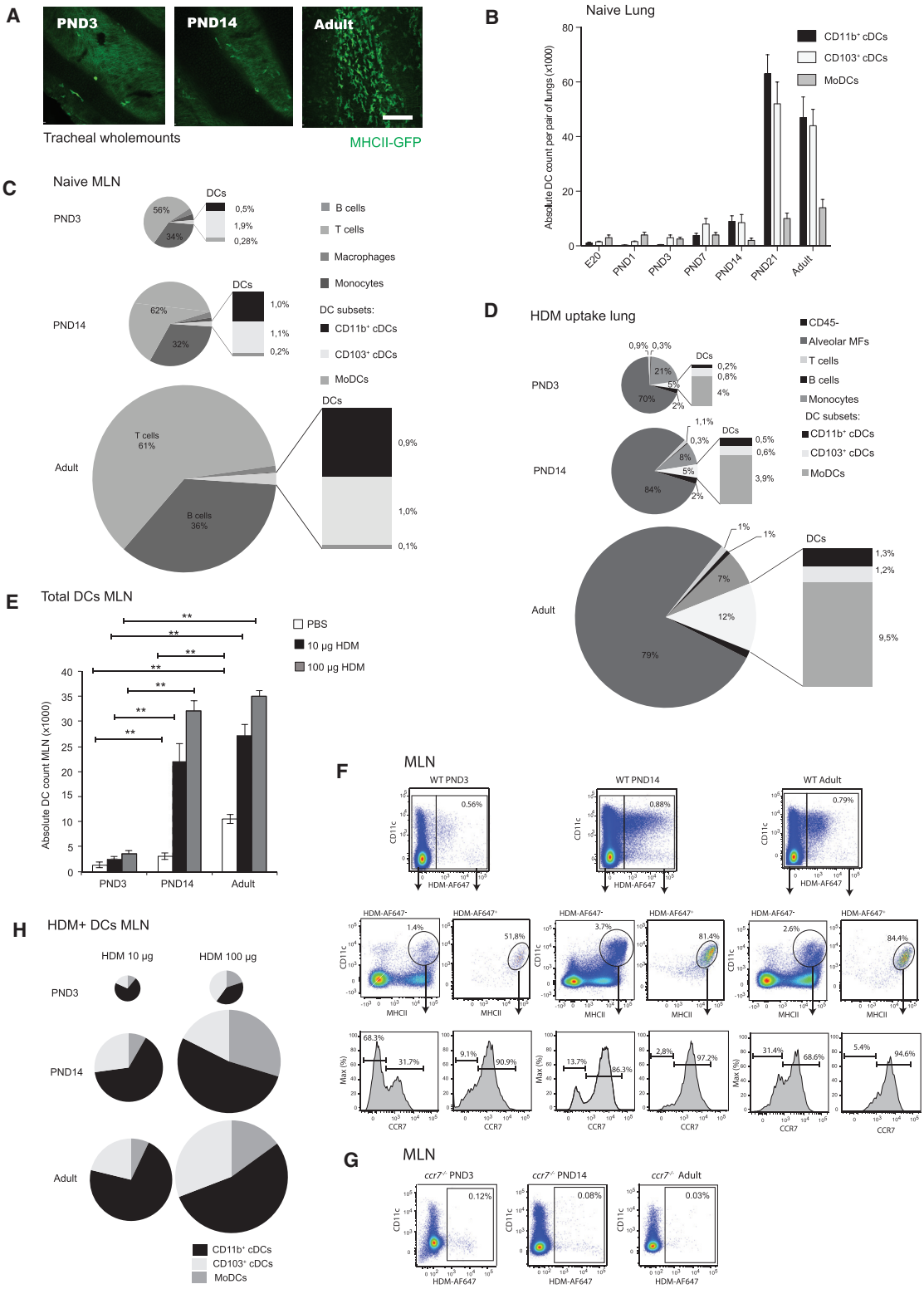

Figure 3. DC Numbers, Antigen Uptake, and Migration in Neonatal, Infant, and Adult Mice

(A) Tracheal whole mounts of naive MHCII-GFP mice at PND3, 14, and adult age. Scale bar represents 20 µM.

(B) Total number of moDCs, CD11b$^+$ cDCs, and CD103$^+$ cDCs in naive fetal, neonatal, infant, and adult lungs.

(C) Pie charts depicting the relative proportions of lymphocytes and myeloid cells within total cells present in naive MLN at PND3, PND14, and adult age.

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MLN (Figure 4J). One of the molecules induced by IL-33 on DCs is OX40L, a TNFR family molecule involved in polarizing naive Th cells into Th2 cells (Salek-Ardakani et al., 2003). We found no constitutive OX40L expression on DCs in naive MLN and lungs but upon HDM exposure, strong induction was observed on migratory CD11b$^+$ cDCs. Expression of OX40L was again most prominent at PND14 (Figure 4K). The CD11b$^+$ cDCs that still migrated to the MLN despite r-sST2 blockade at PND14 had lower OX40L expression (Figure 4L) consistent with previous in vivo findings that OX40L expression of adult pulmonary DCs is controlled by IL-33 (Besnard et al., 2011). Combining the two blocking antibodies did not have an additional effect on migration compared to r-sST2 alone (data not shown). These data indicate that during the innate response to HDM at PND14, IL-33 promotes CD11b$^+$ DC migration via a direct effect on DCs and via the induction of IL-13 production in ILC2s.

Neonatal DCs Preferentially Induce Th2 Cell Polarization

It is well known that the immune system of neonates shows a preferential polarization toward Th2 cell development, caused by delayed production of IL-12p70 by neonatal (cord blood) DCs or defective IFN-γ production by neonatal T cells (Goriely et al., 2001; Upham et al., 2002; Vekemans et al., 2002). Due to practical limitations, studies on the function of neonatal mucosal DCs are missing. We therefore set up an experiment to compare the intrinsic Th2 cell skewing capacity of the three main pulmonary DC subsets at different ages after activation and migration to the MLN, avoiding the use of HDM that has the potential to activate DCs. We administered 100 μg of the harmless antigen ovalbumin (OVA) i.n. to PND3, PND14, and adult WT mice. Three days later we flow-sorted DC subsets from the MLN to stimulate adult OVA-specific T cell receptor (TCR) Tg CD4$^+$ T cells (OT-II cells) in vitro. Each of the neonatal and adult DC subset was proficient in inducing all prototypic Th1 (IFN-γ), Th2 (IL-4, IL-5, IL-13), Th17 (IL-17), and T regulatory (IL-10) cytokines (Figures 5A and S5). However, CD11b$^+$ cDCs and moDCs derived from neonatal mice induced a stronger tendency for Th2 cell-associated cytokine production compared to their adult counterparts (Figures 5A and S5). Also, IL-17 and IL-10 production were slightly increased. As adult T cells were used in the readout, only slightly lower IFN-γ levels were measured. These data show that neonatal DCs have a pro-Th2 cell bias.

IL-33 Causes IL-12p35 Deficiency in Neonatal DCs

We next investigated the reason for the Th2 cell bias of neonatal DCs. IL-33 could promote Th2 cell-mediated immunity via the induction of OX40L on DCs (Figure 4L) and a reduction in IL-12p70 production (Besnard et al., 2011; Kurowska-Stolarska et al., 2008; Rank et al., 2009). Sorted neonatal mediastinal moDCs and CD11b$^+$ cDCs and to a lesser extent CD103$^+$ cDCs expressed less mRNA IL-12p35, when compared to their adult counterparts (Figure 5B). Expression of IL-6 mRNA was identical across age groups.

To study the Th2 cell skewing effect of IL-33, we isolated DC subsets from naive PND14 and adult lung and co-cultured the DCs with OT-II cells in the presence of OVA and increasing concentrations of IL-33 (Figure 5C). Correlating with the highest ST2L expression on PND14 DCs, IL-33 induced strongest IL-4 induction in co-cultures containing PND14 CD11b$^+$ cDCs or moDCs, indicative of Th2 cell polarization.

ST2L expression on PND3 DCs was very low and not upregulated upon HDM exposure, suggesting that ST2L signaling has no role in the well-known IL-12 deficiency of the early postnatal period. To test to what extent IL-33 contributed to the IL-12p35 deficiency in neonatal DCs, we sorted migratory DCs from the MLN of PND3, PND14, and adult mice after treatment with HDM-AF647 with or without r-sST2. Of interest, r-sST2 treatment increased IL-12p35 mRNA levels in adult and PND14 migratory DCs but not in PND3 DCs (Figure 5D). These data indicate that IL-12p35 deficiency in neonatal pulmonary DCs in the early postnatal phase is IL-33 independent. During the phase of alveolarization, however, IL-12p35 is actively suppressed by IL-33 made by epithelial cells.

sST2 Prevents Neonatal Type 2 Immunity to Allergens

Having observed that IL-33 plays a prominent role in ILC2 activation, DC migration, and Th2 cell skewing in early life, we next questioned whether IL-33 blockade through r-sST2 treatment would also prevent asthma in an age-dependent manner. We blocked IL-33 signaling by injecting neonatal and adult mice with r-sST2 during HDM sensitization or challenge, in protocol A (sensitization at PND3), B (sensitization at PND14), and C (sensitization in adulthood). In protocol B, r-sST2 treatment at sensitization led to complete abrogation of the number of eosinophils and lymphocytes in the BAL (Figures 6A and 6B), goblet cell metaplasia in lung epithelial cells (Figure 6C), cytokine production in the MLN (Figures 6D and 6E), and total IgE and HDM-specific IgG1 in the serum (Figure 6F). In protocol A and C, treatment with r-sST2 at time of sensitization resulted in a significant but clearly less-effective reduction in these parameters. In contrast, treatment with r-sST2 during the challenge phase of the protocol had effect on eosinophilia only in protocol A, and not in protocol B or C (Figure S6A), most likely as r-sST2 treatment during challenge in protocol A coincided with the peak of innate immunity to HDM in this period (see PBS-HDM group in protocol A). Th2-cell-associated cytokine production in the MLN (Figure S6B) and serum immunoglobulins (Figure S6C) were not affected.

(D) Pie charts depicting the uptake of Alexa Fluor 647 (AF647)-labeled HDM by CD45$^+$ and CD45$^-$ cells in the lung of neonatal, infant, and adult mice administered with the allergen 72 hr earlier.

(E) Total number of CD11c$^+$MHCII$^+$ in neonatal, infant, and adult MLN 72 hr after allergen challenge.

(F) CCR7 expression on total CD11c$^+$MHCII$^+$ DCs and on HDM-AF647$^+$ CD11$^+$MHCII^{hi} DCs in neonatal, infant, and adult MLN 72 hr after the administration of allergen.

(G) Presence of HDM-AF647-labeled cells in MLN 72 hr after the i.n. administration of allergen (10 μg) to neonatal, infant, and adult *Ccr7*$^{-/-}$ mice.

(H) Pie charts depicting the relative proportion of HDM-AF647$^+$ DC subsets in the MLN of mice 72 hr after 10 μg or 100 μg HDM allergen administration.

Data represent two independent experiments with at least six mice per timepoint. Results are shown as mean ± SEM.

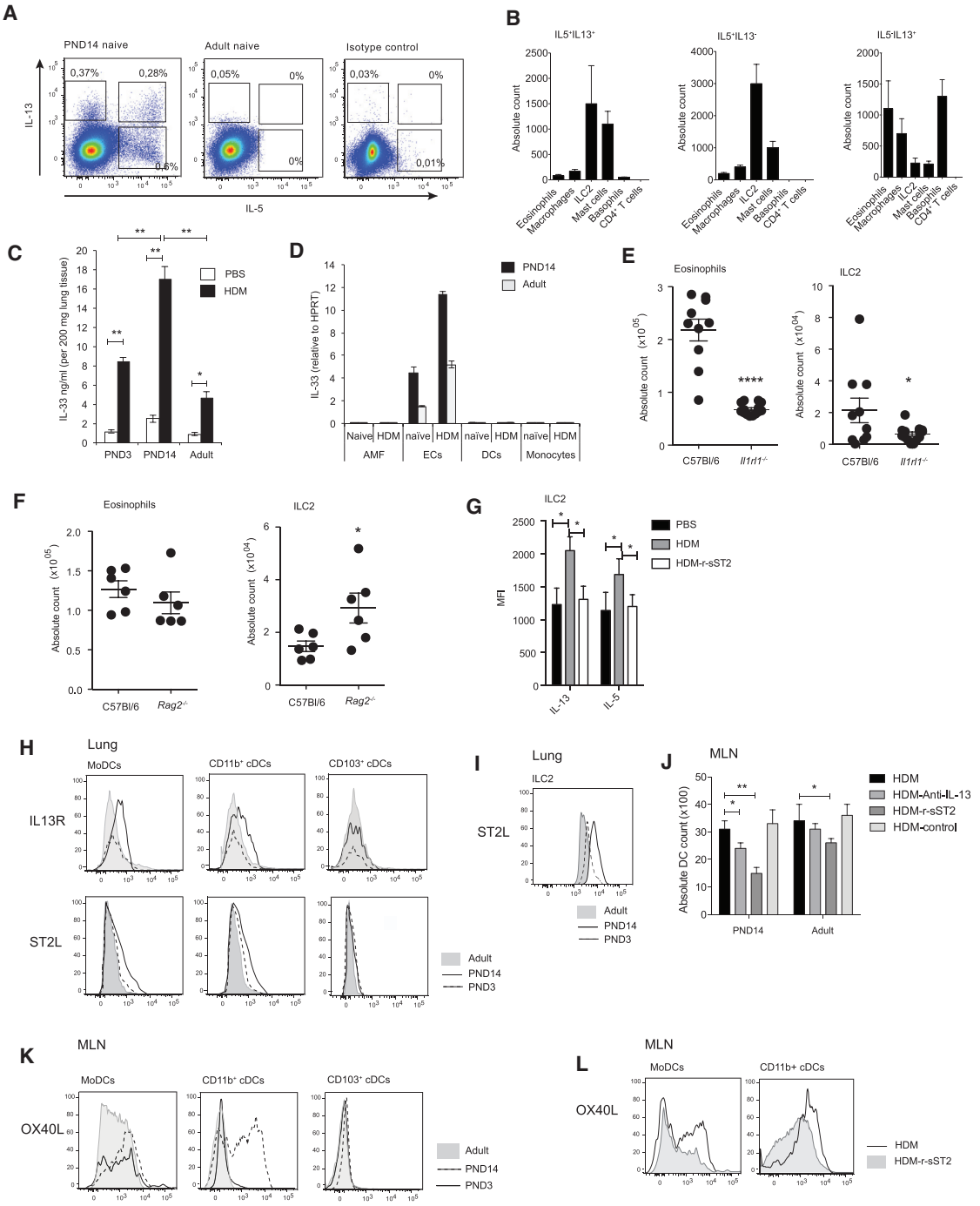

Figure 4. Hyperactive IL-33-ST2L Axis in PND14 Mice

(A) Intracellular IL-5 and IL-13 staining on naive adult and PND14 lungs.

(B) Absolute cell number of IL-5 and IL-13 single- and double-positive cells in the lungs of naive PND14 mice.

(C) IL-33 in lung homogenates 16 hr after PBS or 100 μg HDM i.n. IL-33 concentration was corrected for dry lung weight.

(D) IL-33 mRNA expression in lung cells flow cytometry sorted from naive mice or 6 hr after the administration of HDM (100 μg).

(E) Absolute numbers of eosinophils and ILC2s in the lungs of PND14 C57BL/6 and *Il1rl1*$^{-/-}$ mice.

(F) Absolute numbers of eosinophils and ILC2s in the lungs of PND14 C57BL/6 and *Rag2*$^{-/-}$ mice.

(G) Mean fluorescence intensity (MFI) of IL-13^{+} and IL-5^{+} ILC2s 24 hr after PBS, HDM i.n. (100 μg), or HDM and r-sST2 i.p. (200 μg).

(H) Expression of IL-13R and ST2L on naive DC subsets in the lungs of adult mice (gray solid graph), PND14 mice (black line), and PND3 mice (dashed line).

(I) ST2L expression on lung ILC2s 24 hr after the administration of HDM (100 μg).

(J) Absolute numbers of DCs in the lung draining lymph nodes of PND14 and adult mice 72 hr after i.n. HDM exposure (100 μg). Anti-IL13, r-sST2, and the isotype control were given i.p.

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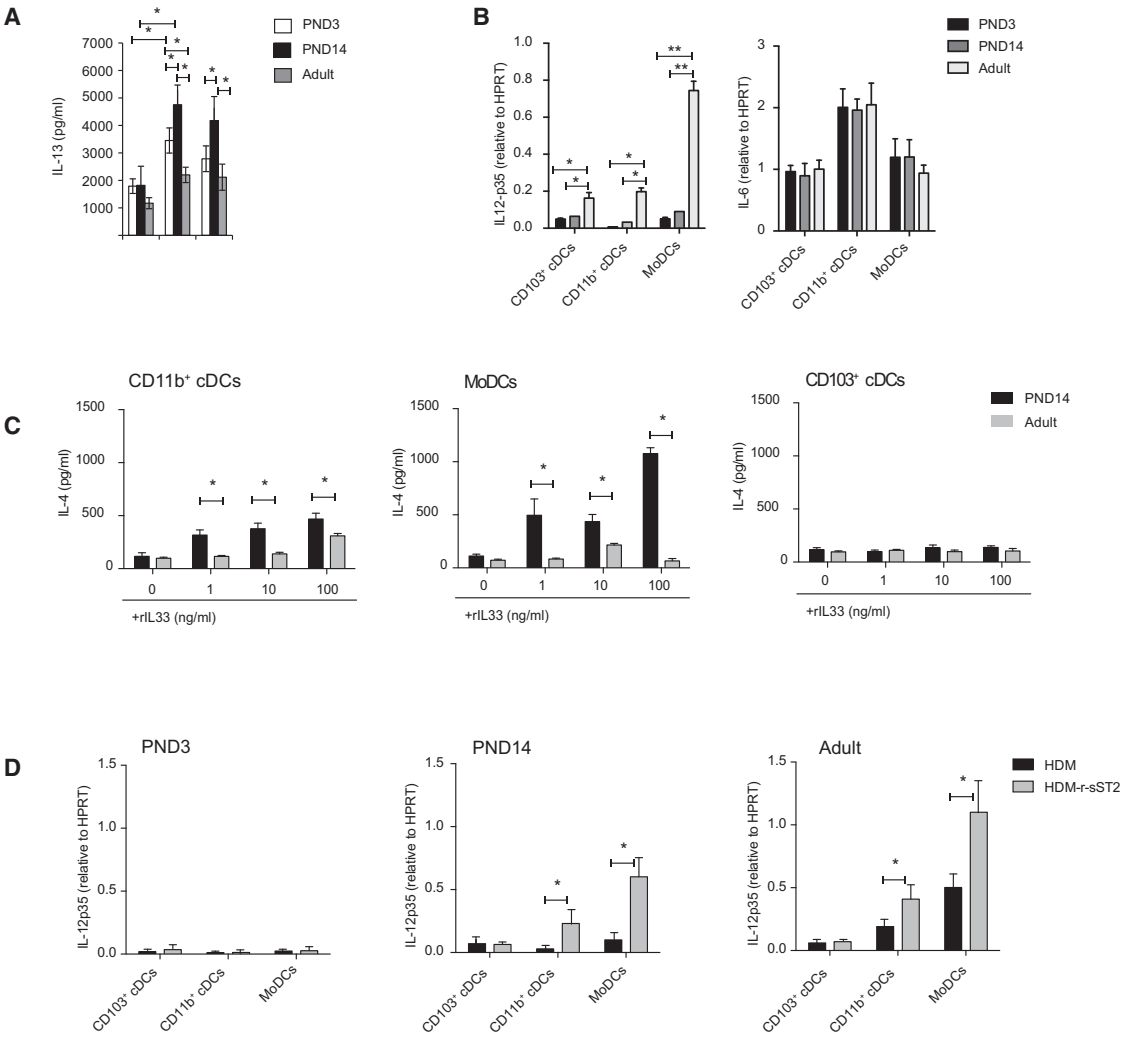

Figure 5. Th2 Cell Skewing Capacity of Neonatal versus Adult DCs

(A) Migratory DCs were sorted from MLNs 3 days after i.n. OVA (100 μg) exposure and co-cultured with naive adult OT-II CD4$^+$ T cells in vitro. After 4 days, IL-13 was measured by ELISA in supernatants. Data represent two independent experiments with at least 6 (adult), 12 (PND14), or 14 (PND3) mice per group. See Figure S5 for data on other cytokines produced.

(B) mRNA IL-12p35 and IL-6 expression determined by RT-PCR in migratory DCs sorted from MLN 3 days after HDM exposure. Data represent three independent experiments with six mice per group.

(C) Cytokine production in supernatants of co-cultures of naive lung-derived PND14 and adult DCs with naive CD4$^+$ T cells from adult OT-II mice, cultured in the presence of OVA protein and different concentrations of IL-33. Data represent two independent experiments with six adult and ten PND14 mice per time point.

(D) mRNA IL-12p35 expression determined by RT-PCR in migratory DCs sorted from MLN 3 days after i.n. HDM exposure with or without r-sST2 i.p.

Results are shown as mean ± SEM. *$p < 0.05$.

Overall, these data indicate that a hyperactive IL-33 axis that coincides with maximal lung remodeling during lung development drives activation of innate and adaptive immunity to allergens in the neonatal period, by driving activation of ILC2s and conventional cDCs.

DISCUSSION

Children under the age of 3 are at risk of developing allergic disease to inhaled allergens, at a time when their lungs are still developing. Based on studies of immune cells obtained from

(K) OX40L expression on migratory DC subsets in the MLN of adult mice (gray solid graph), PND14 mice (thin black line), and PND3 mice (thick black line) 3 days after HDM (100 μg) exposure.

(L) OX40L expression in DCs in lung draining lymph nodes 24 hr after HDM i.n. (100 μg) or HDM i.n. and r-sST2 i.p. (200 μg).

Data represent two independent experiments with at least six mice per time point. Results are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$.

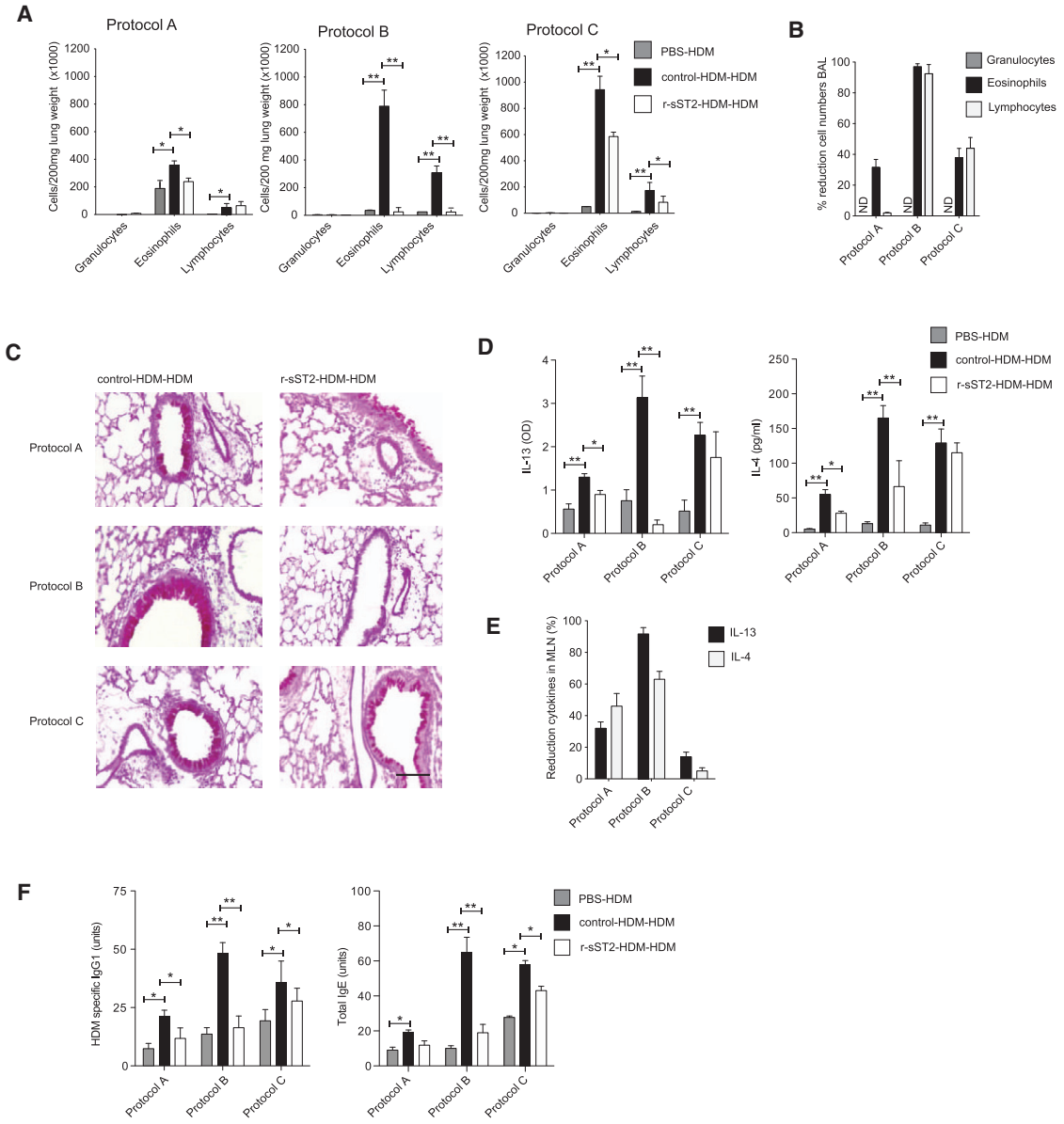

Figure 6. Blockade of IL-33 during Sensitization Is Most Effective in PND14 Mice

PND3 (protocol A) and PND14 (protocol B) and adult WT (protocol C) mice were sensitized on day 0 with HDM or PBS i.n. and in the presence of blocking r-sST2 or control i.p. Subsequently, the mice were challenged with HDM on 5 consecutive days starting 1 week later and sacrificed on day 14 after starting the protocol. See also Figure 2A.

(A) Absolute differential cell counts in BAL fluid determined by FACS 72 hr later.

(B) Reduction in cell numbers in BAL fluid due to r-sST2 treatment at time of sensitization. ND indicates not detectable.

(C) PAS staining of lung sections. Scale bar represents 100 μM.

(D) Cytokines in supernatants of MLN cell cultures, restimulated for 3 days with 15 μg/mL HDM.

(E) Reduction in IL-13 and IL-4 production in supernatants of MLN cell cultures due to r-sST2 treatment at time of sensitization.

(F) Serum Igs. Results show one representative experiment out of two. Six to eight mice/group were used.

Results are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$. See Figure S6 for the effects of r-sST2 administration during challenge.

cord blood, it was proposed that delayed postnatal maturation of Th cell function and developmental defects within the innate immune system that result from feto-maternal adaptation predisposes young children to Th2 cell sensitization (Bot et al., 1998; De Wit et al., 2003; Li et al., 2004; Sarzotti et al., 1996; Sornasse et al., 1996; Tonon et al., 2002). However, a thorough understanding of the allergic sensitization process in the context of postnatal immune maturation and lung development is still lacking. The structural features of postnatal lung development (mainly consisting of formation and remodeling of alveoli) are very similar in humans and mice although the time span is obviously different. The most active phase of alveolar septation

occurs through the second postnatal week in mice and between 2 and 3 years in humans, a time frame where many atopic children also become allergic (Burri, 2006; Wahn and von Mutius, 2001). We here found highly increased production of the epithelial cytokine IL-33 and accumulation of innate type 2 cells during the alveolar period when the lung is maximally remodeling. The spontaneous activation of type 2 cells around this period required extracellular IL-33 and ST2. In this period, innate and adaptive immune responses to HDM were strongly increased through activation of CD11b$^+$ cDCs that migrated vigorously to the MLNs and polarized Th2 cells. HDM-induced IL-33 had a direct effect on CD11b$^+$ cDCs but also stimulated CD11b$^+$ cDCs indirectly via activating innate type 2 cells that produced IL-13. Our finding that IL-13 promotes neonatal DC migration to lung draining lymph nodes is compatible with recent studies employing the model allergen papain in adult mice (Halim et al., 2014, 2016). Although memory Th2 cells have also been found to produce IL-13 after stimulation with epithelial cytokines IL-33 and IL-25 (Endo et al., 2015; Guo et al., 2015), we did not find evidence of this pathway at this very young age, most likely because adaptive T cell immunity becomes fully competent only by the time of weaning. In support, the spontaneous wave of type 2 immunity around PND14 was intact in Rag2-deficient mice, and ILC2 accumulations were even enhanced in these mice, most likely due to increased availability of IL-2 in *Rag2*$^{-/-}$ mice.

Studies in neonatal rodents and humans reported scarcity as well as immaturity of pulmonary DCs, hyporesponsiveness to PAMPs, and low lymphocyte numbers and therefore suggested incompetence of the early postnatal pulmonary immune system to mount adaptive immune responses, an effect that was even exacerbated in germ-free mice (Belderbos et al., 2009; Gollwitzer et al., 2014; Nelson and Holt, 1995; Nelson et al., 1994; Roux et al., 2011; Tschernig et al., 2006). In accordance with these studies, we found that DCs were indeed severely impaired in neonatal lungs, and the few CD11b$^+$ DCs in neonatal lungs during the first post-natal week were sessile CD11b$^+$CD64$^+$ moDCs that closely resemble macrophages (Guilliams et al., 2013; Plantinga et al., 2013). Of the two conventional DC subsets, CD11b$^+$ cDCs developed with slower kinetics compared with CD103$^+$ cDCs and numbers of CD11b$^+$ cDCs remained severely impaired until weaning. The efficiency of PND14 CD11b$^+$ cDCs to drive Th2 cell sensitization in CD4$^+$ T cells was determined by a strong capacity to migrate to the mediastinal nodes in a CCR7-dependent manner. Thus, even during lung development when epithelial barrier function might be incomplete and there might be passive leakage of antigen into the systemic circulation, induction of adaptive immunity occurs by regulated migration of DCs into lung draining nodes, driven by the same chemokine network as in adult mice. Compared to their adult counterparts and compared to CD103$^+$ DCs, neonatal CD11b$^+$ DC subsets also had a strong intrinsic capacity to induce Th2-cell-mediated responses. This Th2 cell skewing capacity was present directly after birth but increased further during the second post-natal week. This specific Th2 cell polarization bias could be explained by two independent but superimposed mechanisms. First, there was increased expression of OX40L, a molecule involved in polarization of Th2 cells, on DCs in the alveolarization phase. Although TSLP is mainly known as the prototypical cytokine-driving expression of OX40L on human and murine DCs (Hammad and Lambrecht, 2015; Ito et al., 2005), we found this increased expression to be IL-33 dependent in the alveolarization phase of lung development. Second, we found that neonatal lung DCs were severely impaired in IL-12p35 expression. IL-12p35 is the rate limiting subunit of IL-12p70, the predominant cytokine driving Th1 cell development. These data are in line with previous work showing that human cord blood DCs (De Wit et al., 2003; Goriely et al., 2001; Tonon et al., 2002) and mouse splenic DCs (Lee et al., 2008) are deficient in IL-12 production. In neonatal lung DCs, IL-12p35 deficiency was strongest in CD11b$^+$ cDCs and CD64$^+$ moDCs, the two main HDM-presenting DC subsets. We could not account the Th2 cell bias to the delayed development of CD103$^+$ cDCs, the mucosal equivalent of splenic CD8α^+CD4$^-$ cDCs that seem to be defective in neonatal spleens (Lee et al., 2008). In neonatal lungs, CD103$^+$ cDCs were most abundant in neonatal lungs and least deficient for IL-12p35, consistent with the idea that CD103$^+$ cDCs have an intrinsic bias to produce more IL-12 (Everts et al., 2016).

Reduced IL-12p70 synthesis in cord blood moDCs has been associated with impaired chromatin remodeling in the IL-12p35 promotor region, suggesting epigenetic regulation of gene transcription (Goriely et al., 2004). Here we identified an additional extrinsic cause for IL-12p35 deficiency in neonatal DCs. In the second postnatal week, IL-12p35 deficiency in lung DCs was caused by a direct and dose-dependent suppressive effect of epithelial IL-33 on IL-12 production (Besnard et al., 2011; Plantinga et al., 2013; Rank et al., 2009).

Altogether, it appears that IL-33 has a high contribution to the development of allergic airway inflammation at young age. This was also shown in a model of sensitization to fungal allergens, in which IL-33 was shown to be high in mice exposed to *Alternaria* allergen (Castanhinha et al., 2015). In our hands, treatment with r-sST2 to block IL-33 at the time of sensitization completely abolished eosinophilic airway inflammation, Th2-cell-associated cytokine production, and GCM particularly when administered in the period of maximal lung alveolarization. Consistently, polymorphisms in *IL1RL1*, coding for ST2 (IL-33R), as well as the *IL33* gene itself have been found associated with asthma and blood eosinophil counts, particularly in childhood asthma (Bønnelykke et al., 2014; Castanhinha et al., 2015; Gordon et al., 2016; Moffatt et al., 2010; Saglani et al., 2013; Savenije et al., 2011; Torgerson et al., 2011; Traister et al., 2015). In asthmatics, polymorphisms in *IL1RL1* also control the relative abundance of the cell-bound IL-33R (ST2L) versus the soluble IL-33 receptor (sST2), that acts as a decoy receptor and antagonist of the IL-33-IL-33R axis (Grotenboer et al., 2013; Traister et al., 2015). It would be interesting to now analyze whether carriers of the *IL33* and *IL1RL1* risk alleles are at increased risk to specifically develop allergic asthma at young age, and whether SNPs associated with high expression of sST2 are associated with protection.

Why would there be spontaneous Th2 cell immunity in the developing lung? We speculate that type 2 immunity controls lung development and/or remodeling. IL-33 boosts the function of ILC2s that can promote tissue remodeling, repair, and homeostasis. Activated ILC2s, mast cells, and basophils produce growth factors such as amphiregulin and epidermal growth factor (EGF) that promote epithelial homeostasis (Groschwitz et al.,

2009; Monticelli et al., 2012). IL-33 is also a known activator of alternatively activated M2 macrophages that control tissue remodeling and postnatal branching morphogenesis of the lung (Jones et al., 2013; Kurowska-Stolarska et al., 2009). We and others did observe increased staining of lung mononuclear cells with the M2 marker Ym1 in the neonatal period (data not shown and Hung et al., 2002). Despite this circumstantial evidence, we were unable to measure gross abnormalities in lung alveolarization in IL-33-deficient animals (I.K., unpublished observations).

In conclusion, our data indicate that neonatal Th2 cell skewing is reminiscent of not just maternal-fetal immune adaptation. High IL-33 production in the postnatal alveolar phase of lung development promotes a wave of type 2 immunity and lowers the threshold for innate immune responses to allergens. It increases the risk for Th2 cell sensitization by boosting the function of DCs and suppressing IL-12 production. Our findings help explain how particular polymorphisms at the *IL1RL1* locus promote or suppress asthma development in childhood. As other genetic polymorphisms seem to control epithelial integrity and genes involved in lung growth, and as respiratory syncytial virus infection at a very young age promotes sensitization while affecting lung growth, it will also be interesting to study whether the IL-33 axis remains hyperactive in some forms of asthma that are accompanied by excessive and altered lung regeneration and remodelling.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 WT mice were obtained from Harlan. MHCII-EGFP mice were provided by H. Ploegh (Harvard Medical School) (Boes et al., 2002). *Ccr7*$^{-/-}$ and *Rag2*$^{-/-}$ mice were derived from Jackson Laboratories. *Il1rl1*$^{-/-}$ mice were housed at Trinity Biomedical Sciences Institute. Mice were housed under specific-pathogen-free conditions. All experiments were approved by the animal ethics committee of Ghent University or Erasmus Medical Center, Rotterdam.

Reagents

HDM extracts were obtained from Greer Laboratories. Recombinant sST2 (r-sST2) was produced and provided by H. Braun (Flanders Institute for Biotechnology) (Willart et al., 2012). Blocking antibody against IL-13 (clone eBio1316H) was obtained by eBioscience. We obtained ELISA Duoset for mIL-13, mKC, mCCL20, and mIL-33 from R&D Systems. APC Cy7-labeled antibody to MHCII, PE-Cy5-labeled, and PE-labeled antibodies to CD3, CD19, and CD8a, PE-Cy7-labeled antibody against CD11c, APC-labeled antibodies against FcεRIα and CD64, PE-Cy7-labeled antibody against CD127, AF647-labeled antibody against EPCAM, biotin-labeled antibody against F4/80, and ELISA sets for mMCP1, mTSLP, and mIL-17A were acquired from eBioscience. FITC-labeled antibody to Ly6C, PE-labeled antibody against Siglec-F, Ly6G, B220, NK1.1, and Ter-119, Horizon V450-labeled antibody against CD11b, PE-TxRed-labeled antibody against CD45, APC Cy7-labeled antibody against cKit, as well as mIL-10, mIL-1β, mIL-4, mIL-5, and mIFN-γ ELISA sets and antibody pairs to mouse IgE, IgG1, and IgG2a in serum were obtained from BD. FITC-labeled antibody to IL-13RA1 was obtained from Antibodies-online. Aqua, CFSE, streptavadin PE-Texas red, OVA Alexa Fluor 488, and OVA AF647 were purchased from Molecular Probes/Invitrogen. HDM (*Dermatophagoidespteronyssinus* extracts, Greer Laboratories) was labeled with an AF647 labeling kit (Invitrogen).

OVA Experiments

DC migration was investigated by injecting 100 μg OVA Alexa Fluor 488 i.n. and dissecting MLNs after 24 hr. Cells were stained for flow cytometry, and DCs positive for Alexa Fluor 488 and highly expressing MHCII and CD11c were considered to be migratory DCs. For DC-OT-II, coculture DCs were sorted from the lungs of naive WT mice and cocultured in a 1:10 ratio with MACS-sorted lymphocytes from LNs and spleen of OT-II TCR Tg mice. 3 days later, supernatants were collected and analyzed on IL-4, IL-5, IL-13, and IFN-γ by ELISA.

Model of HDM-Induced Asthma

HDM extracts were from Greer Laboratories. PND3, PND14, and adult mice were anesthetized with isoflurane, sensitized i.n. with 1 μg HDM in resp. 5, 10, or 40 μL PBS i.n. After 7 days, mice were challenged with 10 μg HDM on 5 consecutive days under anesthesia. 3 days after the last challenge, mice were sacrificed and organs were dissected for analysis. BAL was performed using 3 × 0.7−1 mL EDTA-containing PBS and analyzed, and lungs were inflated with a 1:1 mixture of PBS and OCT solution and snap frozen in liquid nitrogen. Single-cell suspensions of MLNs were prepared by pressing tissue through a 100-μm cell sieve and restimulated in vitro with 15 μg/mL HDM for 3 days. Supernatant was collected from these cultures, and cytokine profiles were assayed by ELISA. In neutralization experiments, IL-33 was blocked at the time of sensitization to HDM by use of 200 μg r-sST2 in 50 (PND3), 100 (PND14), or 150 (adult) μL PBS injected i.p. In other experiments 100 μg r-sST2 was injected i.p. on the first, third, and fifth day of the challenge phase. Recombinant sST2 (r-sST2) was produced and provided by H. Braun (Willart et al., 2012). IL-13 antibody (clone eBio1316H) was from eBioscience.

Early Innate Immune Response to HDM

Mice were i.n. instilled with PBS or 100 μg HDM and sacrificed 16 hr after the injection. Lungs were snap frozen and then homogenized with a tissue homogenizer in 500 μL of cold lysis buffer (20 mM Tris-HCl [pH 8.0], 0.14 M NaCl, 10% glycerol, and 20 mg/mL leupeptin) using a tissue homogenizer (IKA) with the addition of 1% Igepal after homogenization. Samples where then kept on ice for 30 min with agitation each 10 min, followed by a centrifugation to pellet debris. Cleared lysate was quantified for protein concentration with NanoOrange reagent (Invitrogen) according to the manufacturer's protocol.

Cell suspensions were made of the right lung and used for FACS analysis. Innate cytokines were measured by ELISA on lung homogenates, and concentrations were corrected for the protein content.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2016.10.031.

AUTHOR CONTRIBUTIONS

I.K. designed and performed experiments and wrote the manuscript; M.K., M.J.W.B., M.W., J.V.M., M.J.S., M.P., and E.H. performed experiments; R.B. generated key tools; H.H., P.G.F., and R.W.H. designed and supervised part of the experiments; and B.N.L. designed and supervised the study and wrote the manuscript.

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Supplemental Information

Perinatal Activation of the Interleukin-33 Pathway Promotes Type 2 Immunity in the Developing Lung

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Supplemental material

Supplementary figure legends

Supplementary Figure 1, related to Figure 1. Absolute cell counts in naïve lungs of fetal (E20), neonatal, infant and adult mice.

Supplementary Figure 2, related to Figure 2. Protocol A, B and C refer to the protocols described in Fig. 2A. Total ILC2 and $IL5^{+}IL13^{+}$ ILC2 in the lungs of WT and *Ccr7*$^{-/-}$ mice.

Supplementary Figure 3, related to figure 2B. Protocol A, B and C refer to the protocols described in Fig. 2A. Differential cell counts in the BAL of *Ccr7*$^{-/-}$ mice after induction of allergic airway inflammation (for protocol see Fig. 2A). For better comparison we added the WT data of age-matched mice to the graphs (identical data points as in Fig. 2B). Note that the WT and *Ccr7*$^{-/-}$ experiments were done on separate days.

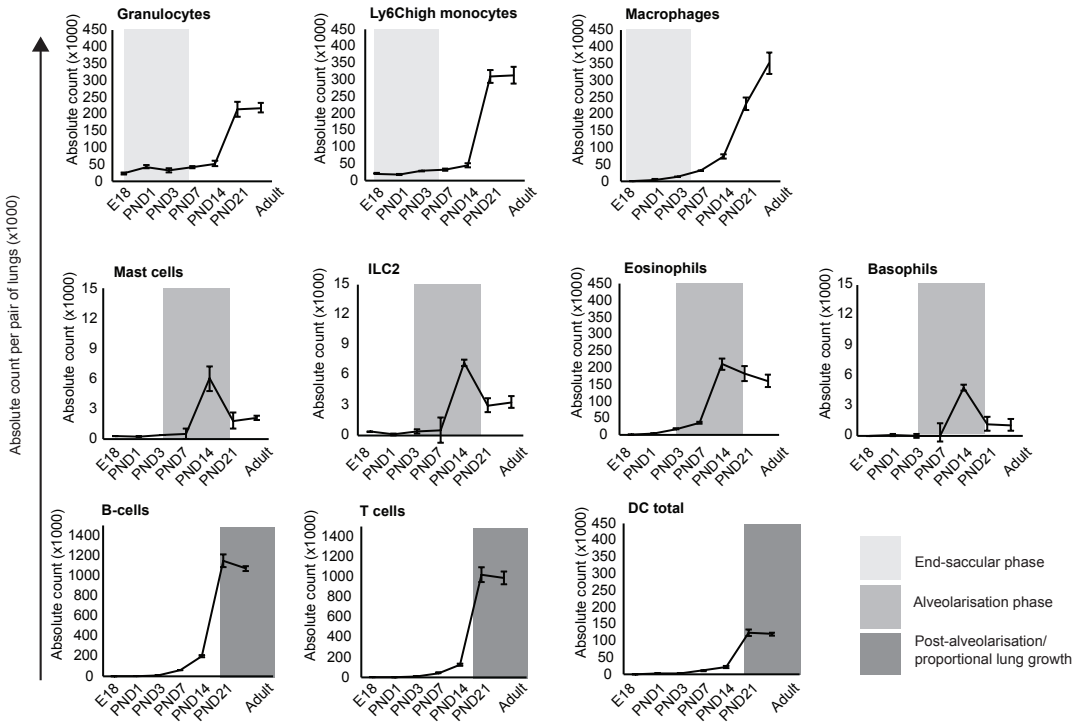
Supplementary Figure 4, related to Figure 2D. Protocol A, B and C refer to the protocols described in Fig. 2A. Cytokine concentration in MLN cells of *Ccr7-/-* mice restimulated for 3 d with 15 µg/ml HDM. For better comparison we added WT data to the graphs (see Fig. 2B). Note that the WT and *Ccr7*$^{-/-}$ experiments were done on separate days.

Supplementary Figure 5, related to Figure 5A. Migratory DC were sorted from MLN 3 days after i.n. OVA (100 µg) exposure and co-cultured with naïve adult OTII $CD4^{+}$ T cells *in vitro*. After 4 days cytokines were measured by ELISA in supernatants. Data represent two independent experiments with at least 6 (adult), 12 (PND14) or 14 (PND3) mice per group.

Supplementary Figure 6, related to Figure 6. Effect of blocking r-sST2 during the challenge phase. Protocol A, B and C refer to the protocols described in Fig. 2A. WT adult, PND14 and PND3 mice were sensitized on day 0 with HDM or PBS i.n. The mice were challenged with HDM on days 7–11. Blocking r-sST2 or isotype control was co-administered on days 7, 9 and 11 i.p. (A) Differential cell counts were determined in the BAL by flow cytometry 72 h later. (B) Cytokines in MLN cells restimulated for 3 d with 15 µg/ml HDM. (C) Total Igs in the serum.

Supplementary figures

S1



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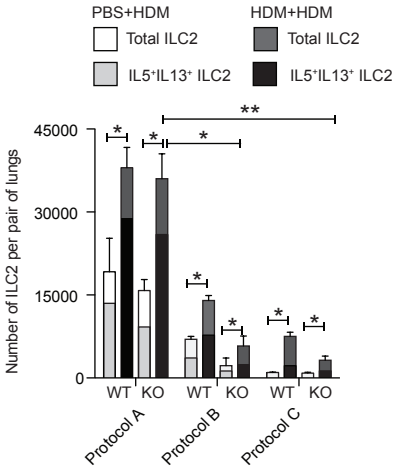

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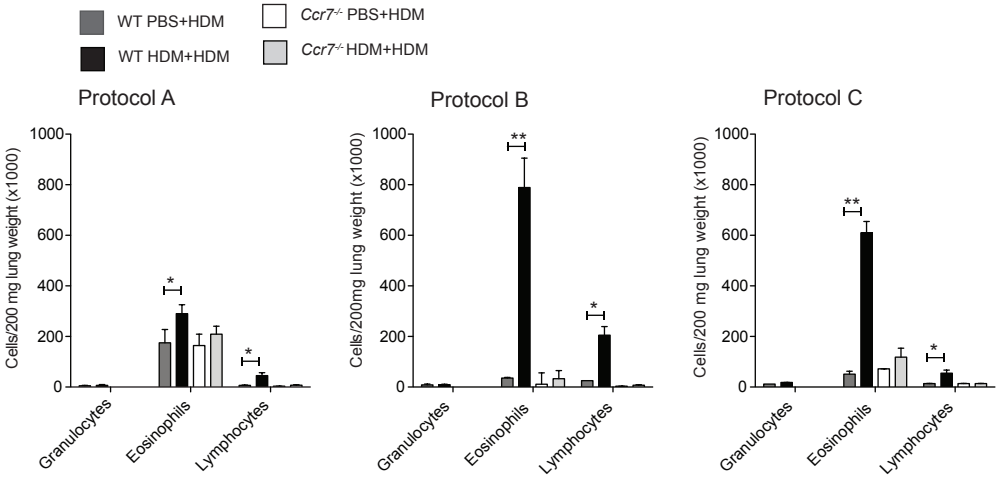

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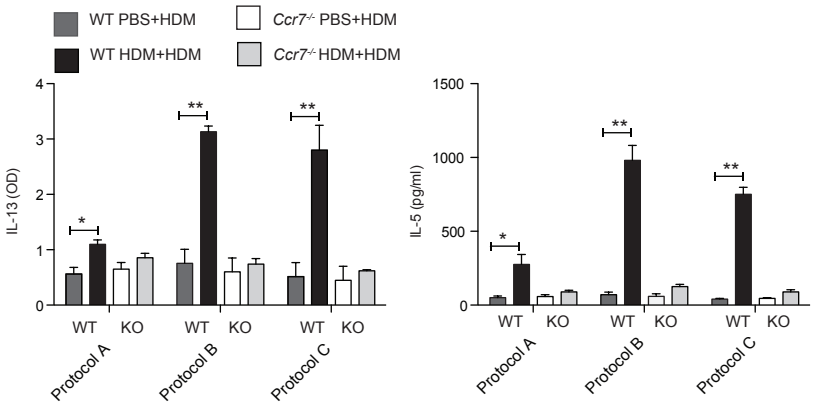
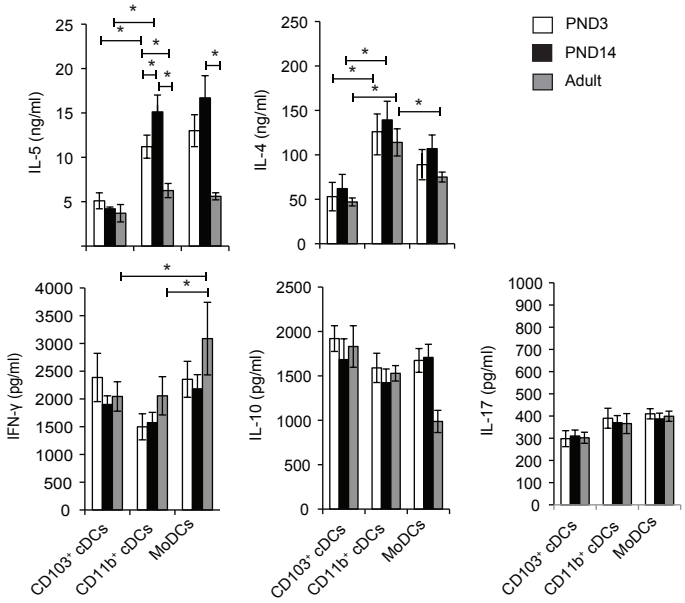


Supplementary figures

S5



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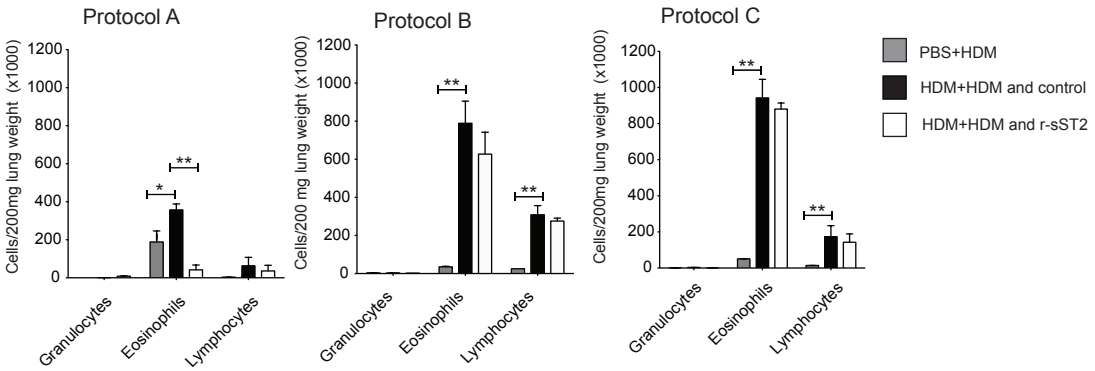
A



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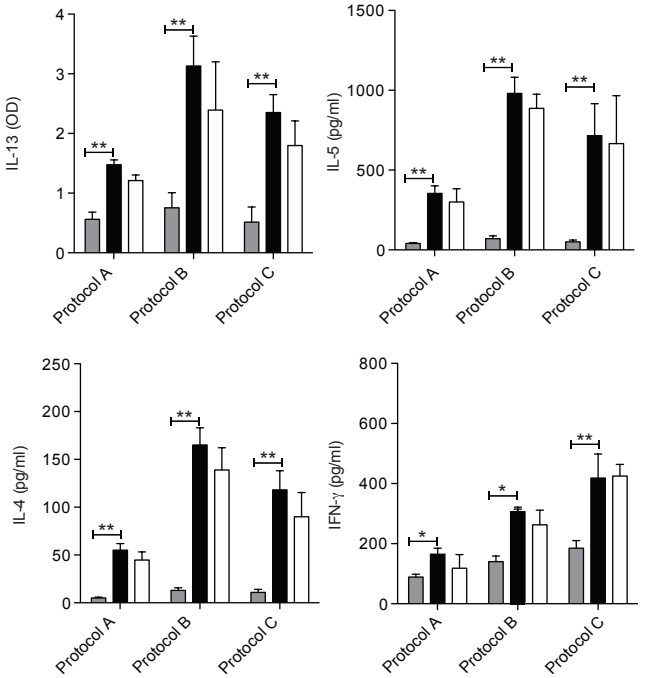
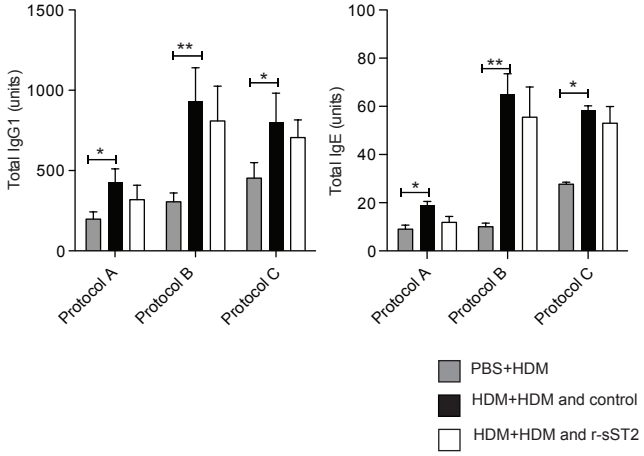
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