

T cells are necessary for ILC2 activation in house dust mite-induced allergic airway inflammation in mice

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Allergic asthma is a chronic inflammation of the airways mediated by an adaptive type 2 immune response. Upon allergen exposure, group 2 innate lymphoid cells (ILC2s) can be rapidly activated and represent an early innate source of IL-5 and IL-13. Here, we used a house dust mite (HDM)-driven asthma mouse model to study the induction of ILC2s in allergic airway inflammation. In BALF, lungs, and lymph nodes, ILC2 activation is critically dependent on prior sensitization with HDM. Importantly, T cells are required for ILC2 induction, whereby T-cell activation precedes ILC2 induction. During HDM-driven allergic airway inflammation the accumulation of ILC2s in BALF is IL-33 independent, although infiltrating ILC2s produce less cytokines in *Il33*^{-/-} mice. Transfer of in vitro polarized OVA-specific OT-II Th2 cells alone or in combination with Th17 cells followed by OVA and HDM challenge is not sufficient to induce ILC2, despite significant eosinophilic inflammation and T-cell activation. In this asthma model, ILC2s are therefore not an early source of Th2 cytokines, but rather contribute to type 2 inflammation in which Th2 cells play a key role. Taken together, ILC2 induction in HDM-mediated allergic airway inflammation in mice critically depends on activation of T cells.

Keywords: Allergy · Asthma · Group 2 innate lymphoid cells (ILC2s) · House dust mite · Th2 cells



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Introduction

Asthma is a heterogeneous disease involving chronic inflammation of the airways and is characterized by episodes of coughing, wheezing, and shortness of breath. Multiple genetic predispositions and environmental factors contribute to asthma development and it is estimated that ~300 million people are affected worldwide. The asthma syndrome is divided into distinct disease entities with specific mechanisms, which have been called asthma endotypes [1, 2]. The most prevalent endotype is allergic asthma, showing a strong association between exacerbations and repeated

exposure to allergens such as pollen, fungi, or house dust mite (HDM). Allergic asthma is thought to be a classic Th2-mediated disease in which the signature effector cytokines IL-4, IL-5, and IL-13 are key orchestrators of persistent inflammation, airway hyper-responsiveness and remodeling, smooth muscle cell hyperplasia, mucous cell metaplasia, and increased angiogenesis (reviewed in [2, 3]).

Although production of IL-5 and IL-13 by a non-T/non-B lymphocyte population in response to IL-25 was first reported by Fort et al. [4], only recently these cells were accurately characterized and defined as a novel cell population that is negative for classic hematopoietic lineage markers and expresses Sca-1, CD117 (c-kit), CD25 (IL-2R α), CD127 (IL-7R α), and T1/ST2 (IL-33R) [5–7]. These innate cells are currently referred to as group 2 innate lymphoid cells (ILC2s) and were found to produce IL-13, which is

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critical for goblet cell hyperplasia and the expulsion of helminths [8]. Activation of ILC2s is sufficient for worm clearance, even in the absence of adaptive immunity [5–7]. Parallel to Th2 cells, development and function of ILC2s are controlled by the transcriptional factor Gata3, which critically regulates the expression of *Il4*, *Il5*, and *Il13* [9–11]. While T cells require priming by antigen presenting cells upon first contact with an antigen and will mount a specific and robust response during a secondary exposure, ILC2s are antigen-independent and are efficiently induced by innate cytokines such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) [5, 6, 8]. In the presence of these proinflammatory cytokines, ILC2s rapidly expand and secrete large amounts of IL-5 and IL-13.

Several reports have shown that ILC2s can be a potent early innate source of type 2 cytokines in experimental asthma. Intranasal administration of papain, a protease allergen, induced eosinophilic airway inflammation in WT and *Rag1*^{−/−} mice lacking T and B cells, but not in *Rag2*^{−/−}*Il2rg*^{−/−} mice that additionally lack ILC2s. Adoptive transfer of WT ILC2s into these mice restored the inflammatory response to papain, supplying further evidence for a central role of ILC2s [12]. In the papain model, ILC2s arise prior to the induction of T cells and ILC2-derived IL-13 promotes migration of activated lung dendritic cells into the draining lymph node and subsequent Th2 differentiation, suggesting an important function of ILC2s during the initiation of inflammation [13]. Other studies using the fungal allergen *Alternaria* have led to similar conclusions, whereby the induction of airway inflammation is heavily dependent on IL-33 signaling and mediated by activated ILC2s rather than Th2 cells [14, 15]. ILC2s were shown to have the capacity to enhance differentiation of naive CD4⁺ T cells to a Th2 phenotype while inhibiting Th1 differentiation in a contact-dependent manner [16, 17]. Together with analyses in helminth infection models showing that Th2 responses are impaired in the absence of ILC2s [18, 19], these findings support a model in which ILC2s are “early sentinel” cells that can be rapidly activated to connect innate and adaptive immunity by acting as an early innate source of type 2 cytokines.

In strong contrast, *Rag1*^{−/−} mice that lack T and B cells fail to develop significant airway inflammation and hyperresponsiveness upon exposure to *Aspergillus* [20]. In allergic airway inflammation driven by OVA in mice, antibody-mediated depletion of CD4⁺ T cells prevented acute inflammation, although airway remodeling remained unaffected [21]. In mouse models of airway inflammation based on the physiologically relevant HDM allergen, Th2 cells are critical and act in close collaboration with IL-21 producing T cells [22]. Blockade of CD28 signaling during challenge in an HDM-induced allergic asthma model strongly diminished airway resistance and inflammatory infiltrates in the lung, supporting a prominent role of T cells [23]. Yet, we have previously shown that ILC2s significantly contribute to the total number of IL-5 and IL-13 producing cells during HDM-induced allergic inflammation in mice [24]. However, given the prominent role of T cells in *Aspergillus*, OVA, and HDM-induced allergic inflammation, it remains unknown whether in these models ILC2s are rapidly

activated and whether they provide a critical early innate source of type 2 cytokines.

In this study, we evaluated the dependence of ILC2s on the activity of T cells in an HDM-induced model for allergic airway inflammation, which is characterized by eosinophilic inflammation and airway hyperresponsiveness that is associated with type 2 cytokines similar to allergic asthma in patients [3]. We found that the induction of ILC2s was critically dependent on the activation of T cells during an acute allergic response to HDM. However, we also observed that the sole activation of Th2—or in combination with Th17 cells—did not induce ILC2s.

Results

Induction of ILC2s and CD4⁺ T cells follows the same kinetics in HDM-mediated airway inflammation

To explore the kinetics of ILC2 and CD4⁺ T-cell activation in allergic asthma, we employed an acute model of allergic airway inflammation: mice were sensitized intranasally with either PBS or HDM and after 7 days challenged with HDM for five consecutive days (Fig. 1A). We used flow cytometry to characterize the immune response at 1, 2, 4, and 6 days after the last challenge. An eosinophilic infiltrate in the BALF accompanied by an influx of CD4⁺ T cells was only observed in HDM-sensitized animals, despite the repetitive exposure of the PBS-sensitized group to HDM for 5 days during the challenge phase (Fig. 1B). The numbers of ILC2s in BALF, defined as lineage-negative CD45⁺Sca-1⁺T1/ST2⁺ lymphocytes that express intracellular Gata3 (Fig. 1C), followed similar kinetics as observed for eosinophils and CD4⁺ T cells, suggesting that activation of adaptive immunity may be a prerequisite for inducing ILC2s (Fig. 1B). Similar trends were found in the lungs and mediastinal LNs (Supporting Information Fig. 1A). The proportion of CD4⁺ T cells and ILC2s that expressed the proliferation marker Ki-67 was consistent with the absolute numbers found in BALF and decreased over time, reflecting a resolution of inflammatory conditions (Fig. 1D). CD4⁺ T cells showed higher proliferation in peripheral tissue than in LNs, while ILC2 proliferation was comparable between the different tissues analyzed (Fig. 1E). In agreement with our previous report [24], we found that—next to Th2 cells—ILC2s are also significant producers of IL-5 and IL-13 in HDM-driven airway inflammation (Fig. 1F and G; Supporting Information Fig. 1B). Similar observations were made at 12 h after the last challenge (Supporting Information Fig. 1C), indicating that no peak of ILC2s occurred before day 1 after the last challenge.

In summary, these data show that the influx of eosinophils, CD4⁺ T cells, and ILC2s in our HDM-induced mouse model of allergic airway inflammation follows similar kinetics and requires previous sensitization. Therefore, induction of ILC2s appears critically dependent on the adaptive immune system, suggesting that ILC2s are not an early source of IL-5 and IL-13 in this model.

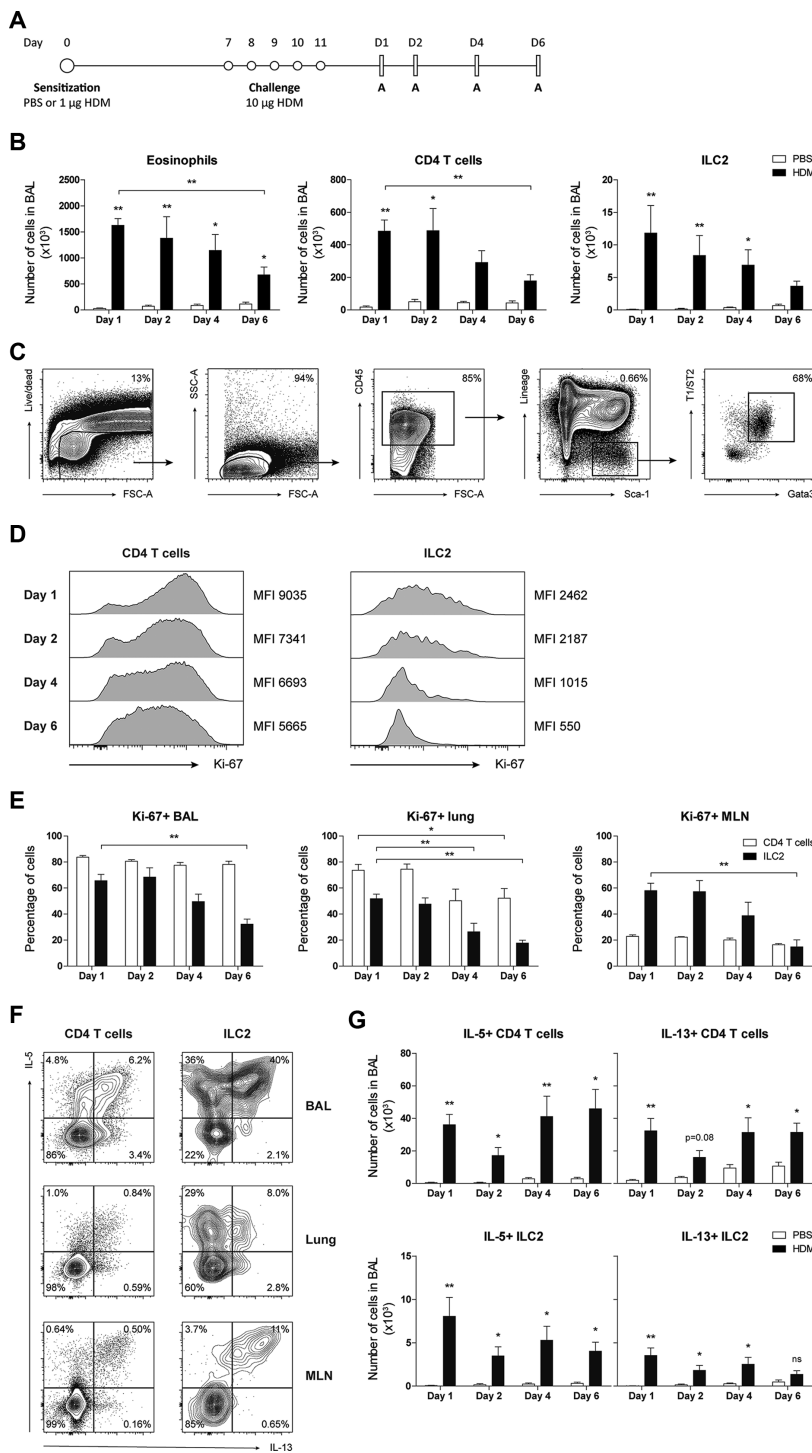


Figure 1. ILC2 induction is critically dependent on activation of the adaptive immune system. (A) Scheme for intranasal HDM treatment of C57BL/6 mice for induction of allergic airway inflammation. Mice were treated with PBS or HDM at indicated time points. Analysis was performed at the indicated time points after the final challenge. (B) Quantification of eosinophils, CD4⁺ T cells and ILC2s in the BALF of HDM-treated mice by flow cytometry at indicated time points after treatment. (C) Flow cytometric identification of CD45⁺Lineage[−] Gata3⁺T1/ST2⁺Sca-1⁺ ILC2s in the BALF of HDM-treated mice one day after the final challenge. (D) Mean fluorescent intensity (MFI) of Ki-67 in CD4⁺ T cells and ILC2s in the BALF, lung and MLN of HDM-treated mice measured by flow cytometry at indicated time points after treatment. (E) Proportion of Ki-67 expressing CD4⁺ T cells and ILC2s in the BALF, lung and MLN at indicated time points after treatment. (F) Flow cytometric analysis of intracellular cytokine production of BALF CD4⁺ T cells and ILC2s in HDM-treated mice one day after the final challenge. (G) Quantification of intracellular IL-5 (left) and IL-13 (right) production by CD4⁺ T cells and ILC2s in the BALF of HDM-treated mice. (B, E and G) Data are shown as mean + SEM of $n = 5$ –6 mice per group of a single experiment and are representative of two independent experiments. (C, D and F) Plots represent combined data from 6 samples of a single experiment (using the concatenate option in FlowJo), representative of two independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$; Mann-Whitney U test.

Induction of ILC2s follows T-cell activation

Next, we determined whether ILC2s participate in an early innate immune response by exposing mice to a single high dose of 100 µg HDM. When we examined the immune cell influx into the BALF over time (Fig. 2A; Supporting Information Fig. 2), we primarily found neutrophils on day 1, which rapidly declined by day 2. The BALF contained low numbers of eosinophils, B cells, CD4⁺ T cells,

or ILC2s across all time points (Fig. 2B). Also at 12 h after the exposure to HDM did we not observe a peak of ILC2s (data not shown). Thus, a single exposure to a high dose of HDM was not sufficient to trigger ILC2 activation.

Next, we aimed to compare the kinetics of ILC2 and T-cell activation during an adaptive HDM-driven immune response. Because multiple challenges of moderate doses of 10 µg of HDM (as in Fig. 1A) would be less optimal to determine the time point of ILC2

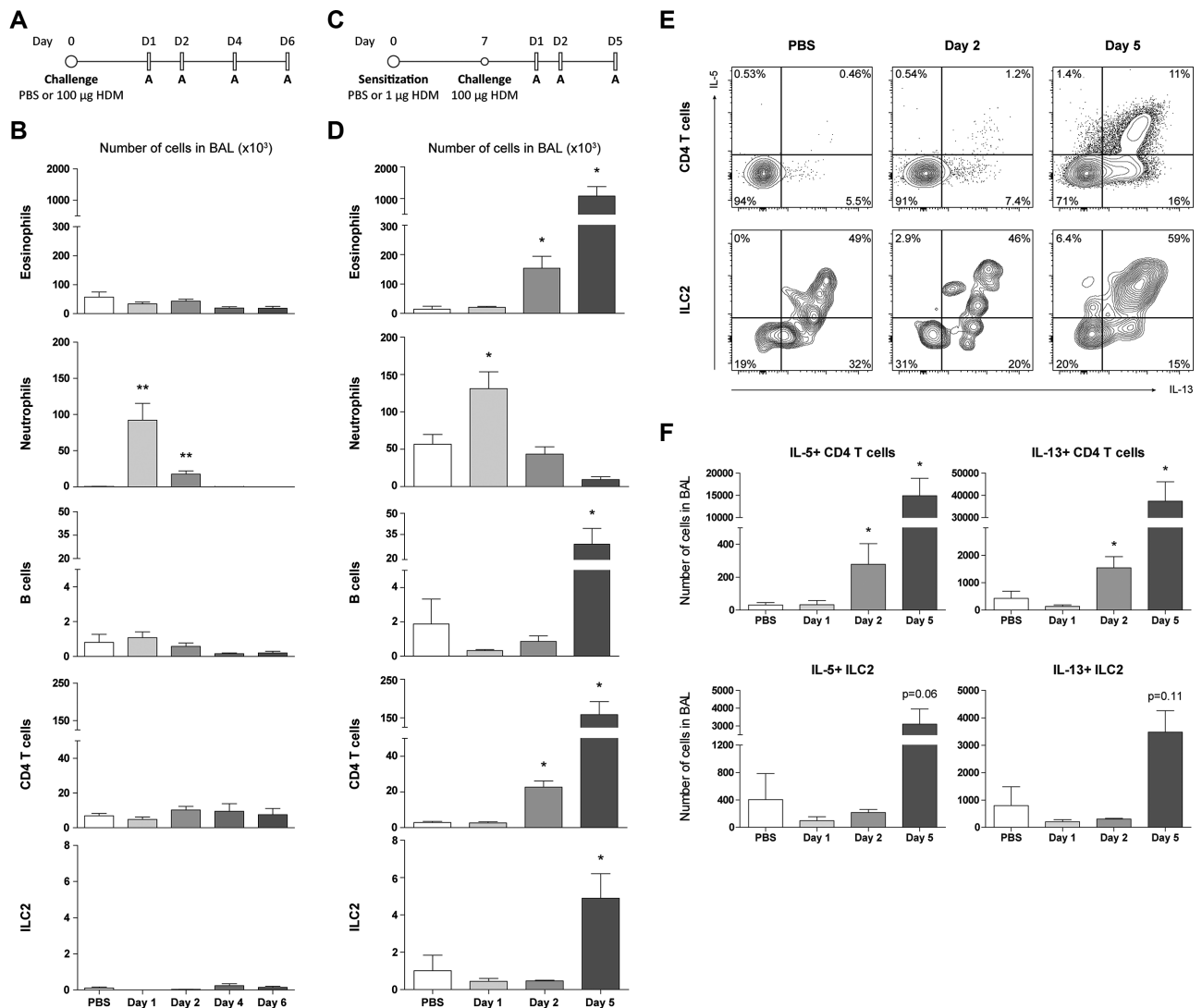


Figure 2. T-cell activation precedes ILC2 induction. (A and B) C57BL/6 mice were treated once with PBS or HDM. Flow cytometry analyses were performed at day 1, 2, 4 and 6 after the challenge. (A) Scheme of the intranasal treatment of C57BL/6 mice for examining the innate response to HDM. (B) Quantification of eosinophils (Siglec-F⁺Gr-1⁺ granulocytes), neutrophils (Siglec-F⁺Gr-1⁺ granulocytes), B cells (CD19⁺ lymphocytes), CD4⁺ T cells and ILC2s in the BALF at the indicated time points. (C and D) C57BL/6 mice were sensitized with PBS or HDM and then challenged with HDM at day 7. (C) Scheme for intranasal treatment of C57BL/6 mice for examining the immune response during the challenge phase of HDM-driven allergic airway inflammation. (D) Quantification of indicated cell populations in the BALF at the indicated time points. (E) Flow cytometric analysis of intracellular cytokine production of BALF CD4⁺ T cells (upper panels) and ILC2s (lower panels) in control PBS-sensitized mice (left) analysed one day after the challenge with HDM, as well as HDM-sensitized mice analysed at day 2 (middle) or at day 5 (right) after the challenge with HDM.

and T-cell activation, we adapted the protocol: we sensitized mice with PBS or HDM prior to a single challenge with a high dose of 100 µg HDM (Fig. 2C). Again, we observed a robust increase of neutrophils in the BALF on day 1, which declined by day 2 (compare Fig. 2B and D), but following the wave of neutrophils, an accumulation of eosinophils and CD4⁺ T cells was seen. Importantly, despite the significant increase of both eosinophils and CD4⁺ T cells at day 2, the numbers of ILC2s in BALF remained low and were in the same range as those in PBS-sensitized mice. Because at day 2 the ILC2 numbers in BALF were not yet increased compared to the PBS control (<1000, Fig. 2D), the ILC2 population was not a major producer of IL-5 and IL-13 despite its high intracellular cytokine content on a per cell basis (Fig. 2E and F).

At day 2 after the HDM challenge, we noticed a small but detectable increase in the numbers of IL-5 and IL-13 producing CD4⁺ T cells (Fig. 2F). The numbers of eosinophils and CD4⁺ T cells continued to rise on day 5, on which day also ILC2s and B cells were significantly increased in the HDM-sensitized and challenged mice compared to the PBS-sensitized group (Fig. 2D). At day 5, both T cell and ILC2 populations in the BALF contained substantial numbers and proportions of IL-5⁺ and IL-13⁺ cells (Fig. 2E and F).

These findings show that at day 2 of the challenge phase of an adaptive HDM-driven allergic response the appearance of eosinophilia in BALF is concomitant with the rise in IL-5 and IL-13 producing T cells, while ILC2 numbers are still unchanged.

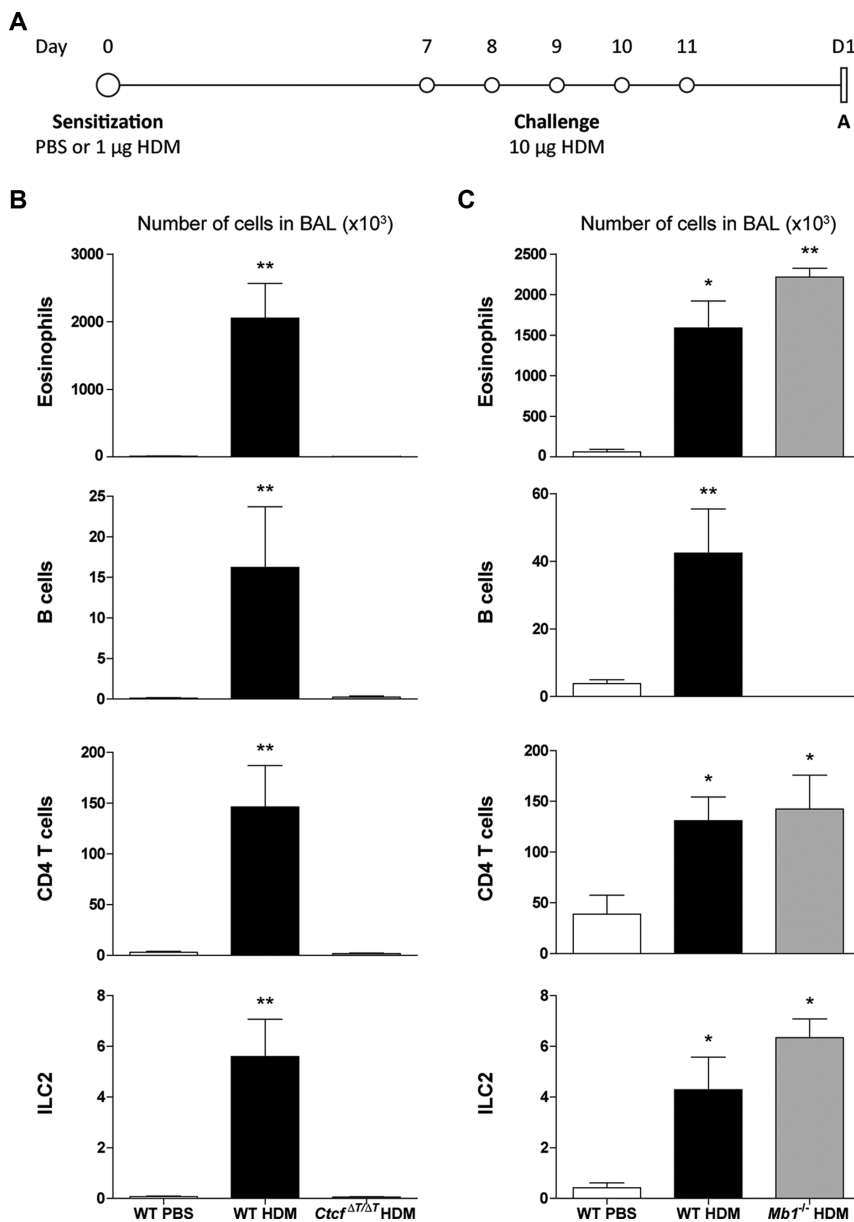


Figure 3. T-cell activation is required for ILC2 induction in HDM-driven allergic inflammation. (A–C) C57BL/6, *Ctcf*^{ΔT/ΔT} and *Mb1*^{-/-} mice were treated with PBS or HDM at indicated time points and analysed one day after the final challenge. (A) Scheme for intranasal treatment of C57BL/6 mice to induce allergic airway inflammation. (B and C) Quantification of eosinophils, B cells, CD4⁺ T cells and ILC2s in the BALF of the different mice strains. (B and C) Data are shown as mean ± SEM of *n* = 4–6 mice per group of a single experiment and are representative of two (C) or three (B) independent experiments. **p* ≤ 0.05, ***p* ≤ 0.01; Mann-Whitney U test.

Thus, the influx of IL-5⁺IL-13⁺ ILC2s in BALF follows T-cell activation.

T cells, but not B cells, are required for the initiation of ILC2s in allergic airway inflammation

In experimental models where ILC2s are a potent early innate source of type 2 cytokines, e.g. papain-driven inflammation, airway eosinophilia can be induced in *Rag1*^{-/-} mice [12]. However, when we sensitized *Rag1*^{-/-} intranasally with HDM and—after 7 days—challenged them with HDM for five consecutive days, we did not see eosinophilic airway inflammation and the numbers of ILC2s in the BALF were in the same range as in PBS controls (<1000 cells; data not shown). To investigate which component

of the adaptive immune system is most important for ILC2 induction, we next employed mice with specific defects in T- and B-cell differentiation.

Mice with a T cell-specific targeted deletion of the ubiquitously expressed transcription factor CTCF (*CD4*^{Cre/+} × *Ctcf*^{fl/fl} mice, hereafter called *Ctcf*^{ΔT/ΔT} mice) have close to normal numbers of T cells in peripheral tissues, but these T cells are unable to become activated and produce cytokines in vitro [25]. We have previously shown that CTCF has a critical role in the control of Gata3- and SATB1-dependent regulation of Th2 cytokine expression in T cells, as well as in anti-CD3 mediated proliferation [25]. The activation and function of ILC2s in IL-33 mediated airway inflammation experiments was not impaired in *Ctcf*^{ΔT/ΔT} (Supporting Information Fig. 3). When we subjected *Ctcf*^{ΔT/ΔT} mice to our HDM-induced allergic inflammation protocol and evaluated

the immune response 1 day after the last challenge (Fig. 3A), we observed a complete absence of inflammation, nor were ILC2s induced in BALF (Fig. 3B).

Mb1^{−/−} mice that are deficient in the expression of Igα/CD79a molecule, which is an essential transducer of B cell receptor signals, lack peripheral B cells due to a developmental arrest of B cell differentiation at the large pre-B cell stage in the bone marrow [26]. HDM-challenged *Mb1*^{−/−} mice displayed similar levels of airway inflammation as WT controls: ILC2 numbers in BALF were comparable (Fig. 3C) and showed similar cytokine production profiles (data not shown).

The proinflammatory cytokine IL-33, produced by epithelial cells upon activation by allergens, has been shown to be an important activator of ILC2s in allergic airway inflammation [27–29]. Our finding that ILC2 induction was T cell-dependent, prompted us to investigate the role of IL-33 in the activation of ILC2s in HDM-driven airway inflammation in vivo. Using *Il33*^{−/−} mice [30], we found that the absence of IL-33 did not affect the severity of HDM-driven inflammation with regard to the numbers of various immune cell populations in the BALF, including ILC2s (Fig. 4A). However, IL-5 and in particular IL-13 production by ILC2s was impaired in *Il33*^{−/−} mice, although this did not translate into a reduction of the numbers of infiltrating eosinophils or CD4⁺ T cells in BALF (Fig. 4C).

In summary, these data indicate that T cells, but not B cells, are required for the induction of ILC2 activation. Moreover, cytokine production by ILC2s but not their influx into the BALF is critically dependent on IL-33.

Eosinophilic airway inflammation provoked by activated Th2 and Th17 cells does not induce ILC2s

We next examined whether transferring activated polarized T cells into naïve mice would be sufficient for ILC2 induction. To accomplish this, OVA-specific OT-II CD4⁺ T cells [31] were cultured in vitro under Th2 and Th17 polarizing conditions. Their polarization into the Th2 and Th17 lineage was confirmed by intracellular expression of the signature transcription factors Gata3 and RORγt, respectively (Fig. 5B). Either Th2 alone or in a 2:1 combination with Th17 cells were transferred intravenously into mice. Subsequently, these mice were intranasally challenged with PBS or HDM and exposed to PBS or OVA aerosols to promote Th2 immunity (Fig. 5A) [32, 33]. Despite a significant eosinophilic inflammation combined with an influx of OT-II CD4⁺ T cells in the BALF, we noticed that ILC2 numbers remained at baseline levels, regardless of treatment or type of Th subset transferred (Fig. 5C). The numbers of IL-5 and IL-13-producing ILC2s in the BALF remained very low (50–100), although they were slightly increased compared to the PBS controls (Fig. 5D). Therefore, ILC2s did not substantially contribute to the total numbers of IL-5 and IL-13-producing lymphocytes: virtually all lymphoid cells that were positive for intracellular IL-5 and IL-13 were CD4⁺ T cells. The majority of these CD4⁺ T cells expressed the OT-II transgenic T-cell receptor, as determined by the expression of the Vα2 and Vβ5 TCR

chains (Fig. 5E). Therefore, we conclude that eosinophilic airway inflammation provoked by transfer of in vitro-activated Th2 and Th17 cells alone is not sufficient to induce ILC2s, suggesting that additional factors derived from other hematopoietic or structural cells are required.

Discussion

ILC2s are potent producers of IL-5 and IL-13 and have been implicated in the pathology of allergic lung inflammation as an early innate source of Th2 cytokines, thereby driving CD4⁺ Th2 responses [12–15, 18]. However, the contribution of ILC2s in HDM-induced allergic inflammation and the mechanisms that link ILC2 activation and the adaptive immune system are not completely understood. In contrast to the reported rapid innate activation of ILC2s, we found in an HDM-mediated mouse model for allergic airway inflammation that ILC2 induction followed T-cell activation and was critically dependent on the adaptive immune response driven by T cells. Absence of IL-33 did not impair the influx of ILC2s in the BALF, although cytokine production by IL-33 deficient ILC2s was substantially reduced. We conclude that ILC2s do not provide an early source of Th2 cytokines in HDM-induced allergic inflammation but instead rely on T cells for instruction and contribute to an inflammatory environment where Th2 cells play a central role.

Without prior sensitization or in the absence of functional T cells, HDM-driven airway inflammation and subsequent ILC2 induction was completely abrogated, paralleling the recently reported findings that T cells are a critical source of IL-4/IL-13 in OVA/alum-induced allergic inflammation [34]. Our results imply that ILC2 activation in HDM-driven allergic inflammation in the lung requires at least two signals, one of the innate-derived signals, e.g. IL-33, IL-25, or TSLP, and one adaptive signal mediated by T cells. These T cell-dependent signals may either be direct, provided by cell–cell contact, or indirect via other cytokines or cells. Several molecules have been suggested as candidates for mediating interactions between T cells and ILC2s. Since ILC2s were found to express MHCII, it has been proposed that they are able to present antigen to naïve T cells, whereby activation of T cells stimulates type 2 cytokine production by ILC2s through IL-2 [16]. Although IL-2 is known to promote ILC2 survival and expansion, as well as the production of type 2 cytokines [5, 35, 36], in our study the introduction of in vitro-activated Th2 alone or in combination with Th17 cells was not sufficient to induce ILC2s to the physiological levels seen in our HDM asthma model. Nevertheless, transfer of Th2 cells did result in the infiltration of large numbers of eosinophils in the BALF. Thus, the presence of a highly inflammatory environment and activated Th2 and Th17 cells that likely produced high amounts of IL-2 after antigen challenge did not activate ILC2s, suggesting that additional signals from other cells are required. It is therefore attractive to speculate that in HDM-mediated allergic airway inflammation, the induction of ILC2s requires both epithelium-derived innate proinflammatory cytokines and T cell-derived IL-2. In this context, it is however

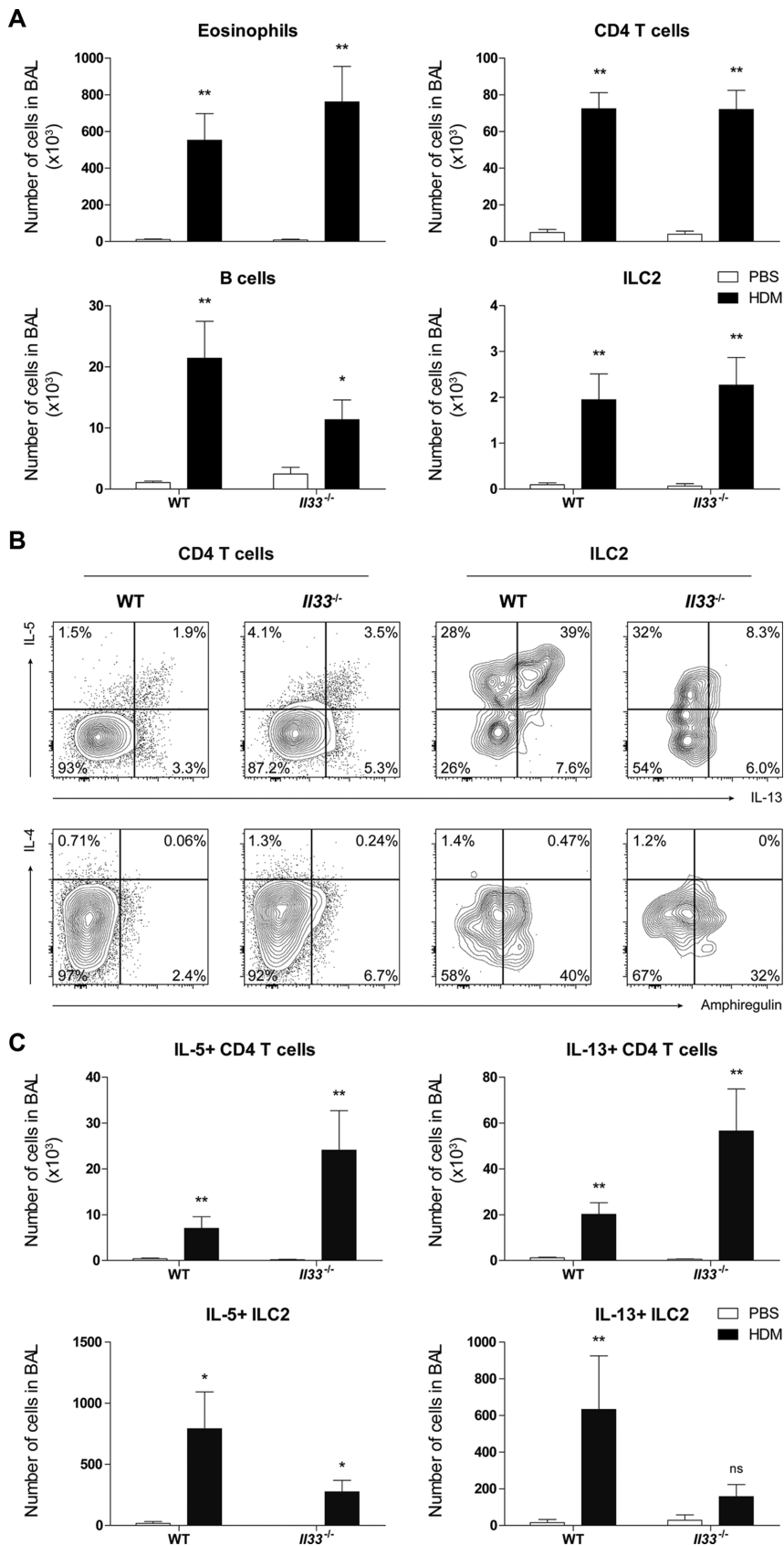


Figure 4. ILC2 influx into the BALF is not impaired by the absence of IL-33. (A–C) WT and $Il33^{-/-}$ mice were treated intranasally with PBS or HDM as described in Fig. 3A and analysed one day after the final challenge. (A) Quantification of the indicated cell populations in the BALF of WT and $Il33^{-/-}$ mice. (B) Flow cytometric analysis of IL-5 (upper panels) and IL-4 (lower panels) intracellular cytokine production of BALF CD4⁺ T cells and ILC2s in HDM-sensitized WT and $Il33^{-/-}$ mice. Plots represent combined data (using the concatenate option in FlowJo) of $n = 5$ –6 mice per group of a single experiment are representative of two independent experiments. (C) Quantification of intracellular IL-5 (left) and IL-13 (right) production by CD4⁺ T cells (upper panels) and ILC2s (lower panels) in the BALF. (A and C) Data are shown as mean $\pm$ SEM of $n = 5$ –6 mice per group of a single experiment and are representative of two independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$; Mann-Whitney U test.

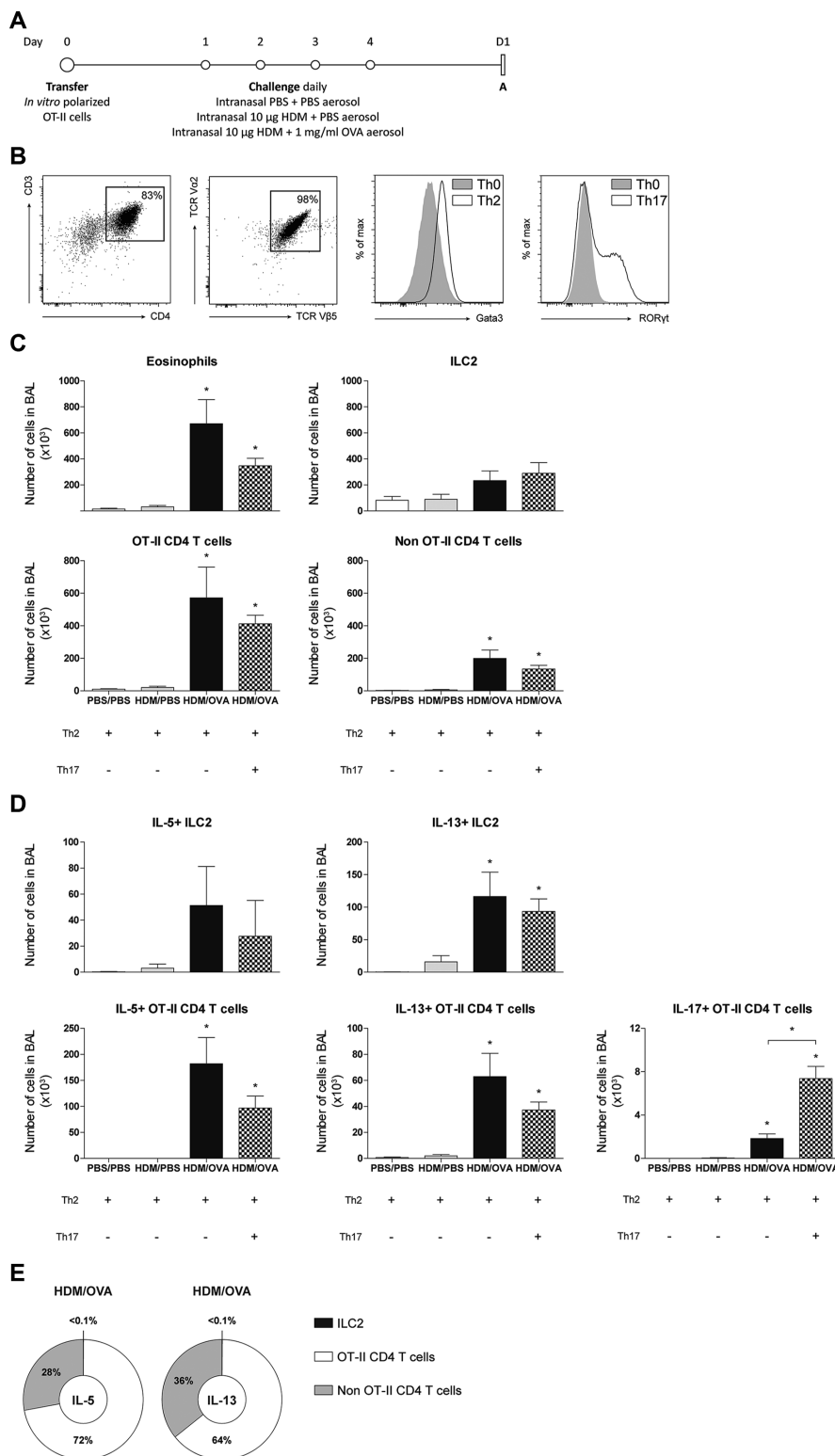


Figure 5. Activation and stimulation of T cells alone is not sufficient to induce ILC2s. (A) Experimental scheme for intravenous transfer of in vitro polarized OT-II CD4⁺ T cells into C57BL/6 mice and subsequent intranasal treatment/aerosol exposure of PBS/PBS, HDM/PBS or HDM/OVA at the indicated time points. Analysis was performed one day after the last challenge. (B) Cultured and purified Th2 and Th17 polarized OT-II CD4⁺ T cells (CD3⁺CD4⁺TCRV α 2⁺TCRV β 5⁺) were analysed for intracellular Gata3 and ROR γ t expression prior to intravenous transfer into WT hosts. OT-II CD4⁺ T cells cultured in absence of polarizing cytokines (Th0) are used as comparison. Plots are representative of three independent experiments. (C) Quantification of indicated cell populations in the BALF of treated mice. (D) Quantification of intracellular IL-5, IL-13 and IL-17 production by OT-II CD4⁺ T cells (lower panels) and ILC2s (upper panels) in the BALF of treated mice. (E) Relative contribution of ILC2s, OT-II CD4⁺ T cells and non OT-II CD4⁺ T cells to IL-5 (left panel) and IL-13 production (right panel) in the BALF of recipients of Th2 polarized OT-II CD4⁺ T cells. The number of cytokine-producing cells from each population is expressed as a percentage of the total number of cytokine-producing cells and were determined by flow cytometry. (C and D) Data are shown as mean $\pm$ SEM of $n = 4$ –5 mice per group from a single experiment and are representative of three independent experiments. * $p \leq 0.05$; Mann-Whitney U test.

interesting that not only T cells, but also group 3 ILCs can be an important source of IL-2 in the lung [35].

It has been shown that ILC2s express a number of chemokine receptors, including CCR2, CCR4, CCR5, and CXCR4 under certain conditions. In particular, CXCR4 is only expressed in ILC2s after

exposure to IL-33 [37]. RNA transcriptome analysis of intestinal ILC2s has also revealed transcripts for CCR8 and its ligand CCL1 [38]. A possible scenario exists where one or several chemokine receptors are not sufficiently induced in the absence of in vivo sensitization and thus leads to a lack of ILC2 accumulation.

Importantly, IL-21 has been proposed to promote type 2 immunity to HDM through a distinct non-Th2/Th17 T-cell population. The ability for ILC2 to produce IL-5 and IL-13 is drastically reduced in HDM-sensitized and -challenged *Il21r^{-/-}* mice, although this is not a cell-intrinsic feature, thus suggesting that IL-21 may indirectly promote ILC2s [22]. OX40-L has also been implicated in the interaction between ILC2s and T cells [17] and more recently, it was shown that ICOS:ICOS-L interaction is critical for ILC2 function through STAT5 signaling, suggesting a possible role for B cells in the maintenance of ILC2s [39]. Our study however, indicates that B cells were dispensable for the initiation of ILC2 recruitment and their proliferation. Although our HDM-driven mouse model provides an acute inflammatory environment in allergic asthma, it does not show features of airway remodeling, which is another important hallmark of asthma [3]. ILC2s potentially provide long-term effects through IL-13 by recruiting alternatively activated macrophages and stimulate TGF- β secretion [40, 41]. In conjunction with ILC2-derived amphiregulin, it is possible that ILC2s regulate the local immune response and restore homeostatic conditions. Because ILC2s have recently been associated with fibrotic diseases where type 2 cytokines are considered the most potent profibrotic factors through direct activation of fibroblasts [42, 43], it is conceivable that ILC2s also contribute to airway remodeling in asthma. In agreement with this, chronic exposure of mice to a cocktail of *Alternaria*, *Aspergillus*, and HDM was shown to induce robust airway inflammation, hyperresponsiveness, and remodeling by a mechanism dependent on adaptive immunity [44].

Our study indicates that the role of ILC2s were less pronounced in HDM-driven allergic airway inflammation than in papain-induced inflammation, in which ILC2s were shown to be critical to mount a robust Th2 cell response by promoting DC migration that is essential to prime naïve T cells [13]. It is conceivable that—compared with papain—HDM-driven allergic airway inflammation is less protease-dependent and is associated with a milder induction of innate cytokine production by epithelial cells. It has been reported that HDM activates epithelial cells through TLR4, which then results in production of innate cytokine such as GM-CSF and the danger signal uric acid [2, 3]. Such differences in the mechanism of epithelial activation could explain the differences between the papain- and HDM-induced allergic airway inflammation models. Our experiments did not provide any evidence that HDM has the capacity to stimulate ILC2s in the BALF, lungs, or mediastinal LNs, in the absence of T-lymphocyte activation, even at high doses. In contrast to the papain model [12] or cytokine-induced airway inflammation [24], HDM was unable to promote eosinophilic inflammation in RAG1-deficient mice in our study. It is therefore unlikely that ILC2s are required for T-cell priming in this model, although more conclusive evidence is required. We noticed that in our acute HDM model the majority of type 2 cytokine-producing cells consist of CD4⁺ T cells rather than ILC2s, but this does not imply that ILC2s are clinically less important in HDM-driven lung inflammation. Particularly, since recent studies in human provided evidence that ILC2s may be respon-

sible for steroid-resistant asthma. ILC2 numbers are increased in sputum and peripheral blood of patients with severe asthma compared to mild asthmatics or healthy controls [45, 46]. Importantly, evidence was found for a role of uncontrolled localized production of IL-5 and IL-13 by a steroid-insensitive population of ILC2s, in agreement with in vitro studies showing that ILC2s are steroid-insensitive in the presence of TSLP [46, 47].

In conclusion, the contribution of ILC2s to asthma is related to the properties of the allergen. In papain-induced inflammation, ILC2s are rapidly induced prior to T-cell activation and ILC2-derived IL-13 is essential for the initiation of T-cell activation and Th2 differentiation [13]. In contrast, in HDM-mediated airway inflammation both epithelium-derived innate proinflammatory cytokines, including IL-33, and T cell-derived signals such as IL-2 and IL-21 are required to induce ILC2 activation. Therefore, although ILC2s have the capacity to support adaptive Th2 response to HDM, they are not an early innate source of type 2 cytokines in HDM-driven allergic inflammation in mice.

Materials and methods

Mice

WT C57BL/6 mice were purchased from Harlan. Transgenic CD4^{Cre/+}, *Ctcf^{fl/fl}*, *Mb1^{-/-}*, *Il33^{-/-}*, and OT-II mouse strains have been described [25, 26, 30, 31, 48] and were bred on a C57BL/6 background and housed at the Erasmus MC Animal Centre. Mice were kept under specific pathogen-free conditions and were ~8–16 weeks old at the time of analysis. All experiments were approved by the Erasmus MC Animal Ethics Committee.

HDM-induced allergic airway inflammation

For HDM-induced allergic inflammation, mice were anesthetized using isoflurane and sensitized intranasally using 1 μ g HDM (Greer, USA) or PBS (GIBCO Life Technologies, USA). Seven days later, mice were challenged daily for five consecutive days with 10 μ g HDM intranasally. Mice were sacrificed for analysis at various days after the last challenge. BALF was obtained by flushing the lungs three times with 1 mL PBS containing 0.5 mM EDTA (Sigma-Aldrich, USA). To investigate the kinetics of the adaptive HDM-induced immune response during the challenge phase, mice were sensitized with 1 μ g HDM or PBS and challenged once with 100 μ g HDM after a resting period of 7 days. The PBS group was sacrificed 1 day after the challenge and the HDM groups were sacrificed at various days for analysis. An innate immune response to HDM was generated by intranasally challenging mice once with 100 μ g HDM and sacrificing the mice at various time points.

OVA-specific allergic airway inflammation

OVA-specific allergic inflammation was induced by transfer of in vitro polarized OT-II CD4⁺ T cells expressing an OVA-specific TCR. In brief, OT-II CD4⁺ T cells were extracted from the spleen and LNs of naïve OT-II transgenic mice by MACS using a CD4⁺ T-cell isolation kit (Miltenyi Biotec, Germany) followed by flow cytometric cell sorting to achieve a high purity (>98%). Naïve OT-II CD4⁺ T cells were cultured in vitro with 10 µg/mL anti-CD3 (BD Biosciences, USA) and anti-CD28 (BD Biosciences) for 3 days under Th2 polarizing conditions: 10 ng/mL IL-4 (Peprotech, USA), 5 µg/mL anti-IL-12 (C17.8), and 5 µg/mL anti-IFN-γ (XMG1.2) or Th17 polarizing conditions: 10 ng/mL IL-12 (R&D Systems, USA), 20 ng/mL IL-6 (R&D Systems), 3 ng/mL TGF-β (R&D Systems), 5 µg/mL anti-IL-4, and 5 µg/mL anti-IFN-γ. CD3 and CD28 stimulation was then removed by replating the cells and the medium was refreshed with the addition of 5 ng/mL IL-2 (R&D Systems) and cultured for four additional days. Th2 and Th17 polarizations were confirmed by intracellular Gata3 and RORγt expression, respectively. A total of 8 million in vitro polarized OT-II CD4⁺ T cells were directly transferred intravenously into WT recipients. Th2 and Th17 cells were injected in a 2:1 ratio where applicable. One day after transfer, mice were subjected to daily intranasal injections of 10 µg HDM or PBS followed by exposure for 30 min to PBS or 1 mg/mL OVA aerosol (Grade III, Sigma–Aldrich) for four consecutive days as previously described [33]. One day after the last challenge the mice were sacrificed for analysis.

Flow cytometry procedures

Single-cell suspensions were prepared from the BALF, lungs, LNs, and spleens, using standard procedures. Lungs, LNs, and spleens were mechanically disrupted using a 100 µm cell strainer (BD Biosciences) in PBS containing 0.5% bovine serum albumin and 5 mM EDTA. Red blood cells from BALF and lungs were lysed using osmotic lysis buffer (8.3% NH₄Cl, 1% KHCO₃, and 0.04% Na₂EDTA in Milli-Q). Flow cytometry surface and intracellular staining procedures have been described previously [24, 49]. Monoclonal antibodies are listed in Supporting Information Table 1. For measurements of cytokine production by flow cytometry, cells were stimulated at 37°C using PMA (Sigma–Aldrich) and ionomycin (Sigma–Aldrich) supplemented with GolgiStop (BD Biosciences), for 4 h. Lineage negative cells were gated as cells not expressing CD3, CD4, CD5, CD8α, CD11b, CD11c, CD19, B220, NK1.1, FcεRIα, TER-119, and Gr-1. Data were acquired using an LSR II flow cytometer (Beckton Dickinson, USA) equipped with three lasers and FACSDiva software (Beckton Dickinson) and analyzed by FlowJo (Tree Star Inc., USA) software.

Statistical analysis

Mann–Whitney *U* tests were used for comparison between two groups and a *p*-value of <0.05 was considered statistically

significant. All analyses were performed using Prism (GraphPad Software, USA).

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Abbreviations: HDM: house dust mite · ILC: innate lymphoid cell · TSLP: thymic stromal lymphopoietin

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**T cells are necessary for ILC2 activation in house dust mite-induced allergic
airway inflammation in mice**

T cells are necessary for ILC2 activation in house dust mite-induced allergic airway inflammation in mice

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Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision

04-Nov-2015

Dear Dr. Hendriks,

Manuscript ID eji.201546119 entitled "Activation of group 2 innate lymphoid cells in house dust mite-induced allergic inflammation is dependent on T cells" which you submitted to the European Journal of Immunology has been reviewed.

The comments of the referees are included at the bottom of this letter. All the referees agree that your study is interesting but it needs some revisions/explanation. A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication. Should you disagree with any of the referees' concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make the requested changes.

You should also pay close attention to the editorial comments included below. **In particular, please edit

your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.**

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referee(s) to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Laura Soto Vazquez

On behalf of
Prof. Marco Colonna

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Reviewer: 1

Comments to the Author

Major comments:

1. The authors showed that T cells are required (Figure 3B) but Th2 cells are not sufficient for the induction of ILC2 activation (Figure 5). Recently, other CD4 T cell subsets including Th17 cells and Th9 cells have been shown to contribute allergic airway inflammation. The authors should examine the effect of co-transfer of Th17 cells or Th9 cells with Th2 cells on ILC2 induction in Figure 5.

Minor comments:

1. Figure 1B, C, E: The authors should examine the numbers of eosinophils, CD4T cells, and ILC2 at 12 hours after the challenge because the peak of ILC2 may occur before day 1.
2. Figure 1F and Figure 5D: The authors should show the data of cytokine-producing cells in bar graphs of the absolute number of cells +/- SEM to provide information on dispersion.
3. Figure 2B: How did the authors count each cell population? Detailed methods should be provided.
4. Have the author confirmed the results of Figure 3B by using other T cell-deficient strain such as TCR β KO mice?
5. Figure 5: Why the authors used CFSE-labeled Th2 cells? If the authors used CFSE to identify the transferred OT-II cells, the authors cannot exclude the possibility that Non OT-II CD4 T cells contain substantial numbers of OT-II cells that vigorously proliferated during experimental period (5 days).
6. In Figure 5C: the authors should show the data of the number of non OT-II CD4 T cells.

Reviewer: 2

Comments to the Author

This paper by Li et al. describes the importance of Th2 cells in the induction of group 2 innate lymphoid cells (ILC2) in allergic lung inflammation triggered by house dust mite (HDM). The authors demonstrated that the induction of ILC2 cells in bronchoalveolar lavage fluid, lung and lymph node is dependent on prior sensitization of the host with HDM followed by activation of T cells by HDM. ILC2 induction is not affected by the lack of IL-33 but cytokine production is dependent on IL-33. The authors concluded that it is likely that ILC2 cells do not provide an early source of type 2 cytokines in HDM-induced allergic lung inflammation. Intriguingly, transfer of Th2 cells alone together with antigen challenge was insufficient in the induction of ILC2, implying that additional stimuli are necessary for the induction of ILC2 cells.

Although data are clear and conclusion was properly drawn based on the experimental results, this paper needs more data regarding additional factors• (2nd line from the bottom of page 7) involved in the induction of ILC2 cells together with T cell activation, which would provide more insights into the mechanisms how ILC2 cells are induced in HDM-induced allergic lung inflammation. In addition, there are several points that need to be clarified as shown below.

- 1) The authors concluded that class II positive ILC2 cells do not produce cytokines by referring to Figure 1H. The fact that a combination of PMA and ionomycin was unable to induce cytokine expression usually means that the cells are not capable of producing cytokines in any physiological stimuli. Are Lin- class II positive cells really ILC2 cells?

2) Contour plot is sometimes misleading because it does not provide the information of the event numbers and even a single dot can be shown as a big mountain. The data should be shown as a dot plot. Or, at least total event numbers should be shown.

3) What does a superscript • on antigens• mean on the 8th line of page 7.

4) 5th line from the bottom of page 8, ref. 5 should be included together with refs. 35 and 36.

5) Refs. 17, 35, and 44 do not have volume and page numbers.

Reviewer: 3

Comments to the Author

The manuscript by Li et al. addresses the role of ILC2 in the induction of airway inflammation in a house dust mite model (HDM).

Following a previous observation by the same group the specific contribution of T cells and innate lymphoid cells is carefully analyzed in consideration of the recent literature on ILC2s.

Despite previous suggestions (Ref 12;13) that ILC2 activation and cytokine production is necessary for and precedes Th2 cell polarization, the authors show that the activation of both subsets is concomitant. Further they suggest that rather ILC2 activation depends on the presence of T cells. Transfer of Th2 polarized T cells, is also not sufficient to rescue ILC2 recruitment and activation, arguing for an ILC2 independent mechanism. The authors also suggest that recruitment and activation of ILC2 are distinct processes, the first one being IL33 dependent the other independent.

These observations challenge previous findings, which suggested that the cytokines produced by ILC2 were necessary to induce Th2 polarization. The observed differences might however be explained by the models used: papain versus HDM.

Several points should however be addressed or better explained by the authors.

Comments:

- Are ILC2 required for priming and after their expansion and recruitment depends on T cells? Once a Th2 immunity is established the amount of cytokines produced in the tissue will depend on the

number of cells present in that tissue. T cells clearly outnumber ILC2 by 2 logs. Therefore the relevance of ILC2 could be earlier during the response rather than later once the response is established.

- Compared to previously published HDM induced asthma protocols, the authors perform multiple challenges. Eosinophilia, CD4 T cell recruitment and activation as well as ILC2 activation is highest on day 1 after the repeated challenges. It could possible that the peak of response already occurred earlier and what is observed is reactivation of effector memory response.
- 4 different models of HDM induction are used making it complicated to extrapolate the conclusions of one experiment and compare them to the others.
- In figure 1 proliferation of ILC2 is observed, based on Ki67. Is this an IL-2 mediated effect of T cell activation?
- Fig 2 eosinophil recruitment is evident and statistical relevant already at day 2 (panel D). What is the source of IL5? ILC2 activation? (panel E day 2): If this is the case T cell activation would amplify the response but not be required.
- Figure 2 why do the authors use a different protocol (dose and challenges) for HDM induces Asthma to prove the earlier activation of T cells?
- Figure 3. This result is interesting however in strong contrast to the existing literature on Rag mice, also reported by the same group. A comment on the findings in the context of the literature should be added in the discussion. What is different in the used CTCF KO mice compared to Rag mice.
- In IL-33 KO mice there is an impaired Th2 immunity reported in case of papain stimulation. Could you please add a comment on this model-based discrepancy? Is the effect IL25/TSLP related? Liying Guo et al NI 2015 (doi:10.1038/ni.3244) please comment on the role of IL33 on Th2 cells.

Specific comments to the presented data:

- In fig 1 details of the Y axes are missing in panels C and E. Check
- Fig 1. In lung tissue there are no MHC-II positive ILC2 cells (panel G). Which cells were used for re-stimulation (panel H lower row middle plots pMHC-II positive cells in lungs)? Maybe it would be better to state "not done" instead of showing questionable contour plots.
- A similar problem in the analysis is evident in the same figure for pMHC-II positive cells in BAL. The authors should either try to more cells in the analysis or just refer to the evident and reasonable limitation in numbers of cells.
- Same comments as before for fig 2 panel E (PBS and day 2 bottom row)
- Day 5 (page 6 of the manuscript last sentence of paragraph "induction of ILC2 follows".) Taken together, influx of ILC2. The observed effect could also depend on proliferation following T cell activation and IL2 production. Please add/ and or comment this hypothesis.
- Figure 4 the facs plots to dot look ideal. A bi-exponential display might help move the cells from the edges. Why are some IL4 stainings stretched (left). ILC2 in WT and IL-33 KO looks like very few

events, it would be good to try improve the data.

- Figure 5. Can HDM alone promote in the absence of OVA the activation of transferred OT2? Is this an the same innate effect of T cells seen by Liying Guo et al NI 2015 (doi:10.1038/ni.3244)

Ref to be added:

Liying Guo et al NI 2015 (doi:10.1038/ni.3244)

First Revision – authors' response

14-Feb-2016

Point-by-point reply

Reviewer 1

Major comments:

1. The authors showed that T cells are required (Figure 3B) but Th2 cells are not sufficient for the induction of ILC2 activation (Figure 5). Recently, other CD4 T cell subsets including Th17 cells and Th9 cells have been shown to contribute allergic airway inflammation. The authors should examine the effect of co-transfer of Th17 cells or Th9 cells with Th2 cells on ILC2 induction in Figure 5.

- We have included an additional experiment in Figure 5 where we examine the effect of co-transfer of in vitro polarized Th2 and Th17 cells on ILC2 induction, and have adapted the text in the Results section accordingly (page 7/8). Furthermore, we have noted that in our hands Th2-polarized OT-II T cells also produced low but detectable amounts of IL-9, which does not seem sufficient to induce ILC2 in vivo.

Minor comments:

1. Figure 1B, C, E: The authors should examine the numbers of eosinophils, CD4T cells, and ILC2 at 12 hours after the challenge because the peak of ILC2 may occur before day 1.

- An additional experiment was performed to compare the response of the indicated cell populations at 12 hours after challenge, comparable to the protocol outlined in Figure 1A, but we did not see a peak of ILC2 before day 1 (shown in Supplementary Figure S1). Furthermore, a 12-hours time point was also included in our 'innate HDM-stimulation' protocol of Figure 2A. Similar to the 24-hours time point, only an increase in neutrophils was found while ILC2 and CD4 T cells remained at baseline levels (mentioned as data not shown on page 5/6).

2. Figure 1F and Figure 5D: The authors should show the data of cytokine-producing cells in bar graphs of the absolute number of cells $\pm$ SEM to provide information on dispersion.

- We now show bar charts indicating absolute numbers of cytokine producing cells (Figures 1G and Figure 5D, respectively).

3. Figure 2B: How did the authors count each cell population? Detailed methods should be provided.

- We clarified the markers used to define each cell population by flow cytometry in the corresponding figure legend (page 19).

4. Have the author confirmed the results of Figure 3B by using other T cell-deficient strain such as TCR β δ KO mice?

- We have performed HDM-exposure experiments according to the protocol described in Figure 1A in Rag1 $^{-/-}$ mice. But, in contrast to what we noticed in wild-type mice, in Rag1 $^{-/-}$ mice this did not result in an inflammatory response and no ILC2 activation was observed (in agreement with our findings in Ctcf Δ T/ Δ T mice). We now mention this experiment in the Results section (page 6).

5. Figure 5: Why the authors used CFSE-labeled Th2 cells? If the authors used CFSE to identify the transferred OT-II cells, the authors cannot exclude the possibility that “Non OT-II CD4 T cells” contain substantial numbers of OT-II cells that vigorously proliferated during experimental period (5 days).

- We agree that it was not clearly stated how the OT-II cells were defined in vivo after transfer and thank the referee for noticing this. We have now performed transfers of OT-II transgenic Th2 cells and Th2/Th17 cells and quantify T cell numbers using TCR V α 2- and TCR V β 5-specific antibodies (see Figure 5C, 5D). Corresponding text in the Results and Materials and Methods sections were revised accordingly (page.... And page, respectively).

6. In Figure 5C: the authors should show the data of the number of non OT-II CD4 T cells.

- A bar chart showing the numbers of non OT-II CD4 T cells has been added in Figure 5C.

Reviewer 2

This paper by Li et al. describes the importance of Th2 cells in the induction of group 2 innate lymphoid cells (ILC2) in allergic lung inflammation triggered by house dust mite (HDM). The authors demonstrated that the induction of ILC2 cells in bronchoalveolar lavage fluid, lung and lymph node is dependent on prior sensitization of the host with HDM followed by activation of T cells by HDM. ILC2 induction is not affected

by the lack of IL-33 but cytokine production is dependent on IL-33. The authors concluded that it is likely that ILC2 cells do not provide an early source of type 2 cytokines in HDM-induced allergic lung inflammation. Intriguingly, transfer of Th2 cells alone together with antigen challenge was insufficient in the induction of ILC2, implying that additional stimuli are necessary for the induction of ILC2 cells.

Although data are clear and conclusion was properly drawn based on the experimental results, this paper needs more data regarding “additional factors” (2nd line from the bottom of page 7) involved in the induction of ILC2 cells together with T cell activation, which would provide more insights into the mechanisms how ILC2 cells are induced in HDM-induced allergic lung inflammation.

- Our findings clearly show that in our HDM-driven airway inflammation model (i) ILC2 are not activated in the absence of T cells, and that (ii) that the presence of activated Th2/Th17 cells in vivo is not sufficient to induce ILC2. We agree that is an intriguing and important issue to identify ‘additional factors’, but we also feel that this is far beyond the scope of our current manuscript. It would imply a large series of reconstitution experiments to demonstrate the importance of e.g. T-cell derived cytokines or other factors that may act either directly on ILC2 or indirectly through other cells. We have expanded the section in the Discussion, where we mention several possible mechanisms, including IL2, IL-21, chemokine(R), next to OX40/OX40L and ICOS/ICOSL. Here, we included also 2 new references.

In addition, there are several points that need to be clarified as shown below.

1. The authors concluded that class II positive ILC2 cells do not produce cytokines by referring to Figure 1H. The fact that a combination of PMA and ionomycin was unable to induce cytokine expression usually means that the cells are not capable of producing cytokines in any physiological stimuli. Are Lin⁻ class II positive cells really ILC2 cells?

- We thank the referee for this helpful comment. Although the MHC class II positive cells identified fulfil our current criteria for gating of ILC2, it is at this point unclear whether we should classify them as genuine ILC2. Clearly, more experiments are required to characterize these cells in more detail. We now feel that the identity of MHC class II positive ILC2 cells is a minor side issue that may distract from the focus of our manuscript and therefore decided to exclude the description of these cells (as was shown in the original Figure 1G and 1H) from the manuscript.

2. Contour plot is sometimes misleading because it does not provide the information of the event numbers and even a single dot can be shown as a big mountain. The data should be shown as a dot plot. Or, at least total event numbers should be shown.

- For reasons of consistency, we decided to show contour plots throughout, but appreciate the comment by this referee. Therefore, we included bar charts with the absolute numbers of cells corresponding to the contour plots, to show the total impact of each population.

3. What does a superscript “1” on “antigens¹” mean on the 8th line of page 7?

- This was an error and has been corrected.

4. 5th line from the bottom of page 8, ref. 5 should be included together with refs. 35 and 36.

- The reference has been added.

5. Refs. 17, 35, and 44 do not have volume and page numbers.

- Volume and page numbers have been corrected in Refs. 17, 34, 35 and 44.

Reviewer 3

The manuscript by Li et al. addresses the role of ILC2 in the induction of airway inflammation in a house dust mite model (HDM).

Following a previous observation by the same group the specific contribution of T cells and innate lymphoid cells is carefully analyzed in consideration of the recent literature on ILC2s. Despite previous suggestions (Ref 12;13) that ILC2 activation and cytokine production is necessary for and precedes Th2 cell polarization, the authors show that the activation of both subsets is concomitant. Further they suggest that rather ILC2 activation depends on the presence of T cells. Transfer of Th2 polarized T cells, is also not sufficient to rescue ILC2 recruitment and activation, arguing for an ILC2 independent mechanism. The authors also suggest that recruitment and activation of ILC2 are distinct processes, the first one being IL33 dependent the other independent.

These observations challenge previous findings, which suggested that the cytokines produced by ILC2 were necessary to induce Th2 polarization. The observed differences might however be explained by the models used: papain versus HDM.

Several points should however be addressed or better explained by the authors.

1. Are ILC2 required for priming and after their expansion and recruitment depends on T cells?

Once a Th2 immunity is established the amount of cytokines produced in the tissue will depend on the number of cells present in that tissue. T cells clearly outnumber ILC2 by 2 logs. Therefore the relevance of ILC2 could be earlier during the response rather than later once the response is established.

- Our data do not support a role for ILC2 in priming of T cells. When we expose naive mice i.n. to HDM, even doses as high as 100 μ g, ILC2 are not induced (Figure 2A).

2. Compared to previously published HDM induced asthma protocols, the authors perform multiple challenges. Eosinophilia, CD4 T cell recruitment and activation as well as ILC2 activation is highest on day

1 after the repeated challenges. It could possible that the peak of response already occurred earlier and what is observed is reactivation of effector memory response.

- We have explored this by including experiments in which we sensitized mice and subsequently perform a single challenge only (outlined in Figure 2C-2F). Whereas at day 2 significant numbers of IL-5/IL-13-producing T cells were induced, numbers of ILC2 cells remained unchanged in BAL. Moreover, we addressed the issue of a very early ILC2 response by including additional experiments with 12-hour time points, as also suggested by Reviewer #1, comment #1 (See suppl. Fig. S1).

3. 4 different models of HDM induction are used making it complicated to extrapolate the conclusions of one experiment and compare them to the others.

- We agree it may be challenging to compare experiments with different methods of HDM induction. The basis of our series of experiments is the standard HDM-driven allergic airway inflammation protocol, consisting of a single sensitization and a challenge phase with 5 daily exposures (Figure 1). The allergic airway inflammation (characterized by T cell activation and eosinophilia) only develops after several challenges, and all next experiment are designed to investigate isolated parts of the disease mechanism in order to explain the observations in Figure 1. They include experiments to study the sensitization and kinetics of the challenge phase (Figure 2), as well as a protocol in which sensitization is essentially replaced by transfer of in vitro activated T cells.

4. In figure 1 proliferation of ILC2 is observed, based on Ki67. Is this an IL-2 mediated effect of T cell activation?

- It is possible that ILC2 proliferation is partly due to IL-2 derived from activated T cells. However, in Figure 5, we show that in vivo transfer of in vitro activated T2 and/or Th17 cells alone do not induce ILC2, arguing against a mechanism in which T-cell-derived IL-2 is sufficient for ILC2 induction.

5. Fig 2 eosinophil recruitment is evident and statistical relevant already at day 2 (panel D). What is the source of IL5? ILC2 activation? (panel E day 2): If this is the case T cell activation would amplify the response but not be required.

- We have added a bar chart depicting the numbers of IL-5 producing T cells and ILC2 (Figure 2F), showing that they are more or less similar at day 2. Nevertheless, only the numbers of IL-5+ T cells (and not ILC2) are increased, compared with day 1 or PBS controls. We have mentioned this in the accompanying text (page 6).

6. Figure 2 why do the authors use a different protocol (dose and challenges) for HDM induces Asthma to prove the earlier activation of T cells?

- Figure 2A,2B: Our aim was to investigate the innate immune response to HDM during the sensitization phase. We reasoned that using 100 μ g of HDM (rather than 1 μ g) would increase the sensitivity of the

experiment, and that if 100 μ g was not sufficient to activate ILC2 in this innate response, then a dose of 1-10 μ g would also lead to the same result.

- Figure 2C-2F: We felt that a protocol with multiple challenges of relatively low doses of HDM would be sub-optimal to determine the time point of activation of T cells and ILC2. To have an optimal resolution, we decided to go for a single challenge with a high dose of HDM, resulting in an eosinophilia in BAL that is quick (already detectable 2 days after the challenge) and strong (at day 5: $\sim 10^6$ eosinophils in the BAL fluid, comparable to 1.5×10^6 eosinophils in our standard HDM-protocol, Figure 1B). The reason why we have adapted the protocol is now explained in the text (page 6, 'next we aimed to compare.....').

7. Figure 3. This result is interesting however in strong contrast to the existing literature on Rag mice, also reported by the same group. A comment on the findings in the context of the literature should be added in the discussion. What is different in the used CTCF KO mice compared to Rag mice.

- The report by Klein Wolterink et al. Eur J Immunol. 2012 employed cytokine-induced airway inflammation mediated by IL-25 and IL-33, which are known to activate ILC2 independently from adaptive immunity. In our current manuscript, we have performed HDM-driven allergic airway inflammation experiments in Ctcf^{-/-} mice as well as in Rag1^{-/-} mice (See also our response to Reviewer #1, comment #4) and found that ILC2 were not induced (<1000 ILC in BAL). We have mentioned this now in the Results section (page 6) and have added further clarification on this issue in the discussion (page 10).

8. In IL-33 KO mice there is an impaired Th2 immunity reported in case of papain stimulation. Could you please add a comment on this model-based discrepancy? Is the effect IL25/TSLP related?

Liying Guo et al NI 2015 (doi:10.1038/ni.3244) please comment on the role of IL33 on Th2 cells.

- Intranasal administration of papain, a protease allergen, induced eosinophilic airway inflammation in WT as well as Rag1^{-/-} mice (Halim et al., Immunity 2012). This is in contrast to HDM, which does not induce inflammation in Rag1^{-/-} mice. Adoptive transfer of WT ILC2 into Rag1^{-/-} mice restored the inflammatory response to papain, supplying further evidence for a central role of ILC2 (Halim et al., Immunity 2012). In the papain model, ILC2 arise prior to the induction of T cells, again in contrast to what we see in our HDM model. Exposure to protease allergen papain is accompanied by a strong release of IL-33 (Kamijo et al. J Immunol. 2013) and would explain the IL-33-dependent Th2 immunity, as memory Th2 cells express high levels of IL-33 receptor (also evident from Guo et al., Nat Immunol. 2015). By contrast, HDM-driven allergic airway inflammation appears protease-independent as it is also induced when HDM protease activity is destroyed by heating procedures. Thus, the protease activity of our HDM is lower than that of papain and does not lead to such a strong increase in IL-33 levels. Instead, it has been reported that HDM activates epithelial cells through TLR4, which then results in production of innate cytokine such as GM-CSF and the danger signal uric acid (Hammad et al. Nat Med. 2009; Kool et al. Immunity. 2011). This difference in the mechanism of activation could explain the discrepancy between papain and HDM induced allergic airway inflammation models. We have explained this in the discussion section (page 10).

Specific comments:

In fig 1 details of the Y axes are missing in panels C and E. Check.

- The figure has been corrected.

Fig 1. In lung tissue there are no MHC-II positive ILC2 cells (panel G). Which cells were used for re-stimulation (panel H lower row middle plots pMHC-II positive cells in lungs)? Maybe it would be better to state "ND" (not done) instead of showing questionable contour plots.

A similar problem in the analysis is evident in the same figure for pMHC-II positive cells in BAL. The authors should either try to more cells in the analysis or just refer to the evident and reasonable limitation in numbers of cells.

- We decided to removed the data on MHC Class II positive cells from this manuscript. See also our response to Reviewer #2 comment #1.

Same comments as before for fig 2 panel E (PBS and day 2 bottom row).

- We have now added a quantification of these small numbers of IL-5+ and IL-13+ ILC2 present.

Day 5 (page 6 of the manuscript last sentence of paragraph "induction of ILC2 follows....") Taken together..... influx..... The observed effect could also depend on proliferation following T cell activation and IL2 production. Please add/ and or comment this hypothesis.

- We have placed more emphasis on the role of IL-2 in the discussion (page 9).

Figure 4 the facs plots to dot look ideal. A bi-exponential display might help move the cells from the edges. Why are some IL4 stainings stretched (left). ILC2 in WT and IL-33 KO looks like very few events, it would be good to try improve the data.

- We have now moved cells from the edges (IL-15/IL-13 profiles) by improving compensation settings in FlowJo analysis. Importantly, this did not significantly impact the data obtained. We have added bar charts to indicate total cell numbers for each population. The 'stretched' nature of the IL-4 stainings originates from considerable background staining and compensation settings, which we always compare with FMO and isotype control stainings.

Figure 5. Can HDM alone promote in the absence of OVA the activation of transferred OT2? Is this an the same innate effect of T cells seen by Liying Guo et al NI 2015 (doi:10.1038/ni.3244).

- In the revised manuscript we have added an experimental group to Figure 5 where we transferred Th2 polarized OT-II cells and only exposed the mice to HDM. No induction of eosinophilic inflammation or production of type 2 cytokines by the transferred cells was observed, similar to the PBS group and in

contrast with the HDM/OVA group. These experiments demonstrate that there is no innate response by memory Th2 cells in our model (Figure 5C).

Second Editorial Decision

11-Mar-2016

Dear Dr. Hendriks,

It is a pleasure to provisionally accept your manuscript entitled "Activation of group 2 innate lymphoid cells in house dust mite-induced allergic inflammation is dependent on T cells" for publication in the European Journal of Immunology.

For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1521-4141/accepted](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted)). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,

Nadja Bakocevic and Laura Soto

on behalf of

Prof. Maria Yazdanbakhsh

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Bobby W. S. Li, Marjolein J. W. de Bruijn, Irma Tindemans, Melanie Lukkes,
Alex KleinJan, Henk C. Hoogsteden and Rudi W. Hendriks

**T cells are necessary for ILC2 activation in house dust mite-induced allergic
airway inflammation in mice**

Supporting information for:

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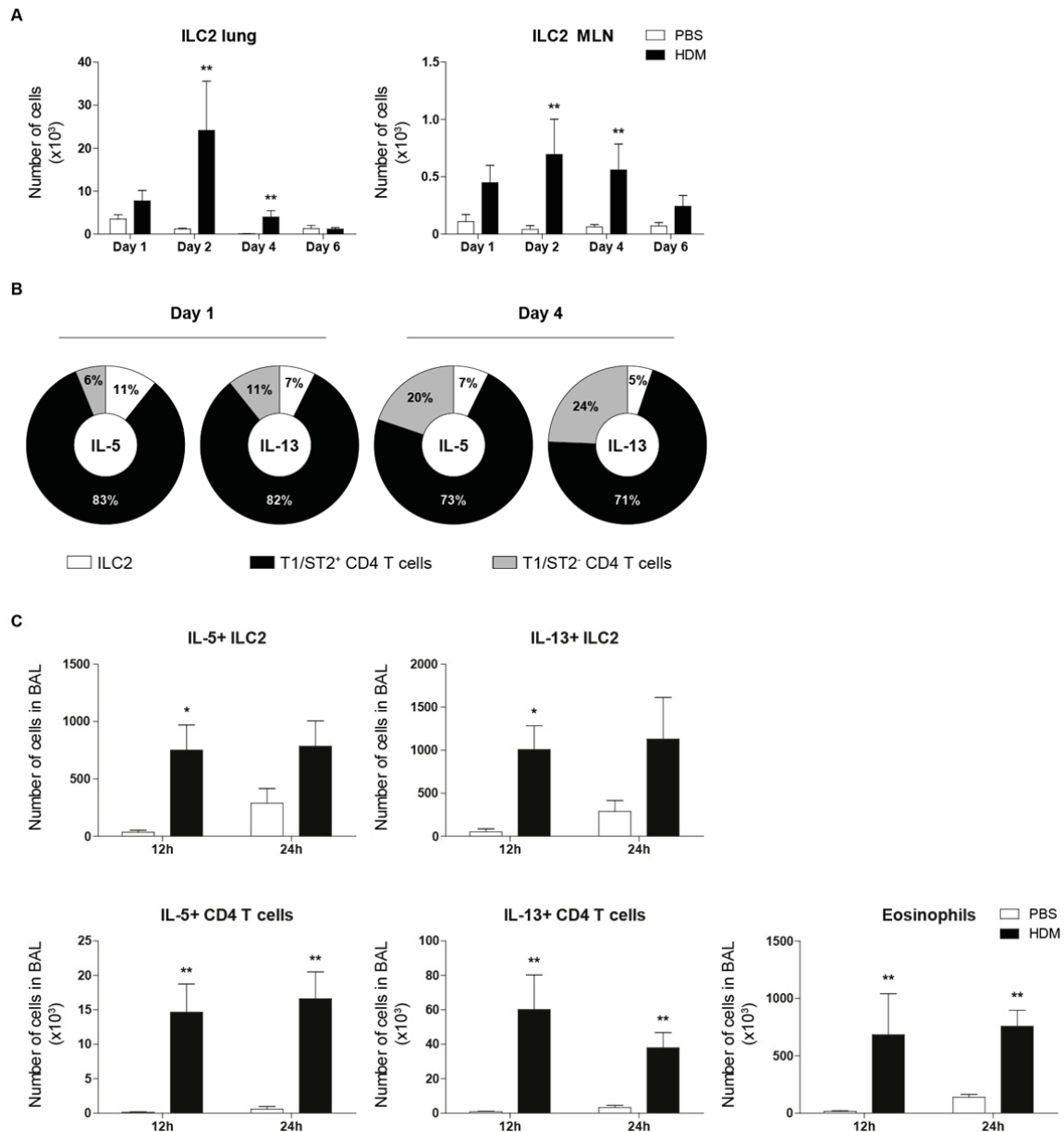
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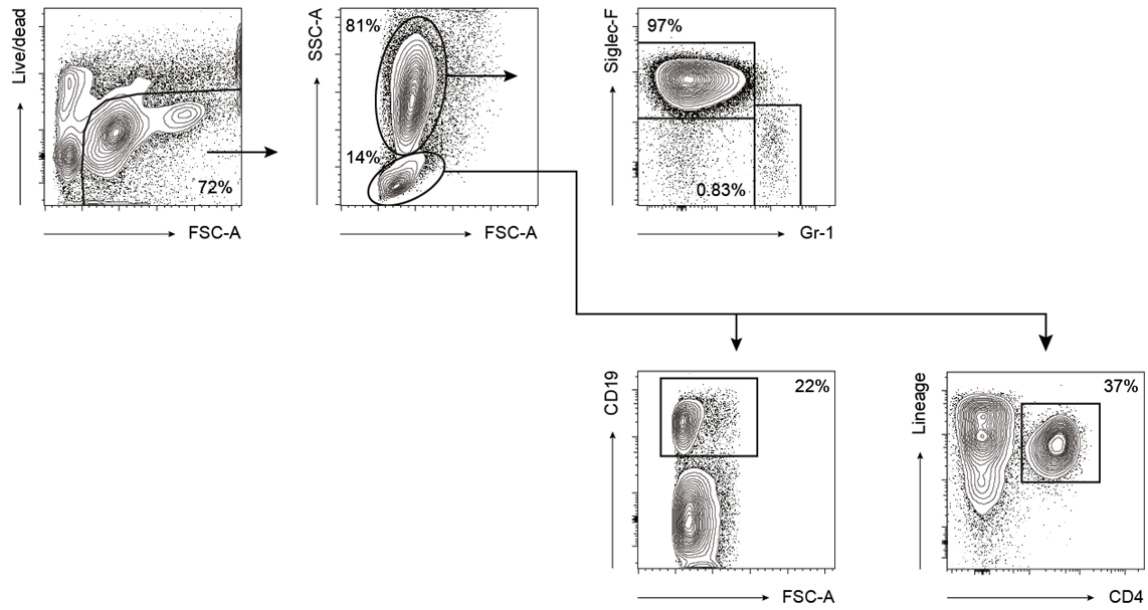
Supporting Information Table 1 Antibodies used for flow cytometry.

Antibody	Conjugate	Clone	Company
Amphiregulin	Biotin	Polyclonal	R&D Systems
B220	PE	RA3-6B2	eBioscience
CD3	PE	145-2c11	eBioscience
CD3	PE-CF594	145-2c11	BD Biosciences
CD4	Alexa Fluor 700	GK1.5	eBioscience
CD4	PerCP-Cy5.5	RM4-5	eBioscience
CD4	Brilliant Violet 605	RM4-5	BD Biosciences
CD5	PE	53-7.3	eBioscience
CD8a	PE	53-6.7	eBioscience
CD11b	PE	M1/70	eBioscience
CD11c	PE	N418	eBioscience
CD19	PE	1D3	BD Biosciences
CD19	PerCP-Cy5.5	eBio1D3	eBioscience
CD19	Biotin	1D3	BD Biosciences
CD25	PerCP-Cy5.5	PC61.5	eBioscience
CD45	PE-CF594	I3/2.3	Abcam
CD45	Pe-Cy7	30-F11	eBioscience
CD127	PE-Cy7	A7R34	eBioscience
CD127	eFluor 450	A7R34	eBioscience
FcεRIα	PE	MAR-1	eBioscience
FoxP3	Alexa Fluor 700	FJK-16s	eBioscience
Gata3	eFluor 660	TWAI-14	eBioscience
Gr-1	PE	RB6-8C5	BD Biosciences
Gr-1	APC-eFluor 780	RB6-8C5	eBioscience
IL-4	Brilliant Violet 711	11B11	BD Biosciences
IL-5	APC	TRFK-5	BD Biosciences
IL-13	eFluor 450	eBio13A	eBioscience
Ki-67	FITC	SolA15	eBioscience
MHCII	Alexa Fluor 700	M5/114.15.3	eBioscience
MHCII	Brilliant Violet 650	M5/114.15.2	BD Biosciences
NK1.1	PE	PK136	eBiosciences
RORγt	PE	Q31-378	BD Biosciences
Sca-1	Pacific Blue	D7	BioLegend
Sca-1	Brilliant Violet 786	D7	BD Biosciences
Siglec-F	PE	E50-2440	BD Biosciences
Streptavidin	APC-eFluor 780		eBioscience
Streptavidin	Brilliant Violet 650		BD Biosciences
Streptavidin	Brilliant Violet 786		BD Biosciences
T1/ST2	Biotin	DJ8	MD Bioproducts
T1/ST2	FITC	DJ8	MD Bioproducts
T-bet	Brilliant Violet 421	O4-46	BD Biosciences
TCR Vα2	APC-Cy7	B20.1	BD Biosciences
TCR Vα2	APC	B20.1	eBioscience
TCR Vβ5	Biotin	MR9-4	BD Biosciences
TER-119	PE	TER-119	eBioscience

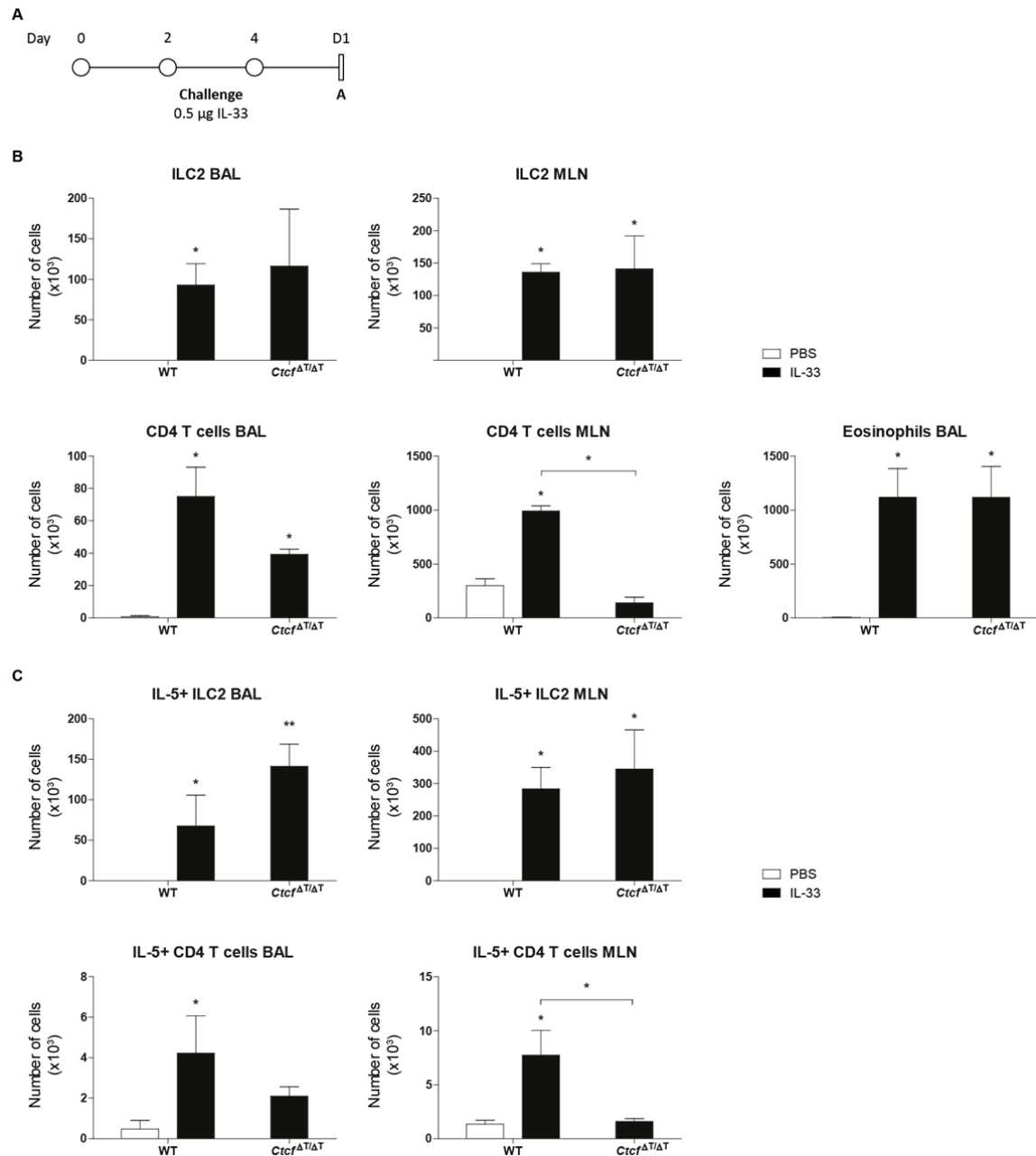


Supporting Information Figure 1. Concomitant induction of ILC2 and CD4⁺ T cells in lung and MLN of HDM-sensitized mice.

(A-C) C57BL/6 mice were treated with PBS or HDM described in Fig. 1A and analysis was performed at indicated time points after the final challenge. (A) Quantification of ILC2 in the lung and MLN of HDM-treated mice by flow cytometry at indicated time points after treatment. (B) Relative contribution of T1/ST2⁺CD4⁺ T cells, T1/ST2⁻CD4⁺ T cells and ILC2 to IL-5 and IL-13 production in the BAL fluid one and four days after treatment. The number of cytokine-producing cells from each population is expressed as a percentage of the total number of cytokine-producing cells and were determined by flow cytometry. (C) Quantification of intracellular IL-5 and IL-13 production by ILC2 and CD4⁺ T cells and the number of infiltrating eosinophils in the BAL fluid 12 hours and 24 hours after treatment. (A-C) Data are shown as mean + SEM of n = 5-6 mice per group and are representative of two independent experiments. * p≤0.05, ** p≤0.01, *** p≤0.001; Mann-Whitney U test.



Supporting Information Figure 2. Gating strategy for the analysis of various immune cell populations. Flow cytometric analysis of eosinophils (Siglec-F⁺Gr-1⁻ granulocytes), neutrophils (Siglec-F⁺Gr-1⁺ granulocytes), B cells (CD19⁺ lymphocytes) and CD4⁺ T cells (Lineage⁺CD4⁺ lymphocytes) in the BAL fluid of HDM-treated mice one day after the final challenge described in Fig. 1A. Data are representative of multiple independent experiments.



Supporting Information Figure 3. Comparable ILC2 response and cytokine production between *Ctrcf*^{ΔT/ΔT} mice and WT mice.

(A) Scheme for intranasal IL-33 treatment of C57BL/6 mice to induce airway inflammation. Mice were treated with PBS or IL-33 at indicated time points. Analysis was performed one day after the final challenge. (B) Quantification of the number of ILC2, CD4⁺ T cells and eosinophils in BAL fluid and MLN. (C) Quantification of intracellular IL-5 and IL-13 production by ILC2 and CD4⁺ T cells in the BAL fluid of IL-33 treated mice. (B-C) Data are shown as mean + SEM of *n* = 4 mice per group and are representative of three independent experiments. * *p* ≤ 0.05, ** *p* ≤ 0.01, *** *p* ≤ 0.001; Mann-Whitney U test.